[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Structural Studies of Ribonuclease. VI. Abnormal Ionizable Groups[1-3]

BY JAN HERMANS, JR., AND HAROLD A. SCHERAGA

RECEIVED OCTOBER 7, 1960

Evidence has been obtained from ultraviolet difference spectra and optical rotation measurements for the presence of abnormal ionizable groups in ribonuclease. If native ribonuclease is brought from *p*H 7 to *p*H 2 below 15°, a small configurational change occurs (small increase in $-[\alpha]$) with essentially no change in optical density. Presumably a group with $pK_{obsd.} = 2.65$ is involved in this transformation. If the *p*H is further lowered to 1 (below 15°), another COO^- group (or groups) near a tyrosyl residue acquires a proton ($pK_{obsd.} = 1.5$), giving rise to a decrease in optical density with essentially no change in $[\alpha]$. Although the COO^- group is near enough to the tyrosyl residue to affect its absorption spectrum, the two groups are not necessarily hydrogen-bonded to each other. However, if one is interested in elucidating the internal configuration of a protein by determining which groups are near each other, it is not necessary to decide whether or not a hydrogen bond exists between the two groups. At elevated temperatures, larger changes in optical density and optical rotation are observed and reflect larger changes in configuration of the molecule. The configurational changes are summarized in Fig. 6. Two possible models, incorporating these abnormally low *pK* carboxyl groups, were used previously to account for the *p*H-dependence of the standard free energy of denaturation. These are also used here to account for the titration curve of native ribonuclease and for the difference titration curve between the native and denatured protein.

Introduction

In the preceding paper of this series[4,5] an investigation of the *p*H-dependence of the reversible configurational change in ribonuclease was reported. By attributing the *p*H-dependence of the denaturation to the presence of specific abnormal ionizable groups in the native molecule, it was possible to account for the *p*H-dependent part of the standard free energy of denaturation with the aid of two possible models. These models are characterized by the particular number and nature of abnormal ionizable groups assumed to be present in them. The present paper, being a continuation of the previous one,[4,5] provides direct evidence for the presence of these abnormal ionizable groups. This evidence was obtained from *p*H-dependent spectral changes and optical rotation changes discussed in the previous paper and also from similar types of measurements not previously reported. The models, which were used[4] previously to explain the denaturation data, are further used here to account for: (1) the titration curve of native ribonuclease, which is known to show certain abnormalities[6] at low *p*H, and (2) the difference titration curve between native and denatured molecules.

Experimental

All of the experimental methods have been described in the preceding paper[4] with the exception of the following.

Titrations.—Direct titrations were performed with a Radiometer TTT1 titrimeter and SBR 2a recorder as previously described.[7] The standardization was carried out with the same buffers as were used in related measurements with the Beckman *p*H-meter.[4] Nitrogen was used to keep the solutions free of CO_2. The initial *p*H was about 2, and the titrations were carried out with 0.8 *N* CO_2-free KOH in about one-half hour. The protein concentration was 25 mg./ml. and 10 ml. was titrated.

Titrations were carried out on the solvent and on the protein solution at 25.0 ± 0.1° and 44.5 ± 0.2°. The amount of acid bound to the protein at any *p*H was computed by subtracting the amount of base required to titrate the solvent from that required to titrate the solution. In order to calculate the average number of protons dissociated per mole, a molecular weight of 13,683[8] was used. Duplicate titration curves agreed with each other to within 0.1 group at all *p*H-values between 2.5 and 4.5.

Results

Effect of *p*H on Difference Spectra.—In the preceding paper[4] the transition from native to reversibly denatured ribonuclease was followed by measuring ultraviolet difference spectra at constant *p*H as a function of temperature. Since the transition occurs at different temperatures when the *p*H is varied (see Fig. V2[9] which is a series of ΔD_{287} *vs.* *T* curves), it is also possible to determine a set of spectrophotometric titration curves (*i.e.*, ΔD_{287} *vs.* *p*H curves) at various temperatures, similar to the one published previously.[10] Such a set is shown in Fig. 1, the ionic strength being 0.08 *M*, half[11] that of the solutions of Fig. V2. The reference solution at all temperatures was at a *p*H between 6 and 7 (measured at those temperatures). It already has been shown in Fig. V2 that the optical density of a *p*H 6.83 solution decreases at temperatures above 43°. Thus, the curves of Fig. 1 at 45, 50 and 55° level off near *p*H 6 at non-zero values of ΔD. These limiting values of ΔD at high *p*H were obtained by adjusting the low *p*H end of the 45, 50 and 55° curves to the values for the 35 and 40° curves at *p*H 1.2, since all the curves of Fig. V2 approach the same asymptote above 43°.

The curve of Fig. V6 has a horizontal portion at low *p*H; thus (in Fig. V2) if a curve were obtainable at a *p*H lower than 0.89, it would be expected to superimpose on the *p*H 0.89 curve. As a result, the largest change in ΔD as the *p*H is lowered from 7 to $\leqq$ 0.89 will be constant from about 0 to about

(1) This investigation was supported by research grant E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service, and by research grant G-6461 from the National Science Foundation.

(2) Presented before the Division of Biological Chemistry at the 134th meeting of the American Chemical Society, Chicago, Illinois, September, 1958.

(3) Part of this work was summarized by H. A. Scheraga, C. Y. Cha, J. Hermans, Jr., and C. L. Schildkraut, "Amino Acids, Proteins and Cancer Biochemistry," Academic Press, New York, N. Y., 1960, p. 31.

(4) J. Hermans, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3283 (1961).

(5) The previous paper should be consulted for nomenclature and details of the experimental work since the previous experiments and their interpretation have a direct bearing on the work of this paper.

(6) C. Tanford and J. D. Hauenstein, *J. Am. Chem. Soc.*, **78**, 5287 (1956).

(7) C. Y. Cha and H. A. Scheraga, *ibid.*, **82**, 54 (1960).

(8) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).

(9) Figures and equations of paper V[4] will be referred to with the prefix V, *e.g.*, Fig. V1 and eq. V2, etc.

(10) H. A. Scheraga, *Biochim. et Biophys. Acta*, **23**, 196 (1957).

(11) These measurements at lower ionic strength were made at an earlier date than those of paper V4 and were not repeated at 0.16 *M* ionic strength.

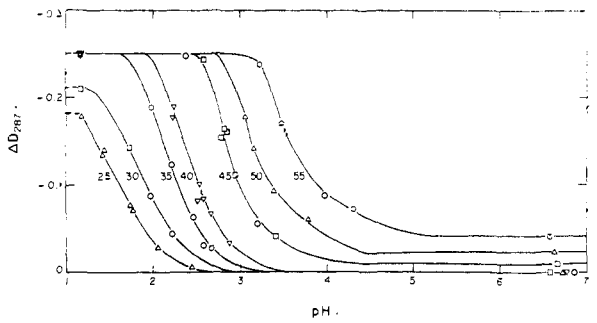


Fig. 1.—Spectrophotometric titration curves at 0.08 M ionic strength and protein concentration 1.90 mg./ml. Temperatures in °C. are indicated. The high pH solution was the reference at $T < 43°$ and its optical density was set equal to zero at each temperature. For $T \geq 43°$ the optical density of the low pH solution (pH 1.2) was set equal to -0.250 and this was taken as the new reference. At 25 and 30° lowering of the pH does not lead to as high a value of $-\Delta D$ as at 35°; this is indicated by dotted lines since the measurements could not be extended to lower pH's at this ionic strength.

17°, will increase from about 17 to about 35° and be constant above 35° (see Fig. V2). Thus, while the 25, 30, 35 and 40° curves approach $\Delta D = 0$ at the high pH end, they do not all have the same limit on the low pH side. Since the ionic strength sets a lower limit to the attainable pH, the low pH end of the 25 and 30° curves of Fig. 1 was not completely realizable and is therefore shown as a dashed line.

If the pK's of the ionizable groups of the denatured protein are not the same as those of the native protein,[12] the value[5] of y (eq. V1), the fraction denatured, will depend on pH. We may represent the difference in the average number of groups ionized, between denatured and native molecules, by the symbol Δr ($\Delta r = r_D - r_N$). It is shown in Appendix II (eq. A13) that this quantity is related to the slope of the curves representing y as a function of pH, by

$$dy/dpH = 2.303y(1 - y)\Delta r \qquad (1)$$

If the curves of Fig. 1 are converted to y $vs.$ pH curves and equation 1 applied to the resulting curves, the data of Table I are obtained. Slopes were taken at all available temperatures at values of $y = 1/4$ and $y = 1/2$. Negative values of Δr indicate that fewer protons are dissociated from the denatured molecule than from the native molecule. It may be noted that the values of $-\Delta r$ at the same pH depend on y, being lower[13] at the lower value of y.

TABLE I

SLOPES OF THE CURVES OF FIG. 1, AND THE VALUES OF Δr, COMPUTED THEREFROM USING EQUATION 1

Temp., °C.	$(dy/dpH)_{y=1/2}$	Δr	$(pH)_{y=1/2}$	$(dy/dpH)_{y=1/4}$	Δr	$(pH)_{y=1/4}$
25	$(-0.75)^a$	(-1.3)	(1.5)	$(-0.64)^a$	(-1.5)	(1.8)
30	$(-0.88)^a$	(-1.5)	(1.8)	$-.66$	-1.5	2.1
35	-1.25	-2.1	2.2	$-.78$	-1.8	2.4
40	-1.29	-2.2	2.5	$-.78$	-1.8	2.7
45	-1.31	-2.2	2.9	$-.58$	-1.3	3.2
50	-0.89	-1.5	3.2	$-.33$	-0.8	3.7
55	-0.67	-1.1	3.7	$-.18$	-0.4	4.5

[a] The computed values of this table at pH 2 and lower are in error for the following reason. Figure V2 shows that at low pH ΔD does not become equal to zero at low temperatures, even though y does (as was found from a comparison between spectral and optical rotation data in Fig. V4). Thus, y is not proportional to ΔD below pH 2, nor $d\Delta D/dpH$ to dy/dpH, as we have assumed in computing the dy/dpH values in this table.

Titration Data.—In order to determine whether the Δr values of Table I are reasonable, titration experiments were carried out. Since the only groups ionizing in the pH-range of interest here (pH 2 to 5) are carboxyls (which in many cases have essentially zero heat of ionization) the titration curve of the *native* molecule should be independent of temperature if no configurational changes occur and if the carboxyl groups are not hydrogen bonded *in only one form*.[14] Any difference found between the titration curves at two temperatures must then be due to the presence of denatured molecules with a different titration curve from that of the native molecule. The temperatures chosen for the comparison were 45 and 25°. The quantity obtained by subtracting the number of protons dissociated from ribonuclease at 25° from that dissociated from ribonuclease at 45° may be designated as Δh. It is easily seen that Δh then should be the product of Δr and y.

At 45° y changes from close to zero at pH 4 to essentially unity at pH 2.4, while essentially no denatured molecules are present at 25° in this pH-range (see Fig. 1). In Fig. 2 open circles represent the values of Δh. The curve drawn in Fig. 2b is that representing the spectral data at 45°, *i.e.*, y as a function of pH at 45°, and was taken from Fig. 1. In Fig. 2a solid circles represent the analog of the spectral curve of Fig. 1 at 45° but at an ionic strength of 0.16 M. The ordinates of the curves of Figs. 2a and 2b are so adjusted that $\Delta D = \Delta D_{max}$ (or $y = 1$) corresponds to $\Delta h = -2.0$ groups, a kind of maximum value of Δr from Table I. With this adjustment the difference in the direct titration curves (represented by open circles) corresponds well with the spectrophotometric titration curve at pH's above[15] 2.5 (*i.e.*, slopes and absolute values are then matched).

The results obtained by applying equation 1 and those obtained by direct titration are thus seen to be in good agreement with each other. The fact

(12) Dissociation constants may differ for many reasons, *e.g.*, if ionizing groups act as donors or acceptors in hydrogen bonds or if electrostatic interactions are different when the configuration of the molecule has changed or if hydrophobic regions surround the ionizable group in one of its configurations but not in the other.

(13) It is interesting to note that at $y = 3/4$ the value of $-\Delta r$ is still higher. At this value of y the curves in Fig. 1 are not well defined by experimental points. We do, however, possess a series of measurements of ΔD as a function of pH at 45° (and ionic strength 0.16 M), which do define the curve near $y = 3/4$. (These data are represented by the filled circles of Fig. 2a.) From the slope at $y = 3/4$ one obtains $-\Delta r = 2.5$ at pH 2.7, which is higher than any of the $-\Delta r$ values of Table I.

(14) Actually, a configurational change has been shown to occur[4] and, further, it will be shown below that a carboxyl group(s) is probably hydrogen bonded *in both* of its forms (*i.e.*, as COOH and as COO^-). See also Appendix I.

(15) Below pH 2.5 the direct titration data approach a $-\Delta h$ value of 3 and do not coincide at all with the spectral data. However, this is in agreement with the increase in $-\Delta r$ with y at constant pH, which was noted[13] in Table I.

The inflection point of Fig. 2 is in reality the inflection point of the y $vs.$ pH, not the r $vs.$ pH curve. Therefore, the pH at the inflection point of Fig. 2 is *not* to be interpreted as the $pK_{obsd.}$ of the abnormal group(s).

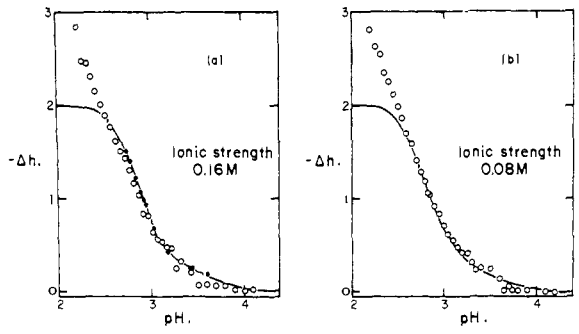


Fig. 2.—The difference in groups titrated at 45° and 25° (open circles) compared with the spectrophotometric titration curve at 45°. The spectrophotometric data were so represented that they coincide with the titration-curve points at high *p*H and that they level off at low *p*H at two groups difference (*i.e.*, $\Delta D = \Delta D_{max}$ corresponds to $\Delta h = -2$). The curve in *b* was taken from Fig. 1, the spectrophotometric data in *a* (filled circles) were determined in a separate experiment but are entirely analogous. In Fig. 2a there is a point (not shown) on the horizontal portion at *p*H 0.9.

that at one *p*H different values of Δr are found, depending on the value of y (see Table I), can best be explained by assuming that more than one denatured form of ribonuclease exists (each with a different titration curve) and that the distribution of the denatured material over these forms is a function of *p*H and temperature and therefore varies with y. As a simplification we shall assume that at values of $y \leqq 1/2$ only one denatured form exists,[16] even though small variations occur in Δr as a function of y at constant *p*H at low y-values (see Table I).

The good agreement between direct titration data and the results of Table I, based on eq. 1, shown in Fig. 2, provides confidence in the reasonableness of the Δr values of Table I. These Δr values will be compared later with those computed theoretically for different models.

Difference Spectrum at Low Temperatures.—When a solution of ribonuclease at *p*H 0.9 and ionic strength 0.16 *M* is cooled below 15°, essentially all of the molecules are in the native form (see Fig. V4). Nevertheless, as may be seen in Fig. V2, this solution has a negative difference spectrum with respect to a solution at *p*H 6.8 at the same temperature (*i.e.*, $\Delta D \sim -0.06$ at 10°) and also (from Fig. V3) a higher negative specific rotation than does a solution of *p*H 6.8 at the same temperature. The form of the difference spectrum under these conditions is *slightly* different from that accompanying the configurational change (Fig. V1) and is shown in Fig. 3, the peaks occurring at 288.5 and 281 mμ instead of at 287 and 280 mμ as in Fig. V1.

The value of this optical density difference at 288 mμ has been determined at various temperatures, *p*H's and ionic strengths. Data at 10° are shown in Fig. 4. Changing the temperature to 0° or to 15° did not affect the data noticeably *indicating that the heat of ionization is essentially zero.* The observed spectrophotometric titration curves again correspond to the ionization of groups on the protein molecule. Two curves have, therefore, been drawn in Fig. 4 corresponding to the equation

$$pH - \log \frac{\Delta D_{288}}{(\Delta D_{288})_{max} - \Delta D_{288}} = pK_{obsd.} \qquad (2)$$

adjusting the value of $pK_{obsd.}$ to obtain the best agreement with the experimental data. Since the value of $(\Delta D_{288})_{max}$ cannot be obtained at these ionic strengths, the arbitrary choice of -0.074 is supported by a single measurement at the higher ionic strength of 1.3.

It may be noted that the pK_{obsd} increases from 1.53 to 1.67 when the ionic strength increases from 0.16 to 0.32 *M*. This is the increment to be expected on the basis of general electrostatic interactions[17] for a sphere of 17.1 Å. radius[18] with no change in configuration upon ionization in a molecule of net positive charge of 18.[6,19] Using the appropriate electrostatic interaction factor a value of 2.48 is obtained for the $(pK^H)^5$ when the charge on the molecule is zero. Thus, the low-temperature

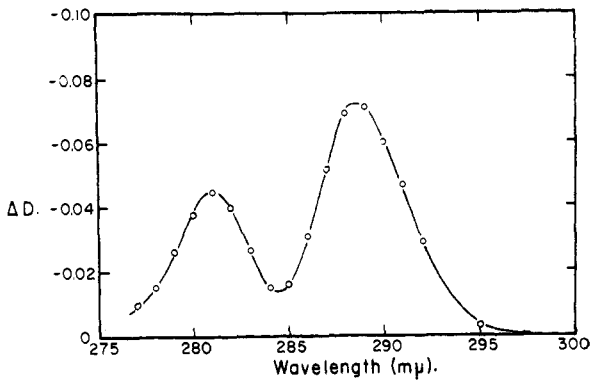


Fig. 3.—Difference spectrum at ionic strength 0.32 *M*, *p*H 0.60 against a reference solution of zero optical density at *p*H 6.5, both at 0° (protein concentration 1.90 mg./ml.).

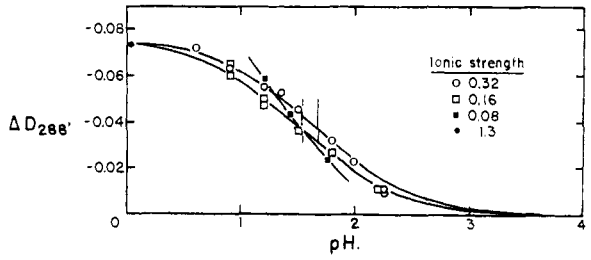


Fig. 4.—Optical densities of solutions at various ionic strengths as a function of *p*H at 10° compared with solutions of the same ionic strength and concentration at *p*H = 6 to 7. The two curves are of the form $pH - \log \frac{x}{(1-x)} = pK$, where $x = \Delta D/\Delta D_{max}$, with $\Delta D_{max} = 0.074$ and with pK's chosen so that they fit the data at ionic strength 0.32 and 0.16, respectively. The vertical lines indicate the value of pK (where $x = 1/2$).

(16) The hypothesis of the occurrence of only one form of reversibly denatured ribonuclease below, but more than one above, the transition temperature, was made in the preceding paper[4] to explain the asymmetry (skewness) of the curves representing y *vs.* T at constant *p*H.

(17) See, *e.g.*, I. M. Klotz, in "The Proteins" (Ed. by H. Neurath and K. Bailey), IB, Academic Press, Inc., New York, N. Y., 1953, p. 965.

(18) C. Tanford, J. D. Hauenstein and D. G. Rands, *J. Am. Chem. Soc.*, **77**, 6409 (1955).

(19) See the next subsection for evidence that the configuration does not change *in this range of pH and temperature.*

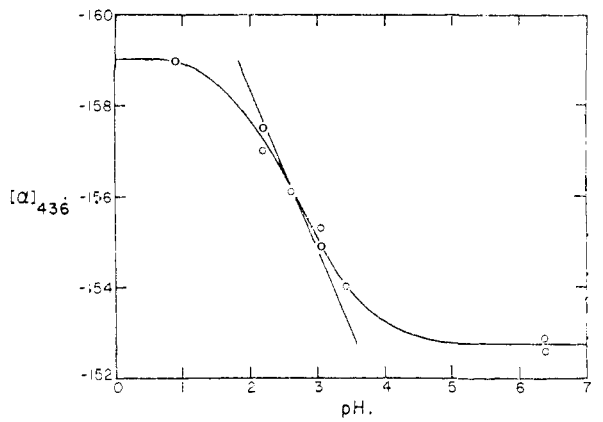


Fig. 5.—Optical rotation as a function of pH at 15°, ionic strength 0.16 M. The curve was drawn through the experimental points. The line is the tangent of maximum slope to the curve representing the variation of $[\alpha]_{436}$ between the observed limits, following the equation describing the ionization of a monovalent weak acid with $pK = 2.65$.

spectrophotometric titration curve indicates that there is at least one carboxyl group in the native molecule with a low pK^{H} of 2.5.

It should be pointed out that the data at still lower ionic strength (0.08 M) do not fit into this simplified scheme. If a curve is drawn to fit the three experimental points (according to eq. 2), agreement is obtained only with a value of $(\Delta D_{288})_{max}$ which is much higher than 0.074. Unfortunately, no data could be obtained below pH 1.2 at this ionic strength, and the origin of this discrepancy is unexplained.

It is important to note the absence of a similar difference spectrum at temperatures ($T > 43°$; see Fig. V2) where all the molecules are in the unfolded form. We may conclude from this that the group whose ionization produces the difference spectrum at 10° is in all likelihood one (or two) which contributes to the pH-dependence of the equilibrium between native and unfolded ribonuclease, and thus to the value of $-\Delta r$, which has a maximum of about two groups. Further support for this conclusion was provided in the preceding paper,[4] where the presence of this group (or these two groups) with $pK_{obsd.} = 1.5$ (at 0.16 M ionic strength and pH 1.5) and zero heat of ionization was used to account for the pH-dependence of the standard free energy of denaturation.

Effect of pH on Optical Rotation at Low Temperature.—We shall now show that the low temperature optical density changes reported in the previous subsection are not accompanied by changes in the configuration of the molecule. Since the specific rotation at 15° is different at pH 0.9 from that at pH 6.8 (see Fig. V3), it was necessary to determine whether the ionization (with $pK_{obsd.} = 1.5$) is accompanied by a change in $[\alpha]_{436}$. At an ionic strength of 0.32 M and temperature of 15°, the value of $-[\alpha]_{436}$ is 159 (*i.e.*, 8 units greater than that at pH 7, see Fig. V3) and independent of pH in the range 0.7 to 1.8, even though ΔD_{288} *is* changing (see Fig. 4). This shows that the pH-dependence of $[\alpha]$ does *not* follow that of ΔD shown in Fig. 4. Thus, apparently no configurational change accompanies the ionization of the abnormally strong group with $pK_{obsd.} = 1.5$. *The fact that the ionization of this group is accompanied by a change in the tyrosyl ultraviolet absorption spectrum implies that there exists some direct interaction between a tyrosyl group and the carboxyl group with abnormally low pK.* The alternative explanation for the occurrence of the change in spectrum, namely, that the ionization of the abnormally strong group can trigger a configurational change in which a tyrosyl group is also involved, has thus been ruled out.[20,21,23]

The optical rotation measurements, just cited, provided evidence that one or more carboxyl groups (with $pK_{obsd.} = 1.5$) are responsible for the low temperature difference spectrum in the pH range of 0.8 to 1.8 without any accompanying configurational change. At a somewhat higher pH (1.8 to 4) there is a small change in optical rotation *at low temperature*, as can be seen from Fig. 5. These measurements indicate the possible presence of a carboxyl groups(s) with $pK_{obsd.} = 2.65$, this group being involved in a configurational change. This small configurational change is not accompanied by any change in ΔD (Fig. 4).

It is remarkable that at *high* temperatures, where all the molecules are completely unfolded, there is also a change in optical rotation with pH, as can be seen from Fig. V3, and that this change is of about the same magnitude as the one at *low* temperatures, just discussed. It would, therefore, appear that the two configurational changes, namely, the one under discussion and the one forming the subject of the preceding paper,[4] occur to a great deal independently of one another. This point needs further investigation, however, and our conclusion should be regarded as a tentative one.

Discussion

Schematic Representation of the Configurational Changes.—To avoid confusion by the rather large number of different causes (*i.e.*, configurational changes and ionizations) which lead to various effects (changes in optical density and optical rotation) when the pH or temperature is changed, we shall make use of Fig. 6, which schematically represents the various forms in which the ribonuclease molecule can occur. Helical and random portions of the molecule are indicated by the usual symbols.

(20) See also S. J. Leach and H. A. Scheraga, *J. Biol. Chem.*, **235**, 2827 (1960).

(21) We have demonstrated here the effect of the ionization of a COOH group on the absorption of a nearby tyrosyl residue. From the point of view of its effect on the spectrum, this neighboring charge effect is indistinguishable from a tyrosyl–carboxyl hydrogen bond. However, if one wishes to elucidate the internal configuration of a protein by determining which side-chain groups are near each other, it is not necessary to decide whether or not a hydrogen bond exists between the two groups. This kind of charge effect, in which the three-dimensional folding brings *remote* groups near each other, is different from the one discussed previously,[20] based on experiments of Donovan, *et al.*,[22] on low molecular weight model compounds. In the latter cases, attention was focused[20] on charge effects due to groups which were nearby *in the polypeptide chain*.

(22) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *Biochim. et Biophys. Acta*, **29**, 455 (1958).

(23) In the Discussion section and in Appendix I it will be shown how the experimentally observed zero value for the heat of ionization of the abnormal carboxyl group with $pK^{H} = 2.5$ supports the conclusion that no configurational change accompanies the ionization of this group below 15°.

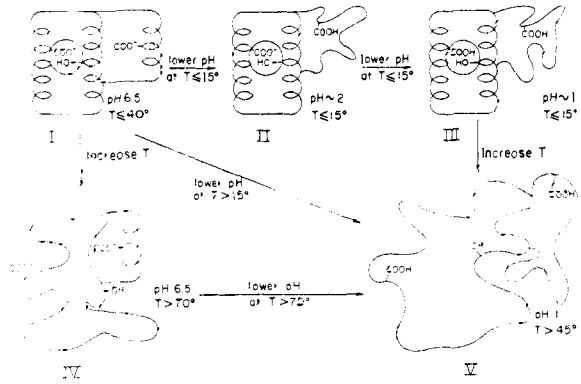


Fig. 6.—Schematic representation of the various forms of ribonuclease. A particular combination of temperature and pH, where any form predominates, is indicated, as are the changes in pH and/or temperature which will reversibly transform one form into others. Helical and random portions are indicated by the usual symbols; the circle in forms I, II and III indicates an unspecified interaction, which lowers the pK of at least one carboxyl group and increases the pK of at least one tyrosyl group. The pictures are not meant to resemble the actual structure of ribonuclease on any but these points. Also, the *whole* molecule is not represented, but only those portions pertinent to the present discussion.

The circled region implies the existence of interactions, which presumably stabilize the molecule and which modify the pK of the ionizing groups participating in these interactions. The following five forms may exist:

(I) Native ribonuclease, with two helical regions, one consisting (in the diagram only) of two large helices, the other of one small helix. Somewhere in the large helical region there is a zone of interaction, with (at least) one carboxyl and one tyrosyl group. The carboxyl and tyrosyl groups in the interaction zone are close but not necessarily hydrogen bonded to each other. No other features of the ribonuclease molecule are meant to be represented in the diagram.

(II) At low temperature and pH the small helical region becomes random (with an ionization of a group with $pK_{\text{obsd.}} = 2.65$), and we observe the small change in $[\alpha]_{436}$ of Fig. 5. No spectral change accompanies the transformation of I to II since the tyrosyl-carboxyl interaction zone is unaffected.

(III) At still lower pH and low temperature, the carboxylate ion in the circled region takes up a proton ($pK_{\text{obsd.}} = 1.5$); no configurational change occurs, however. The proximity of the tyrosyl group to the newly introduced proton gives rise to the change in optical density of Fig. 4.

(IV) If the temperature is raised at high pH, the large helical portion of I unfolds, and the change in D of Fig. V2 is observed because the tyrosyl residue is no longer in the circled region, *i.e.*, no longer abnormal[24]; also, the change in optical rotation of Fig. V3 is observed. A small helical region is retained in IV because the high-temperature limiting value of $-[\alpha]_{436}$ at pH 6.5 is less than that at pH 0.9 (see Fig. V3) by an amount corresponding to the total change observed in Fig. 5. This conclusion is tentative, however (see above).

(V) At low pH the small helical portion of IV can unfold giving rise to an additional increase in $-[\alpha]_{436}$ (Fig. V3); also the COO^- groups take up protons in the transformation of IV to V *without* a change in optical density since the tyrosyl and carboxyl groups in IV are already far apart. Form I can be converted to form V by lowering the pH at temperatures above 15°.

(24) In the transformation I → III, the tyrosyl OH group remains near the COOH group and a small value of ΔD is observed. In the transformation I → IV, the interacting tyrosyl and carboxyl groups are removed far apart from each other and a large ΔD results. As discussed below, this larger value of ΔD may be the result of both the rupture of a tyrosyl-carboxylate ion hydrogen bond and the removal of the tyrosyl group from a hydrophobic region (which surrounds the tyrosyl and carboxyl interaction zone in form I) to an aqueous medium in form IV.

Titration Curves.—Tanford and Hauenstein[6] have published measurements of the direct titration of ribonuclease in 0.15 M KCl. The data in the range where the carboxyl groups titrate (pH 1 to 6) involve certain abnormalities, which indicate that an undetermined number of groups have a pK which is lower than that which is normally found ($pK^0 = 4.6$). Since we have found evidence, which in part determines the extent of these abnormalities, we have computed titration curves (with eq. V, A4) in the region pH 1 to 7, using different sets of ionizing groups, according to models A, B, C_1 and C_2 described in Table V-II. A group with $pK_{\text{obsd.}} = 2.65$ ($pK^H = 3.65$), whose *possible* existence was inferred above, was included in models B, C_1 and C_2 since it improves the agreement with the experimental titration data; it was omitted from model A since it makes the agreement worse. While this group does affect the titration curve, it does not affect $\Delta F_H{}^0$ of the preceding paper very much.

In computing the titration curve of ribonuclease at 25°, 0.15 M ionic strength, we have to take into account the configurational change. From the data in Fig. V2 we can see that the fraction denatured at pH 2.0 and 25° is only 0.09, so that the correction for the presence of the denatured form is only a minor one since the measured titration curve does not descend below pH 1.8.

Since not very much is gained by inspection of the graphs containing calculated and measured titration data, the data are presented in Table II as

TABLE II

MEASURED VALUE[5] OF h MINUS COMPUTED VALUE OF h
(Absolute largest values)

Model	In pH-range 1.8 to 2.5	In pH-range 3 to 4.5
A	−1.2	+0.2
B	− .5	+0.4
C_1	+ .2	+1.0
C_2	− .5	+0.4

the absolute largest value which the differences attain in two pH-ranges. Above pH 4.5 all curves are in good agreement with the experimental data (within ± 0.2 group). Below pH 4.5 models B and C_2 give the best agreement.

Difference in Titration Curves.—The difference in numbers of groups titrated, Δr, was obtained by subtracting the computed titration curve for fully native molecules from that of fully denatured molecules. The theoretical curves for models A, B, C_1 and C_2 are shown in Fig. 7. The open circles are

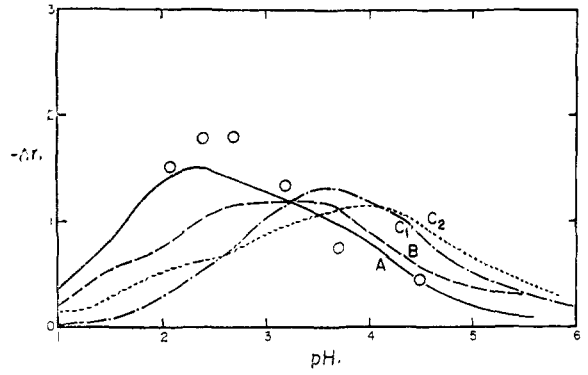


Fig. 7.—Number of groups ionized on the denatured ribonuclease molecule minus that on the native molecule for four models. Open circles are experimental points, taken from Table I, *i.e.*, from the slopes of the curves giving fraction denatured (y) as a function of pH, at $y = 1/4$.

the data[25] of Table I, with $y = 1/4$. It should be pointed out that the experimental Δr values are subject to an error of at least 15%. Thus, curve A can be said to be in fairly good agreement with the experimental data and curve B to be in reasonable agreement with them; the curves for models C_1 and C_2 apparently give poor agreement. The fact that the experimental data were obtained at ionic strength 0.08 M, but the curves calculated at 0.16 M introduces little discrepancy because of this same large experimental error.

In paper V[4] we conclude from denaturation data that models C_1 and C_2 can be excluded and that no choice could be made between models A and B. The comparison between the models on the basis of the titration curve and the difference titration curve presented in this paper leads to the same conclusion.

Tyrosyl Groups.—It previously has been shown[18,26] from spectrophotometric titration data that 3 of the 6 tyrosyl groups of ribonuclease have an abnormally high pK. Since the unfolding of the molecule (transformation I to IV or V) is accompanied by a change in the ultraviolet absorption spectrum, it is simplest to assume that one or more of the 3 abnormal tyrosyl groups (circled region of I in Fig. 6) have become normal. The exact number could be ascertained in principle by determining the number of groups (by direct spectrophotometric titration) which remain abnormal in the denatured protein, *i.e.*, at temperatures above 75°. Unfortunately, even though such solutions are stable below pH 7, there is a rapid[27] irreversible denaturation accompanied by precipitation, above pH 7 at 75°. Since the tyrosyl groups ionize above pH 8, it is therefore impossible to titrate them[28] at 75°.

(25) If our models were strictly applicable (*i.e.*, if, as already pointed out, only one denatured form of ribonuclease existed), Δr should be independent of y. However, as shown in Table I, Δr depends on y. Therefore, the choice of $y = 1/4$ is somewhat arbitrary.

(26) D. Shugar, *Biochem. J.*, **52**, 142 (1952).

(27) J. Hermans, Jr., and H. A. Scheraga, unpublished results.

(28) The presence of 3 abnormal tyrosines was deduced from the interpretation of the irreversible titration of these groups at room temperature at high pH.[18,26] It may thus appear that an irreversible change of configuration is required in order to normalize any of these groups. Yet we observe a *reversible* normalization of abnormal tyrosyl group(s) at *low* pH. This apparent discrepancy may be resolved as follows. The transformation I to IV is reversible. However, at high pH, the *reversibly* denatured form then undergoes modification to irreversibly denatured material. In support of the postulated existence of a reversibly denatured form at high pH at room temperature, the following may be pointed out: Reversibly denatured material occurs in measurable amounts at room temperature at pH 1 but is absent at pH 7. Theoretically, it is to be expected that the equilibrium constant for unfolding should also increase, at high pH and room temperature, to values where reversible denaturation is appreciable (eq. V, A23, in which the pK of the tyrosyl groups in the native form is abnormally high but in the denatured form normal). While we cannot make these measurements at high pH *in solution*, such a decrease of the transition temperature, *both* at low *and* high pH, has been observed with ribonuclease films.[29]

(29) A. Nakajima and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 1575 (1961).

(30) C. Bigelow and M. Ottesen, *Biochim. et Biophys. Acta*, **32**, 574 (1959).

(31) C. Bigelow, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, **31**, 305 (1960).

(32) J. Hermans, Jr., and H. A. Scheraga, paper presented before the Division of Biological Chemistry at the 138th meeting of the American Chemical Society, New York, N. Y., September, 1960, p. 42C. The comments made here in the text were based on experiments performed *after* the submission of the ACS abstract and supersede the statements made in the published abstract.

(33) M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **76**, 6305 (1954).

Alternatively, it might be possible to determine the number of tyrosyl groups which become normal upon reversible denaturation at low pH and high temperature from the value of the change in molar extinction ϵ at 287 mμ, which is about −1800 for complete reversible denaturation (Fig. 1). Recent data of Bigelow, *et al.*, indicate a decrease of about 900 in ϵ_{287} for every tyrosyl group becoming normal, following various changes in the ribonuclease molecule which lead to complete or partial normalization of these groups.[30,31] From this it would follow that of the three groups which are abnormal in native ribonuclease, one is still abnormal in the reversibly denatured molecule. Recent experiments in this Laboratory[32] with low pH irreversibly heat denatured ribonuclease (which is indistinguishable from reversibly denatured ribonuclease by spectral measurements,[32] *i.e.*, has $\Delta\epsilon_{287} = -1800$ with respect to inative ribonuclease), have failed to establish unequivocally the number of abnormal tyrosyl groups in this derivative. In light of the findings of Bigelow and Ottesen,[30] it would still appear most likely, however, that one abnormal tyrosyl group per molecule is present in both reversibly and irreversibly denatured ribonuclease.

Nature of the Abnormality of the Tyrosyl and Carboxyl Groups.—Having attributed the abnormality of one or more tyrosyl residues to the presence of neighboring carboxyl groups with $pK_{obsd.} = 1.5$, it is now appropriate to attempt to account for the abnormal pK's of the tyrosyl and carboxyl groups.

In order to make clear the difficulties involved, it is instructive to consider the theoretical parameters describing the hydrogen bond between a tyrosyl group and a carboxylate ion.[33] Since the hydrogen bonding constant[33] K_{ij} is approximately 1, the pK of the carboxyl group is lowered 0.3 unit, that of the tyrosyl group is raised by the same amount and the heat of ionization of the carboxyl group is changed from zero to −3000 cal./mole. In contrast, it is observed for ribonuclease that the pK^H of the carboxyl group is 2 units lower than pK^0 and

that the heat of ionization of the carboxyl group is zero.

It can be seen in Appendix I that these particular theoretical values are calculated because K_{ik}, the hydrogen bonding constant for the tyrosyl–carboxyl bond (*i.e.*, OH $\cdots$ COOH) is very small. In Appendix I it is shown that, by assuming that this bond is also a strong one (with the tyrosyl–carboxylate ion bond still stronger, of course), the theoretical heat of ionization can become zero even though a hydrogen bond exists. Such a case was encountered in salicylic acid,[34] where the pK of the carboxyl group is 1.5 units lower than that of *p*-hydroxybenzoic acid, but the heats of ionization in both compounds are similar (+ 1.5 kcal./mole). In salicylic acid the spatial restraints imposed on the donor (OH) and acceptor (COOH, COO^-) groups account for the great stability of the hydrogen bond.[34] We postulate here that some additional interaction imposes this restraint on the tyrosyl–carboxyl bond in ribonuclease. Specifically, we suggest that the tyrosyl–carboxyl hydrogen bond is embedded in a hydrophobic region which coöperates with the hydrogen bond in stabilizing the native structure. The presence of this hydrophobic region provides the additional constraints to increase the abnormality of the pK's of the tyrosyl and carboxyl groups (over and above those expected for a hydrogen bond alone[33]). When the carboxyl group is ionized (transformation I to III), a small perturbation of the tyrosyl spectrum occurs ($\Delta D \sim -0.07$). If, however, part of the molecule is grossly unfolded (transformation I to IV) then not only does the hydrogen bond rupture, but also the tyrosyl group is brought from a hydrophobic region to an aqueous one; both effects combined give rise to the large observed blue shift[20,30,31] ($\Delta D \sim -0.25$). It is configurational restraints of the kind proposed here (*i.e.*, an interaction between specific tyrosyl and carboxyl groups and the associated hydrophobic interactions) which, hopefully, will some day provide enough information to enable us to deduce the nature of the folding of the polypeptide chain of native ribonuclease *in solution*.

Appendix I

Magnitude of K^H (Hydrogen Bonding).—In the preceding paper[4] it was shown how the *p*H-dependent part of the standard free energy of denaturation could be related to the changes in pK occurring upon unfolding. Specifically, if a general electrostatic interaction is neglected, *i.e.*, if $w_D = w_N = 0$, we have (from eq. V, A22) that the *p*H-dependent part of the free energy of unfolding is given by

$$-\frac{1}{RT}\Delta F^0{}_H = \sum_i \ln\left(\frac{1 + K_i{}^0/H}{1 + K_i{}^H/H}\right) \qquad (A1)$$

where $K_i{}^0$ and $K_i{}^H$ are defined in the previous paper[4] and H is the hydrogen ion activity. The abnormality (*i.e.*, $K_i{}^H \neq K_i{}^0$) may be due to local electrostatic interactions, hydrophobic bonding or hydrogen bonding. Since, in this paper, we are discussing the nature of the abnormality of the ionizing group, the following treatment, where hydrogen bonding is suggested as the origin of the abnormality, is included.

For a heterologous single hydrogen bond[33]

$$-\frac{1}{RT}\Delta F^0{}_H = \ln(1 - x_{ij}) \qquad (A2)$$

where x_{ij} is the fraction of the molecules having the ijth hydrogen bond.[35] In the Discussion section it is indicated that the interaction responsible for the low pK of the abnormal carboxyl group ($pK_{obsd.} = 1.5$) in ribonuclease is still present at low *p*H, *i.e.*, that there is hydrogen bonding between donor DH and acceptor AH, with constant K_{ik} (but with K_{ik} smaller than K_{ij}) as well as between DH and A. In that case eq. A2 becomes (according to the method of ref. 33) for a non-ionizable donor (at low *p*H)

$$\begin{aligned}-\frac{\Delta F^0{}_H}{RT} = \ln(1 - x_{ij} - x_{ik}) &= \ln(1 + K^0/H) - \\ &\ln[1 + K_{ik} + (1 + K_{ij})K^0/H] \\ &= -\ln(1 + K_{ik}) + \ln(1 + K^0/H) - \\ &\ln\left[1 + \frac{1 + K_{ij}}{1 + K_{ik}} \cdot \frac{K^0}{H}\right] \qquad (A3)\end{aligned}$$

where it is written as the sum of a *p*H-independent contribution and of two terms which vanish at very large values of H. The general electrostatic effect has obviously been neglected in this treatment.

In addition, the presence of a hydrogen bond modifies the pK of the acceptor group. The strong bond to the ionized form A tends to increase the concentration of molecules having the form A, instead of AH; the group thus is more strongly ionized and the pK is lower than normal.

$$K^H = \frac{1 + K_{ij}}{1 + K_{ik}} K^0 \qquad (A4)$$

Thus, eq. A3 can be written

$$-\frac{\Delta F^0{}_H}{RT} = -\ln(1 + K_{ik}) + \ln\frac{1 + K^0/H}{1 + K^H/H} \qquad (A5)$$

By noting that the first term on the right hand side of this equation is *p*H-independent, it is seen that eq. A1 (for one hydrogen bond) and A5 are identical as far as the *p*H-dependence of ΔF^0 is concerned.

According to eq. A4, the observed difference in pK for the abnormal carboxyl group, *i.e.*, $pK^0 - pK^H = 4.6 - 2.5 = 2.1$, is then equal to $\frac{1 + K_{ij}}{1 + K_{ik}}$ or

$$\frac{1 + K_{ij}}{1 + K_{ik}} = 125 \qquad (A6)$$

from which it follows that

$$K_{ij} \gg 1 \qquad (A7)$$

Regarding the enthalpy of ionization of the abnormal group, it is seen that, with this inequality

$$\begin{aligned}\Delta H^H = -R\frac{\partial \ln K^H}{\partial(1/T)} &= \Delta H^0 - R\frac{\partial \ln K_{ij}}{\partial(1/T)} + \\ &R\frac{\partial \ln(1 + K_{ik})}{\partial(1/T)} \qquad (A8)\end{aligned}$$

Now $\Delta H^0 = 0$ for the normal carboxyl group and the second term on the right hand side of eq. A8 is equal to $\Delta H_{ij}{}^0$, the heat of formation of the ijth hydrogen bond. We thus have

$$\Delta H^H = \Delta H_{ij}{}^0 - \frac{K_{ik}}{1 + K_{ik}}\Delta H_{ik}{}^0 \qquad (A9)$$

From this equation we can deduce:

(1) If the ikth hydrogen bond is weak, *i.e.*, $K_{ik} \ll 1$, then ΔH^H is equal to $\Delta H_{ij}{}^0$, *i.e.*, large and negative.[33]

(2) If the ikth hydrogen bond is strong, then ΔH^H can approach zero as is observed for the abnormal group with $pK^H = 2.5$. Since the ikth bond is weaker than the ijth bond (*i.e.*, experimentally $K^H < K^0$, and hence $K_{ij} > K_{ik}$, see eq. A4), one would expect $|\Delta H_{ij}{}^0| \geq |\Delta H_{ik}{}^0|$; thus, if ΔH^H is actually zero, both $K_{ik} \gg 1$ and $\Delta H_{ij}{}^0 = \Delta H_{ik}{}^0$ must hold. The equality of the two heats of hydrogen bond formation might seem surprising, in view of the large difference between the equilibrium constants for formation of the hydrogen bond but is in agreement with data on salicylic acid. The analogy between the structural effect in salicylic acid and in ribonuclease is elaborated in the last subsection of the Discussion section.

(34) J. Hermans, Jr., S. J. Leach and H. A. Scheraga, *J. Am. Chem. Soc.*, to be submitted.

(35) M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, **78**, 5793 (1956).

Appendix II

Denaturation and Change in Hydrogen-ion Binding.—When, in a certain *p*H-range, the ratio of native to denatured molecules is *p*H-dependent, denaturation must be accompanied by a change in the average number of hydrogen ions dissociated from the protein molecules. Using equation V, A11 of the preceding paper,[4] we have

$$\frac{1}{RT}\frac{d\ \Delta F^0_H}{d\ln H} = \frac{1}{RT}\left(\frac{d\ \Delta F^0_h}{d\ln H}\right)_D - \frac{1}{RT}\left(\frac{d\ \Delta F^0_h}{d\ln H}\right)_N = r_D - r_N \quad (A10)$$

Converting the standard free energy to an equilibrium constant, we obtain

$$d\ln K_{obsd.}/d\ln H = r_N - r_D \quad (A11)$$

Since

$$K_{obsd.} = y/(1-y) \quad (A12)$$

according to eq. V3, we can write

$$dy/dpH = -2.303\ dy/d\ln H$$
$$= -2.303\ (dy/d\ln K_{obsd.})\ (d\ln K_{obsd.}/d\ln H)$$
$$= 2.303\ y\ (1-y)(r_D - r_N) \quad (A13)$$

Thus the rate of change of the fraction of the molecules in the denatured state with *p*H is directly proportional to the difference in proton binding between native and denatured molecules. It should be pointed out that equation A13 is quite general, *i.e.*, no assumption regarding special models has been made in deriving it.

[CONTRIBUTION FROM THE DEPARTMENT OF ORGANIC CHEMISTRY, UNIVERSITY OF ATHENS, ATHENS, GREECE, AND THE LABORATORY OF BIOCHEMISTRY, NATIONAL CANCER INSTITUTE, NATIONAL INSTITUTES OF HEALTH, PUBLIC HEALTH SERVICE, U. S. DEPARTMENT OF HEALTH, EDUCATION AND WELFARE, BETHESDA, MD.]

Studies on Arginine Peptides. III. On the Structure of Tricarbobenzoxy-L-arginine[1]

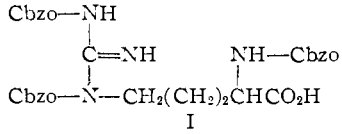
BY LEONIDAS ZERVAS, MILTON WINITZ AND JESSE P. GREENSTEIN[2]

RECEIVED FEBRUARY 10, 1961

The structural elucidation of N^{α},N^{ω},N^{ω}-tricarbobenzoxy-L-arginine has been achieved. Thus, conversion of this compound to the *p*-nitrobenzyl ester, followed by the treatment with propionic anhydride, led to N^{α},N^{ω},N^{ω}-tricarbobenzoxy-N^{ω}-propionyl-L-arginine *p*-nitrobenzyl ester. Catalytic hydrogenolysis of the latter yielded N^{ω}-propionyl-L-arginine which, when subjected to the action of acetic anhydride, was converted to N^{α},N^{ω}-diacetyl-N^{ω}-propionylanhydroarginine. Cleavage of the latter with water led to *N*-acetyl-*N'*-propionylurea and DL-α-acetylaminopiperidone which, upon identification, established the structure of the starting material. The relation of the structure of tricarbobenzoxyarginine to its utility as a peptide intermediate is considered.

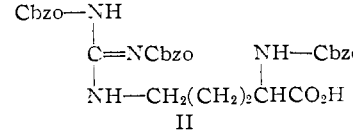
Introduction

Previous studies in this series[3,4] were concerned with the preparation of N^{α},N^{ω},N^{ω}-tricarbobenzoxy-L-arginine and the utility of this and related compounds as intermediates in the synthesis of N- and C-terminal arginine peptides. The triacylated arginine derivative was secured readily by treatment of L-arginine with an excess of carbobenzoxy chloride in a highly alkaline aqueous medium and behaved, in its reactions, as a pure and discrete chemical entity. However, its exact structural assignment posed a somewhat puzzling problem, for although the position of one of the carbobenzoxy substituents on the α-nitrogen atom could be established with certainty, the positions of the other two carbobenzoxy groups on the nitrogen atoms of the guanidino nucleus permitted the assignment of one or the other of two equally plausible structures, as represented by formulas I and II, respectively. It is evidence leading to the



I

unequivocal designation of I as the correct structure with which the present communication is primarily concerned.[5]

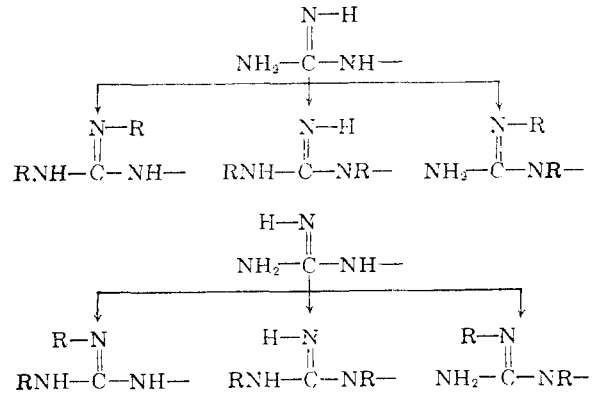


II

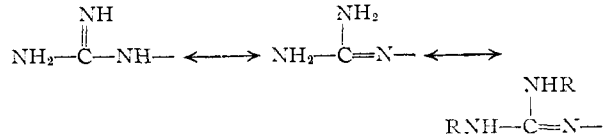
Results and Discussion

In an earlier communication,[3b,4b] the preferential cleavage of one of the two N^{ω}-carbobenzoxy sub-

(5) Examination of the structure of the guanidino group indicates that diacylation of this moiety may, theoretically at least, lead to a number of *cis-trans* isomers, as in what follows



In addition, resonance may lead to the possibility



The present study is directed toward demonstrating the positions of the two acyl substituents on the nitrogen atoms of the guanidino moiety, leaving the stereochemical aspects of the problem for future consideration.

(1) A summary of this paper was presented at the 2nd European Peptide Symposium, Munich, September, 1959.

(2) Deceased February 12, 1959.

(3) (a) L. Zervas, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **65**, 573 (1956); (b) L. Zervas, M. Winitz and J. P. Greenstein, *J. Org. Chem.*, **22**, 1515 (1957).

(4) (a) L. Zervas, T. Otani, M. Winitz and J. P. Greenstein, *Arch. Biochem. Biophys.*, **75**, 290 (1958); (b) L. Zervas, T. Otani, M. Winitz and J. P. Greenstein, *J. Am. Chem. Soc.*, **81**, 2578 (1959).