# Steady-State Kinetic Study of Action of Ribonuclease A, Involving a Conformational Change between 30 and 40 °C†

R. R. Matheson, Jr.,‡ and H. A. Scheraga*

ABSTRACT: The steady-state kinetics of the reaction of ribonuclease A with cyclic cytidine 2′,3′-phosphate as substrate are investigated as a function of temperature at pH 5 and ionic strength 0.1 M. The results suggest, but cannot prove, that a conformational change near 32 °C is involved in the rate-limiting step of the reaction mechanism. This conformational change is proposed to be the same one that was observed in studies of the free enzyme and of enzyme–inhibitor complexes near the same temperature.

Ribonuclease A (RNase A)[1] is among the most thoroughly studied of all proteins (for a review, see Richards & Wyckoff, 1971). The reversible thermal unfolding of RNase A is a popular subject for investigations of the pathways of protein folding (Burgess & Scheraga, 1975; Hagerman & Baldwin, 1976), and the mechanism of its enzymatic action has also been the object of extensive study (Findlay et al., 1961; Witzel, 1963; Deavin et al., 1966; Hammes, 1968a,b; Harris et al., 1969; Roberts et al., 1969a). Thus, it is quite surprising that the temperature dependence of the reaction catalyzed by RNase A has not been thoroughly explored. The temperature dependence of the RNase-catalyzed digestion of RNA was examined by Kalnitsky & Resnick (1959), while the reactions involving cyclic uridine phosphate (Cheung & Abrash, 1964) and cyclic cytidine phosphate (Takahashi et al., 1969) as substrates have been examined over rather limited temperature ranges. The digestion of RNA is a very complicated process. The studies involving the nucleotide substrates were not intended primarily to investigate the temperature dependence of RNase A activity and, consequently, did not cover the entire range of temperature (from 0 °C to the point of denaturation). We are not aware of any previous report of the complete temperature profile of RNase A activity toward a small-molecule substrate.

We were motivated to undertake such an investigation by the observation in a related study (Matheson & Scheraga, 1979) of an apparently limited conformational change in the structure of RNase A at pH 5 in the temperature range of 30–40 °C. The nonspecific surface labeling technique (Matheson et al., 1977) which was used in that study demonstrated that the overall exposure of Ala and Met residues to the solvent begins to increase in this temperature range. Less compelling evidence from the same study suggested that the 13 N-terminal residues of RNase A were also affected somewhat. Since His-12 is necessary for catalytic activity (Crestfield et al., 1963b; Kenkare & Richards, 1966; Hofmann et al., 1970), this raised the possibility that the enzymatic activity of RNase A might also be influenced by temperature between 30 and 40 °C in a manner other than the usual effect of temperature on a single reaction rate. This suggested conformational change occurs about 25 °C below the temperature $T_m$ of the main denaturational transition and precedes any detectable changes in optical rotation or ultraviolet absorption at pH 5 (Hermans & Scheraga, 1961).

In this paper, we demonstrate that the enzymatic activity of RNase A toward cyclic cytidine 2′,3′-phosphate (C>p) does indeed show a dependence on temperature that may suggest a structural change in the enzyme between about 30 and 40 °C. The characteristics and possible significance of the structural change will be discussed.

## Experimental Section

*Materials.* Bovine pancreatic ribonuclease was purchased from Sigma (type II-A) and purified by column chromatography on carboxymethylcellulose (Whatman CM52) by the procedure of Taborsky (1959). Fractions corresponding to Taborsky's fraction D (RNase A) were pooled, dialyzed, lyophilized, and stored at 4 °C. Solutions of RNase A were prepared from the lyophilized enzyme by dissolving the protein in the buffered solution of interest (10–20 mg/100 mL) and heating to 60 °C for 5 min in order to dissociate aggregates (Crestfield et al., 1963a). The concentration of RNase A in this stock solution was determined ($\epsilon_{278}$ 9700, Sage & Singer, 1962), and dilutions were then made volumetrically from this stock solution in the appropriate buffer.

Ribonucleic acid (Sigma, type X1, lot 35B-8530) and cyclic cytidine 2′,3′-phosphate (P-L Biochemicals) were used as received. The concentrations of these substrates were determined by dilution of stock solutions made from weighed amounts of the solid material.

Buffers were prepared by using 0.05 N 2-(*N*-morpholino)ethanesulfonic acid (Sigma) and 0.05 N potassium acetate (Mallinckrodt). In each case, the ionic strength was adjusted to a constant value of 0.1 M by addition of the appropriate amount of KCl. The pH of all solutions was measured at 25 °C by using a Radiometer type PHM4c pH meter equipped with a Radiometer GK2302B combined electrode.

*Methods.* All spectroscopic measurements were made with a Cary Model 14 spectrophotometer. The temperature was controlled by a Haake type F water bath and was monitored with a thermistor inserted directly into the cuvette both before and after measurement. At temperatures above 15 °C, the maximum difference in the temperatures observed before and after a measurement was 0.1 °C. Below 15 °C, the drift was as great at 0.8 °C. The temperature reported is the average of those measured before and after the spectroscopic measurement.

The activity of RNase A toward RNA was measured by

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[1] Abbreviations used: RNase A, bovine pancreatic ribonuclease A; C>p, cyclic cytidine 2′,3′-phosphate; Mes, 2-(*N*-morpholino)ethanesulfonic acid; $v_i$, the initial velocity of reaction; RNA, ribonucleic acid; $K_m$, the Michaelis constant; $T_m$, the temperature at which a thermally induced conformational change is 50% complete.

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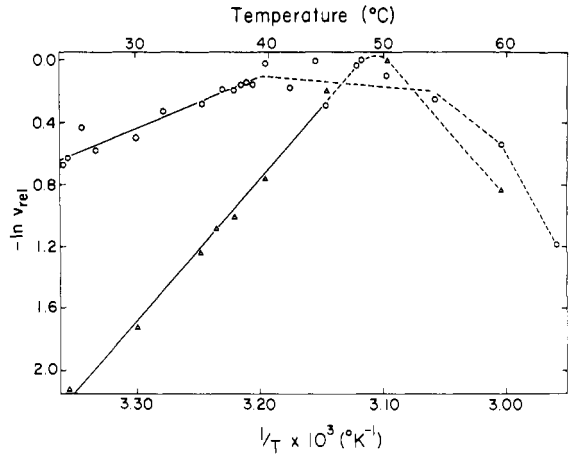


FIGURE 1: Arrhenius plot for the enzymatic activity of RNase A toward RNA (△) and C>p (○) measured in 0.05 M acetate buffer, pH 5.03 and ionic strength 0.1 M. The RNase A concentration was $4.9 \times 10^{-8}$ M, while the substrate concentrations were 1.4 mg/mL (RNA) and 0.4 mg/mL (C>p). In order to permit convenient measurement and plotting on the same scale for both substrates with the same enzyme concentration, the data are plotted as the observed velocities relative to the maximum velocity. Thus, $\ln v_{rel}(T) = \ln v_i(T) - \ln v_{max}$, where $v_{rel}$ at temperature $T$ is the value whose natural logarithm is plotted, $v_i(T)$ is the observed initial velocity at temperature $T$, and $v_{max}$ is the largest initial velocity observed in the temperature range studied. Each point represents the mean for a duplicate set of measurements. The solid lines correspond to the best straight line (as determined by the method of least squares) through the experimental points in the temperature range over which the line extends. The dashed curves are intended to be only guides to the eyes and have no theoretical or experimental significance.

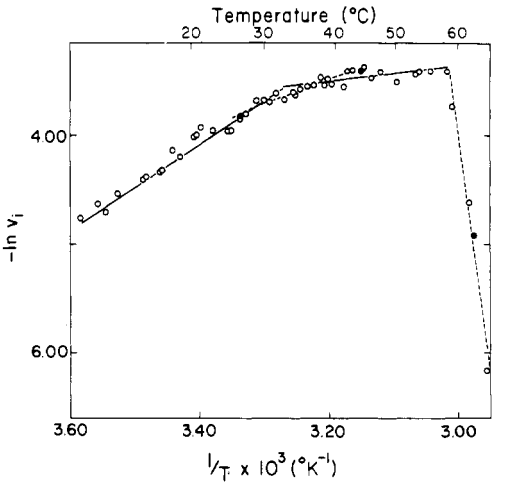


FIGURE 2: Arrhenius plot for the enzymatic activity at pH 5.03 (Mes buffer, ionic strength 0.1 M) of RNase A ($2.2 \times 10^{-7}$ M) toward 0.63 mM C>p (○). The filled circles (●) represent measurements made with a C>p concentration of 1.2 mM. Each point represents the mean for a triplicate set of measurements. The data were obtained in three independent sets of experiments (by using different enzyme and substrate solutions), and the error symbols represent the standard deviation in $-\ln v_i$ for all nine measurements obtained from the three sets at the indicated temperatures. The standard deviation for the initial velocities was 2.5% and the standard deviation for $-\ln v_i$ was 1.8%. The solid lines correspond to the best straight lines through the experimental points in the temperature range over which the line extends. The manner in which each line and its temperature range were determined is described in the Results section. The dashed curves are intended to be guides to the eye and have no theoretical or experimental significance. The apparent discontinuous junction of the solid lines most likely arises from the procedure used in the analysis of the data, but such a discontinuity cannot be supported by the data. In reality, there is an ambiguous (presumably smoothly curving) temperature dependence between about 29 and 35 °C.

a modification of the method of Kunitz (1946). Two milliliters of a solution containing the RNA was equilibrated in the cuvette, while 1.0 mL of a solution of RNase A was equilibrated in a syringe which was thermostated by the same water bath. After allowing 10 min (15 min for temperatures above 50 °C) for thermal equilibration, the contents of the syringe were discharged into the cuvette and mixing was accomplished by repeatedly filling and discharging the syringe. The decrease in optical density at 300 nm was recorded, and the initial velocity, $v_i$, was taken to be the slope of the recorder trace measured directly on the chart paper at times between 0.5 and 3 min after mixing. Final concentrations of RNA and RNase A were in the range of 1–2 mg/mL and 0.27–0.68 μg/mL, respectively.

The activity of RNase A toward C>p was monitored by the increase in optical density of 286 nm (Crook et al., 1960). Some experiments were conducted as described for RNA. Other measurements (including all of those made at temperatures below 20 °C) were made by equilibrating 3.0 mL of a solution of RNase A in the thermostated cuvette. After 10–15 min, the substrate was added from a concentrated stock solution with the aid of a Hamilton syringe. The volume added was always less than 30 μL (1% of the total volume), and stirring of the solution in the cuvette was accomplished manually for 20–30 s. As with RNA, $v_i$ was measured directly from the chart recorder trace. This trace was never perfectly linear, as also observed by Herries et al. (1962). However, the curvature was fairly small for times less than 3 min after mixing. We have arbitrarily selected the nearly linear trace obtained between 1 and 2 min as a good measure of $v_i$. Comparison of this approximation to $v_i$ with the more exact and laborious estimate proposed by Herries et al. (1962) gave the same value (within 1%) when checked at 25 °C. The enzyme concentration was varied over the range 0.1–3.0 μg/mL.

## Results

Kalnitsky & Resnick (1959) have reported the temperature dependence of ribonuclease activity toward RNA at pH 5 and ionic strength 0.06 M. They observed a linear Arrhenius plot between 30 and 50 °C, characterized by an energy of activation, $E_a$, of 20.5 kcal/mol. We have confirmed their observations (by using a different assay and slightly higher ionic strength) and the $E_a$ which characterizes the Arrhenius plot for RNA presented in Figure 1 is 18.8±0.5 kcal/mol. For comparison, the enzymatic activity of the identical solution of RNase A toward C>p is also presented in Figure 1. It is clear that the C>p data are not describable by a linear Arrhenius plot; i.e., $E_a$ is not independent of temperature in the range of 30–50 °C. Even over the temperature range for which an approximately linear behavior is seen (23–39 °C as indicated by the solid line through the open circles in Figure 1), the fit is not particularly good (the calculated correlation coefficient being only 0.934).

The temperature dependence of the activity of RNase A toward C>p at pH 5 is shown in greater detail in Figure 2. The Mes buffer used in the experiments summarized in this figure was selected because of its excellent spectroscopic properties, absence of chelation with metals, and insensitivity of its p$K$ to temperature (Perrin & Dempsey, 1974). A few measurements were made at pH 5 in the 0.05 M acetate buffer used in the experiments summarized in Figure 1 and comparable results were obtained. As in Figure 1, the Arrhenius plot is not linear in the temperature range (6–66 °C) examined. It is important to make sure that saturation of the enzyme is maintained at all temperatures; otherwise, $v_i$ will be sensitive to substrate concentration in a manner which will

vary with temperature (Gibson, 1953). The Michaelis constant, $K_m$, for C>p is known to be a strong function of temperature at least at pH 6 (Erman & Hammes, 1966). Some measurements were made at higher substrate concentrations and the results are shown in Figure 2. Since no significant differences were observed at any temperature, we can conclude that the substrate concentration used routinely was sufficiently high to ensure that we are always measuring a velocity close to the maximal velocity.

The Arrhenius plot of Figure 2 can be divided conveniently into four temperature regions. At temperatures below room temperature, the plot of $-\ln v_i$ vs. $1/T$ is insensibly different from a straight line. Between this initially linear region and a second apparently linear region, which occurs roughly between 40 and 55 °C, is a region of curvature. Finally, at higher temperatures, the data fall off rapidly since this is the temperature range in which RNase A unfolds at pH 5 (Kalnitsky & Resnick, 1959; Hermans & Scheraga, 1961).

Accepting the hypothesis that the Arrhenius plot of Figure 2 is linear at low temperatures, we desire to know how good the fit to a straight line is and at what temperature it breaks down. To accomplish this we have performed the following calculation. Starting with the points at the lowest temperature, we computed the best fitting straight line (as judged by the method of least squares) for the first $n$ points ($n \geq 8$). We then calculated the difference between the predicted (i.e., from the best fitting straight line for the $n$ points) and observed values of $\ln v_i$ for the $n$ temperatures at which measurements were made. The signs of the differences between the predicted and observed values of $\ln v_i$ were then analyzed by the run test for randomness (Crow et al., 1960) at the 90% level of significance. If the distribution of signs was random at this level of significance, then we concluded that the differences might have been due simply to experimental uncertainty and repeated the calculation, this time using the first $n + 1$ data points to define the straight line. The largest value of $n$ for which the null hypothesis of randomness was consistent with the data was taken to be the line that best described the data. This procedure eliminates any constraints on the intervals at which data are taken, and its reliability can be assessed by testing the data at a more stringent confidence level and by computing the correlation coefficient for the final line.

Application of this objective test to the data of Figure 2 resulted in the conclusion that the Arrhenius plot is linear in the temperature range 6.1 to 33.6 °C. The correlation coefficient for the best fit line through the data in this range is 0.982 ± 0.003 [the error limits on the correlation coefficient being calculated as recommended by Kendall & Stuart (1963)], the corresponding Arrhenius activation energy, $E_a$, is 7.4 ± 0.9 kcal/mol, and the intercept is 8.6 ± 0.8. An identical procedure, which commenced at 58.6 °C and extended a line to lower temperatures, led to the conclusion that the data in the range 33.6–58.6 °C are linear with a correlation coefficient of 0.889 ± 0.006, $E_a$ = 1.7 ± 0.2 kcal/mol, and intercept of −0.8 ± 0.1. The small correlation coefficient is partially a result of the small slope of the best fit line and is a good example of how misleading this quantity can be. Nevertheless, reference to Figure 2 shows that linearity is obeyed quite closely in this temperature range. The uncertainties reported are always the 95% confidence limits.

The activity of RNase A toward C>p increases steadily but only very slowly between 30 and 48 °C. Thus, since the change in $\ln v_i$ is small, many points are needed to distinguish any trend from the experimental uncertainty. This is evident from the fact that, although the run test (at the 90% level of significance) indicates that the data should be fit by two straight lines which meet at 33.6 °C, at the 95% level of significance the regions of linearity overlap. The longest line (largest value of $n$) that is consistent with the run test for randomness and that begins at low temperatures includes points up to and including 35.1 °C. Starting at high temperatures, the longest such line includes points as low as 28.6 °C. The fact that the longest straight lines meet at 33.6 °C when the data are tested at the 90% level of significance is probably fortuitous. When the data are analyzed at the 95% level of significance, we see that there is a region of ambiguity of approximately 6 °C from 29 to 35 °C in which we cannot say which line best describes the data. Presumably, there is actually a smooth and continuous change in the value of $E_a$ throughout this region. We will take this region of overlap as an approximate measure of the temperature range of curvature in the Arrhenius plot. It should be emphasized that the temperature range 29–35 °C does not contain points which lie in a notably more curved pattern than those in other temperature ranges. This is simply a region of ambiguity which results from the attempt to fit the data to two straight lines. We report the curvature as being between 29 and 35 °C, although this probably overstates the precision of the data.

The Arrhenius plot above about 59 °C shows a steep decline in $E_a$. As we remarked earlier, this is the temperature range in which the cooperative, thermal unfolding of RNase A is observed by other techniques at pH 5 (Kalnitsky & Resnick, 1959; Hermans & Scheraga, 1961). The data taken in the region of unfolding permit estimation of the thermodynamic parameters for the unfolding of RNase A, if a two-state transition between fully active and completely inactive enzymatic forms is assumed (Kalnitsky & Resnick, 1959). If we choose the high-temperature (i.e., 33.6–58.6 °C) linear portion of the Arrhenius plot of Figure 2 as the base line, then we calculate the midpoint of the transition, $T_m$, to be 60 ± 3 °C, $\Delta H°$ = 91 ± 12 kcal/mol and $\Delta S°$ = 273 ± 80 eu. These estimates are in good agreement with earlier ones (Foss & Schellman, 1959; Tsong et al., 1970).

## Discussion

The following four questions are provoked by the data presented above. Do the nonlinear Arrhenius plots of RNase A activity toward C>p at pH 5 and ionic strength of 0.1 M indicate a conformational change around 32 °C, or is there an alternative explanation? If there is such a conformational change, where is it situated in the RNase A molecule? Are there inferences that can be drawn about the catalytic mechanism of RNase A based upon these data? And why is no such behavior observed when RNA is the substrate? We shall consider each question in turn.

We have already ruled out the possibility that the nonlinear Arrhenius plots might be due to the failure to maintain saturation of RNase A at elevated temperatures. By using two different techniques for mixing the enzyme and substrate and by using two different buffers, we have eliminated the possibilities that a peculiar artifact of mixing or a specific buffer effect is the cause of the nonlinear plots. While nonlinear Arrhenius plots are not infrequently reported in enzyme kinetic studies (Massey, 1953; Smith et al., 1955; Saint-Blancard et al., 1977), their existence and significance must be considered carefully (Kistiakowsky & Lumry, 1949). Besides artifacts, a number of other possible causes of nonlinear Arrhenius plots must be considered before a conformational change in the enzyme can be postulated with confidence. Dixon & Webb (1958) mention four such possibilities. A phase change in the solvent can clearly be ruled out since

Table I: Evidence for a Conformational Change of RNase A in the Temperature Range 29–45 °C at pH 5–6.5

| temp range (°C) | solvent | residues or site involved | technique | ref |
|---|---|---|---|---|
| 35–40 | 0.2 M phosphate; pH 6.02 | phosphate binding site | chromatography | Hirs (1962) |
| 40–45 | 0.01 M $CaCl_2$; pH 6.5 | Tyr-25-Cys-26 | proteolysis | Rupley & Scheraga (1963) |
| 32–41 | 0.2 M NaCl (pH not reported) | His-12 & His-119 | NMR | Roberts et al. (1969b) |
| 40–45 | 0.2 M acetate; pH 5.5 | His-48 | NMR | Zaborsky & Milliman (1972) |
| 30–40 | 0.01–0.1 M KCl; pH 5 | Met-13, Ala-19, & Ala-20 | nonspecific surface labeling | Matheson & Scheraga (1979) |
| 29–35 | 0.05 M Mes; pH 5 | active site | steady-state kinetics (C>p substrate) | this work[a] |

[a] Strictly speaking, "this work" applies to the enzyme–substrate complex, whereas all other entries in this Table pertain to the enzyme itself.

the region of nonlinearity in our case is from about 29–35 °C where dilute, aqueous solutions near pH 5 show no such change. The existence of two parallel reactions with different temperature coefficients cannot be the cause since this situation would lead to a higher activation energy at higher temperature in contrast to observation. A third general possibility, viz., reversible inactivation of the enzyme, implies that a conformational change must occur, unless the binding of buffer ions is both temperature dependent and inactivating which is not likely for the buffers used here. The fourth possible explanation for a "break" in an Arrhenius plot, which is cited by Dixon & Webb, is that the rate-controlling step (or the nature of the step) changes with temperature. This possibility can never be distinguished from a conformational change of the enzyme–substrate complex on the basis of only steady-state kinetic data. However, other evidence for a conformational change of the enzyme itself around 30–40 °C is available. Some of this evidence is summarized in Table I. Since a conformational change is indicated from other experiments on the enzyme in the absence of substrate, we believe that a conformational change in the RNase A/C>p complex is a more likely explanation than a change of rate-determining steps. Of course, a conformational change itself might cause a temperature-dependent change of rate-determining steps.

Another possible cause of a nonlinear Arrhenius plot could be the temperature dependence of the $pK_a$ of some ionizable group involved in the rate-determining step but not in any conformational change. The imidazole groups of His-12 and -119 are the most likely suspects in such a case since both are involved in the catalytic process and imidazole has an enthalpy of ionization, $\Delta H_i$, of around 8 kcal/mol (Martin, 1964). However, while we do not know the exact $pK_a$ of the histidines in the active site complex, we may suppose that they lie in the range 6–8 at 25 °C (see Meadows et al., 1969). Thus, an increase in temperature to 35 °C (i.e., passing through the range of nonlinearity in the Arrhenius plot) will lower the average degree of protonation only slightly. For a group with a $pK_a$ of 6 at 25 °C, the decrease is from 0.91 to 0.86. It is improbable that such a small fractional change in protonation of the imidazoles could be the *sole* cause of the sharp change in slope of the data plotted in Figure 2. However, this point can and should be investigated further by studying the Arrhenius plots for RNase A activity over a range of pH values. We have not undertaken such an extended investigation for two reasons. The value of $K_m$ for C>p increases rather dramatically with pH above pH values of 5 (Crook et al., 1960). Thus, saturation of the enzyme is no longer maintained at substrate concentrations that are suitable for activity studies which use the absorbance of the solution at 286 nm as a probe (as we did). Of course, this difficulty can be circumvented either by making many measurements at convenient concentrations and extrapolating to infinite substrate concentration or by using a different assay, but our purpose in this work did not justify the extra labor required. Secondly, there is evidence that ribonuclease may show allosteric behavior under certain conditions (Walker et al., 1975, 1978a,b), typically at higher pH and higher substrate concentrations than those employed in this work. Since the allosteric behavior is sensitive to enzyme preparation, pH, ionic strength and buffer composition, an investigation at higher pH values would have to be a very extensive one. Hence, while studies of the temperature dependence of RNase A activity toward C>p over a range of pH are desirable, they will require much labor. None of this labor is required to demonstrate that the temperature dependence of the $pK_a$ of some catalytically important group is probably not the explanation for the nonlinear Arrhenius plots at pH 5.

Thus, based on the elimination of the most obvious reasonable alternatives, and the observation of a conformational change in the *free* enzyme, the hypothesis may be advanced that a conformational change of the enzyme–substrate complex is responsible for the Arrhenius plots of Figures 1 and 2. The simplest assumption is that the conformational change of the native enzyme is the same in the presence or absence of substrate. Thus, the inferences about the structural changes, which have resulted from work on the free enzyme, can be considered to be approximately valid for the changes which occur in the enzyme–substrate complex as the temperature is increased. Evidence in support of this assumption can be found in the correspondence of the temperature range in which the conformational change is observed for the free enzyme (see Table I) and the temperature range of curvature in the Arrhenius plots.

The structural changes involved in the conformational change of the free enzyme and enzyme–inhibitor complexes appear to be localized in the portion of the polypeptide chain near His-48 and Lys-41, and in the portion between Asp-14 and Tyr-25 (French & Hammes, 1965; Meadows et al., 1969; and references cited in Table I). These two sections of the backbone are adjacent to one another in the three-dimensional structure of RNase S (Wyckoff et al., 1970). The structural changes observed by nonspecific surface labeling (Matheson & Scheraga, 1979) are concentrated in the latter region.

Meadows et al. (1969) have described the conformational change as one which involves the opening and closing of a "hinge". If this "hinge" motion is indeed the same motion which gives rise to the curved Arrhenius plots, then it must be involved in the rate-determining step over the temperature range examined. Of all the mechanisms that have been proposed for the action of RNase A, that of Hammes (1968a,b) most clearly requires a conformational change as an intimate component of the mechanism. This was first pointed out by Roberts et al. (1969a). While our results cannot prove that such a motion is in fact involved in the enzymatic reaction, they do indicate that such a conformational change is far from implausible.

Finally, we consider the different behavior when RNA is the substrate. We can suggest at least two reasons for this

difference. First, the large RNA molecule may stabilize the enzyme against a conformational change. Second, the reaction with RNA involves two steps, viz., the cleavage of the chain, accompanying formation of the cyclic phosphate, and the hydrolysis of the latter; only the second step is involved when the substrate is C>p. With RNA, the first step is the rate-limiting one (Richards & Wyckoff, 1971).

## Conclusions

The activity of RNase A toward C>p is characterized by a nonlinear Arrhenius plot. The properties of the Arrhenius plot can be explained by assuming that they arise because of a conformational change around 32 °C. Such a conformational change is known and rather well characterized for the free enzyme and for enzyme–inhibitor complexes.

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