formate (0.983 ml, 7.5 mmol) were added to a solution of BOC-L-isoleucine (1.73 g, 7.5 mmol) in tetrahydrofuran (75 ml) at −40°. The mixture was stirred at −20° for 1 hr. A solution of L-leucyl-L-leucyl-L-leucyl methyl ester trifluoroacetate (from treatment of 3.54 g of tripeptide XXVIII with trifluoroacetic acid) in dimethylformamide (15 ml) was added, followed by triethylamine (0.712 g, 7.5 mmol) and the mixture stirred at −20° for 30 min and at 4° overnight. The usual work-up gave 1.1 g (25%) of impure product. Elution from a column of silica gel by chloroform and recrystallization from ethyl acetate gave pure product, mp 240° dec, $[\alpha]^{24}\text{D}$ −43.5° (*c* 0.2, trifluoroethanol).

Anal. Calcd for $C_{30}H_{56}N_4O_7$ (584.8): C, 61.62; H, 9.65; N, 9.58. Found: C, 61.62; H, 9.36; N, 9.32.

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Polynucleotides. IX.[1] Temperature Dependence of Kinetics of Complex Formation in Equimolar Mixtures of Polyriboadenylate and Polyribouridylate[2]

R. D. Blake,[3] L. C. Klotz, and J. R. Fresco

Contribution from the Program in Biochemical Sciences, Frick Chemical Laboratory, Princeton University, Princeton, New Jersey 08540. *Received November 15, 1967*

Abstract: The kinetics of formation of poly (A + U) in equimolar mixtures of poly A and poly U in 0.01 $M\,\text{Na}^+$, as a function of temperature, have been followed spectrophotometrically at specific (isochromic) wavelengths near 280 mμ. It was observed that the second-order rate constant, k_1, for the formation of poly (A + U) decreases with increasing temperature, approaching zero within 0.2° of the temperature corresponding to the inflection point of the absorbance–temperature profile. This behavior is similar to that previously reported by Ross and Sturtevant (1960) on the basis of measurements at 259 mμ, where the validity of quantitative interpretation is in doubt. Some observations on the transient formation of poly (A + U + U) are also reported. The kinetics of formation of both complexes are discussed in relation to hypothetical schemes of elementary processes for helix formation. The model proposed by Saunders and Ross (1960) and by Flory (1961), and amended by Kallenbach, Crothers, and Mortimer (1963) is found to be inadequate. In the Appendix, Crothers, Davidson, and Kallenbach present a more complex model that anticipates the observed temperature dependence of the kinetics and allows the conclusion that more than a single base pair is needed to establish a stable nucleus for helix growth.

Ross and Sturtevant[4,5] have reported that in equimolar[6] mixtures $(0.50X_U)$[7] of poly A and poly U the apparent second-order rate constant, k_1^{app}, for the formation of poly (A + U)

$$\text{poly A} + \text{poly U} \xrightarrow{k_1^{\text{app}}} \text{poly (A + U)} \qquad (1)$$

first increases with increasing temperature and then *decreases* linearly, approaching zero in the vicinity of the dissociation temperature (T_m). Such unusual temperature dependence has been explained by Ross and Sturtevant[5] in terms of a general model for helix formation[8,9] that involves a sequence of elementary processes.

The formation of poly (A + U) may be conveniently described according to the following specific illustration of that model. The sequence is initiated by a bimolecular *nucleation* step

$$[\text{A}]_n + [\text{U}]_n \underset{k_u}{\overset{k_o}{\rightleftarrows}} [(\text{A} + \text{U})]_\alpha \{[\text{A}]_{n-\alpha} + [\text{U}]_{n-\alpha}\} \qquad (2)$$

where $[\text{A}]_n$ and $[\text{U}]_n$ are concentrations of poly A and poly U residues, respectively, here assumed to be of equal chain length n. k_o is the second-order rate constant for nucleation, which for the sake of simplicity is treated here as a conserted event. k_u is the corresponding first-order rate constant for the opposing (strand separation) reaction. In this model, nucleation involves the synergic reaction of an unknown number of neighboring residues, α.

Nucleation is followed by a multistep, first-order, "zipping-up" process or helical growth, which may be described by eq 3 where k_f and k_b are the first-order forward and opposing rate constants, respectively. While eq 3 implies that the formation of one (A + U) base

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[1] Paper VIII: R. D. Blake, J. Massoulié, and J. R. Fresco, *J. Mol. Biol.*, 30, 291 (1967).

[2] This investigation was supported by grants from the National Institutes of Health (GM-07654), The American Heart Association, and the National Science Foundation (GB-6664). We thank the Princeton University Computer Center (supported in part by the National Science Foundation (GP-579)) for their cooperation.

[3] U. S. Public Health Service Predoctoral Trainee, 1963–1967.

[4] P. D. Ross and J. M. Sturtevant, *Proc. Natl. Acad. Sci. U. S.*, **46**, 1360 (1960).

[5] P. D. Ross and J. M. Sturtevant, *J. Am. Chem. Soc.*, **84**, 4503 (1962).

[6] Concentration refers to *nucleotide residue* concentrations.

[7] The following abbreviations have been used: poly A = polyriboadenylate; poly U = polyribouridylate; poly (A + U) = the two-stranded helix containing one strand of poly A and one of poly U; poly (A + U + U) = the three-stranded helix containing one strand of poly A and two of poly U; T_m = the temperature at the midpoint of the appropriate absorbance change; $T_{m_{3\rightarrow 2}} = T_m$ of the dissociation of the three-stranded to the two-stranded helix, whereas $T_{m_{2\rightarrow 1}}$ signifies dissociation of the latter to the homopolymers; T_i = the temperature of the inflection point of an absorbance–temperature profile; X_U = the mole fraction of U residues in mixtures of poly A and poly U.

[8] M. Saunders and P. D. Ross, *Biochem. Biophys. Res. Commun.*, **3**, 314 (1960).

[9] P. J. Flory, *J. Polymer Sci.*, **49**, 105 (1961).

$$[(\mathrm{A}+\mathrm{U})]_{\alpha}\{[\mathrm{A}]_{n-\alpha}+[\mathrm{U}]_{n-\alpha}\} \underset{k_b}{\overset{k_f}{\rightleftarrows}}$$

$$[(\mathrm{A}+\mathrm{U})]_{\alpha+1}\{[\mathrm{A}]_{n-\alpha-1}+[\mathrm{U}]_{n-\alpha-1}\} \underset{k_b}{\overset{k_f}{\rightleftarrows}}$$

$$[(\mathrm{A}+\mathrm{U})]_{\alpha+2}\{[\mathrm{A}]_{n-\alpha-2}+[\mathrm{U}]_{n-\alpha-2}\} \underset{k_b}{\overset{k_f}{\rightleftarrows}} \ldots \text{etc} \qquad (3)$$

pair constitutes a step, this assumption does not affect the end result for long chains. Obviously for quantitative formation of poly (A + U), $k_f > k_b$.

The over-all rate of formation of an (A + U) base pair, d[(A + U)]/dt, must reflect a combination of the processes described by eq 2 and 3. Assuming that $k_u = k_b$ this rate of formation has been shown[8,9] to be given by

$$\mathrm{d}[(\mathrm{A}+\mathrm{U})]/\mathrm{d}t = k_o[\mathrm{A}][\mathrm{U}](1 - k_b/k_f) \qquad (4)$$

In other words, given the particular assumptions of this derivation, the observed over-all apparent second-order rate constant, k_1^{app}, should correspond to

$$k_1^{\mathrm{app}} = k_o(1 - k_b/k_f) \qquad (5)$$

However, Kallenbach, Crothers, and Mortimer[10] have argued that it is quite possible that $k_u > k_b$ since the final opposing strand separation step ought to be accompanied by a larger free energy change. In this case the expression for k_1^{app} differs from eq 5. The actual kinetic equations derived by those authors contain errors which are corrected in the Appendix.

It will be appreciated that as $(T_m - T) \rightarrow 0$, $k_f \rightarrow k_b$, so that the factor $(1 - k_b/k_f)$ in eq 5 decreases the observed k_1^{app}. Conversely, as $(T_m - T)$ increases, $k_f \gg k_b$, so that $(1 - k_b/k_f) \rightarrow 1$. Thus, this simple model for helix formation appears to account qualitatively for the negative dependence of k_1^{app} on temperature and for k_1^{app} falling to zero in the neighborhood of the T_m. It will be shown below (see Discussion) that eq 5 does not, however, account quantitatively for the variation of k_1^{app} with temperature. The extent to which the model must be made more complex in order to fit the results is considered in the Appendix.

The kinetics against which the model had previously been evaluated were determined from the increase in hypochromicity at 259 mμ.[5] It is now appreciated that measurements at this wavelength do not distinguish between the formation of an (A + U) base pair and the successive reaction to form an (A + U + U) base triplet.[1,11,12] In fact, a recent reexamination of the kinetics of interaction of poly A and poly U at specific wavelengths where each reaction can be followed independently[13] has revealed the rapid transient formation of (A + U + U) base triplets to a substantial extent when equimolar ($0.50X_U$) amounts of poly A and poly U are mixed under solvent and temperature conditions that are also thermodynamically favorable to poly (A + U + U). The ambiguity introduced thereby is likely to be further aggravated at the elevated temperatures and ionic strengths (>0.2 M) used in some of the studies of Ross and Sturtevant,[5] since these conditions support poly (A + U + U) as the *equilibrium* product *even in equimolar mixtures* of poly A and poly U.[1,11,12,14] It is evident, therefore, that k_1^{app} values obtained under such conditions at 259 mμ may not simply reflect the rate of poly (A + U) formation. The availability of specific isochromic wavelengths[1] for separately following the formation of each complex has made it possible to circumvent these difficulties.

In the present work the temperature dependence of the kinetics of poly (A + U) formation has been studied (at both 259 mμ and the isochromic wavelengths) under that limited range of temperatures and ionic strengths in which formation of poly (A + U + U) cannot occur (even incidentally), *i.e.*, at temperatures between $T_{m_{2\to1}}$ and $T_{m_{3\to2}}$ in $[\mathrm{Na}^+] < 0.1\ M$.[1,11,15] Under such conditions, the formation of (A + U) base pairs proceeds with second-order kinetics beyond 90% reaction.[13] In further experiments, below $T_{m_{3\to2}}$, where the transient formation of poly (A + U + U) does occur, the kinetics of both reactions have been followed simultaneously (at the isochromic wavelengths only), and their respective rate constants determined.

Experimental Section

Materials and Methods. Poly A and poly U were prepared and characterized as previously.[1,13] All experiments were carried out in 0.01 M Na$^+$; anions were Cl$^-$ and 0.005 M cacodylate, pH 7.0. Kinetic measurements were made spectrophotometrically as previously.[13]

Kinetic Analysis. A more detailed description of the kinetic analysis used here has been presented previously.[13] Values of $[(\mathrm{A}+\mathrm{U})]_t$ and $[(\mathrm{A}+\mathrm{U}+\mathrm{U})]_t$ were calculated directly from absorbance measurements at 283.5 and 280.0 mμ, respectively, according to the expressions

$$[(\mathrm{A}+\mathrm{U})]_t = [(D_{t,0.5} - D_{0,0.5})/(D_{\infty,0.5} - D_{0,0.5})][\mathrm{A}]_0 \qquad (6)$$

$$[(\mathrm{A}+\mathrm{U}+\mathrm{U})]_t = 0.67[(D_{0,0.5} - D_{t,0.5})/(D_{0,0.67} - D_{\infty,0.67})][\mathrm{A}]_0 \qquad (7)$$

where $D_{t,0.5}$ is the absorbance of a $0.50X_U$ mixture at time t, $D_{0.0.5}$ is the absorbance at time $t = 0$, $D_{\infty,0.5}$ is the absorbance at "infinite" time (completed reaction), and $[\mathrm{A}]_0$ is the initial (residue) concentration of poly A. Values of [A] and [U] were then calculated by subtraction.

The denominators of eq 6 and 7 are slightly temperature dependent, primarily because of the variation in absorbance of poly A with temperature. This dependence on temperature was determined at 283.5 mμ for poly A, poly U, and poly (A + U), and is shown in terms of the difference in molar extinction coefficients, $(\epsilon_{\infty,0.5} - \epsilon_{0.0.5})$, in Figure 1. Above about 26° the *observed* difference begins to deviate from a *projected* difference (dashed line) due to the helix → coil transition of poly (A + U) (with $T_{m_{2\to1}} = 37.3°$). Consequently, for kinetics above 26° the projected (dashed) line was used for $(D_{\infty,0.5} - D_{0,0.5})$ in eq 6.

Once concentrations of A, U, (A + U), and (A + U + U) were determined as a function of time, analyses for rate constants were carried out from the least-squares slope and intercept of the linear forms of the rate expressions[13]

$$\frac{-\mathrm{d}[\mathrm{A}]/\mathrm{d}t}{[(\mathrm{A}+\mathrm{U})]} = k_1\frac{[\mathrm{A}][\mathrm{U}]}{[(\mathrm{A}+\mathrm{U})]} - k_{-1} \qquad (8)$$

$$\frac{\mathrm{d}[(\mathrm{A}+\mathrm{U}+\mathrm{U})]/\mathrm{d}t}{[(\mathrm{A}+\mathrm{U}+\mathrm{U})]} = k_2\frac{[(\mathrm{A}+\mathrm{U})][\mathrm{U}]}{[(\mathrm{A}+\mathrm{U}+\mathrm{U})]} - k_{-2} \qquad (9)$$

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(10) N. R. Kallenbach, D. M. Crothers, and R. G. Mortimer, *Biochem. Biophys. Res. Commun.*, **11**, 213 (1963).

(11) C. L. Stevens and G. Felsenfeld, *Biopolymers*, **2**, 294 (1964).

(12) J. Massoulié, R. D. Blake, L. C. Klotz, and J. R. Fresco, *Compt. Rend.*, **259**, 3104 (1964).

(13) R. D. Blake and J. R. Fresco, *J. Mol. Biol.*, **19**, 145 (1966). In fact, the transient intermediate has been isolated chromatographically; *cf.* C. Larsen, *Compt. Rend.*, **262**, 1985 (1966).

(14) T. H. Miles and J. Frazier, *Biochem. Biophys. Res. Commun.*, **14**, 129 (1964).

(15) J. R. Fresco in "Informational Macromolecules," H. J. Vogel, V. Bryson, and J. O. Lampen, Ed., Academic Press Inc., New York, N. Y., 1963, p 121.

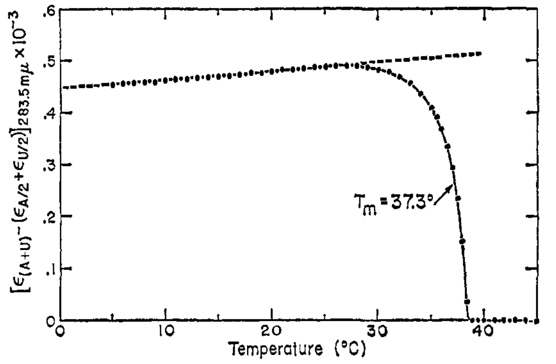

Figure 1. The calculated difference in molar extinction coefficients between poly A and poly U and poly (A + U), ($\epsilon_{(A+U)} - [\epsilon_A + \epsilon_U]/2$), at 283.5 mμ as a function of temperature. The poly (A + U) samples for these experiments were aliquots of a mixture allowed to equilibrate for at least 1 week at 22° to assure quantitative formation of the complex.

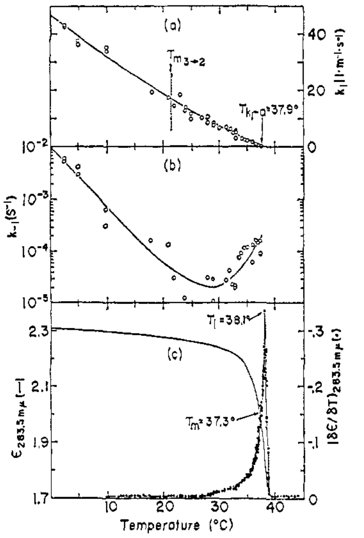

Figure 2. Temperature dependence of the poly A-poly U interaction in 0.01 M Na$^+$, pH 7.0: a, variation of k_1 with temperature; b, variation of k_{-1} with temperature; c, absorbance–temperature profile for poly (A + U) (solid line) and the derivative of that profile (points).

k_1 determined by this procedure corresponds to k_1^{app} discussed earlier; *above* $T_{m_{3\rightarrow 2}}$ it was found to be the same (within ±3%) as the least-squares slope of the *initial* kinetics plotted according to the standard integrated second-order expression

$$[(A + U)]_t/[A]_0([A]_0 - [(A + U)]_t) = k_1 t \qquad (10)$$

that applies when the initial concentration of poly A, $[A]_0$, is equal to that of poly U, $[U]_0$. This agreement provides assurance that the analysis using eq 8 is valid.

Most of the calculations were made on an IBM 7094 computer with a program that sequentially prescribes two principal calculations. The actual input involves absorbances at about 80 time intervals, which are converted to concentrations according to eq 6 and 7. The derivative in (8) (or (9)) at time t is approximated from the slope of the line connecting the concentration at time $(t - 1)$ and $(t + 1)$. The linear least-squares slope, k_1 (or k_2), and intercept, k_{-1} (or k_{-2}), of eq 8 (or 9) is first calculated.

In addition, to provide a visual comparison of the observed kinetics with the theoretical kinetics prescribed by the rate expressions 8 and 9 (*cf.* ref 13 for expressions for d[U]/dt and d[(A + U)]/dt required below $T_{m_{3\rightarrow 2}}$) these rate expressions were numerically integrated simultaneously with the constants empirically obtained.

Finally, the integrated form of the single-term rate expression for the formation of poly (A + U), eq 10, was calculated for the *initial* kinetics.

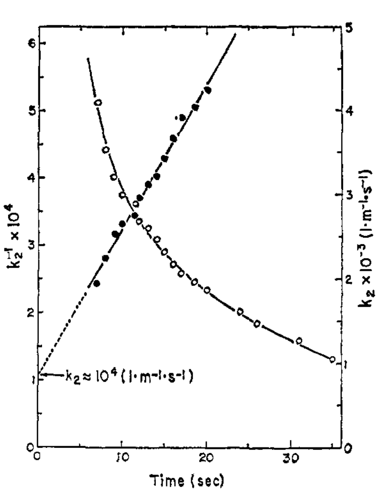

Figure 3. Time dependence of the "rate constant," k_2, for formation of poly (A + U + U) at 2.5° in 0.01 M Na$^+$, pH 7.0. Variation of k_2 (open circles) and $1/k_2$ (filled circles).

Results

Kinetics of Poly (A + U) Formation. Figures 2a and 2b show the experimentally determined k_1 (l. mol^{-1} sec^{-1}) and k_{-1} (sec^{-1}) values, respectively, as a function of temperature. As previously noted,[4,5,13] k_1 decreases with increasing temperature and extrapolates to zero in the neighborhood of T_m. Thus, $k_1 \rightarrow 0$ at 37.9 ± 0.1°, whereas $T_{m_{2\rightarrow 1}}$ = 37.3° for the absorbance–temperature profile of the poly (A + U) formed (Figure 2c, line with no points), and the derivative of that profile (Figure 2c, points) indicates an inflection point for the transition, at T_i = 38.1 ± 0.1°. The closeness of these temperatures is not unexpected[16a] (*cf.* Discussion).

Whereas Ross and Sturtevant[4] noted a linear dependence of k_1 on temperature, the data in Figure 2a suggest a gentle curvature. There appears to be a continuity between data *above* $T_{m_{3\rightarrow 2}}$ and those in the temperature range favorable to the transient formation of poly (A + U + U), *i.e.*, *below* $T_{m_{3\rightarrow 2}}$ = 21.5° of a 0.67X_U mixture in this [Na$^+$].

Figure 2b shows a negative dependence of k_{-1} on temperature approximately to the temperature of onset of the helix → coil transition of poly (A + U); thereafter the dependence becomes positive.

Kinetics of Poly (A + U + U) Formation. At temperatures below $T_{m_{3\rightarrow 2}}$, the determination of k_1 and k_{-1} requires the simultaneous calculation of k_2 and k_{-2}. The kinetics of poly (A + U) formation, plotted according to eq 8, were linear at any temperature below $T_{m_{2\rightarrow 1}}$, showing that k_1 is a true constant over the entire course of the reaction. However, the kinetics of poly (A + U + U) formation (transient) were *not* similarly linear, as would be predicted by eq 9. Instead, as can be seen from Figure 3, the apparent k_2 decreases rapidly with the extent of reaction. Extrapolation of $1/k_2$ to t = 0 indicates an initial value of $k_2 = 10^4$ l. mol^{-1} sec^{-1}. By contrast, when poly U was added to *preformed* poly (A + U), a 10^{-4}–10^{-6} times slower rate of poly (A + U + U) formation (nontransient) was observed—too slow to measure quantitatively at this ionic strength.

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(16) (a) D. M. Crothers and B. H. Zimm, *J. Mol. Biol.*, **9**, 1 (1964); (b) J. Applequist and V. Damle, *J. Am. Chem. Soc.*, 87, 1450 (1965).

Discussion

The simple mechanism implicit in the formalized rate eq 8 offers no provision for explaining either the negative dependence of k_1 on temperature or that k_1 goes to zero in the neighborhood of T_m. It must be concluded, therefore, that k_1 is a complex collection of constants. On the other hand, the hypothetical scheme of elementary processes given by eq 2 and 3[8,9] undoubtedly represents a more meaningful physical description of the events leading to the formation of poly (A + U). Indeed, a *qualitative* explanation for the negative dependence can be rationalized on the basis of the resultant rate expression of Saunders and Ross[8] and of Flory[9] given by eq 4. On the basis of the following argument, however, it would appear that the limited case of expression 4 is *not* sufficient to explain *quantitatively* these extraordinary kinetics.

If the equilibrium constant for the coil → helix transition of what is assumed to be a single base pair (zipping-up process), K^{-1} (following the notation of Saunders and Ross[8]), is given by

$$K^{-1} = k_f/k_b \tag{11}$$

then

$$K^{-1} = e^{-\Delta F/RT} \tag{12}$$

where ΔF is the change in free energy upon adding an (A + U) base pair to a helical region (see eq 3). At the temperature where $k_1 \rightarrow 0$ and $K^{-1} \rightarrow 1$, that is, effectively at T_m[16]

$$\Delta F = \Delta H - T_m \Delta S = 0 \tag{13}$$

so that

$$\Delta S = \Delta H/T_m \tag{14}$$

Assuming that ΔS (and ΔH) is constant with temperature,[17] the variation of ΔF with temperature is given simply by

$$\Delta F(T) = \Delta S(T_m - T) \tag{15}$$

Consequently, (12) becomes

$$K^{-1} = e^{\Delta S(T_m - T)/RT} \tag{16}$$

Direct calorimetric measurements of the heat of the transition of poly (A + U)[18,20-22] indicate a ΔS of ~23 eu. With this value for ΔS it can be calculated from eq 16 that K (*i.e.*, k_b/k_f) = 0.20 at 0°. Now, from eq 5 (which, it should be recalled, is dependent upon eq 4) it can be calculated that $k_0 = 58.7$ (where k_1^{app} was obtained from Figure 2a). Since k_0 should increase with increasing temperature, as is usual for simple chemical processes, one would predict, on the basis of eq 5, that $k_1 > 58.7(1 - 0.319) = 40.0$ at 10° (where $K = 0.319$). But, from Figure 2a it can be seen that k_1 is only 31 at that temperature. From this analysis, it would appear that the model serving as the basis for eq 5 must be inadequate. Extensions of that model are considered in the Appendix.

By choosing a suitable [Na$^+$] that would give both a slow enough rate of reaction to be observed without a stopped-flow apparatus, and a T_m of about 40°, it had been hoped that the maximum in k_1 at some lower temperature, as might be anticipated by the treatments of the mechanistic scheme given by eq 2 and 3, would become apparent. This expectation was not realized. Ross and Sturtevant[5] did observe such a maximum in two different [Na$^+$] at a $(T_{m_{2\rightarrow1}} - T)$ of about 35°.[23] While it has been shown above that their observations at 259 mμ contain inherent uncertainties, these would not qualitatively alter the maxima which they detected.

It is seen in Figure 2b that just below 30° k_{-1} increases with decreasing temperature. Taken at face value, this dependence would suggest consequences that are physically unreal. It is more likely, therefore, that the observed k_{-1} arises from complex determinants of the kinetics of interaction of poly A and poly U strands as reaction proceeds and the way these affect the analysis for this constant. Thus, early in the reaction, helix growth undoubtedly results in transient "dangling ends" due to different lengths of A and U strands and the random occurrence of sites of helix nucleation. It may be presumed that at the higher temperature range (30–37°), such imperfections undergo realignment ("annealing") very rapidly; otherwise, the net increase in (A + U) base pairs could not remain exclusively rate controlling, as has been observed. Moreover, in this temperature range k_{-1} is independent of the extent of reaction. In this connection, it will be recalled that the annealing process cannot be detected by the spectrophotometric procedure for monitoring the kinetics, and also, as can be seen from eq 8, that the

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(17) With regard to ΔH, it has been reported[16] "that the variation of ΔH with temperature is greater in 0.5 M NaCl ($d\Delta H/dt \cong +110$ cal deg^{-1}) than in 0.1 M NaCl ($d\Delta H/dT) \cong +42$ cal deg^{-1})." If we extrapolate to 0.01 M NaCl (the salt concentration used in the present work), ΔH is clearly going to be even *less* temperature dependent, *i.e.*, $d\Delta H/dT \cong +8$ cal deg^{-1}, which is effectively zero, since it is well within the experimental error of those calorimetric measurements. Concerning ΔS, it had been reported previously[19] "that for poly (A + U), the heat of the transition at T_m is independent of the value of T_m . . . . ", so that ΔS_{T_m} would have to *decrease* slightly with increasing temperature (*cf.* eq 14). However, recent results with an adiabatic calorimeter[20,21] indicate a small temperature dependence of ΔH_{T_m}, such that $d\Delta H_{T_m}/dT_m \cong +60$ cal deg^{-1}. From this latter relationship we calculate $\Delta S_{T_m} \cong 23.7$ eu in 0.01 M NaCl. Moreover we can estimate from the available data[21] that between 0.018 and 0.104 M Na$^+$, $d\Delta S_{T_m}/dT_m \cong 0.08$ eu deg^{-1}, which is effectively zero.

(18) P. D. Ross and R. L. Scruggs, *Biopolymers*, **3**, 491 (1965).

(19) M. A. Rawitscher, P. D. Ross, and J. M. Sturtevant, *J. Am. Chem. Soc.*, **85**, 1915 (1963).

(20) E. Neumann and T. Ackermann, *J. Phys. Chem.*, 71, 2377 (1967).

(21) H. Krakauer and J. M. Sturtevant, *Biopolymers*, **6**, 491 (1968).

(22) Although under the conditions of Ross and Scruggs[18] the transient formation of some (<20%) poly (A + U + U) would undoubtedly occur,[13] the available data suggest that this does not seem to affect the apparent thermodynamics. Thus, they measured the heat of formation of poly (A + U + U) from the interaction of poly (A + U) and poly U in 0.1 M Na$^+$, and found that $\Delta S = 11.5$ eu at the T_m, which is just half that for the formation of poly (A + U). *A priori* this is what one would expect for the complexing of a single polynucleotide random coil into a helical conformation. Incidentally, since the thermally induced strand displacement reaction undergone by poly (A + U), 2 poly (A + U) → poly (A + U + U) + poly U, only occurs at [Na$^-$] > 0.2 M,[1,11,12] the T_m measured by Ross and Scruggs[18] must, in fact, be that for poly (A + U) (and not that for poly (A + U + U)).

(23) It was actually reported[5] that the maximum rate occurred at 39° below T_m in 0.5 M Na$^+$ and 42° below T_m in 0.25 M Na$^+$. However, at the time these measurements were made it was not known that poly (A + U) does not dissociate directly at such ionic strengths, but instead enters into a "strand displacement reaction"[14] at intermediate temperatures, yielding poly (A + U + U), with little or no loss of hypochromicity at 259 mμ.[1,11,12] At still higher temperatures this poly (A + U + U) then dissociates directly to the homopolymers with $T_{m_{3\rightarrow1}}$ and with an observable loss of hypochromicity at 259 mμ. Thus, the T_m values reported by Ross and Sturtevant[5] must correspond to $T_{m_{3\rightarrow1}}$. The $(T_{m_{2\rightarrow1}} - T)$ of ~35° noted above was calculated by us simply by extrapolating the variation of $T_{m_{2\rightarrow1}}$ with [Na$^+$] (from a region in which the strand displacement reaction does not occur).

The lack of recognition of the strand displacement reaction also explains why the variation in k_1^{app} with temperature observed by Ross and Sturtevant (ref 5, Figure 2) extrapolates to zero in the vicinity of that "expected" for $T_{m_{2\rightarrow1}}$ rather than to the $T_{m_{3\rightarrow1}}$ they observed.

analysis for k_{-1} is not independent of that for k_1. The inversion in the temperature dependence of k_{-1} as temperature is lowered below 30° could be ascribed then to a relative slowing in the rate of annealing, which would alter k_1 slightly as the reaction proceeds. The nature of the computation for k_{-1} is such that this slight time dependence of k_1 results in a relatively large increase in k_{-1}. Thus, the inversion seen for k_{-1} with temperature can be viewed as a computational artifact.

The reason why k_2 changes with time and, therefore, why the kinetics of poly (A + U + U) formation do not appear to conform to the simple empirical treatment, as does the rate of poly (A + U) formation, is not obvious from the available information, but may be accounted for by the following simple hypothesis. The formation of poly (A + U + U) can be envisioned as occurring by *two* possible sequences of elementary processes—one with and one without a rate-determining nucleation step. That involving nucleation is expected to occur when poly U is added to preformed poly (A + U). Since the interacting species, poly (A + U) and poly U, must overcome both unfavorable electrostatic and entropic potentials in order to nucleate, one predicts for this mechanism a much slower rate of formation of poly (A + U + U) than of poly (A + U); this was observed in the experiment in which preformed poly (A + U) was mixed with poly U.

In the alternative, more rapid mechanism of poly (A + U + U) formation, not involving a special nucleation step, poly A and poly U can be envisioned to interact to form poly (A + U) by nucleation of a long strand of poly A with strands of poly U at several points. In this case, a U strand will form poly (A + U + U) whenever it finds itself overlapping some poly (A + U) that had previously formed further along the chain. Conversely, two (or more) poly A strands could nucleate to a single poly U strand, but they would remain partially unbonded and soon pick up additional U strands, since poly (A + A + U) does not occur. In any event, this initial formation of poly (A + U + U) by intramolecular annealing would be expected to occur both very rapidly (to an extent that would depend upon the ratio of the actual *number of A:U strands* and the length of poly A strands) and not to persist, but instead decrease with time. Both these predictions are fulfilled in Figure 3. It would appear, then, that most of the expectations of the two different processes for poly (A + U + U) formation have already been realized.

Implicit in this scheme for formation of poly (A + U + U) is that the "overlapping" U strand of poly (A + U + U) is *parallel* to the poly U strand of poly (A + U). This agrees with the conclusion of Miles[24] on the basis of infrared spectral measurements. The alternative possibility exists that a U strand that is "too long" will hairpin back *antiparallel* to itself (that is, bonding with the poly (A + U) to which that U strand had already contributed).

If the rationale proposed here for transient formation of poly (A + U + U) in equimolar mixtures of poly A and poly U[13] should prove correct, then the very high value for k_2 at $t = 0$ (10^4 l. mol^{-1} sec^{-1}) under the conditions of Figure 3 is actually that for the intramolecular growth process

$$[(\mathrm{A+U+U})]_x[(\mathrm{A+U})]_y[\mathrm{U}]_z \xrightarrow{k_{2_{t=0}}} [(\mathrm{A+U+U})]_{x+1}[(\mathrm{A+U})]_{y-1}[\mathrm{U}]_{z-1} \xrightarrow{k_{2_{t-0}}} \ldots \text{ etc} \qquad (17)$$

From previous work[13] it can be estimated that about 15% of the A residues in the complexes initially formed have become transiently involved in helical regions of poly (A + U + U) in the relevant experiments described here.

Acknowledgments. We are pleased to acknowledge helpful discussions with Drs. B. M. Alberts, D. M. Crothers, N. Davidson, and B. H. Zimm.

Appendix

Steady-State Kinetics of Double Helix Formation[25]

A simple kinetic mechanism for formation of a double helical complex between two single strands is given in eq 2 and 3 of the body of this paper. Our concern here is with the kinetic consequences of this and similar mechanisms. We rewrite the reaction in slightly altered notation, also making more explicit the nucleation process

$$\mathrm{A+U} \underset{\gamma_0 k_b}{\overset{\beta\gamma_0 k_f}{\rightleftharpoons}} (\mathrm{AU})_1 \underset{\gamma_1 k_b}{\overset{\sigma_1\gamma_1 k_f}{\rightleftharpoons}} (\mathrm{AU})_2 \underset{\gamma_2 k_b}{\overset{\sigma_2\gamma_2 k_f}{\rightleftharpoons}} \ldots \underset{\gamma_{i-1} k_b}{\overset{\sigma_{i-1}\gamma_{i-1} k_f}{\rightleftharpoons}} (\mathrm{AU})_i \underset{\gamma_i k_b}{\overset{\sigma_i\gamma_i k_f}{\rightleftharpoons}} \ldots \underset{\gamma_{n-1} k_b}{\overset{\sigma_{n-1}\gamma_{n-1} k_f}{\rightleftharpoons}} (\mathrm{AU})_n \qquad (\mathrm{A1})$$

where the subscript i is equal to the number of hydrogen-bonded base pairs joining the two strands. The parameter β is defined by Applequist and Damle[26] in such a manner that the equilibrium constant K_{01} for *formation* of an isolated base pair joining the two strands, the postulated first step of the reaction, is

$$K_{01} = \beta k_f / k_b \qquad (\mathrm{A2})$$

where k_f and k_b are, as previously defined, the rate of formation and breakage of hydrogen-bonded pairs at the ends of a long helical segment. The parameter γ_0 is multiplied into the forward and reverse rate constants for the first step because it is not legitimate to assign the same rate of opening to an isolated base pair as to a pair at the end of a helical sequence. (Designation of such parameters by γ follows the notation of Schwarz.[27]) The quantities γ_i and σ_i are introduced to allow for the fact that formation of the first few base pairs in a helical segment may not have the same rate and equilibrium constants as those characteristic of a long helix. By definition

$$\lim_{i\to\infty} \sigma_i = 1 \qquad \lim_{i\to\infty} \gamma_i = 1 \qquad (\mathrm{A3})$$

The maximum number of base pairs joining the two strands is n. In passing, it should be mentioned that the kinetic mechanism (A1) is inaccurate in that it neglects the annealing processes that are necessary to correct for initial misalignment of the two strands.

Under conditions such that formation of additional base pairs is strongly favored, that is, when the equilibrium constant K

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(24) T. H. Miles, *Proc. Natl. Acad. Sci. U. S.*, **51**, 1104 (1964).

(25) The Appendix was written by D. M. Crothers, Department of Chemistry, Yale University, New Haven, Conn.; N. Davidson, Department of Chemistry, California Institute of Technology, Pasadena, Calif.; and N. R. Kallenbach, Department of Biology, University of Pennsylvania, Philadelphia, Pa.

(26) See ref 16b.

(27) G. Schwarz, *J. Mol. Biol.*, **11**, 64 (1965).

$$K = k_b/k_f \qquad (11)$$

is considerably less than 1, the reaction can be regarded as a transformation between the two forms A + U and $(AU)_n$

$$A + U \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (AU)_n \qquad (A4)$$

for which the phenomenological kinetic equation is given by eq 8 of the body of the paper. Several kinds of experiments might be imagined which fit these conditions. One is that reported in this paper: determination of the rate of complex formation between separated polynucleotide strands, yielding the second-order rate constant k_1. An experiment which would give the apparent dissociation rate constant k_{-1} would be exemplified by measurement of the rate of melting of double-stranded oligonucleotides above their melting temperature. (It would be necessary to study oligonucleotides in order to preserve the condition that $K < 1$ above the melting zone.) A third kind of experiment would be to measure the relaxation kinetics of double helix formation between oligonucleotides, for which the relaxation time τ corresponding to the simple kinetic mechanism, eq A4, is given by

$$1/\tau = k_1([A] + [U]) + k_{-1} \qquad (A5)$$

(where concentrations here refer to those for the *oligomers*). Such experiments have been carried out by Pörschke, in collaboration with Eigen, for complex formation between adenylic acid oligomers[28] and oligomers of A and U.[29]

Considering the kinetic mechanism, eq A1, in light of the simplification represented by eq A4, it is natural to apply the steady-state approximation to eq A1 to calculate the rate constants k_1 and k_{-1} in terms of kinetic parameters of eq A1. Denoting by $R_{i,i+1}$ the rate of transformation of material between the states i and $i + 1$, we obtain from eq A1 the set of equations

$$\begin{aligned}
R_{01} &= \beta\gamma_0 k_f[A][U] - \gamma_0 k_b C_1\\
R_{12} &= \sigma_1\gamma_1 k_f C_1 - \gamma_1 k_b C_2\\
&\vdots\\
R_{i,i+1} &= \sigma_i\gamma_i k_f C_i - \gamma_i k_b C_{i+1}\\
&\vdots\\
R_{n-1,n} &= \sigma_{n-1}\gamma_{n-1} k_f C_{n-1} - \gamma_{n-1} k_b C_n \qquad (A6)
\end{aligned}$$

where C_i is the concentration of $(AU)_i$. The steady-state assumption requires that all the R_{ij} be equal, by which we define R

$$R \equiv R_{01} = R_{12} \ldots = R_{i,i+1} = \ldots = R_{n-1,n} \qquad (A7)$$

Multiplying both sides of each equation for $R_{i,i+1}$ (except R_{01}) in eq A6 by $\gamma_0 K^i/\gamma_i \prod_{m=1}^{i} \sigma_m$ and adding all equations together gives

$$RS = \beta\gamma_0 k_f[A][U] - \frac{\gamma_0 K^{n-1} k_b}{\prod_{m=1}^{n-1} \sigma_m} C_n \qquad (A8)$$

where

$$S = 1 + \sum_{i=1}^{n-1} \gamma_0 K^i/\gamma_i \prod_{m=1}^{i} \sigma_m \qquad (A9)$$

Equation A8 is the general expression for the rate R of the transformation and is of the form required by eq A4. Hence one may write for the rate constants k_1 and k_{-1}

$$k_1 = \beta\gamma_0 k_f/S \qquad (A10)$$

and

$$k_{-1} = \gamma_0 K^{n-1} k_b/S \prod_{m=1}^{n-1} \sigma_m \qquad (A11)$$

It should be obvious that the series S contains far too many parameters for rigorous evaluation, forcing some simplifying assumptions. The simplest possible condition is that $\sigma_i = 1, \gamma_i = 1$ for all $i \geq 0$. Equations A10 and A11 then reduce to

$$k_1 = \beta k_f(1 - K) \qquad (A12)$$

and

$$k_{-1} = k_b K^{n-1}(1 - K) \qquad \text{(case I)} \qquad (A13)$$

respectively. These are the solutions obtained by Saunders and Ross.[8]

The next level of complexity can be introduced by observing that γ_0 is likely to be considerably larger than 1, since the opening of an isolated base pair does not involve loss of the stacking free energy and hence probably proceeds faster than the opening of a pair at the end of a helical sequence. Assuming that $\gamma_0 \gg 1$, and that $\sigma_i = 1, \gamma_i = 1$ for all $i \geq 1$, eq A10 and A11 reduce to

$$k_1 = \beta k_f(1 - K)/K = K_{01} k_f(1 - K) \qquad (A14)$$

and

$$k_{-1} = k_b K^{n-2}(1 - K) \qquad \text{(case II)} \qquad (A15)$$

respectively. This is the case considered by Kallenbach, Crothers, and Mortimer.[10] We take this opportunity to note that the kinetic equation in that paper is incorrect because of an erroneous boundary condition (the condition $C_{N+1} = 0$ in ref 10) applied to the relevant difference equation.

In the two cases considered thus far in this Appendix, all of the difficulty associated with nucleating a helix was assigned to formation of the first base pair, with formation of subsequent pairs characterized by values of the kinetic and equilibrium constants found for a long helix. In fact, the data reported in the body of this paper show that this simplification is not consistent with experiment, since neither case I nor case II adequately predicts the temperature dependence of the apparent second-order rate constant (see below). It seems reasonable that the second base pair formed should be weaker than the nth, expressed in terms of reaction scheme A1 by $\sigma_1 < 1$, but it does not seem possible to say *a priori* whether this instability results from a formation rate constant less than k_f or a dissociation rate constant greater than k_b. In the former instance $\gamma_1 \approx 1$, and in the latter $\gamma_1 \gg 1$. We present here two further sets of equations for the rate constants based on these limiting extremes.

Suppose that the term $K^i/\gamma_i \prod_{m=1}^{i} \sigma_m$ in the sum S is a maximum when $i = \alpha$ (α being the number of "nu-

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[28] D. Pörschke, Diplomarbeit, University of Göttingen, 1966.

[29] M. Eigen in "Nobel Symposium No. 5," S. Claesson, Ed., Almquist and Wiksell Publishers, Stockholm, 1967, pp 354–363.

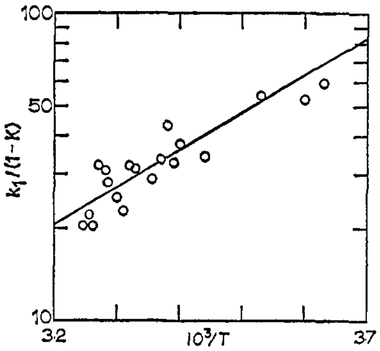

Figure 4. Variation of $k_1/(1-K)$ (on log scale) with $1/T$. Values of k_1 are those shown in Figure 2a; K was calculated from the van't Hoff equation, assuming -7.3 kcal/mol nucleotide pairs as the heat of base-pair formation. The slope of this figure, equal to $-\Delta H^*/R$, gives $\Delta H^* = -6$ kcal.

cleating" residues). Let, in the first instance, $\sigma_i = 1$, $\gamma_i = 1$, for $i \geq \alpha$. As previously, we suppose that $\gamma_0 \gg 1$. Neglecting the terms in S for which $i < \alpha$, and assuming $K^{n-\alpha} \ll 1$, we obtain

$$k_1 = k_f K_{01}(1 - K)\prod_{m=1}^{\alpha}\sigma_m/K^{\alpha-1} \qquad \text{(A16)}$$

and

$$k_{-1} = k_b K^{n-\alpha-1}(1 - K) \qquad \text{(case III)} \qquad \text{(A17)}$$

For case IV suppose again that $K^i/\gamma_i\prod_{m=1}^{i}\sigma_m$ is a maximum when $i = \alpha$, but let $\gamma_\alpha \gg 1$. For simplicity, let $\sigma_i = 1, \gamma_i = 1$, for $i > \alpha$. Again, $\gamma_0 \gg 1$ and $K^{n-\alpha} \ll 1$. Then, neglecting terms with $i < \alpha$

$$k_1 = k_f K_{01}(1 - K)\prod_{m=1}^{\alpha}\sigma_m/K^{\alpha} \qquad \text{(A18)}$$

and

$$k_{-1} = k_b K^{n-\alpha-2}(1 - K) \qquad \text{(case IV)} \qquad \text{(A19)}$$

In cases III and IV, the series S is assumed to have a maximum term when the index $i = \alpha$. Qualitatively this means that the major kinetic barrier in the transformation is between $(\mathrm{AU})_\alpha$ and $(\mathrm{AU})_{\alpha+1}$. In this sense $\alpha + 1$ may be taken as a measure of the number of base pairs required for successful nucleation.

The best means for distinguishing among the cases I through IV is by comparing with experiment the predicted temperature variation of the apparent second-order rate constant. All of the expressions for k_1 contain a factor $(1 - K)$ which can be evaluated from calorimetric data (see eq 16). It is therefore expedient to divide the measured rate constant by $(1 - K)$ and examine the temperature variation of the ratio. Thus, we define

$$\frac{\partial \ln (k_1/(1 - K))}{\partial T} = \frac{\Delta H^*}{RT^2} \qquad \text{(A20)}$$

where ΔH^* is a heat, which will be a combination of activation and reaction enthalpies. In particular, the theoretical expressions for the rate constant disclose that

$$\begin{aligned} \Delta H^* &= \Delta H^{\ddagger} && \text{(case I)} \\ \Delta H^* &= \Delta H^{\ddagger} + \Delta H_{01} && \text{(case II)} \\ \Delta H^* &= \Delta H^{\ddagger} + \Delta H_{01} + \Delta H_{1,\alpha-1} && \text{(case III)} \\ \Delta H^* &= \Delta H^{\ddagger} + \Delta H_{01} + \Delta H_{1,\alpha} && \text{(case IV)} \end{aligned} \qquad \text{(A21)}$$

where $\Delta H^{\ddagger}$ is an activation energy associated with the major kinetic barrier. The nature of this barrier depends on the particular case. In case I, $\Delta H^{\ddagger}$ refers to the formation of the first (isolated) base pair between two strands. In case II, $\Delta H^{\ddagger}$ is the activation energy for formation of the second base pair; in case III, it refers to the step between pairs α and $\alpha + 1$; and in case IV, to the step between pairs $\alpha + 1$ and $\alpha + 2$. ΔH_{ij} is the reaction enthalpy for transformation from a state with i hydrogen bonds to one with j bonds. Some rough estimates of these quantities are possible. It is unlikely that $\Delta H^{\ddagger}$ is larger than a few kilocalories, and this must be positive because the reaction is postulated to be a simple elementary step. We expect that ΔH_{01}, which is the heat for formation of an isolated base pair, would be small because no stacking energy is gained and the interbase hydrogen bonds are formed at the expense of hydrogen bonds to water. Electrostatic repulsion terms, which are large in the low salt concentrations of the present experiments, might even make ΔH_{01} positive. We therefore expect that $\Delta H^{\ddagger} + \Delta H_{01}$ will not exceed a few kilocalories and will probably be positive. The other enthalpies in eq A21 can be approximated by assuming the measured[20,21] heat of base-pair formation, ΔH_f, known to be negative and of the order of -6 to -8.5 kcal/mol depending on conditions. Hence

$$\Delta H_{1,\alpha-1} = (\alpha - 1)\Delta H_f \qquad \text{(case III)} \qquad \text{(A22)}$$

and

$$\Delta H_{1,\alpha} = \alpha\Delta H_f \qquad \text{(case IV)} \qquad \text{(A23)}$$

Figure 4 shows a plot of log $(k_1/(1 - K))$ *vs.* $1/T$, using the data of Figure 2a and assuming $\Delta H_f = -7.3$ kcal/mol[20,21] to calculate K. The slope yields $\Delta H^* = -6$ kcal. Given the values of ΔH^* discussed above, it is not possible to account for this result on the basis of cases I or II, since these both predict ΔH^* should be positive. For case III, $\alpha \cong 2$, and for case IV, $\alpha \cong 1$ (assuming in both instances that $\Delta H^{\ddagger} + \Delta H_{01} = 0$). It is not possible to distinguish between cases III and IV on the basis of these simple considerations. Nevertheless, there is the qualitative conclusion that more than a single base pair is needed to establish a stable nucleus for helix growth. A similar conclusion has been reached by Eigen for complexes formed from oligomers.[29]