and suggests, instead, that the $\rho_1$-locus is involved in binding of the substrate.

It is clear that the attempt to incorporate the conformationally constrained molecules into the theory has done more to demonstrate the striking differences between these compounds and the more conventional acylated $\alpha$-amino acid derivatives than to clarify the theory. These compounds represent a new and challenging tool for elucidating the specificity of $\alpha$-chymotrypsin action. Only when more compounds like these have been studied and more detailed information is available concerning the effects of reaction parameters other than enzyme and substrate concentration on the kinetic constants obtained in the presence as well as absence of various types of inhibitors can one hope to integrate completely this class of compounds into a general scheme.

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[CONTRIBUTION NO. 1696 FROM THE STERLING CHEMISTRY LABORATORY OF YALE UNIVERSITY, NEW HAVEN, CONNECTICUT]

# On the Kinetics and Mechanism of Helix Formation: The Two Stranded Poly (A + U) Complex from Polyriboadenylic Acid and Polyribouridylic Acid[1a]

BY PHILIP D. ROSS[1b] AND JULIAN M. STURTEVANT

RECEIVED JULY 5, 1962

The kinetics of formation of the poly (A + U) double-stranded helical complex from random coil polyriboadenylic acid and polyribouridylic acid has been studied as a function of ionic strength, temperature and polymer concentration by means of the spectrophotometric stopped-flow technique. The reaction follows complex kinetics, initially of second order form, then deviating toward first order. The exact form followed depends both upon the ionic strength and upon the polymer concentration used. At high salt concentrations, the rate of formation of the poly (A + U) complex is found to pass through a maximum about 40° below the helix melting temperature (65° in 0.25 $M$ NaCl). This latter result provides support of the similar theories for the kinetics of helix–coil transformations recently proposed by Flory and by Saunders and Ross. The significance of the parameters appearing in these theories and limitations on their quantitative interpretation are discussed.

## Introduction

Equimolar quantities of the synthetic polynucleotides, polyriboadenylic acid (poly A) and polyribouridylic acid (poly U), when combined in aqueous salt solutions at neutral pH react to form an ordered 1:1 complex, poly (A + U).[2-3] X-Ray studies have shown the poly (A + U) fiber to have a two-strand helical structure similar to that of naturally occurring deoxyribonucleic acid (DNA).[2b]

In a previous paper,[4] using the stopped-flow technique[5] to observe the kinetics of the optical density change at 259 m$\mu$ accompanying the formation of the poly (A + U) complex, Ross and Sturtevant found the reaction to follow complex kinetics, initially of second order form, then deviating toward first order. The reaction rate decreased with increasing temperature and approached zero rate in the vicinity of the helix "melting" temperature[6] $T_m$.

Concurrent with this work, two essentially similar theories of the kinetics of helix–coil transformations were proposed: one by Flory[7] who likened the process to a biased one-dimensional random walk to which he applied the solution of the gambler's ruin problem of probability theory, and the other by Saunders and Ross who based their argument on a kinetic model for the reaction.[8] One of the consequences of both of these theories is that at some temperature below the helix melting temperature, the rate of helix formation should pass through a maximum. The present investigation was undertaken in order to obtain evidence which might be used to test these theories.

By increasing the salt concentration, $T_m$ is raised, thus permitting examination of the kinetics of the reaction at temperatures farther below $T_m$ than was possible at the lower salt concentrations previously employed. This investigation, under conditions where the reaction is more rapid, was made possible by the construction of a new stopped-flow apparatus[9] capable of studying reactions in the millisecond range.

Through a detailed study of the kinetics of suitable model systems, it is hoped that an increased understanding of the mechanism of helix-coil transformations may be gained. Such knowledge is of fundamental chemical interest and may have application in the interpretation of similar phenomena occurring in macromolecules of biological interest.

## Experimental

The polyribonucleotides, poly A and poly U, were synthesized enzymatically using polynucleotide phosphorylase isolated from *M. lysodeikticus* and the appropriate monomer diphosphates (Sigma Chemical Company).[10] The preparations were deproteinized by successive chloroform emulsi-

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[1] (a) This research was carried out at Yale University during the tenure, by P. D. R. of a United States Public Health Service Postdoctoral Fellowship, 1959–1961. This support and the support given by grants from the National Institutes of Health (RG-4725) and the National Science Foundation (G-9625), are gratefully acknowledged. (b) Inquiries regarding this paper should be addressed to P. D. Ross, NIAMD, National Institutes of Health, Bethesda 14, Maryland.

[2] (a) R. C. Warner, *Fed. Proc.*, **15**, 379 (1956); (b) A. Rich and D. R. Davies, *J. Am. Chem. Soc.*, **78**, 3548 (1956); (c) H. T. Miles, *Biochim. et Biophys. Acta*, **30**, 324 (1958), **45**, 196 (1960).

[3] G. Felsenfeld and A. Rich, *Biochim. et Biophys. Acta*, **26**, 457 (1957).

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

[5] B. Chance in "Investigations of Rates and Mechanisms of Reactions," ed. S. L. Friess and A. Weissberger, Interscience Publishers, Inc., New York, N. Y., 1953, p. 690.

[6] See, for example, P. Doty, *et al.*, *Proc. Natl. Acad. Sci. (U. S.)*, **45**, 482 (1959).

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

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

[9] J. M. Sturtevant, unpublished work.

[10] R. F. Steiner and R. F. Beers, "Polynucleotides," Elsevier Pub. Co., Amsterdam, 1961, pp. 374–378.

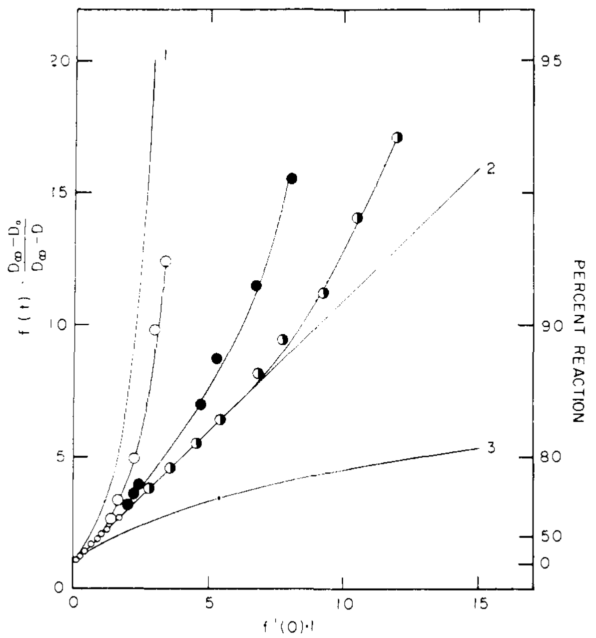

Fig. 1.—Dimensionless plot illustrating the time course of the formation of the poly (A + U) complex in 0.025 *M* NaCl at 25° as measured by the change in optical density *D* at 259 mμ. The reciprocal of the fraction unreacted is plotted against the product of the initial rate of change of this function by the time. The curves labeled 1, 2, and 3 are, respectively, the universal curves for simple first, second, and third order reactions (equal initial concentrations). Polymer phosphate concentrations: ○, $1.5 \times 10^{-5}$ *M*; ●, $6 \times 10^{-5}$ *M*; ◐, $1 \times 10^{-4}$ *M*. The smaller circles illustrate that data are obtained commencing at abscissa values of 0.1.

fications (until a clear interface was obtained) followed by several extractions with water saturated phenol. The resulting solution was then dialyzed against several changes each of NaCl-ethylenediaminetetraacetic acid, NaCl, and finally exhaustively dialyzed against deionized distilled water, and lyophilized. Values obtained for the mean sedimentation coefficients $s^{w}_{20}$ in 0.1 *M* NaCl were 4.6 for the poly A and 7.2 for the poly U.

The polymers were dissolved in either 0.25 or 0.50 *M* NaCl solution which also contained 0.01 *M* sodium cacodylate buffer at pH 7. The solutions were adjusted to equal monomer molarity using the molar extinction coefficients cited in the literature.[8] This procedure was checked by spectral analysis after alkaline hydrolysis, and by attainment of a minimum absorbancy at the expected equivalence point for the 1:1 complex in mixing experiments conducted at constant total phosphorus concentration.[8]

The kinetics of the optical density change at 259 mμ (approximately 28% at 25°) accompanying the formation of the poly (A + U) complex was observed by the spectrophotometric stopped-flow technique[5] in a new apparatus[9] capable of examining reactions with half-lives as short as three milliseconds. The temperature was controlled and measured with an accuracy of ±0.05°.

All kinetic measurements were performed within two hours of the preparation of the individual polymer solutions. At each salt concentration, the reported rate constants represent data from at least three sets of independently prepared solutions; usually four kinetic runs were made with each set of solutions. It is estimated that the rate constants are subject to an uncertainty of about 6%.

## Results and Discussion

**Kinetic Form.**—The time course of the optical density change at 259 mμ accompanying formation of the poly (A + U) two strand helical complex from equimolar solutions of poly A and poly U is illustrated by a novel dimensionless plot in Fig. 1. This plot is very convenient in all cases where reactants are initially present in equal concentrations, since any irreversible reaction of a given order (and reversible reactions in the case of first order) will follow the same universal curve. The curves for first, second and third order reactions are shown in the figure. The kinetics is of complex form, initially close to second order and then deviating toward first order behavior in the final stages of the reaction. This is consistent with the view that the poly (A + U) complex is formed by a nucleation process consisting of the formation of a few hydrogen bonded base pairs followed by a coöperative growth of ordered helical regions from these nuclei.

At low ionic strength (0.025 *M* NaCl) a dependence of kinetic form upon polymer concentration is evident (Fig. 1). Deviation from second order behavior occurs at earlier extents of reaction as the concentration of reactants is lowered. This result suggests that at low polymer concentrations a larger fraction of the final base pairing is achieved by growth from nuclei, rather than by the formation of new nuclei, than is the case at high polymer concentrations, where the primarily second order nucleation process occurs much more readily. In the case of the higher salt concentrations (0.25–0.50 *M* NaCl) and at all temperatures employed in this investigation the kinetic form is apparently independent of polymer concentration and closely follows second order kinetics to beyond 80% reaction, similar to the curve shown for the highest polynucleotide concentration in Fig. 1. When equimolar solutions of the polymers are combined, the initial rate is directly proportional to the square of the monomer concentration. These results are in agreement with those reported previously for the formation of the poly (A + U) complex at lower salt concentrations.[4]

**Apparent Second Order Rate Constants.**—From the kinetics of the optical density change and the initial nucleotide concentration it is possible to calculate apparent second order rate constants, $k_{\text{app}}$. Since the exact relationship between the fractional optical density change and helical content is unknown at present,[11] it has been assumed in this paper that any deviations from proportionality are negligible. In a recent discussion of this problem, Applequist[12] concludes that the nonlinearity of hypochromism *vs.* extent of base pairing is probably not significant for a highly ordered helical structure as in DNA or, presumably, poly (A + U). If small deviations from linearity in the direction suggested exist, then the correspondence between optical density change and the extent of base pairing would be worst in the initial stages of the reaction.[13] Since the apparent second order rate

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[11] Contradictory results appear in the literature for experiments having possible bearing on this problem (A. Rich and I. Tinoco, *J. Am. Chem. Soc.*, **82**, 6409 (1960); M. N. Lipsett, L. A. Heppel and D. F. Bradley, *J. Biol. Chem.*, **236**, 857 (1961)).

[12] J. Applequist, *J. Am. Chem. Soc.*, **83**, 3158 (1961).

[13] Throughout this investigation there have been indications of a lag phase during the first few milliseconds of the reaction. This might be due to a pre-steady-state phase or possibly to nonlinearity in the hypochromism as the first few base pairs combine. The effect is so small as to render careful study of it very difficult.

constants are obtained from data covering the major portion of the reaction, these deviations are reduced, so that the values of $k_{\mathrm{app}}$ and the over-all kinetic form may be considered to be a fair reflection of the actual poly (A + U) complex formation process.

**Effect of Ionic Strength.**—The effect of salt concentration on the rate of poly (A + U) complex formation is given in Table I, where the apparent second order rate constants, $k_{\mathrm{app}}$, calculated from the initial rates at 25° are listed, along with values of $T_{\mathrm{m}}$, the midpoint of the thermal transition as determined by optical density measurements at 259 mμ.

TABLE I

POLY (A + U) COMPLEXES. EFFECT OF SALT CONCENTRATION ON THE APPARENT SECOND ORDER RATE CONSTANT OF FORMATION AT 25°, AND THE HELIX MELTING TEMPERATURE

| NaCl, $M$ | $10^{-5}k_{\mathrm{app}}$ l. mole$^{-1}$ sec.$^{-1}$ | $T_{\mathrm{m}}$, °C. |
|---|---|---|
| 0.50 | 13.1 | 72.6 |
| .25 | 6.1 | 66.0 |
| .10 | 0.98 | 58.1 |
| .05 | 0.15 | 51.2 |

All solutions contained 0.01 $M$ Na cacodylate buffer, pH 7.0. These results illustrate the general trend of an increase in rate with increasing ionic strength as would be expected for a reaction between two species of similar charge type. In the region of 0.25 $M$ NaCl to 0.50 $M$ NaCl, the effect of ionic strength on the rate is much less pronounced than in the more dilute salt solutions. This behavior parallels the leveling out of $T_m$ with increasing salt concentration also shown in Table I. It should be noted that the rate constants cited above are not strictly comparable, due to variation of the rate with the degree of supercooling, *i.e.*, the magnitude of $T_{\mathrm{m}} - T$ where $T$ is the temperature at which the reaction was carried out.

**Effect of Temperature.**—The rate of formation of the poly (A + U) complex as measured by the apparent second order rate constant is displayed in Fig. 2 as a function of the temperature at which the reactants were mixed. The striking result is obtained that the rate of complex formation passes through a maximum as the temperature at which the reaction is carried out is lowered from the helix melting temperature.[14,15] The temperature of the rate maximum, $T_{\mathrm{max}}$, for the formation of the poly (A + U) complex is approximately 34° in 0.5 $M$ NaCl and 24° in 0.25 $M$ NaCl. Thus, the maximum rate occurs 39° below $T_{\mathrm{m}}$ in 0.5 $M$ NaCl and 42° below $T_{\mathrm{m}}$ in 0.25 $M$ NaCl. It is interesting that the rate maximum occurs at 0.88 $T_{\mathrm{m}}$ when temperature is expressed in °K. A rate maximum between 0.8 and 0.9 $T_{\mathrm{m}}$ is characteristic of the kinetics of bulk crystallization of linear polymers proceeding by a homogeneous nucleation mechanism.[16]

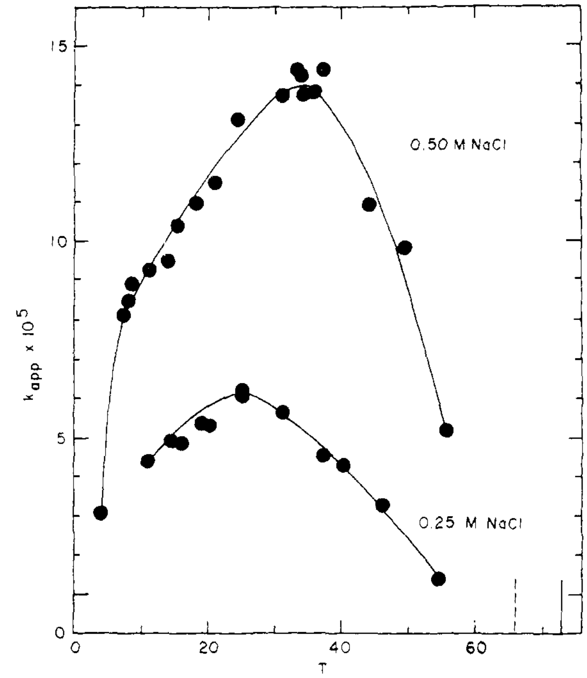

Fig. 2.—The effect of temperature on $k_{\mathrm{app}}$, the apparent second order rate constant (liter mole$^{-1}$ sec.$^{-1}$) for the formation of the poly (A + U) complex. The two vertical lines rising from the abscissa denote $T_m$ for the two salt concentrations (see Table I); polymer phosphate concentration $3.6 \times 10^{-5}$ $M$.

The existence of a maximum in the rate *versus* temperature profile for the formation of the poly (A + U) helical complex is in accord with the essentially similar theories of the kinetics of helix-coil transformations proposed independently by Flory[7] and by Saunders and Ross.[8] This effect was predicted explicitly by Flory,[7] and is implicit in the Saunders–Ross model which now will be discussed.

**Kinetic Model.**—The model proposed by Saunders and Ross[8] for the kinetics of helix–coil transformations as applied to the formation of the poly (A + U) complex consists of a bimolecular initiation step, characterized by rate constant $k_i$ to form a nucleus consisting of an unknown number of nucleotide pairs (eq. 1). Helical growth proceeds

$$\mathrm{A} + \mathrm{U} \underset{k_{\mathrm{b}}}{\overset{k_{\mathrm{i}}}{\rightleftharpoons}} \mathrm{C}_1 \qquad \text{first step} \qquad (1)$$

$$\mathrm{C}_1 \underset{k_{\mathrm{b}}}{\overset{k_{\mathrm{f}}}{\rightleftharpoons}} \mathrm{C}_2 \underset{k_{\mathrm{b}}}{\overset{k_{\mathrm{f}}}{\rightleftharpoons}} \cdots\cdot \mathrm{C}_i \underset{k_{\mathrm{b}}}{\overset{k_{\mathrm{f}}}{\rightleftharpoons}} \mathrm{C}_{i+1} \qquad \text{remaining steps} \qquad (2)$$

from these nuclei by the intermediate steps represented in eq. 2 characterized by the forward rate constants $k_{\mathrm{f}}$ and backward rate constants $k_{\mathrm{b}}$. Assuming a large number of intermediate steps and applying the steady state approximation gives, upon simplification[8]

$$\text{rate} = k_{\mathrm{i}}[\mathrm{A}][\mathrm{U}](1 - k_{\mathrm{b}}/k_{\mathrm{f}}) \qquad (3)$$

where the quantities [A] and [U] denote the mono-

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[14] If the reciprocal half-time, which is independent of any assumptions about the kinetic form of the process, is used as a measure of the rate of the reaction, a similar rate-temperature profile is obtained.

[15] Recently, a similar rate maximum in the renaturation kinetics of heat denatured DNA has been reported (J. Marmur and P. Doty, *J. Mol. Biol.*, **3**, 585 (1961), and L. F. Cavalieri, T. Small and N. Sarker, *Biophysic. J.*, **2**, 339 (1962)).

[16] J. D. Hoffman and J. I. Lauritzen, *J. Research Natl. Bur. Standards*, **65**A, 297 (1961).

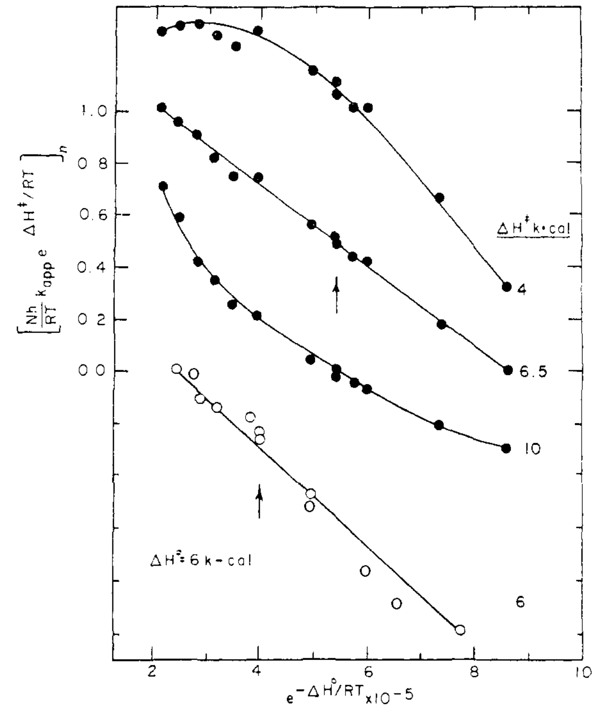

Fig. 3.—Test of eq. 6 assuming $\Delta H^\circ = 6$ kcal. and various values for $\Delta H^*$. The ordinate function has been normalized between the first and last points chosen for analysis[18] and the different curves have been vertically displaced from one another for clarity. The arrows denote the temperature of the rate maximum: ●, 0.5 $M$ NaCl; ○, 0.25 $M$ NaCl.

molar concentrations of the respective polynucleotides.

Thus, the over-all rate of helix formation is simply the rate of the initiation process multiplied by the factor $(1 - k_b/k_f)$. Since $k_b/k_f$, interpreted as the dissociation constant of a helical base pair, increases to approach unity at the helix melting temperature, the factor $(1 - k_b/k_f)$ should decrease with increasing temperature and the rate of the reaction will become smaller as the temperature approaches the helix melting temperature. This accounts for the negative temperature coefficient of reaction rate observed on the high temperature side of Fig. 2 and in the earlier work[4] at lower salt concentrations. At temperatures far removed from the helix melting temperature, $T_m$, the rate expression becomes dominated by the more usual exponential dependence of $k_i$ upon temperature. This is borne out by the positive temperature coefficient observed on the left side of Fig. 2. At intermediate temperatures these two opposing effects balance, and the rate of complex formation is predicted by this theory to pass through a maximum which is the salient experimental result presented in Fig. 2. We conclude that the theory is in qualitative accord with the observed rate behavior.

It is of interest to make a very tentative quantitative interpretation of the experimental results obtained in 0.25 $M$ NaCl and 0.50 $M$ NaCl in terms of the theory. According to transition-state theory, the initiation rate constant $k_i$ is given by equation (4)

$$k_i = (RT/Nh)e^{\Delta S^*/R}\, e^{-\Delta H^*/RT} \qquad (4)$$

where $N$ is Avogadro's number, $h$ is Planck's constant and $\Delta S^*$ and $\Delta H^*$ are, respectively, the entropy and enthalpy of activation for the initiation process. Introducing the empirical rate law for the observed (initial) rates

$$\text{rate} = k_{app}[A][U] \qquad (5)$$

and assuming a normal temperature dependence for the equilibrium constant $k_b/k_f$, eq. 3 after introduction of eq. 4 and 5 becomes

$$\frac{Nh}{RT} k_{app}\, e^{\Delta H^*/RT} = e^{\Delta S^*/R} - e^{\Delta S^*/R}\, e^{\Delta S^\circ/R}\, e^{-\Delta H^\circ/RT} \qquad (6)$$

where $\Delta S^\circ$ and $\Delta H^\circ$ are standard entropy and enthalpy changes, presumably to be associated with the dissociation of a base pair in the helix. A plot of the left side of eq. 6 against $e^{-\Delta H^\circ/RT}$ for suitable choices of the two enthalpy parameters, $\Delta H^*$ and $\Delta H^\circ$, (the two entropy parameters are assumed not to change with temperature), should give a straight line.

Unfortunately, it is not known what values to choose for $\Delta H^*$ and $\Delta H^\circ$. If a value of 6 kcal./mole is assumed for $\Delta H^\circ$,[17] it is possible to obtain the expected straight line behavior with eq. 6 over a 40° temperature range which includes the rate maximum (Fig. 3).[18] This fit is not unique, since other combinations of values of $\Delta H^*$ and $\Delta H^\circ$ below about 10 kcal./mole can be found to give a satisfactorily linear fit. It is therefore not possible to attach much significance to the actual values of the enthalpy parameters arrived at as shown in Fig. 3. Rather, Fig. 3 should be regarded as illustrating that eq. 6 is sufficient to represent the experimental results, and suggesting that a value for $\Delta H^*$ can be obtained once $\Delta H^\circ$ is known definitely. It appears that both these quantities are probably less than 10 kcal./mole. Several difficulties remain, however, concerning the interpretation of $\Delta H^*$. It is not at all clear to what size unit $\Delta H^*$ refers. Also, it is not known how the different extents of intramolecular organization in the polynucleotides (especially poly A) at different temperatures may contribute to the activation enthalpy. Finally, the model[7,8] itself is certainly oversimplified in representing such a complex process in terms of a single rate constant and an equilibrium constant. The model does not consider multi-site nucleation and does not account for the complex kinetic behavior observed in the final stages of the reaction or at low salt concentration. If in a more exact theory, $k_i$ (or what we now interpret as $k_i$) turns out to be a complex rate constant, then it is clearly without significance to interpret $\Delta H^*$ as the activation energy for

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(17) No calorimetric data are presently available for the heat of dissociation of poly (A + U). A value of $\Delta H^\circ$ of about 6 kcal. per base pair has been found for the formation of the poly (A + U) complex (M. Rawitscher, P. D. Ross and J. M. Sturtevant, unpublished work; R. F. Steiner and C. Kitzinger, "Abstracts 6th Annual Meeting Biophysical Society," Washington, D. C., Feb. 14-16, 1962).

(18) The 4° point for 0.5 $M$ NaCl has been omitted from this analysis because some of the precipitous drop in rate observed is possibly due to complications arising from the transition to the ordered poly U structure (M. N. Lipsett, *Proc. Natl. Acad. Sci.* (*U. S.*), **46**, 445 (1960)). The highest temperature point at each salt concentration also has been omitted.

any single process. The simple model, however, does provide a useful qualitative picture of the kinetics of helix formation as a nucleation and growth phenomenon.

[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS 14, MINN.]

# Further Studies on the Biosynthesis of Tropic Acid[1]

BY MARY L. LOUDEN AND EDWARD LEETE

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The administration of L-phenylalanine-1-$C^{14}$ and DL-phenylalanine-2-$C^{14}$ to *Datura stramonium* plants in separate experiments led to the formation of radioactive hyoscyamine and hyoscine which were labeled solely in their tropic acid moieties. Systematic degradation of the side chain of the radioactive tropic acids indicated that the carboxyl group was derived from the carboxyl group of phenylalanine, while the hydroxymethyl group arose from the $\alpha$-carbon of the amino acid. Administration of sodium bicarbonate-$C^{14}$ to *Datura* plants resulted in general labeling of the alkaloids. Plausible biogenetic schemes for tropic acid are discussed.

In our previous work on the biosynthesis[2] of tropic acid[3] (I), DL-phenylalanine-3-$C^{14}$ was administered to two-month old *Datura stramonium* plants resulting in the formation of radioactive hyoscyamine and hyoscine which were labeled specifically at C-2 of their tropic acid moieties. This result has been recently confirmed by the work of Underhill and Youngken.[4] We had originally considered that the hydroxymethyl group (C-2) of tropic acid was derived from a one-carbon source. However, the administration of sodium formate-$C^{14}$,[3] formaldehyde-$C^{14}$,[3] methionine-methyl-$C^{14}$[5] or serine-3-$C^{14}$[4] to *D. stramonium* plants did not lead to tropic acid labeled at C-2.

We thus shelved the problem of the origin of the hydroxymethyl group and fed phenylalanine-2-$C^{14}$ to three-month old *Datura* plants, fully expecting to obtain tropic acid which would be labeled on its carboxyl group. Radioactive tropic acid was indeed obtained and the degradative scheme which was used to determine the activities on the side chain carbons of tropic acid is illustrated in Fig. 1. Dehydration of tropic acid by refluxing with concentrated aqueous potassium hydroxide[6] yielded atropic acid (II) which was oxidized in alkaline solution with sodium metaperiodate and a catalytic amount of osmium tetroxide yielding formaldehyde collected as its dimedone derivative, and phenylglyoxylic acid isolated as its oxime (III). This oximino acid was refluxed in water yielding benzonitrile and carbon dioxide,[7] collected as barium carbonate. The benzonitrile was hydrolyzed with potassium hydroxide yielding benzoic acid which was subjected to the Schmidt reaction affording aniline, assayed as its benzoyl derivative, and carbon dioxide, collected as barium carbonate. We were agreeably surprised to discover that the tropic acid derived from phenylalanine-2-$C^{14}$ was labeled solely on the hydroxymethyl group (*cf.* Table I).

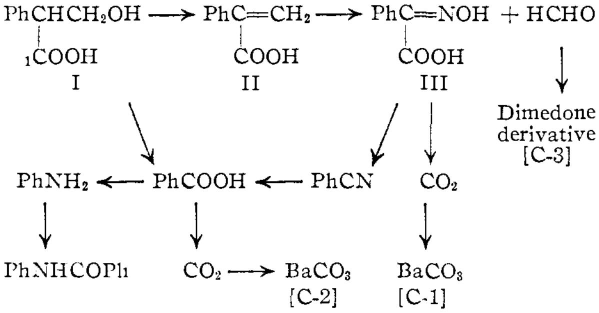

Fig. 1.—Degradative scheme for the radioactive tropic acid.

While our work was in progress, Goodeve and Ramstad[8] reported that the administration of tryptophan-3-$C^{14}$ to *D. stramonium* yielded radioactive hyoscyamine which was labeled specifically on the carboxyl group of its tropic acid moiety. This was an astonishing result and difficult to reconcile with our observations. It seemed unlikely that tropic acid was being formed in the same plant by two quite different biosynthetic mechanisms, and we interpreted their work as follows. The radioactive tryptophan could undergo metabolic breakdown in the plant yielding some radioactive carbon dioxide. This could then participate in a carboxylation reaction with a metabolite derived from phenylalanine leading to carboxyl labeled tropic acid. We attempted to test this hypothesis by feeding sodium bicarbonate-$C^{14}$ to *Datura* plants in the hope that we would obtain preferential incorporation of carbon-14 into the carboxyl group of tropic acid. Radioactive hyoscyamine and hyoscine were obtained. However, assay of the degradation products of the alkaloids (*cf.* Table I) indicated that the distribution of activity between the tropic acid and the tropane bases was almost proportional to the carbon content of the two halves of the alkaloids. The distribution of activity in the side chain of

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[1] Part of this work has been published in preliminary communications: E. Leete and M. L. Louden, *Chemistry & Industry*, 1405 (1961); M. L. Louden and E. Leete, *J. Am. Chem. Soc.*, **84**, 1510 (1962). This investigation was supported by a research grant, MY-2662 from the National Institute of Mental Health, U. S. Public Health Service.

[2] We accept with enthusiasm the suggestion of Rapoport (F. R. Stermitz and H. Rapoport, *J. Am. Chem. Soc.*, **83**, 4045 (1961)) that the word biosynthesis should describe *in vivo* experimental studies on the mode of formation of natural products, whilst the word biogenesis should be applied to the hypothetical schemes which organic chemists are so fond of creating to rationalize the formation and structures of natural products.

[3] E. Leete, *ibid.*, **82**, 612 (1960).

[4] E. W. Underhill and H. W. Youngken, *J. Pharm. Sci.*, **51**, 121 (1962).

[5] L. Marion and A. F. Thomas, *Can. J. Chem.*, **32**, 1116 (1954).

[6] J. W. Baker and A. Eccles, *J. Chem. Soc.*, 2125 (1927).

[7] A. Ahmad and I. D. Spenser, *Can. J. Chem.*, **39**, 1340 (1961).

[8] A. M. Goodeve and E. Ramstad, *Experientia*, **17**, 124 (1961).