COOPERATIVE ASPECTS OF SMALL ION, SMALL MOLECULE INTERACTIONS WITH NUCLEIC ACIDS

POTASSIUM-POLYRIBOADENYLIC ACID, POLYRIBOURIDYLIC ACID

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ABSTRACT Ionic conductivity measurements vs. temperature were made on KCl solutions of K-polyriboadenylic acid + K-polyribouridylic acid (K–poly-A + K–poly-U) in regions of temperature and composition where the helix-coil transition occurred. It was possible to relate the measurements to a differential helix-to-coil binding of K^+ to nucleotide. The results were, within experimental error, the same as those obtained from a limited number of differential KCl activity coefficient measurements and from a theoretical interpretation of polymer free-boundary electrophoretic mobilities. It was concluded that the alkali ion-phosphate interaction in polynucleotides must be regarded as cooperative in nature and several criteria for recognition of such phenomena were given. A brief outline for a proposed statistical mechanical model for binding was presented.

BACKGROUND

In the past, much information concerning the in vitro structure of duplex nucleic acids has been inferred from theoretical models. The procedure has been to develop a one-dimensional, statistical mechanical model for the order-disorder process (helix-coil transition) and to fit it in parametric form to an experimental curve such as an optical hypochromism-temperature profile (melt curve). The resulting parametric values have been related through the interpretive model to thermodynamic properties of the helix and coil forms of the polymer. The relative magnitudes of the energy values obtained have been further interpreted as information concerning the sources of thermodynamic stability of the in vitro helix.

The procedure just outlined is correct, in principle, and recent calculations (Montroll and Goel, 1966) suggest that ultimately one may hope to derive statistical distribution of nucleotide base pairs from the theoretical interpretation of the experimental data represented by the melt curves. In practice, oversimplification has oc-

curred. For example, it has been found convenient to account for the known effects of solution cationic strength on the experimental melt curves by an artifice. The assumption is made that the results depend only on the ionic strength at the start of an experiment and that a derived quantity such as an order-disorder enthalpy can merely be labeled with the ionic strength as a characterizing index.

It is the purpose of the present work to show that (*a*) cation binding to polynucleotides is variable during the course of a pH or thermally induced order-disorder transition; (*b*) the variability is thermodynamically significant in comparison to other interactions such as hydrogen bonding; (*c*) methods are possible for improving the earlier theoretical lines of investigation.

In the following, the term *conformation* will be used to indicate in vitro polymer structure. It is used to distinguish solution structure from the more familiar Watson-Crick model for hydrated fiber crystals.

INTRODUCTION

Many studies have been made of the binding of small ions and molecules to nucleic acids. In most experiments these ions and molecules have been quaternary solutes in aqueous solutions where the primary-to-tertiary solutes are the nucleic acid, alkali, and hydrogen ions. Among them have been alkali and alkaline earth metal ions, amines, monomeric nucleotides, polypeptides, and many organic dyes. Summaries of results are available in the literature (Steiner and Beers, 1961; Felsenfeld and Miles, 1967). In many cases the binding appears to depend upon the polymer conformation and is quantitatively different for coil and helix forms of the nucleic acid. Although it does not appear to have been generally recognized, this result has a corollary: in ranges of temperature and solvent composition which place the polymer in or near a helix-coil transition, the addition of a quaternary solute may influence or completely determine the final polymer conformational state. The implications in terms of equilibrium and kinetic binding studies are obvious.

This class of interactions may be termed "cooperative" in the same sense that a first-order thermodynamic phase transition is a cooperative process. The present report is a preliminary study of these interactions using a model system (K–poly-A, poly-U; KCl) at neutral pH. The notation indicates K salts of the polymers with added KCl in the solvent. Here polymer conformation is known to be dependent upon KCl concentration, but quantitative data on the extent and variation in ion-polymer binding heretofore has not been available for the helix-coil transition regions. In the discussion of binding in this simple system, it is possible to give alternative criteria for the occurrence of cooperative binding in more complex systems and to suggest methods for theoretical analysis of conformation and binding experiments.

Two-strand helix conformation may be conveniently monitored by measuring optical absorbance, A, at 260 nm. The hypochromism fraction will be defined as

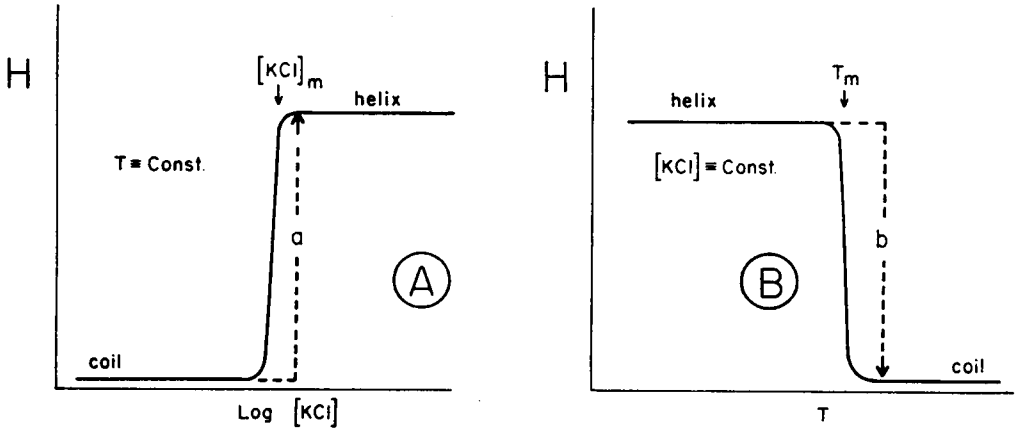

FIGURE 1. A Schematic representation of hypochromism fraction H vs. added salt concentration at constant temperature. B H vs. temperature at constant added salt concentration.

$H = \{A(T, [KCl])\text{-}A(coil)\}/\{A(helix)\text{-}A(coil)\}$. Fig. 1 shows schematically the behavior of polymer conformation, in the system (K–poly-A, poly-U; KCl), with varying salt at constant temperature, and with varying temperature at constant salt concentration. For equimolar amounts of poly-A and poly-U, these curves are independent of polymer concentration (Felsenfeld and Huang, 1960) and, for molecular weights above 100,000, almost independent of the degree of polymerization (Steiner and Beers, 1961). The two profiles are related to one another by $T_m \propto \log[KCl]_m$ (Stevens and Felsenfeld, 1964). The hypochromism probably arises from the influence of base-base stacking upon the monomer UV optical absorption coefficient (Rich and Tinoco, 1960). Profiles for K–poly-(A + U) with other conformation criteria such as NMR observation of base hydrogen pairing, viscosity, etc., appear to be congruent with the curves of Fig. 1.

The curves of Fig. 1 give no direct measure of binding although they are presumptive evidence of the possibility of occurrence of differential binding across a helix-coil transition. Alkali ion-DNA binding studies have been made using equilibrium dialysis (Shack, Jenkins, and Thompsett, 1952), polymer free-boundary electrophoresis (Ross and Scruggs, 1964), counterion activity values (Lyons and Kotin, 1964), and ionic conductivity-temperature profiles (Felsenfeld and Huang, 1961). A summary (Steiner and Beers, 1961; Felsenfeld and Miles, 1967) of available evidence leads to these conclusions: the primary role of alkali ion in changing polymer conformation is through neutralization of internal repulsion between charged phosphate groups in the backbone; in regions of approximately constant conformation, the charge neutralization (and binding) is not stoichiometric and (for helical DNA) is about 0.5 alkali ion per phosphate and much less for Cl^- byion; the fraction of bound counterions is greater for the helix than for the coil form. In anticipation of the present results, these conclusions are in qualitative agreement with

experiments on the duplex structure, K–poly-(A + U) and for both Na-DNA and K–poly-(A + U), one may approximately relate the fraction of bound counterion to conformation by a phenomenological expression, $f_B = A'(1 - H) + B'$ where A′ and B′ are constant.

To illustrate the diversity of situations possible with ion binding, the experiments for (Mg-DNA; $MgCl_2$) may be mentioned. Counterion activity measurements, for Mg-DNA solutions with no added $MgCl_2$, and an observed decrease in T_m for solutions with added $MgCl_2$ led to these conclusions (Lyons and Kotin, 1964, 1965): on a charge basis, Mg is bound almost stoichiometrically to both native and denatured DNA; the binding is slightly greater for the denatured form; the addition of $MgCl_2$ may be expected to favor the coil form of the polymer. Results of Venner and Zimmer (1966) where T_m was found to increase with Mg^{++} concentration, are not necessarily in conflict with these conclusions, since they were representative of a mixed systen, (Na, Mg-DNA; NaCl, $MgCl_2$). The apparent conflict reflects the difficulty in interpreting experiments where more than one kind of ion may bind cooperatively.

In the following, measurements of the (K–poly-A, poly-U; KCl) ionic conductivity-temperature profile are presented. With appropriate account of polymer conductivity contributions, the measurements lead to values of differential ion binding across the thermal transition. The total change in binding from helix to coil is related to the conformational transition (*b*) shown in Fig. 1 B.

There are inherent theoretical difficulties in treating single ion activities (see Discussion) which make it desirable to check the analysis by other methods. Total change in binding at constant temperature was measured by observing differential KCl activity and (through electrophoretic mobility) apparent differential phosphate charge neutralization. These experiments are related to coil-to-helix conformational change (*a*) shown in Fig. 1 A.

METHODS AND MATERIALS

Ionic Conductivity

Measurement of ionic conductivity of polymer solutions was made with a Jones Bridge (Leeds & Northrup Co., Philadelphia, Pa., model 578880) at 1000 cps and electric fields of 0.1 v/cm. The bridge was modified to measure directly the difference in resistance between two conductivity cells, one containing polymer in salt solution (R_x) and one containing only salt solution (R_r). The modified bridge gave results reproducible to ±1%.

The platinum electrodes of the cells were cleaned with aqua regia and given a short electrolysis treatment in contact with 3% chloroplatinic acid-0.02% lead acetate solution. The resulting surfaces were not visibly different from bright Pt but both cell constants were approximately the same as for completely blackened electrodes. A plot of conductivity at 60 cps and at 1000 cps indicated negligible polarization effects for measurements at 1000 cps. The mode of preparation was used to reduce possible catalytic decomposition of polymer by platinum black.

The cells were immersed in a water bath with short term temperature stability of ±0.01°C. Measurements were made from 25°C to temperatures above each helix-coil transition and values of $R_3 = R_r - R_x$ and R_r were recorded after temperature equilibration.

It may be shown that

$$(R_x^0/R_r^0)(R_3/R_x - R_3^0/R_x^0) = \left[\frac{\sigma_x/\sigma_r}{\sigma_x^0/\sigma_r^0} - 1\right], \tag{1}$$

where zero superscripts indicate 25°C values. The σ_x , σ_r are specific conductivities of polymer and reference solutions at $T > 25°$. From this one has

$$\left[\frac{\sigma_x/\sigma_r}{\sigma_x^0/\sigma_r^0}\right] - 1 = \frac{\sigma_x - \sigma_r[\sigma_x^0/\sigma_r^0]}{\sigma_r[\sigma_x^0/\sigma_r^0]} = \frac{\sigma_x - \sigma_x'}{\sigma_x'}. \tag{2}$$

Here σ_x' is the conductivity of a salt solution whose room temperature conductivity value is equal to that of the polymer solution at room temperature.

Thus, a fractional change in conductivity of the polymer solution solely due to temperature-induced changes in polymer mobility and ion binding will be

$$\left(\frac{\delta\sigma}{\sigma}\right)_{T > T_m} = \frac{\sigma_x - \sigma_x'}{\sigma_x'} = (R_x^0/R_r^0)[R_3/R_x - R_3^0/R_x^0]. \tag{3}$$

Two single-lot samples each of poly-A (K salt) and poly-U (K salt) were obtained from Calbiochem, Los Angeles, Calif. Pair A had molecular weights by end-group analysis of $M_A \sim M_U \sim 50{,}000$. Pair B had $M_A \sim 300{,}000$, $M_U \sim 100{,}000$ as determined by sedimentation velocity. Calf thymus DNA (Na salt) from Calbiochem was deproteinated by repeated shaking with 50% chloroform solution.

Phosphate concentrations were determined by optical absorption taking ϵ (260 mμ, 1 cm) of 10,400 (poly-A, $\mu = 0.1$), 9900 (poly-U, $\mu = 0.1$) (Stevens and Felsenfeld, 1964), and 7000 (DNA, $\mu = 0.2$)/M-cm (Lyons and Kotin, 1964).

For K binding measurements the samples were dialyzed against the K salt of EDTA and then against KCl solutions. For Mg binding studies the samples were dialyzed against $MgCl_2$ Magnesium chloride solutions were prepared from a stock solution which had been standardized by titration against the hydrated Na salt of EDTA using Eriochrome Black T as indicator. Dialysis was done at 4°C with 100:1 volume ratios. To avoid complications of possible buffer ion binding all solutions were unbuffered but maintained in the region of pH 6–8 by use of KOH and exclusion of atmospheric CO_2.

KCl Activity

Difference measurements were made for mean KCl activity in the presence of K–poly-A or K–poly-U and in the presence of the mixture *K*-poly-(A + U). The following electrochemical cell was used.

K ion specific electrode	K polymer and added KCl	2 N KCl, AgCl; Ag.	(4)

The ion specific electrode was a glass Beckman cationic electrode (model 39137) (Beckman Instruments, Inc., Palo Alto, Calif.) with a cracked-glass AgCl reference electrode (Leeds & Northrup, model 117147). Electromotive force of the cell was measured with a Beckman research pH meter (model 1019) with a precision of ±0.05 mv.

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The cell eMF was represented by the phenomenological expression:

$$\epsilon = \epsilon_0 + k' \log a_{KCl} \tag{4a}$$

and

in the presence of poly-A or poly-U, k' was 50 $\pm$ 5 mV/decade. Hydrogen ion concentration was held three to four orders of magnitude below alkali ion concentration.

In these experiments equimolar, equal volume samples of poly-A and poly-U were dialyzed against a common KCl solution. One of the samples (either poly-A or poly-U) was placed in a thermally controlled cup at 25 $\pm$0.1°C and a measurement ϵ (coil) was made. The other sample was added and ϵ (helix) was determined giving

$$\delta\epsilon = \epsilon \text{ (coil)} - \epsilon \text{ (helix)} = k' \log [a^{C}_{KCl}/a^{H}_{KCl}]. \tag{5}$$

For small changes

$$\delta\epsilon \cong k'[\log e] \frac{\delta(a_{KCl})}{\langle a_{KCl} \rangle}. \tag{6}$$

It was found that atmospheric CO_2 affected ϵ. Experiments with Tris-HCl buffer solutions indicated that the mechanism was through changes in the liquid junction potential (which is included in ϵ_0) rather than by direct hydrogen ion interference. Each polymer solution was given identical exposures to the atmosphere and [ϵ_0 (coil) $-$ ϵ_0 (helix)] appeared to be negligible in accord with Equation 6.

Free Boundary Electrophoresis

Electrophoretic mobilities for poly-A, poly-U, and poly-(A + U) were measured at 0°C using the Perkin-Elmer (model 38) Tiselius apparatus (Perkin-Elmer Corp., Norwalk, Conn.). Boundaries were established between the polymer solution and supporting electrolyte solutions which were the final outer dialysis bath solutions in the polymer sample preparation. The conductivity of the supporting electrolyte was measured with the Jones Bridge and used for computing mobilities. The computational equations were conventional (Shedlovsky, 1946) and will not be given here.

RESULTS AND ANALYSIS

Conductivity

Fig. 2 shows the raw data for the resistance-temperature profile of K–poly-(A + U). As indicated, the thermal transition is reversible. Fig. 3 (curve A) shows the replotted curve according to Equation 2 for pair A of the polymer samples. Fig. 3 (curve B) is a similar treatment of data taken with the polymer samples, pair B.

The fractional conductivity change per phosphate, $[(\delta\sigma/\sigma)_{T > T_m}]/[P]$, in going from helix to coil is insensitive to degree of polymerization and for a given polymer

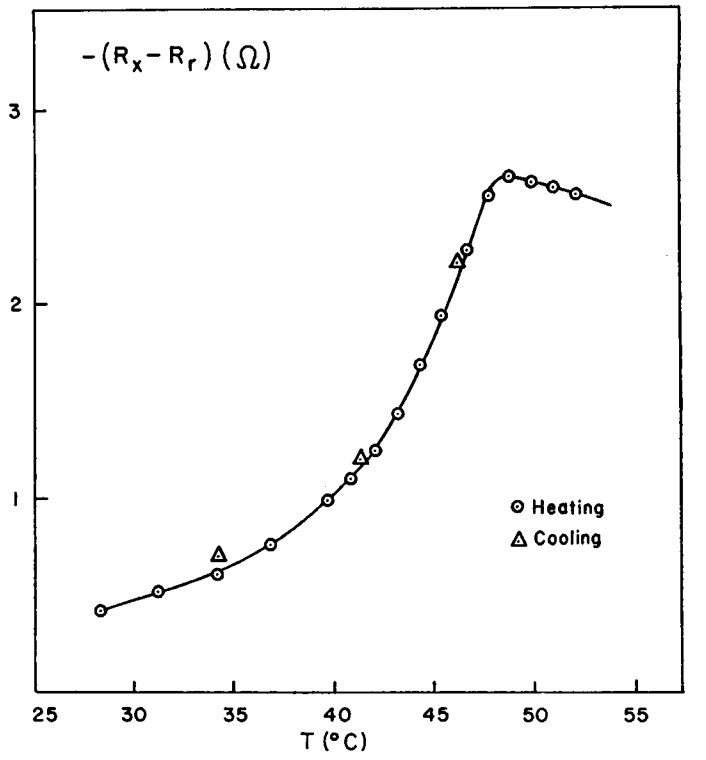

FIGURE 2 Differential resistance between polymer containing cell and salt containing reference cell; [*P*] ~ 0.01 M; [KCl] = 0.05 M.

sample and fixed value of [KCl], the shape of the transition is independent of polymer phosphate concentration. The shape of the $\delta\sigma/\sigma$ vs. T curves for pair A and B lots were congruent, within experimental error, with their respective absorbance (A_{260}) vs. T profiles although the latter curves were taken with [P] ~ 10^{-4} M.

The approximately constant regions of Fig. 3 on either side of the transition (particularly those of curve B) suggest that except for temperatures where large polymer conformational changes are occurring, the *ratio* of polymer mobility to small ion mobility is nearly constant. That is, the effect of temperature on mobility through solvent viscosity is, with small error, the same for both kinds of molecules. This approximation will be used in the following analysis to apply corrections to conductivity data at T_m which are obtained from 0°C polymer/ion mobility ratios.

In analyzing data from Fig. 3 one wishes to interpret the specific conductivity changes in going from helix to coil as changes is bound counterion concentration. This approach has implicit within it an operational definition of polymer-ion binding. That is, those counterions which remain within the polymer domain for times long compared to collisional processes and subsequently migrate with the polymer in

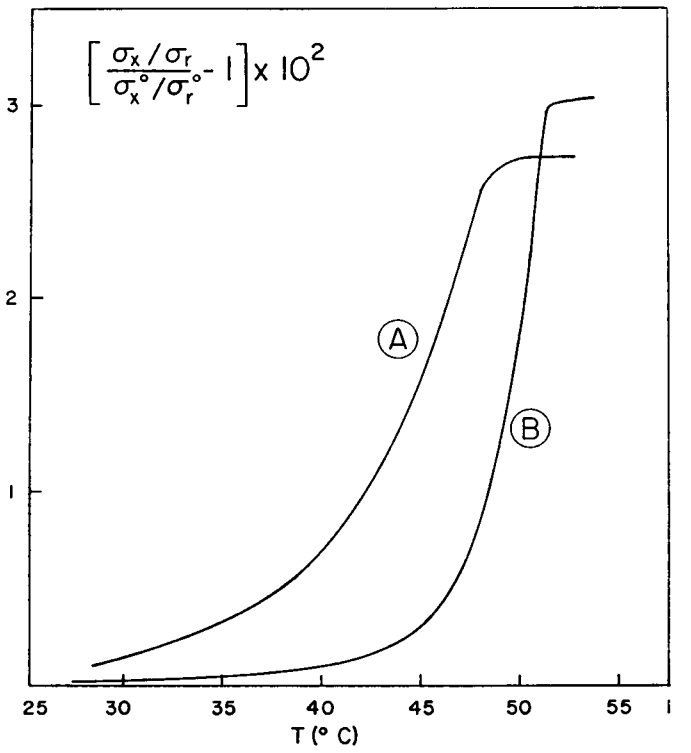

FIGURE 3 Fractional change in specific conductivity of polymer solution with temperature; [KCl] = 0.05 M. (A) Pair A samples of poly-*A* and poly-*U*, [*P*] = 0.011 M. (B) Pair B samples, [*P*] = 0.0118 M.

conductivity or electrophoresis are regarded as bound (Huizenga, Grieger, and Wall, 1950). The following assumptions are made.

a 1 The individual monomeric units of the polymer may be assigned a mobility, frictional coefficient, and charge which are dependent upon conformation but not upon degree of polymerization. This is justified partially by the similar results of pair A and B of Fig. 3 and partially by results obtained in electrophoresis (see below).

a 2 The conventional assumptions are made that the small ion activity coefficients in the presence of the polymer are the same as for a pure KCl solution and that $\langle \lambda_{KCl} \rangle = \lambda_K = \lambda_{Cl}$ (Lyons and Kotin, 1964).

a 3 The charge-carrying population is arbitrarily divided into polymer plus bound counterion, free counterion, and added salt (Wall, Grieger, Huizenga, and Doremus, 1952; Inman and Jordan, 1960). It should be noted that assumptions *a* 2 and *a* 3 are not consistent with the usual definition of the mean stoichiometric activity (Harned and Owen, 1943). For the present work, activity is defined in the following way. If C_K alkali ions are present and a fraction α of them are bound, the

free alkali ion activity is $\lambda_K(1 - \alpha)C_K$ where λ_K is the coefficient for pure KCl solutions at a concentration $(1 - \alpha)C_K$.

Assumption *a* 2 is similar to assumptions which have been termed "non-thermodynamic" (Bates, 1954) and will be discussed in more detail later. Assumption *a* 3 merely implies that the bound fraction of counterion remains in the polymer environs for times long compared to collisional times.

An outline of the analysis will be given here and a more detailed discussion is reserved for the Appendix. Defining i_H, i_C as fractional charges per phosphate for helical and coil segments [where $i_C = (\frac{1}{2})(i_A + i_U)$], the polymer contribution to conductivity in the helix to coil changes is

$$\Delta\sigma_p = [P](i_C - i_H)\bar{\mu}_p . \tag{7}$$

Here the average polymer mobility is $\bar{\mu}_p = \mu_{AU} \cong (\frac{1}{2})(\mu_A + \mu_U)$, a result from electrophoresis. The final analysis will depend on the assumption that $\bar{\mu}_p/\bar{\mu}_+$ is nearly independent of temperature (see above).

The free counterion contribution is

$$\Delta\sigma_+ = [P](i_C - i_H)\bar{\mu}_+ \tag{8}$$

and $\bar{\mu}_+$ is taken, in line with assumption *a* 2, to be that for K^+ in pure KCl solution.

A simplified relation between Equations 7, 8, and 3 can be given which contains the more important parameters:

$$\Delta i = i_C - i_H \approx \frac{2[KCl]}{(1 + \bar{\mu}_p/\bar{\mu}_+)[P]} \left(\frac{\delta\sigma}{\sigma}\right)_{T > T_m} . \tag{9}$$

Equation 9 is accurate to about 20%. For computational purposes Equation A 8 (see Appendix) was used. All measurements for 0.004 M < [P] < 0.005 M and 0.025 M < [KCl] < 0.05 M fell in the range of $\Delta i = 0.22 \pm 0.02$ electron/phosphate ion. With the present definition of bound counterion, Δi is numerically equal to the differential binding in units of K^+ ion/P^- ion.

Exploratory measurements of $(\delta\sigma/\sigma)$ vs. T for Na-DNA have been reported (Felsenfeld, 1962), Using Equation 9 and DNA electrophoretic data (Ross and Scruggs, 1964) the results indicate $\Delta i \sim 0.13$ Na^+ ion/P^- ion.

KCl Activity

Fig. 4 shows typical emf values for the electrochemical cell containing K–poly-A and K–poly-U. The features of curve A are: (*a*) emf in the cell containing poly-U at temperature equilibrium and time zero; (b) slow changes in $\Delta\epsilon$ due to atmospheric effects; auxiliary experiments indicate that most of the slow increase in $\Delta\epsilon$ with time may be accounted for by H_2O evaporation and, hence, increase in KCl concentration; (*c*) addition of poly-A and decrease in $\Delta\epsilon$ due to difference in ion

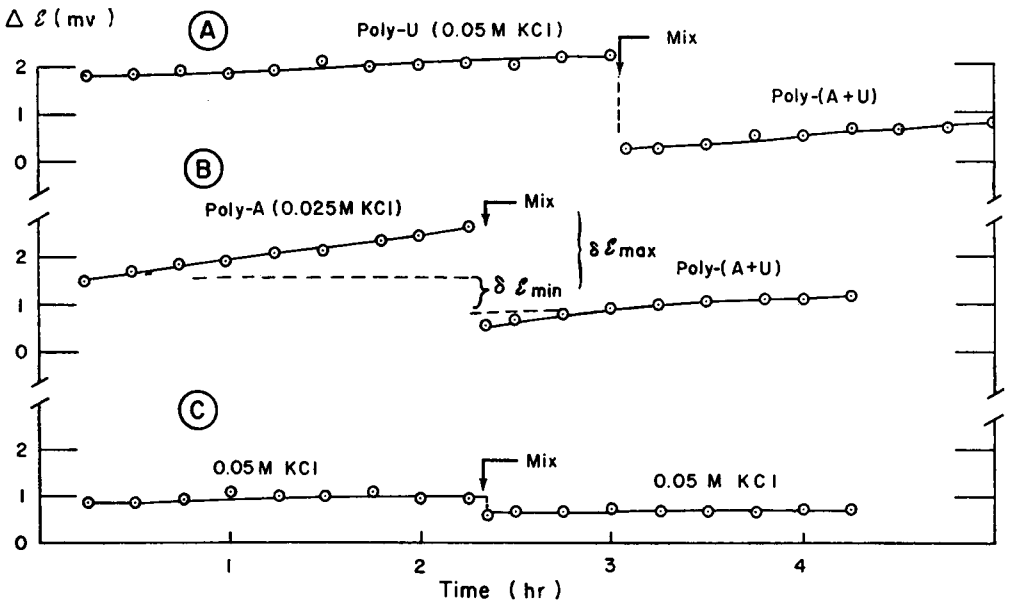

FIGURE 4. Change in electrochemical cell potential with time and with formation of helical *K*-poly-(*A* + *U*) (see text). (A) [*P*] = 0.0195 M. (B) [*P*] = 0.016 M. (C) Blank mixing run with no polymer; $T = 25°C$.

binding from coil to helix. The precision at 0.05 M KCl is considerably less than for conductivity and the measurement requires relatively high phosphate concentrations. At 0.025 M KCl (curve B) the evaporation effects are more pronounced and it is difficult to choose a method for determining $\delta\epsilon$. The curve shows a possible minimum and maximum value. Incidentally, the slow rise after mixing to the equilibrium line in curve B is the result of the long poly-A–poly-U reaction rate to form the helix (Blake and Fresco, 1966). At lower salt and with a controlled atmosphere, the method could be used for kinetic studies.

For analysis the following should be assumed.

b 1 Changes in emf reflect K^+ activity changes and the byion-polymer interaction may be neglected. This is justified by results obtained with other polyelectrolytes (Ascoli, Botré, and Liquori, 1960; Lyons and Kotin, 1964; Rice and Nagasawa, 1961).

b 2 The small ion activity coefficients are the same as for pure KCl solution, $\langle\lambda_{KCl}\rangle = \lambda_K = \lambda_{Cl}$, and activity is defined as before. Although this is formally the same as assumption *a* 2 above, the failure of the assumption will affect this experiment and the conductivity experiment in different ways.

With these conditions Equation 6 may be written

$$\delta\epsilon = k'(\log e)\frac{\delta(C_+ + C_-)}{(C_+ + C_-)} = k' \log e \frac{\delta C_+}{\langle C_+ + C_-\rangle}. \qquad (10)$$

A correction for the contribution to C_+ of free counterion may be made by writing $C_+ + C_- = 2[\mathrm{KCl}] + (\frac{1}{2})[\mathrm{P}](2 - i_C - i_H)$ and one has

$$\frac{\delta C_+}{[\mathrm{P}]} = \frac{\{2[\mathrm{KCl}] + (1/2)[\mathrm{P}](2 - i_C - i_H)\}\delta\epsilon}{k'[\mathrm{P}] \log e}. \qquad (11)$$

Here, as in Equation 9, [KCl] is taken to be the concentration of KCl in the outer dialysis solution during sample preparation and δC_+ is the change in K^+ concentration in the helix-to-coil direction.

Because of the large volumes of high concentration polymer solutions required to cover the electrodes, only a few observations could be made. Measurements were made in the same [P] and [KCl] ranges as for the conductivity runs. The precision was so poor as to require statistical treatment of the results. 10 entries (taking ϵ_{max} and ϵ_{min} as equally probable) averaged without respect to [KCl], [P], or order of mixing gave $\delta C_+/[\mathrm{P}] = +0.24$ K^+ ion/P^- ion with a standard deviation of ± 0.07 K^+ ion/P^- ion.

Two activity experiments with Na-DNA, with no added salt, have been reported in the literature. In the first (Ascoli, Botré, and Liquori, 1960) an electrochemical cell similar to that of the present experiment was used. The emf of the cell containing N-DNA was plotted as a function of temperature to which the DNA had previously been exposed. Using the data for $[\mathrm{P}] \sim 6.5 \times 10^{-3}$ M and Equation 11 above, $\delta C_+/[\mathrm{P}]$ is estimated to be 0.15–0.2 K^+ ions/P^- ion where the difference is between the native and denatured (presumably part coil and part helix) forms.

In the second experiment (Lyons and Kotin, 1964), Na counterion activity was measured in the presence of native and denatured DNA. For $[\mathrm{P}] \sim 3 \times 10^{-3}$ M, $\delta C_+/[\mathrm{P}]$ is estimated to be 0.2 K^+ ion/P^- ion.

Free Boundary Electrophoresis

Briefly, it was found that the ascending boundary was hypersharp and moved faster than the diffuse descending boundary. The computed μ_{asc} was greater than μ_{des} and the difference between them was greater at lower ionic strengths; at fixed ionic strength the difference goes to zero at infinitely dilute polymer concentrations. These phenomena appear to be common in electrophoretic studies of polyelectrolytes (Ross and Scruggs, 1964; Constantino, Liquori, and Vitagliano, 1964) and a preliminary discussion of their probable causes has been given (Rice and Nagasawa, 1961). Although it is likely that the differences ($\mu_{des} - \mu_{asc}$) are due to polymer-polymer interactions it must be borne in mind that artifacts such as ionic conductivity and pH discontinuities at the boundaries may play a role.

We have taken the view (Ross and Scruggs, 1964) that, at high polymer concentrations, the theoretically significant value is μ_{des} and have used it for computational purposes.

As with the KCl activity apparatus, the sample size requirements permitted only a few runs. 15 observations on the three polymers fell in the range $1.15 < \mu_{des} < 1.65 \times$

10^{-4} cm²/v sec (with 0.025 M < [KCl] < 0.2 M). The higher mobilities were associated with the lower ionic strength and conversely. The approximations, $\bar{\mu}_p = \mu_{AU} \cong \mu_A \cong \mu_U$ were good to 5% at each value of ionic strength. For 0.025 M KCl, $\bar{\mu}_p = 1.6 \times 10^{-4}$ cm²/v sec and for 0.5 M KCl, $\bar{\mu}_p = 1.5 \times 10^{-4}$ cm²/v sec.

One may calculate the polymer contribution correction to the ionic conductivity data. Taking $\bar{\mu}_+ = \lambda/F$ where F is the Faraday and λ is the equivalent conductance of K^+ from tables (Harned and Owen, 1943), one has $\bar{\mu}_p/\bar{\mu}_+ = 0.20$ (0.025 M KCl) and 0.21 (0.05 M KCl). Errors in $\bar{\mu}_p$ of 20% lead to errors of only 5% in Δi computed from Equation 9.

In addition to calculation of polymer contributions to conductivity the electrophoretic mobilities lead, through a theoretical model, to another method of determining Δi.

One assumes the following.

c 1 The nucleic acids are free drained in electrophoresis.

c 2 Henry's equation relating the polymer mobility to the zeta potential is applicable:

$$\mu = \frac{\zeta/DC}{\eta}$$

where D and η are the solvent dielectric constant and viscosity and C is a polymer-shape–dependent term (Abramson, Moyer, and Gorin, 1942).

c 3 Gorin's equations for ζ and C are valid.

Assumptions *c* 1 and *c* 2 have been discussed previously (Ross and Scruggs, 1964). Assumption *c* 3 implies that in calculating the electrostatic potential about the polyion, $e\psi$, one may take the linear Debye-Hückel treatment of the Poisson-Boltzmann equation. Analytically this requires $e\psi/kT \ll 1$. Although the linear equation for poly-A, poly-U, and poly-(A + U) gives $e\psi/kT \sim 1$ at the polymer radius, in practice, it turns out that the linear approximation leads to results for ζ which are within a few per cent of those obtained by computer solution of the complete Poisson-Boltzmann equation for $e\psi/kT < 1$ (Kotin and Nagasawa, 1962; Wall and Berkowitz, 1957). The reasons for this fortuitous result appear to be due to cancellation of higher order terms in the Debye-Hückel expansion (Abramson, Moyer, and Gorin, 1942). Additional insight into this problem is given by a discussion of the contrast in polyelectrolyte properties as treated by the Debye-Hückel and McMillan-Mayer theories (Manning and Zimm. 1965).

With these considerations, the unneutralized charge per phosphate is

$$i = \mu\pi F'[\underline{k}(\rho + r_i)]\overline{\Delta l}/4A, \tag{12}$$

where

$$A = K_0[\underline{k}(\rho + r_i)]/\underline{k}(\rho + r_i)K_1[\underline{k}(\rho + r_i)] + \ln\left(\frac{\rho + r_i}{\rho}\right). \tag{13}$$

Here $\underline{k}$ is the characteristic Debye reciprocal radius, ρ is the polymer radius, r_i is the hydrated alkali radius, $\overline{\Delta l}$ is the projected distance along the polymer axis per two phosphate charges. The value of r_i and the shape-dependent functions K_0, K_1, and F' have been tabulated (Abramson, Moyer, and Gorin, 1942).

The polymer radii, and the charge distribution, $\overline{\Delta l}$, were taken from low angle X-ray scattering data (Witz and Luzzati, 1965). For neutral poly-A, $\rho = 6$ A and $\overline{\Delta l} = 7.0$ A/two phosphates; for poly-U, $\rho = 6$ A and $\overline{\Delta l} = 7.8$ A/two phosphates. Similar data is not available for poly-(A + U). X-ray diffraction on fibers of poly-(A + U) (Sasisekharan and Sigler, 1965) and natural two-stranded RNA's (Arnott, Hutchinson, Spenser, Wilkins, Fuller, and Langridge, 1966) indicates that poly-(A + U) is slightly larger than DNA and as an estimate ρ was taken to be 10.5 A with $\overline{\Delta l} = 3.5$ A/2 phosphates. Uncertainties in ρ of 10% lead to 5% uncertainties in i.

Taking $\Delta i = i_{AU} - (\frac{1}{2})(i_A + i_U)$, Equation 12 gives for 0.05 M KCl, $\Delta i = 0.21$ electron/phosphate ion. Here additive errors due to the computation of ζ with the linearized Poisson-Boltzmann equation tend to cancel. The cancellation cannot occur for the results $i_U \cong 0.7$ electron/phosphate ion and $i_A \cong 0.5$ electron/phosphate ion and the values are probably too high.

Preliminary Results for Other Systems

Measurements have been made of the ionic conductivity ratio vs. T profile for the (Mg–poly-A, poly-U; $MgCl_2$)system. Qualitatively they agree with the "phase diagram" (Stevens and Felsenfeld, 1964) for the mixed Na, Mg salt case. For polymer phosphate at 0.1 M and 7×10^{-4} M $MgCl_2$, equimolar mixtures of Mg–poly-A and Mg–poly-U appear to be in the duplex form at room temperature. At a higher temperature the polymer converts to triplex helix with a drop in specific conductivity ratio and at some still higher temperature converts to coil with an increase in specific conductivity ratio. Since no electrophoretic mobility data is yet available for the Mg polymers, the data yield only estimates of the differential Mg binding. For two- to three-strand conversion it gives $\delta C_{++}/[P] \sim -0.01$ Mg^{++}/phosphate and for the three strand-coil transition $\delta C_{++}/[P] \sim 0.02$ Mg^{++}/phosphate. Solutions with initial stoichiometry of Mg–poly-A + 2 Mg–poly-U show only the triplex helix-to-coil transition.

At higher $MgCl_2$ or phosphate concentration, some portion of the polymer is salted out and solutions become turbid. This effect has been reported for DNA (Lyons and Kotin, 1965) and RNA (Millar and Steiner, 1966). The specific conductivity ratio vs. T profiles are still qualitatively the same but the apparent $\delta C_{++}/[P]$ is lower than for nonturbid solutions. Whatever may be the state of the precipitated polymer, it does not transform with temperature in the same way as the polymer in solution.

In preliminary results with K-DNA (0.01 M phosphate and 0.05 M KCl) changes in

specific conductivity ratio vs. T give an estimated $\delta C_{++}/[P] \sim 0.2$ K^+ ion/P^- ion. The uncertainty is set here by the present lack of polymer coil mobility data.

DISCUSSION

Results

It is obvious from the results of Fig. 3 that the portion of the alkali-polymer interaction which is revealed by conductivity experiments is cooperative in nature. Cooperativity here is to be taken in the same sense as that usually applied to the the hydrogen bonding and stacking interactions present in the helix (Hill, 1959; Crothers and Zimm, 1964) which are known to be cooperative. Furthermore, the binding and consequent phosphate charge neutralization are crucial in the formation of the helix. Hence, the nature of the binding may be regarded as established and the absolute magnitudes of the numbers of ions bound are of only secondary importance. A discussion of the accuracy of the experimental determination is important, however, in making plausible the extension of conductivity-electrophoretic methods to more complex binding experiments.

In all experiments in which one attempts to measure the properties of single ionic species in solution there are inherent uncertainties which arise from the fact that a single ion activity has no well-defined thermodynamic significance. Bates (1954) has given an excellent discussion of this point. As an example, consider the determination of hydrogen ion activity by electrochemical means. One may measure an emf which is related to hydrogen ion activity but the theoretical computation usually requires knowledge of a diffusion potential at a liquid junction which may neither be directly measured nor precisely computed. A reasonable nonthermodynamic assumption is made concerning the diffusion potential and auxiliary experiments are then performed to check the error in pH which is introduced by the assumption. Recently it has been suggested that the single ion activity be regarded as defined by the combined measurement plus the assumptions (Frank, 1963). From this standpoint, the single ion activity is a useful theoretical concept and its determination then explicitly depends upon the subtlety and precision of the experimental estimation of the diffusion potential.

In the computations above leading to Equation 9, the effect of polymer conformation on the mobilities of the ions of the added salt was neglected. The differential conductivity term here is (see Appendix)

$$\Delta\sigma_{KCl} \cong [KCl](\mu_+^H - \mu_+^C + \mu_-^H - \mu_-^C). \quad (14)$$

If assumption *a* 2 is true this term should be zero and Equation 9 is valid. Rather than attempt to measure $\Delta\sigma_{KCl}$, we have chosen to evaluate Δi in two other ways each of which has a different nonthermodynamic aspect.

In the KCl activity measurement in the presence of polymer helix and coil, the

uncertain quantity is the diffusion potential, ΔE_{diff}, at the liquid junction of the electrochemical cell. It may not be calculated exactly but an expression which indicates the form of the diffusion potential has been given (Milazzo, 1963). The boundary was assumed to be an infinite plane between two solutions of salts of different activity and whose ions are univalent. One has for the present experiment

$$\Delta E_{diff} = A''(\mu_+^H - \mu_-^H) - B''(\mu_+^C - \mu_-^C). \quad (15)$$

Here A'' and B'' are constant for any given experiment and depend upon the geometry of the reference electrode, the Nernst constant, and the logarithm of the ratio of the mean KCl activity inside and outside the reference electrode.

If assumption *b* 2 is true then $\Delta E_{diff} = 0$ (Equation 14). Although this kind of assumption is conventionally used for small ion solutions (Bates, 1954) it should be noted that it has not been completely investigated for polyelectrolyte solutions (Ise and Okubo, 1966).

Finally, the calculation of Δi from electrophoretic mobility and polyelectrolyte theory has as uncertainties the approximations mentioned above and the statistical mechanical assumptions implicit in the Poisson-Boltzmann equation.

Within the limitations set forth in the Results section, the magnitude of differential ion binding from helix to coil was found to be the same for the three methods of determination. Because of their diverse origin it seems likely that the uncertainties just discussed lead to relatively small contributions and that the possible mischance of their being large and of equal magnitude can be ignored.

Theoretical Considerations

In mathematical treatments of polyelectrolytes, which do not possess highly ordered internal structure, two points of view have developed. In the "site binding" model the ion-polymer interaction is taken to be similar to that in simple weak electrolytes. The "ion atmosphere" model which was used (Wall et al., 1952) to explain results obtained with polyacrylates appears to be also suitable for DNA (Inman and Jordan, 1960). The analysis of the poly-(A + U) data is consistent with this model. A detailed discussion and comparison of the two models has been given elsewhere (Katchalsky, Alexandrowicz, and Kedem, 1964). In their primitive forms, neither model accounts for possible polymer conformational changes and, perhaps because of this, neither is completely successful even for slightly ordered polyelectrolytes.

At present there exists no general theoretical method of characterizing cooperative binding of small ions or molecules to nucleic acids. However, there have been a number of specialized calculations in the literature which indicate the way a theory might develop. Only an outline of such a program can be given at present:

I. Assume the polyelectrolyte to be a separate phase containing polymer, bound ions, and water. The definition of binding is the pragmatic one used in this work.

At fixed and well-defined polymer conformation the polymer phase is in thermodynamic equilibrium with the solvent and secondary solutes and the activities for all species in the two phases are defined. An extensive discussion of the thermodynamics of such systems has been given (Hill, 1963, 1964). For temperatures or solvent compositions which place the polymer in or near a helix-coil transition, the polymer must be considered in a complex state in that helix and coil forms may coexist in equilibrium with each other and with the solvent phase. In this case the concept of a polymer-ion equilibrium constant, which is of central importance to the site binding model, is meaningless.

II. Upon recognizing the inadequacy of thermodynamics, one might introduce statistical mechanical considerations to account for the conformational changes.

Parts I and II of the program are in accord with earlier theories for slightly ordered polyelectrolytes. Lifson's (1957) titration curve calculation represents the polymer with an Ising model whose statistical states are the enumeration of all possible ways of distributing charge. Polymer conformation in this case is formally accounted for by the fact that different degrees of binding are reflected as shifts in the statistical distribution.

Hill (1963, 1964) has discussed the equilibrium between two isomeric states of a macromolecule. The results are dependent upon degree of polymerization and are not strictly applicable to the K–poly-A, poly-U case. However, a short digression will be made to present Hill's preliminary results since they lead to a more formal definition of cooperativity in binding.

Consider a polymer of N segments with a fraction of them, θ, in the helical conformation. Usually it is assumed that $\theta \equiv H$. Assume that in the transition region all aspects of a *segment* in the helical (or coil) form, including stacking, hydrogen and ion binding, etc., are indistinguishable from its properties when the *whole* polymer is in helical (or coil) form. One may write a constant $K' = \bar{\theta}/(1 - \bar{\theta})$ and using Hill's results obtain:

$$\partial \ln K'/\partial(1/T) = \frac{\Delta H \cdot N}{R}\left[\frac{(\overline{\theta^2} - \bar{\theta}^2)}{\bar{\theta}(1 - \bar{\theta})}\right] \qquad (16)$$

At constant temperature and composition θ has an average, $\bar{\theta}$, and the heat for the complete transition is ΔH, which can be calorimetrically measured. Thermal profiles of either conductivity or hypochromism may be used for experimental values of $\bar{\theta}$ and hence K'. Without cooperativity, K' would be a mass action equilibrium constant and Equation 16 would be the slope of a van't Hoff plot with the square-bracketed term equal to unity. In cooperative processes, the statistical fluctuations in the bracketed term are large and temperature dependent. Experimentally (using data from Fig. 3 B) the term is of the order of 10 in the transition region.

The data in the Results section are sufficient to construct a thermal profile of $K^+ - P^-$ binding. An analysis similar to that just presented shows that the statistical

fluctuations are temperature dependent and that the expression corresponding to the bracketed term in Equation 16 is greater than unity. The latter conclusion is reached by observing that a computed "heat of binding" (with the bracketed term unity) has a maximum value at T_m of 50 kcal/mole.

Returning to the problem of finding a polymer model, it appears that Lifson's or Hill's model is not complex enough for the K–poly-A, poly-U case. Insight as to the additional requirements for the model is provided by a calculation of the hydrogen ion titration of a duplex polymer (Steiner, 1960). All interactions except that of proton binding were included in a basic Ising model similar to Hill's (Hill, 1959) and one binding site per nucleotide pair was assumed. This introduced the same two parameters which were used to characterize the thermal transition (Hill, 1959; Crothers and Zimm, 1964). Partition functions for protons bound to helical segments were taken to be different from those for protons bound to coil segments. A weighting term was explicitly introduced into these functions to express the probability that the binding of a proton to a segment changes the segment from coil to helical form or vice versa (in Steiner's notation, a hydrogen bond pair is formed or broken). These provisions appear to be the key assumptions which permit the model to exhibit cooperative binding.

In this connection, it is to be noted that no direct discussion of cooperativity was given in the original paper (Steiner, 1960). The prime concern there was to investigate the range of parameters which would cause sharpening of the curves of fraction of bound sites vs. hydrogen ion activity. "Sharpening" may be taken to mean that the binding curve is completed over a hydrogen ion activity range which is narrow compared to the range predicted by a Langmuir isotherm. Since the Langmuir isotherm is a consequence of the mass action law, this definition of cooperativity seems compatible with that derived from Equation 16.

The model is mathematically complicated but some general conclusions could be drawn about parameter values which permit sharpening of titration curves. The parameters characterizing hydrogen bonding and base stacking had the same restrictions as those in the basic model for description of the thermal profile. That is (in appropriate solvent composition regions) the binding model must exhibit a sharp hypochromism-thermal profile for fixed composition. It was further found that the ratio λ''/λ' must be small compared to unity. Here $\lambda'' = \lambda X_r$, $\lambda' = \lambda X_h$ with X_h, X_r being respectively partition functions for protons bound to helical and coil segments and λ is the absolute hydrogen ion activity. The ratio could be made small by requiring the proton interaction be preferentially strong to a helical (or coil) segment and/or by assuming that proton binding produces a high probability of changing the conformation of the segment.

With the following reservations, the model appears suitable for an initial description of a cooperative, alkali-ion binding curve. (*a*) The references to a definite binding site are meaningless and must be deleted. In this case the λ'', λ' corresponding to

binding of the "long dwell time" portion of the counterion population may be related to activities of ions in the polymer "phase" (Lifson, 1957; Hill, 1963, 1964). (*b*) Account must be made for the "short dwell time" (i.e. unbound) counterions. (*c*) The interpretation of hydrogen bonding and stacking parameters must be reconsidered since, in the basic models, they are taken to be phenomenological functions of alkali ionic strength.

With these restrictions it appears that heats of hydrogen bonding, ion binding, and base stacking could be realistically determined and that the program for derivation of base sequences (Montroll and Goel, 1966) could be carried out.

Comments and Implications

A number of measurements have been made of the rate of reaction of poly-A + poly-U to form poly-(A + U) (Ross and Sturtevant, 1962; Blake and Fresco, 1966; Jakabhazy and Fleming, 1966). Where the final composition and temperature are remote from a helix-coil region, experiments give fairly good agreement with a bimolecular reaction model. The reason for this rather surprising result is not obvious at present. For cases where final composition and temperature are in or near a transition zone, the data are fitted very poorly by mass action theory.

Jakabhazy and Fleming (1966) have found that a plot of initial reaction rate of (Mg–poly-A)–(Mg–poly-U) vs. pH exhibits a sigmodal shape near the pK of poly-A. It is nearly congruent with the (Mg–poly-A conformation)-pH profile as determined by specific Kerr constant and presumably would be accurately congruent with a (Mg–poly-(A + U) conformation)-pH profile.

In this laboratory, crude rate measurements have been made for K–poly-A + K–poly-U in the region near T_m. Separate samples were heated in a temperature-controlled observation compartment of a Beckman DU (Beckman Instruments, Inc.). They were then rapidly mixed and returned to the instrument and the reaction was followed by observing A_{260} vs. time. Defining the rate for helix formation as the reciprocal of a $1/e$ time constant, the rate follows the conformation-temperature profile. It is immeasurably long (>10 min) at $T = T_m - 2°C$ and immeasurably short (<1 min) at $T = T_m + 2°C$.

For these transition zone experiments, the forward rate may be expressed phenomenologically as a linear function of $(\theta_f - \theta_i)$, the difference between the helical content of the final and initial equilibrium states. Obviously a cooperative theory for the kinetics of nucleic acid reactions must be developed. For polypeptide helix-coil kinetics this has been done by Schwartz (1965).

Wang (1955) has found that helical DNA is hydrated to the extent of about 10 molecules of H_2O per nucleotide pair. In view of the results of recent studies of "hydrophobic bonds" (Némethy and Scheraga, 1962) and work indicating preferential hydration of helical DNA in trifluoroacetate solutions (Tunis and Hearst, 1968) it is likely that the hydration is different for the coil form. Work is in progress in this laboratory to see if this is indeed true.

The dye, acridine orange, binds differently with native and denatured DNA (Steiner and Beers, 1961). It is likely that many of the apparently anomalous results obtained with dye-nucleic acid solutions can be understood if the binding process were to be considered as cooperative in nature.

Recent hypochromism (A_{260}) studies of phage DNA have led to the conclusion that DNA in vivo has a helical content which is intermediate in value to the helical content of the in vitro coil and helical forms (Tikchonenko, Dobrov, Velikodvorskaya, and Kisseleva, 1966). One may speculate that a state of lowered helical content would facilitate replication and that protein and ion binding which favors the coil form in vitro, may be an important part of the mechanism for controlling that state in phage.

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APPENDIX

Contributions to the ionic conductivity *difference* from helix to coil for polymer, free counterion, and added salt are

$$\Delta\sigma_p = -[\mathrm{P}](\mu_{\mathrm{AU}} i_{\mathrm{AU}} - (\tfrac{1}{2})(\mu_{\mathrm{A}} i_{\mathrm{A}} + \mu_{\mathrm{U}} i_{\mathrm{U}})) \tag{A 1}$$

$$\Delta\sigma_+ = [\mathrm{P}](\mu_+^{\mathrm{AU}}(1 - i_{\mathrm{AU}}) - (\tfrac{1}{2})[\mu_+^{\mathrm{A}}(1 - i_{\mathrm{A}}) + \mu_+^{\mathrm{U}}(1 - i_{\mathrm{U}})]) \tag{A 2}$$

$$\Delta\sigma_{\mathrm{KCl}} = [\mathrm{KCl}]((\mu_+^{\mathrm{AU}} + \mu^{\mathrm{AU}}) - (\tfrac{1}{2})[(\mu_+^{\mathrm{A}} + \mu_-^{\mathrm{A}}) + (\mu_+^{\mathrm{U}} + \mu_-^{\mathrm{U}})]). \tag{A 3}$$

Implicit in these equations is the electrophoresis result (Ross and Scruggs, 1964) that a monomer unit may be assigned a charge and frictional coefficient. Explicit recognition, in superscripts, is given to the possibility that polymer-ion collisions may affect small ion mobilities. Concentration dependence of small ion mobility will be introduced later.

The electrophoresis results give $\mu_{\mathrm{AU}} \cong (\tfrac{1}{2})(\mu_{\mathrm{A}} + \mu_{\mathrm{U}}) = \bar{\mu}_P$ and $i_{\mathrm{C}} = i_{\mathrm{A}} \cong i_{\mathrm{U}}$. Assumption *a* 3 of the Results section implies $\mu_\pm^{\mathrm{AU}} = \mu_\pm^{\mathrm{A}} = \mu_\pm^{\mathrm{U}} = \bar{\mu}_\pm$ and

$$\Delta\sigma_p \cong -[\mathrm{P}]\bar{\mu}_p(i_{\mathrm{H}} - i_{\mathrm{C}}) \tag{A 4}$$

$$\Delta\sigma_+ \cong [\mathrm{P}]\bar{\mu}_+(i_{\mathrm{C}} - i_{\mathrm{H}}) \tag{A 5}$$

$$\Delta\sigma_{\mathrm{KCl}} = 0. \tag{A 6}$$

The average conductivity near T_m is

$$\sigma = \langle\sigma_p + \sigma_+ + \sigma_{\mathrm{KCl}}\rangle$$

$$\cong \bar{\mu}_+\{2[\mathrm{KCl}] + \frac{[\mathrm{P}]}{2}(2 - (1 + \bar{\mu}_p/\bar{\mu}_+)(i_{\mathrm{H}} + i_{\mathrm{C}}))\}. \tag{A 7}$$

A fractional *difference* $\Delta\sigma/\sigma$ may be computed from Equations A 4–A 7. For a fractional *differential*, $\delta\sigma/\sigma$, account should be made of the nonlinear relationship between specific conductivity and ion concentration: $\sigma \cong AC + BC^{(1/2)}$ (Harned and Owen, 1943). Since the polymer contribution to Equation A 7 is small one may correct for nonlinearity by introducing a term $\beta \cong 0.9$ which may be computed from KCl conductivity tables.

$$\delta\sigma/\sigma \cong \beta[\mathrm{P}](1 + \bar{\mu}_p/\bar{\mu}_+)(i_\mathrm{C} - i_\mathrm{H}) \Big/ \Big\{2[\mathrm{KCl}] + \frac{[\mathrm{P}]}{2}(2 - (1 + \bar{\mu}_p/\bar{\mu}_+)(i_\mathrm{H} + i_\mathrm{C}))\Big\}. \quad (\mathrm{A}\,8)$$

With a maximum error of 20%, one has for simplicity

$$\delta\sigma/\sigma \approx \frac{[\mathrm{P}](1 + \mu_p/\mu_+)(i_\mathrm{C} - i_\mathrm{H})}{2[\mathrm{KCl}]}, \quad (\mathrm{A}\,9)$$

and

$$\Delta i = i_\mathrm{C} - i_\mathrm{H} \approx \frac{2[\mathrm{KCl}](\delta\sigma/\sigma)_{T>T_m}}{(1 + \mu_p/\mu_+)[\mathrm{P}]}. \quad (\mathrm{A}\,10)$$

REFERENCES

ABRAMSON, H. A., L. S. MOYER, and M. H. GORIN. 1942. *In* Electrophoresis of Proteins. Reinhold Publishing Corp., New York. 125.

ARNOTT, S., F. HUTCHINSON, M. SPENSER, M. H. F. WILKINS, W. FULLER, and R. LANGRIDGE. 1966. *Nature (London)*. **211**:227.

ASCOLI, F., C. BOTRÉ, and A. M. LIQUORI. 1960. *J. Mol. Biol.* **3**:202.

BATES, R. G. 1954. *In* Electrometric pH Determinations. John Wiley and Sons, Inc., New York. Chap. 3.

BLAKE, R. D., and J. R. FRESCO. 1966. *J. Mol. Biol.* **19**:145.

CONSTANTINO, L., A. M. LIQUORI, and V. VITAGLIANO. 1964. *Biopolymers.* **2**:1.

CROTHERS, D. M., and B. H. ZIMM. 1964. *J. Mol. Biol.* **9**:1.

FELSENFELD, G. 1962. *In* The Molecular Basis of Neoplasia. University of Texas Press, Austin. 104.

FELSENFELD, G., and S. HUANG. 1960. *Biochim. Biophys. Acta.* **37**:425.

FELSENFELD, G., and S. L. HUANG. 1961. *Biochim. Biophys. Acta.* **51**:19.

FELSENFELD, G., and H. T. Miles. 1967. Annual Review of Biochemistry. P. D. Boyer, editor. Annual Reviews, Inc., Palo Alto.

FRANK, H. S. 1963. *J. Phys. Chem.* **67**:1554.

HARNED, H. S., and B. B. OWEN. 1943. *In* The Physical Chemistry of Electrolytic Solutions. Reinhold Publishing Corp., New York. Chap. 1.

HILL, T. 1959. *J. Chem. Phys.* **30**:383.

HILL, T. L. 1963. *In* Thermodynamics of Small Systems. W. A. Benjamin, Inc., New York. Part I. 104.

HILL, T. L. 1964. *In* Thermodynamics of Small Systems. W. A. Benjamin, Inc., New York. Part II. 39.

HUIZENGA, J. R., P. F. GREIGER, and F. T. WALL. 1950. *J. Amer. Chem. Soc.* **72**:2636.

INMAN, R. B., and D. O. JORDAN. 1960. *Biochim. Biophys. Acta.* **42**:421.

ISE, N., and T. OKUBO. 1966. *J. Phys. Chem.* **70**:3025.

JAKABHAZY, S. Z., and S. W. FLEMING. 1966. *Biopolymers.* **4**:793.

KATCHALSKY, A., Z. ALEXANDROWICZ, and O. KEDEM. 1964. *In* Chemical Physics of Ionics. Solution B. E. Conway, and R. G. Barradas, editors. John Wiley and Sons, Inc., New York. 295.
KOTIN, L., and M. NAGASAWA. 1962. *J. Chem. Phys.* **36**:873.
LIFSON, S. 1957. *J. Chem. Phys.* **26**:727.
LYONS, J. W., and L. KOTIN. 1964. *J. Amer. Chem. Soc.* **86**:3634.
LYONS, J. W., and L. KOTIN. 1965. *J. Amer. Chem. Soc.* **87**:1781.
MANNING, G. S., and B. H. ZIMM. 1965. *J. Chem. Phys.* **43**:4250.
MILAZZO, G. 1963. *In* Electrochemistry. N. V. Uitgevers Mij. Elsevier, Amsterdam. [1st English edition.] 138.
MILLAR, D. B., and R. F. STEINER. 1966. *Biochemistry.* **5**:2289.
MONTROLL, E. W., and N. S. GOEL. 1966. *Biopolymers* **4**:855.
NÉMETHY, G., and H. A. SCHERAGA. 1962. *J. Phys. Chem.* **66**:773.
RICE, S. A., and M. NAGASAWA. 1961. *In* Polyelectrolyte Solutions. Academic Press, Inc., New York. Chap. 9.
RICH, A., and I. TINOCO. 1960. *J. Amer. Chem. Soc.* **82**:6409.
ROSS, P. D., and J. M. STURTEVANT. 1962. *J. Amer. Chem. Soc.* **84**:4503.
ROSS, P. D., and R. L. SCRUGGS. 1964. *Biopolymers.* **2**:79, **9**:234.
SASISEKHARAN, V., and P. B. SIGLER. 1965. *J. Mol. Biol.* **12**:296.
SCHWARTZ, G. 1965. *J. Mol. Biol.* **11**:64.
SHACK, J., R. J. JENKINS, and J. M. THOMPSETT. 1952. *J. Biol. Chem.* **198**:85.
SHEDLOVSKY, T. 1946. *In* Physical Methods of Organic Chemistry. Interscience Publishers, Inc., New York. **2**: Chap. XXI.
STEINER, R. F. 1960. *J. Chem. Phys.* **32**:215.
STEINER, R. F., and R. F. BEERS. 1961. *In* Polynucleotides. N. V. Uitgevers Mij. Elsevier, Amsterdam. Chap. 10.
STEVENS, C. L., and G. FELSENFELD. 1964. *Biopolymers.* **2**:293.
TIKCHONENKO, T. I., E. N. DOBROV, G. A. VELIKODVORSKAYA, and N. P. KISSELEVA. 1966. *J. Mol. Biol.* **18**:58.
TUNIS, M. B., and J. E. HEARST. 1968. *Biopolymers.* **6**:1325.
VENNER, H., and C. ZIMMER. 1966. *Biopolymers.* **4**:321.
WALL, F. T., and J. BERKOWITZ. 1957. *J. Chem. Phys.* **26**:114.
WALL, F. T., P. F. GRIEGER, J. R. HUIZENGA, and R. H. DOREMUS. 1952. *J. Chem. Phys.* **20**:1206.
WANG, J. H., 1955. *J. Amer. Chem. Soc.* **77**:258.
WITZ, J., and V. LUZZATI. 1965. *J. Mol. Biol.* **11**:620.