Tritium–Hydrogen Exchange Studies of Polynucleotides. Double-Stranded Polyriboadenylic Acid*

Morton P. Printz

ABSTRACT: The tritium–hydrogen exchange of polyriboadenylic acid was studied as a function of the pH, sodium ion concentration, temperature, and method of preparation of the helical form. Poly A is known to form a double-stranded helix upon protonation of the bases. The helical form contains 3.7–4 measurable, exchangeable hydrogens per nucleotide pair. This value agrees with the number of hydrogen-bonded hydrogens in the model of Rich *et al.* which was based upon X-ray diffraction studies of fibers. The exchange data offer support for the existence, in aqueous solution, of the hydrogen bonding pattern proposed for that model. Protonation, which in the Rich *et al.* model results in the formation of an interstrand salt bond, is important in stabilizing the structure. Exchange can only be measured when approximately 40–45% of the bases are protonated.

This level of protonation is a lower limit for a stable structure. When there is *less* than one protonated base/base pair, all the hydrogens exchange with the same rate constant and the exchange rate is markedly dependent upon the sodium ion concentration. Increasing the salt concentration results in an increased exchange rate. When the fraction of bases protonated is *greater* than 0.5, salt does not affect the kinetics of exchange, provided the helix is formed under conditions which minimize aggregation. The observed exchange rate constant is clearly of the Kk_3 type, that is, the rate is controlled both by the chemical exchange step (k_3) and by structural factors (K_L). The chemical exchange exhibits specific base catalysis. Therefore, the data presented in this paper are direct evidence for both Kk_3 limited exchange and for specific acid–base catalysis in nucleic acids. K_L, the equilibrium constant for hydrogen bond labilization, is pH dependent when there is less than one protonated base/base pair, but pH invariant when there is more than one. The observation of a pH dependence for K_L is significant with respect to the use of hydrogen exchange for the dynamic structure of natural nucleic acids since it shows that K_L may be environment sensitive.

Hydrogen exchange is a potentially useful probe of the elements of the dynamic conformation of proteins and nucleic acids. In previous publications we demonstrated that proton exchange measurements can easily be obtained with structured nucleic acids, and that exchange rates for helical DNA are in the time range of minutes (Printz and von Hippel, 1965, 1968). It was inferred from these studies that a prime requisite for measurement of exchange (on this time scale) was the involvement of the labeled hydrogen in a hydrogen bond. This correlation was based on the total number of measurable, exchangeable hydrogens in native calf thymus DNA compared with renatured (heat-denatured) DNA, on the results with poly A at neutral pH, and from preliminary measurements on helical polynucleotides (Printz and Trout, 1968). In addition, we concluded from the results with calf thymus DNA that the variation of the exchange kinetics with pH correlated with the fractional extent of titration of the bases and that the exchange mechanism was of the Kk_3 type.

To gain further understanding of the mechanism(s) of exchange of nucleic acids, we undertook an intensive study of the tritium–hydrogen exchange of helical homopolynucleotides. In this paper we report on our studies of the acid form of poly A. We previously had reported that poly A contained four measurable hydrogens when in the double-stranded form (von Hippel and Printz, 1965). Other exchange studies of poly A were mainly concerned with measuring the exchange of the C-8 proton (Shelton and Clark, 1967; Shelton, 1968; Maslova *et al.*, 1969). This hydrogen exchanges very slowly with a half-life of days at pH's below 7 and room temperature.

Poly A forms an ordered structure which is a function of the temperature, pH, and ionic strength (Steiner and Beers, 1957, 1959; Fresco and Doty, 1957; Ts'o *et al.*, 1962b). This structure has characteristics that have been ascribed to double-stranded helical polynucleotides. A model based on X-ray diffraction data was postulated and consists of a double-stranded, parallel chains structure with four hydrogen bonds per base pair (Rich *et al.*, 1961). In addition, two ionic bonds/base pair were postulated to explain the pH dependence of helix formation.

In studying this (and subsequent) polynucleotides, we are concerned with answering the following questions. What is the contribution of the various conformational restraints to limiting the exchange rate (*i.e.*, hydrogen bonding, intra- and intermolecular association, solvent structure, etc.)? Is there specific catalysis of the exchange by hydrogen and/or hydroxyl ion, and can we deduce the exchange mechanism? Finally, can we define the structural effects which limit the exchange rates in nucleic acids? We show that the acid form of poly A is uniquely suited to contributing answers to these questions. The number of measurable hydrogens was found to agree with that predicted on the basis of the Rich *et al.* model. Thus we confirm the hydrogen

* Contribution from The Rockefeller University, New York, New York 10021. *Received November 26, 1969.* This work was supported in part by U. S. Public Health Service Grant No. A. M. 02493.

bonding pattern for that model and show that it exists in aqueous solution. Both the analysis of the kinetics and the agreement between the predicted and observed numbers of hydrogens reinforce the conclusion that hydrogen bonding in native nucleic acids is essential for measuring exchange rates in seconds or minutes. A mechanism of exchange is deduced and shown to exhibit both conformational restraints and specific base catalysis. It is shown that the conformational constraint on the exchange rate is pH dependent when the fraction of bases titrated is <0.5. Presumably, this is a reflection of "long-range" cooperativity in the titration of the base pairs.

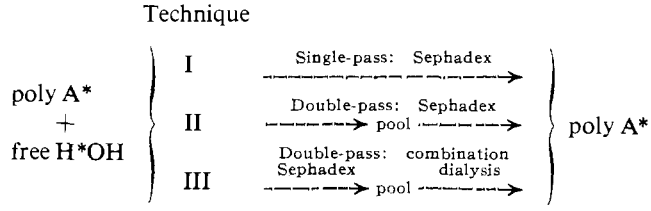
Experimental Section

Materials and Methods. All polynucleotides were obtained from Miles Laboratories (Elkhart, Ind.). Solutions were prepared by dissolving the fibrous material and dialyzing it against 0.001 M EDTA buffer (neutral pH) to remove divalent metal ion. For the tritium exchange separations it was necessary to sonicate the commercial polymer preparations in order to lower the molecular weight and thus reduce the viscosity of solutions of the double-stranded form. Because sonication must be done when the polymer is helical, all preparations were first dialyzed to low pH (pH 5, 0.1 or 0.05 M Na^+) and then sheared as described previously (Printz and von Hippel, 1965). The samples were then dialyzed against EDTA to remove metal contaminants which might have been introduced by the sonicator probe. Finally, the samples were dialyzed to the desired solvent conditions as described in the Results.

Polymer concentrations were determined spectrophotometrically (with a Zeiss PMQII) using as a reference a base molar extinction of 10,500 at 257 nm in a pH 7.5, 0.1 M sodium ion buffer (Ts'o *et al.*, 1962a). Concentrations at all acid pH's were determined from the absorbance at 251 nm. All chemicals were reagent grade, and aqueous solutions were prepared using water that was distilled and then deionized with a mixed bed resin. Only Visking dialysis tubing was used. Membranes were cleaned by first soaking them in three changes of distilled water, then washing them (with stirring) in three changes of 1% EDTA–pH 7 buffer. Finally, they were extensively washed with deionized water and stored at 2°.

Melting curves were obtained with a Gilford Model 2000 automatic spectrophotometer and accessory bath equipment. The solutions were degassed with purified helium and sealed in cuvets with silicone rubber. No corrections were made for thermal expansion.

Tritium–Hydrogen Exchange. The tritium–hydrogen exchange experiments were done essentially as described previously (Englander, 1963; Printz and von Hippel, 1968). We always measure the rate of back-exchange, *i.e.*, the rate with which "labeled" hydrogens exchange off the polynucleotide. Thus the first step in an experiment consisted of equilibrating a sample with 8–10 mCi/ml of tritiated water (specific activity of 1000 mCi/ml, New England Nuclear Corp.). Equilibration generally took place at 0° in order to minimize the exchange at the C-8 position as well as the possibility of depurination. The back-exchange was begun by reducing the free tritium concentration by a factor of 10^3–10^5. In order to measure bound hydrogen (10^{-4} M) in the great excess (10^2 M) of hydrogen present in water, a total reduction in free tritium of 10^8 is necessary. The basic experimental problem then is one of achieving "perfect" separation. A variety of techniques have become available for this purpose, and those used in the experiments described in this paper are diagrammed below.



In technique I, used only for early time points, the complete separation is done in one Sephadex column. Variable time points are obtained by stopping the flow after the polynucleotide has moved approximately one-fourth the distance through the column. Techniques II and III were those customarily used since less stringent separation is required on each pass. In both techniques, the experiment was started by quickly passing 0.4–0.6 ml of the tritiated sample through a Sephadex column (at 0°). In passage through the first column the sample was diluted by a factor of 3–5. The bulk of the effluent, known as the pool, was collected, quickly vortexed, and maintained at 0° unless otherwise stated. Sampling of the pool took place at various times, depending upon the number of points desired, the exchange kinetics, and the method of separation to be used. The exchanged tritium was removed either by a Sephadex column (technique II) or by continuous dialysis in a slightly modified dialyzer (Englander and Crowe, 1965; technique III). With the latter, dialysis took place in an ice-jacketed cylinder with a ratio of outside:inside volumes of approximately 600. The outside volume was sampled and quantitatively analyzed for free tritium during the experiment, and corrections were applied to the counts of the inside solution when necessary. We found that the dialyzer was inefficient for a complete experiment and required a first step gel filtration. All these techniques gave equivalent results.

In order to evaluate exchange results quantitatively, data must be obtained under rigorously controlled conditions of temperature and pH. To this end, and to reduce the amount of material used for each experimental point, 1-cm diameter glass columns were used for all Sephadex separations. These columns had outer jackets, open at the top, which were filled with ground ice to maintain the temperature of the gel at 0°. The temperature was frequently monitored with a thermistor–bridge circuit having a sensitivity of 0.1°. During a complete exchange experiment the temperature was controlled so that it was never higher than 0.3–0.5°.

Solutions were analyzed for radioactivity and polynucleotide concentration as reported previously (Printz and von Hippel, 1968). No evidence was found for quenching of the counting rate by the presence of the polymer. Since the molarity of hydrogen in water is in great excess over that on the polymer, the exchange follows first-order kinetics. Thus, the data are plotted on a semilog scale and expressed as the number of hydrogens per nucleotide pair (H/np) remaining unexchanged at time t. In general, the average

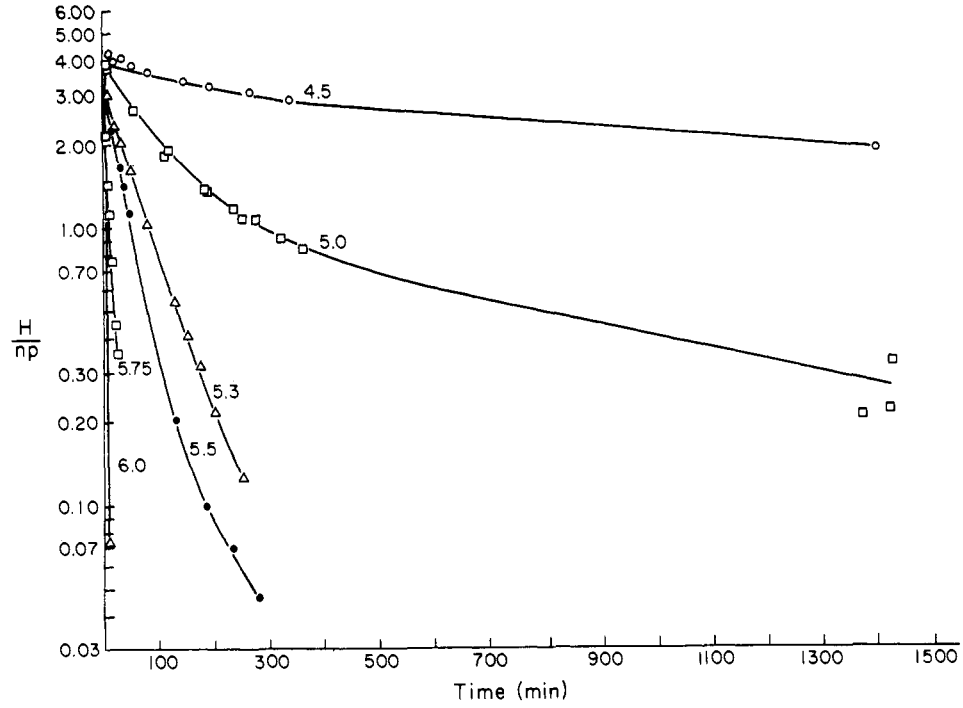


FIGURE 1: Tritium–hydrogen exchange curves as a function of pH at 0°. The numbers next to each curve indicate the pH of the back-exchange. The solvent consisted of 0.1 M NaCl, 0.01 M NaAc, and 10^{-4} M EDTA.

error of a measurement is 5%, the exceptions occurring at very low numbers of hydrogens remaining (*e.g.*, 0.1–0.2 H/np) or for points at times earlier than 50 sec. In these extremes a 10% error should be assumed, although in fact a much smaller error may be present. In all the calculations we assume the absence of an equilibrium isotope effect. Justification for this assumption comes from previous studies on DNA and tRNA (Printz and von Hippel, 1965; Englander and Englander, 1965).

Results

Variation of pH. The pH dependence of the exchange was studied at constant sodium ion concentration using a single preparation of poly A. The sample was prepared by dialyzing a sonicated, neutral pH solution to pH 5.0 (0.05 M NaCl, 0.01 M NaAc, 10^{-4} M EDTA)[1] and tritiating the bulk of the sample. Experiments at various pH's were accomplished by changing the pH of the exchanging sample in passage through the first Sephadex column. Thus, exchange took place at the pH of the buffer in the first-pass separation. The pH jump took place over a time scale of 15–20 sec and at least an additional 400 sec elapsed before the first second-pass measurement. Thus, we assumed that any relaxation processes would have reached completion (Wetmur and Davidson, 1968). All the data were collected using exchange technique II. The results of these experiments are shown in Figure 1. The kinetics are barely measurable at pH 6 and show a marked decrease in exchange rate as the pH is lowered. The curves become nonlinear at pH values below 5.5. This suggests that with decreasing pH an increasing fraction of the measurable hydrogens are exchanging with very slow kinetics. The early parts of the exchange curves are replotted in Figure 2. There, the effect of pH variation on initial exchange kinetics is more readily apparent. Table I lists the initial rate constants at the various pH's, showing a 1000-fold decrease in the initial rate constant in going from pH 6 to 4.5. Also listed in Table I are the fraction of the bases protonated ($1 - \alpha$) and the approximate T_m, both sets of data obtained from the literature (Holcomb and Timasheff, 1968; Massoulie, 1965). The degree of protonation is given for 0°, and this was obtained by plotting the published values as a function of temperature and extrapolating (from 3°) to 0°. The T_m data have been corroborated by melting experiments in this laboratory.

The exchange curves in Figure 2 permit an easy extrapolation to zero time. The extrapolated number of H/np is the total number of exchangeable hydrogens measurable by this experimental technique. There are 3.7–4 H/np measurable from pH 5.75 and lower. This number would agree with the Rich *et al.* (1961) model which contains 4 hydrogen bonds per nucleotide pair.

Variation of Salt Concentration. The thermodynamic stability of the helical form was shown to be dependent upon the ionic strength as well as the pH and temperature (Massoulie, 1965). The ionic strength effects arise from modifying the balance between the attractive potential of base pair formation (due to the ionic linkage between the base and phosphate) and the repulsive potential present in all double-stranded nucleic acids and arising from interchain inter-

[1] Abbreviations used are: NaOAc, sodium acetate buffers consisting of the given molarity of sodium acetate with added acetic or hydrochloric acid; EDTA, disodium salt of ethylenediaminetetraacetic acid; NaCac, cacodylic acid buffers consisting of the given molarity of cacodylic acid with sodium hydroxide added to adjust the pH.

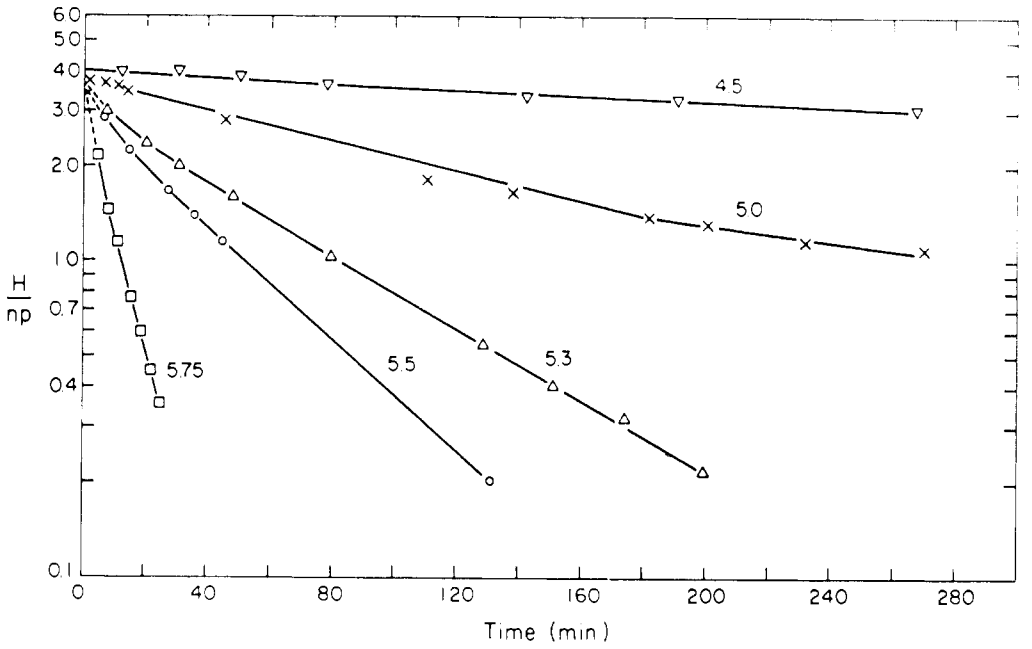


FIGURE 2: The initial parts of the exchange curves in Figure 1 replotted to show the magnitude of the variation of exchange rate with pH.

actions of the phosphates (Holcomb and Timasheff, 1968; Guschlbauer and Vetterl, 1969). This balance is obviously pH dependent, since the number of phosphate–phosphate interactions is reduced with decreasing pH (*i.e.*, increasing degrees of protonation of the bases). While most model amide systems show minimal effects of ionic strength on hydrogen exchange kinetics (Klotz and Frank, 1965), DNA shows a significant dependence (Printz and von Hippel, 1968). Alterations in exchange rate may be expected under the following circumstances: (1) when the exchange kinetics are dependent upon structural stability and the latter is altered by variations in ionic strength, (2) when the exchange occurs *via* an ionization mechanism and the pK of the ionizing group is markedly altered by ionic strength variation, and (3) if there are multiple (but recognizably different) pathways for exchange and salt variation alters the contribution of the various mechanisms to the observed exchange process.

TABLE I: The Variation of the Apparent and Corrected Initial Rate Constants (k_i) as a Function of pH.

pH	$k_i \times 10^4$ min^{-1}	$k_i \times 10^4$ min^{-1} corrected[a]	T_m[b]	$(1 - \alpha)$[c]
6.0	6900		18	0.44
5.75	1067		29	0.47
5.5	260		39	0.50
5.3	161		45	0.55
5.0	54.5	99.2	59	0.62
4.5	4.95	30.0	80	0.73

[a] The initial rate of exchange was corrected for the presence of the very slowly exchanging hydrogens as described in the text. [b] Values were taken from Massoulie (1965). [c] $(1 - \alpha)$, the fraction of bases protonated, was obtained by plotting published values (Holcomb and Timasheff, 1968) as a function of temperature and extrapolating to 0°.

The first experiment to test for ionic strength effects was conducted in a manner identical with that for the pH series. A preparation of poly A in 0.05 M Na^+ solution, pH 5.0, was tritiated, and the salt concentration was varied in the exchange solvent by a sudden alteration during the passage through the first column. The results of this salt-jump experiment at pH 5.0 disclosed that the initial exchange kinetics were the same for all the NaCl concentrations varying between 0.02 and 0.61 M. However, the number of very slowly exchanging hydrogens varied with the salt concentration. We found that this behavior was due, in part, to changes in the state of aggregation induced by the sudden alteration in the salt concentration. It had been noted earlier (Fresco and Doty, 1957; Steiner and Beers, 1957, 1959) that the acid form of poly A aggregates. It was shown that the aggregation was a function of both the polynucleotide concentration and the ionic strength (Steiner and Beers, 1959). Fresco and Klemperer (1959) proposed that the extent of aggregation may be reduced by a gradual change from conditions optimum for a random coil (*i.e.*, neutral pH and high ionic strength) to those optimum for the double-stranded helix (namely, low pH). We found that this was indeed the technique to use to minimize aggregation.

Using a stepwise dialysis procedure to prepare our samples, we repeated the above experiment. Separate samples of poly A were dialyzed against EDTA to remove trace metal ions and then against 0.01 M NaCac buffer, pH 7.7, to ensure that the polymer was dissociated into single strands and that the ionic strength was low. Next the samples were dialyzed at low ionic strength to pH 5.0, followed by dialysis

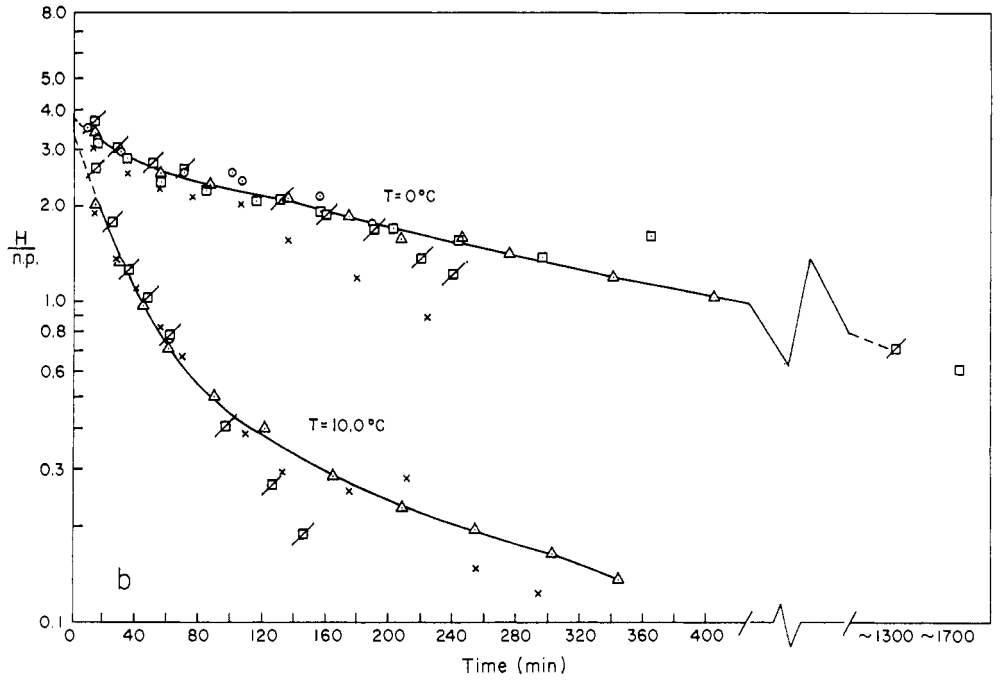


FIGURE 3: Tritium-hydrogen exchange curves at pH 5.0 as a function of NaCl. The back-exchange was measured both at 0° (top) and 10° (bottom). The solvent consisted of 0.01 M NaOAc, 10^{-4} M EDTA, and variable quantities of NaCl. The symbols ×, ⊙, △, □, and ⊠ represent 0.02, 0.06, 0.14, 0.31, and 0.61 M Na^+, respectively.

at constant pH against the desired ionic strength buffer. The solutions were kept at 0° at all times. All the samples were tritiated independently and separate molar extinction coefficients were determined for each. The results of the exchange experiments at the various salt concentrations are shown in Figure 3. We found that with this preparation technique there were no significant differences among the exchange curves, although the salt concentration varied almost 20-fold. The initial exchange kinetics were essentially identical with those found in the earlier experiment where aggregation was not minimized. All the curves are nonlinear and show the presence of approximately the same number of slowly exchanging hydrogens. We conclude that there is no significant effect of NaCl concentration on the exchange kinetics at pH 5.0, 0°, when aggregation tendencies are carefully controlled. In order to more definitively establish that NaCl was not affecting the exchange of the very slow hydrogens we repeated the experiments at a higher temperature. The increased temperature accelerated the exchange rate and brought the terminal kinetics into a more easily measurable time range. In these experiments the first-pass column was maintained at 0°, but the pool dialysis was conducted at 10°. By using vigorous stirring, a high surface:volume ratio, and an outside:inside volume ratio of 500–1000, we established conditions which favored very rapid temperature equilibration. The results of these experiments are given as the lower curve in Figure 3. Since the first column was at 0°, the extrapolation point is the same for both sets of curves. Because of the increased exchange rate, we could follow the reaction down to 0.1 H/np. Still there is no separation in the terminal region among the different ionic strength solutions. Thus, variations in salt concentration (and in T_m of the sample) did not affect the exchange kinetics when the sample was prepared by stepwise dialysis and the pH was kept constant at 5.0.

At pH 5.0 the structure is quite stable and the fraction of bases titrated (both at 0° and 10°) is approximately 0.6. Under these conditions variations in salt concentration did not affect the observed exchange. However, we found that salt effects on the exchange rate are manifested at a lower level of protonation (higher pH). For this experiment a sonicated poly A solution was dialyzed first against a neutral pH EDTA buffer, and then against 10^{-3} M NaCac at pH 7.6. Next, three samples were dialyzed exhaustively at low temperature and in a stepwise manner into one of the following solvents. Sample 1 (S1) was dialyzed against a pH 6.0 solution containing 0.0015 M Na^+, sample 2 (S2) against one containing 0.065 M Na^+, pH 5.9, and sample 3 (S3) against one containing 0.205 M Na^+, pH 5.9. All three solvents contained 10^{-4} M EDTA. The samples were dialyzed, tritiated, and kept at 0°. The results of the exchange experiments are presented in Figure 4a. S2 exhibits very fast exchange kinetics, markedly different from the curve for S1, the lowest salt concentration solution. In Figure 4b the melting curves show that S3 has a continuous absorbancy increase with temperature, while S2 shows a small "transition." S1 appears the most stable, with no hypochromicity until the temperature is greater than 30°. Above 30° there is a gradual increase in absorbancy until all three curves overlap at approximately 75°. S2 shows only 30% of the transition absorbancy change of that of S1. The most typical exchange behavior was found in the lowest salt concentration, *i.e.*, almost 3 H/np and nonlinear exchange kinetics. Since S2 exhibited only 30% of the hyperchromic transition of S1, we would predict only about 1 H/np measurable in S2. If the exchange curve for S2 in Figure 4a is extrapolated

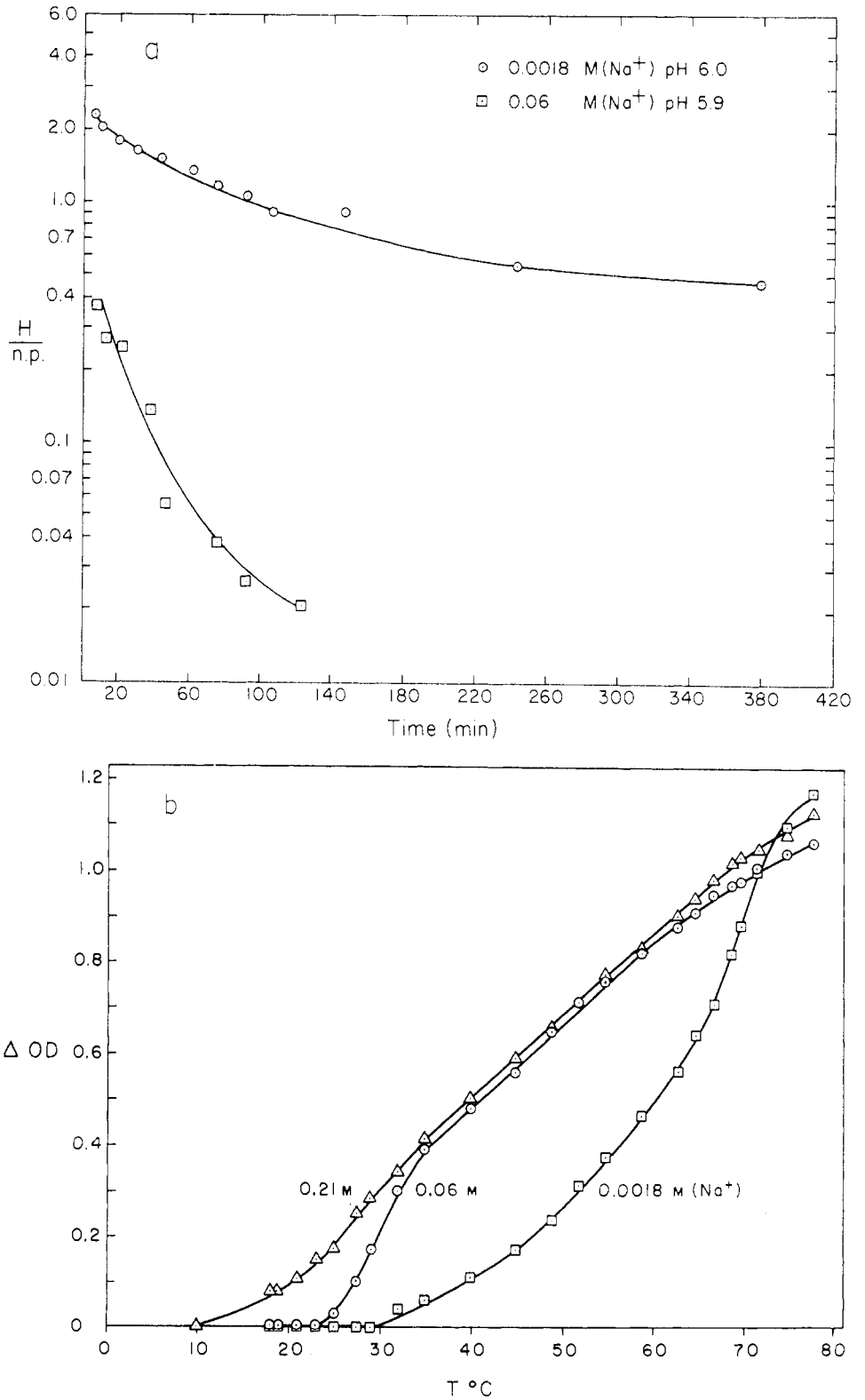


FIGURE 4: Tritium–hydrogen exchange curves and thermal transition profiles of helical poly A at "high" pH and low ionic strength. (a) Exchange curves at 0° of S1, containing 0.001 M NaCl, 0.001 M NaCac, and 10^{-4} M EDTA (pH 6.0), and S2, 0.06 M NaCl, 0.01 M NaCac, and 10^{-4} M EDTA (pH 5.9). (b) The increase in optical density (ΔOD) of S1, S2, and S3 at 251 nm as a function of the temperature; S3 contained 0.20 M NaCl, 0.01 M NaCac, and 10^{-4} M EDTA (pH 5.9). The total sodium ion concentration is listed beside each curve. The starting absorbancies were 0.670 for S1, 0.672 for S2, and 0.640 for S3.

to the zero time ordinate, it would intersect somewhat below 1 H/np. This result is not too surprising since, under these solvent conditions, the stability of the helix is marginal. Thus, these results show that when the fraction of bases protonated approached 0.4 there was a marked effect of NaCl on the exchange kinetics; increasing NaCl increases the exchange rate.

Initial Exchange Rate. The exchange curves of poly A at pH values below 5.5 show the presence of a large number of very slowly exchanging hydrogens. This observation will be the subject of a subsequent publication; however, it should be noted that studies of natural nucleic acids do not indicate that a large fraction of their measurable hydrogens exchange with such slow kinetics. In order to consider the exchange mechanism for the "typical" hydrogen in poly A, *i.e.*, those exchanging rapidly, we must correct the initial part of the exchange curves for the contribution of the slowly exchanging hydrogens. All the data we have obtained indicate that there is a marked difference in the initial and terminal exchange rates. As a result of this difference in rates we were able to correct graphically for the presence of the very slow hydrogens. This was done by constructing a linear curve from the last few data points and then subtracting this estimated exchange curve from the original data. The resulting corrected curve was essentially linear down to approximately 10% of the total number of hydrogens. We estimate that the corrected curves yield rate constants with a standard error less than 10%. Using a new method of analysis of exchange curves that gives a statistically best fit to the experimental data (Laiken and Printz, 1970), we obtained a class of slow hydrogens similar in size to that estimated by the above graphical procedure.

By correcting (as outlined above) the data in Figure 3 (*i.e.*, at 0° and 10°), we were able to calculate the net activation energy for the exchange of these "typical" hydrogens. It may easily be shown that at equivalent extent of exchange, the ratio of rate constants of the two corrected curves is equal to the inverse ratio of the times; thus $k^{10}/k^{0} = t^{0}/t^{10}$. We made several calculations and found that $t^{0}/t^{10} = 3.5$–5. Thus the apparent activation energy is approximately 18–20 kcal/mole. Once we define more accurately the terminal exchange kinetics, we will be able to determine whether the exact value is on the high or low side of the range given above.

If the exchange of the bases exhibits hydrogen or hydroxyl ion catalysis (as the present data do), then one must consider the temperature dependence of K_w, the ionization constant of water. The ΔH of ionization of water is 14.5 kcal/mole. Correcting the observed activation energy for the contribution of the ionization of water, we find that there is an excess of 3–5 kcal/mole required for the exchange. This additional energy requirement is probably a result of structurally imposed limitations on the exchange.

Discussion

The random coil to helix conversion occurs abruptly for poly A when approximately 40% of the bases are protonated. The onset of helix formation is therefore a function of the pH, temperature, and ionic strength (Fresco and Doty, 1957; Steiner and Beers, 1957, 1959; Massoulie, 1965). The model proposed for poly A and based on X-ray diffraction studies (Rich *et al.*, 1961) consists of a double-stranded helix with parallel chains. In the proposed structure there are four hydrogen bonds per base pair with each base donating both hydrogens of the 6-amino group to the opposing base. One of these hydrogens is accepted at the N-7 position, while the other bonds with the "neutral" oxygen of the phosphate. Most important was the postulate of an ionic salt linkage between the protonated base and the ribose-phosphate backbone.

From the data reported here there are a total of 3.7–4 H/np directly measurable at all pH values below 6. This is in close agreement with the number predicted from the Rich *et al.* model. However, there is a total of 8 potentially exchangeable hydrogens in an rA:rA base pair. Four of these are from the exocyclic amino groups and two are from the 2′-OH, while there are two at the C-8 position. The C-8 proton was shown to have kinetics of exchange at acid pH's which should have precluded any significant tritiation of the polymer during the incubation (Shelton, 1968; Shelton and Clark, 1967). If the ribose hydroxyl hydrogen is freely accessible to the solvent then its exchange rate, being diffusion controlled, should be too fast to measure. We observed no evidence of 2′-OH exchange in our studies with poly C, poly G, rRNA, or tRNA (Printz and Trout, 1968). In the case of poly A there is additional evidence which shows that the 2′-OH is not essential for helix stability (Bobst *et al.*, 1969) and, therefore, we would not expect any retardation of its exchange rate due to structural involvement. Thus, we believe that only amino hydrogens are being measured. The agreement between our data in aqueous solution and the Rich *et al.* model for the fibrous state is the first demonstration of the validity of the hydrogen-bonding pattern of this model. Furthermore, these hydrogen bonds are stable in aqueous solution.

The observed agreement in the number of measurable hydrogens and the number of hydrogen-bonded hydrogens in the Rich *et al.* (1961) model does not prove that the kinetics of exchange of nucleic acids are measurable only when there is stable hydrogen bonding. However, all the evidence gathered to date supports this conclusion. This includes the agreement between the number of measurable hydrogens for both DNA at neutral pH (Printz and von Hippel, 1968) and poly C at acid pH (M. P. Printz and H. P. Williams, unpublished data) with the number of hydrogen-bonded hydrogens in their respective models. In addition, we have from this paper both the observation of no detectable exchange at pH's where poly A is single stranded and the results as discussed above. Furthermore, recent studies in this laboratory show that formylation, which disrupts interbase hydrogen bonds, causes a major loss in the number of measurable hydrogens. Finally, there is definitive experimental evidence that, in model systems similar to nucleic acids, hydrogen bonding slows exchange by several orders of magnitude below diffusion controlled rates (Rose and Stuehr, 1968). The conclusion, therefore, is that involvement of purine and pyrimidine bases in stable hydrogen bonding is necessary to slow the intrinsic exchange rate of the exo- and endocyclic amino hydrogens so that the rates are measurable by present experimental techniques.

Mechanism of Exchange. Proton exchange of nucleic acids in an aqueous solvent follows first order kinetics. If a single class of exchange sites is present, then the experi-

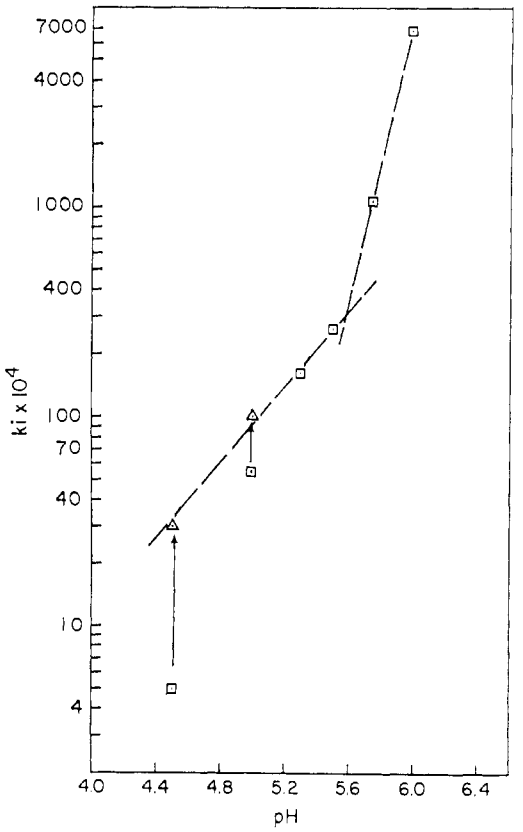


FIGURE 5: The variation of the initial rate constant (k_i) with pH. Both the uncorrected (□) and corrected (△) rate constants are given.

mental data (hydrogens remaining unexchanged) on a semilog plot will be linear, as represented by

$$\log H_t = \log H_i - k_i t \quad (1)$$

Here H_t is the number of unexchanged hydrogens at time t while H_i is the total number of hydrogens and k_i the observed rate constant for the ith class. Typical experimental exchange data for proteins and natural nucleic acids are nonlinear, implying multiple classes of sites or a distribution of exchange rates. With self-associating polynucleotides such as poly A and poly G (Printz and Trout, 1968), the terminal region of an exchange curve appears to exhibit "history" effects. That is, the number of very slowly exchanging hydrogens may, in part, be a function of the history of the sample. The presence of a large number of slow hydrogens significantly altered the observed initial exchange kinetics for the pH 5.0 and 4.5 curves. However, since there was a large difference between the apparent initial and terminal rates it was possible to make graphically a correction to the initial kinetics for the presence of the slow hydrogens. The correction was described above. Our discussion will therefore be restricted to the corrected initial rate data, and we will consider this exchange to be represented by a single observed rate constant. (We have shown that even for a situation where there are multiple exchange contributions, the observed curve may be represented by a single mean rate constant and the standard deviation of the distribution of rate constants (Laiken and Printz, 1970).)

For specific acid or base catalysis, *i.e.*, catalysis by (H^+) or (OH^-), we would expect a linear relationship between k_i and catalyst concentration. Thus, for base catalysis, which the present situation appears to be, we may write

$$k_i = K_L k_{OH}(OH^-) = K_L k_{OH} K_w/(H^+) \quad (2)$$

Here, K_L represents all terms arising from structural limitations on the exchange rate, k_{OH} is the catalytic rate constant for hydroxyl ion, K_w is the ionization constant of water, and (H^+) or (OH^-) represents hydrogen or hydroxyl ion concentration. From this relationship, a plot of log k_i *vs.* pH should have a slope of +1. Plotting the corrected initial rate constants from Table I *vs.* pH we obtain the results shown in Figure 5. The resultant plot is definitely biphasic with the slope at high pH approximately +2 while the slope at pH values below 5.5 is +1. Thus there appear to be two pH dependent processes controlling the exchange rate of these hydrogens.

In the usual kinetic treatment of hydrogen exchange curves of macromolecules, three limiting situations may prevail. These have been discussed in great detail in several recent reviews and will only be presented here briefly. For further information see Hvidt (1964), Hvidt and Nielsen (1966), or Englander (1967).

A. If exchange takes place from an open, unhindered site into the solvent and no "labilizing" equilibrium is involved, then the observed rate constant (k_i) should equal the rate constant due solely to the chemical exchange step. If this step exhibits (as in the present situation) specific base catalysis, then we may write

$$k_i = k_{OH}(OH^-) = k_3 \quad (3)$$

B. If a prior equilibrium is involved such as

$$\text{stable H} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{labile H} \xrightarrow{k_3} \text{exchange}$$

and the relationships between the rate constants are such that $k_2 \gg k_1$ and $k_2 \gg k_3$ (the situation for a stable, native structure), then the observed rate constant may be written as

$$k_i \cong k_1 k_3/k_2 = K_L k_3 = K_L k_{OH}(OH^-) = K k_3 \quad (4)$$

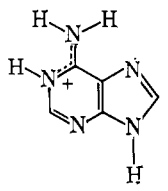
where K_L is the equilibrium constant for labilization of the hydrogen. Until we know more about the actual molecular events which occur during exchange of a hydrogen-bonded hydrogen we cannot rigorously define K_L (see below).

C. Finally, if a prior equilibrium exists as in B, but the rate constants are such that $k_3 \gg k_2$ while $k_2 > k_1$, *i.e.*, any labilized hydrogen is exchanged before it can return to a stable form, then

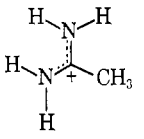
$$k_i = k_1 \quad (5)$$

A slope of +1 from the data plotted in Figure 5 indicates that the observed chemical exchange step (k_3) is base catalyzed. For this reason we may safely rule out a k_1 mechanism as describing our experimental data. An additional argument

is that the estimated values of k_2 and k_3 do not satisfy the primary kinetic requirement for a k_1 mechanism, namely, $k_3 \gg k_2$. A k_3 mechanism implies exchange from a structurally unconstrained site. If we assume a k_3 mechanism is operative, then substitution of k_i and (OH^-) into eq 3 would give us an estimate of k_{OH}. At pH 5.0 the observed rate constant is $\sim 10^{-2}$–10^{-3} min^{-1} and (OH^-) $\cong 10^{-9}$; therefore k_{OH} would need to be 10^6 to 10^7 M^{-1} min^{-1}. Even at pH 6.0 where k_i is $\sim 7 \times 10^{-1}$ min^{-1}, k_{OH} would be 7×10^7 for a k_3 process. These estimates for k_{OH} are quite low in light of the similarity between adenine and amidine.



adenine



amidine

It was shown that proton exchange of the amidinium cation (and its dimethyl derivative) was base catalyzed (Neuman *et al.*, 1962). The estimated rate constant at 33° for the amidinium cation was 2.1×10^{12} min^{-1}. This exchange rate is nearly maximum and implies a diffusion-controlled process. Since the purine ring structure should allow for a greater electron delocalization than the amidine, we would expect the exocyclic amino of adenine to have as acidic a pK as that of amidine. The intrinsic proton exchange rate is a function of the difference between the pK of the acceptor group and that of the donor group (Eigen, 1964). The more positive that difference is, the more likely that the exchange will be diffusion controlled. In this system the amino group is the donor and the hydroxyl ion the acceptor. Thus the more acidic the amino function, the faster the exchange rate would be (in the absence of other limiting factors). We conclude that a value of 10^6–10^7 for k_{OH} is, for an unconstrained group, much slower than the expected catalytic constant of 10^{10}–10^{12} M^{-1} min^{-1}. Therefore, the data are best fit by a Kk_3 mechanism, *i.e.*, one where the exchange is determined by both chemical and structural constraints.

Consideration of K_L. Given that the exchange follows a Kk_3 process, we must explain why the kinetics for this homopolynucleotide show a marked biphasic pH dependence. It is unlikely that the variation in k_i is due solely to the k_3 term. From (2) k_i should vary only with the first power of (OH^-) if K_L is pH invariant. Since the slope for the k_i–pH curve at pH values below 5.5 is +1, we conclude that in this pH range K_L is invariant with pH. For pH values above 5.5, the slope is approximately +2. This pH dependence is reconciled either by K_L becoming a function of (OH^-), or by k_3 becoming a function of (OH^-)^2. We may readily demonstrate that the latter is *not* the situation. If $k_3 = k_{OH} \cdot (OH^-)^2$, then substituting this relation into (4) and solving for K_L we find that at pH 6, $K_L = 10^3$–10^4. This is clearly impossible since K_L is presumably a measure of the *lability* of the base pair and, for a stable, native structure, should be much less than 1. On the other hand, if we assume a normal k_3–OH dependence and substitute (similar values for the parameters) into (4), we find that K_L at pH 6 is approximately 10^{-4}–10^{-5} (at 0°). This value of K_L is more reasonable and approximates the equilibrium constant for base pair denaturation (Crothers, 1968). The magnitude of K_L is very dependent upon the assumed value of k_{OH}. For these calculations we used the maximum value expected (10^{12} M^{-1} min^{-1}). A similar calculation for the other pH values indicates that K_L is pH invariant from 5.5 and lower, and averages 0.8–0.9 $\times$ 10^{-5}. K_L increases by a factor of over 2 in going from pH 5.5 to 5.75, and by a factor of 7–10 at pH 6.0.

There are two additional indications that K_L varies with pH. The first involves the effect of NaCl on the exchange kinetics. In Figure 3 it was shown that at pH 5.0 variation in NaCl over a 20-fold range did not affect the exchange kinetics. However, at pH 5.9–6.0 there was a marked effect as shown in Figure 4a. The effect of NaCl on the kinetics may be a result of both an increased cooperativity among the bases and an increased lability of the base pairs when the extent of titration is less than 0.5. These effects would be reflected in k_i *via* the term K_L since it is a measure of structural lability. The transition point in the k_i–pH curve where K_L becomes pH invariant occurs at a pH where the extent of titration is approximately 0.5. That is, when all the base pairs have, on the average, one titrated base there is a loss in the dependence of K_L on pH. The molecular process which causes this transition may involve either the presence of one ionic bond/base pair or a change in the cooperativeness of the titration of the bases. The exchange kinetics of DNA also exhibit a marked NaCl dependence (Printz and von Hippel, 1968). It was postulated then that the salt was affecting the exchange kinetics by altering the extent of reversible titration of the bases, well accepted as a highly cooperative process. The exchange rate of *all* the measurable hydrogens of DNA is increased by changes in NaCl that promote the formation of ionized bases, either anionic or cationic. In the DNA structure ionized bases cannot form stable base pairs; thus, K_L is increased with increasing titration. The exchange of poly A is increased by changes in NaCl that promote the formation of *un-ionized* bases. In this case, un-ionized bases cannot form stable base pairs and K_L is increased. Since *all* the exchangeable hydrogens appear equally affected, the correlation between exchange rate and titration must be in terms of structural effects. Thus, we conclude that K_L is that which is modified. It has also been shown that the exchange of polyglutamic acid is markedly salt dependent in the range of the helix–coil transition, and salt invariant outside of that pH range (Leichtling and Klotz, 1966).

If K_L is a measure of structural lability then we may be able to relate k_i to T_m. If this is the case, then when K_L becomes invariant with pH, k_i should also be invariant with T_m. It has been clearly established that the T_m is proportional to pH for the acid form of poly A (Holcomb and Timasheff, 1968; Massoulie, 1965). From the published data the slope of a T_m *vs.* pH plot is −44.4°. Thus, we may write

$$T_m = A\text{pH} + B = A \log 1/(H^+) + B \qquad (6)$$

where A is the proportionality constant (−44.4°) and B is a constant equal to ~250. Rearranging we get

$$1/(H^+) = 10^{(T_m - B)/A} \qquad (7)$$

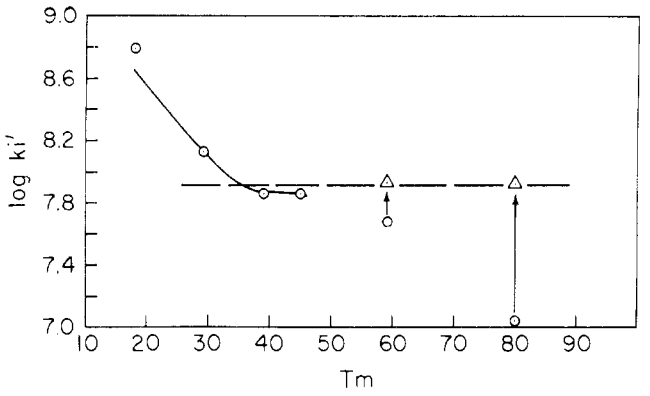


FIGURE 6: The variation of k_i' (see text) with T_m. The k_i' for both the uncorrected (⊙) and corrected (△) initial exchange rates are given.

Now since k_i also shows an inverse dependence on (H^+), we may rearrange (2) in terms of $1/(H^+)$, equate with (7), and, expressing the resulting equation in a logarithimic form, we get

$$\log k_i = \log (K_L k_{OH} K_w) + T_m/A - B/A \quad (8)$$

Thus a plot of log k_i *vs.* T_m should be linear with an inverse slope equal to A (*i.e.*, $-44.4°$). We obtain a dependence of k_i on T_m; however, of more interest is what happens if we correct k_i for the (OH^-) dependence due to the k_3 term. This may be done by redefining k_i as k_i', namely from (4).

$$k_i' = k_i/(OH^-) = K_L k_{OH} \quad (9)$$

If then k_i' is substituted for k_i in (8) and log k_i' plotted *vs.* T_m, then a linear dependence with a negative slope will be obtained *only* if K_L is pH dependent. The results of this analysis are shown in Figure 6. It is clear from Figure 6 that below pH 5.3 (*i.e.*, $T_m > 30°$) K_L is invariant with pH and T_m. For pH values above 5.5 (*i.e.*, $T_m \leqq 30°$), K_L varies with pH and T_m. A similar dependence between T_m and the rate of exchange has been obtained by McConnell and von Hippel (1970) for calf thymus DNA at neutral pH.

Although K_L varies somewhat between pH 5.5 and 6.0, it is still $\ll 1$ (at 0°). Therefore, the exchange kinetics must reflect a small change in the rate of fluctuation of base pairs between "stable" and "labile" states. We are unable to define these states. K_L may be an equilibrium constant for a single base pair denaturation (*i.e.*, titration) or it may be a measure of the frequency (or mobility) of small "breathing" loops. The equilibrium constant is equal to the ratio of forward and reverse rate constants. Clearly, for K_L to be pH dependent means that k_1 and/or k_2 must be pH dependent. Without knowledge of the molecular basis of K_L we are unable to predict how the rate constants may be changing with pH. Work is in progress to define the relationship between K_L and the dynamic structure. Such knowledge may be valuable in the development or proof of helix–coil transition theories. Hydrogen exchange is especially useful for these studies since it is most sensitive to the fraction of time a base pair is in a labile state.

In conclusion, we have direct evidence for the Rich *et al.* (1961) model for poly A in aqueous solution. We show that the exchange of this homopolynucleotide is of the Kk_3 type and exhibits specific base catalysis. We find that the structural term may be sensitive to changes in the solvent. The effect of variation in NaCl concentration on exchange kinetics is *via* modification of the structural term (K_L) and not by directly influencing the chemical exchange rate. These results may now be considered in the search for the underlying controls of the exchange of the natural, heterobase nucleic acids.

Acknowledgments

I wish to acknowledge the excellent technical assistance of Miss Judy Trout and Miss Hazel Williams. I also wish to thank Dr. Arnold Wishnia (SUNY at Stony Brook) for calling to my attention the reference on amidinium ion exchange.

References

Bobst, A. M., Cerutti, P. A., and Rottman, F. (1969), *J. Amer. Chem. Soc. 91*, 1246.
Crothers, D. M. (1968), *Biopolymers 6*, 1391.
Eigen, M. (1964), *Angew. Chem. Int. Ed. Engl. 3*, 1.
Englander, S. W. (1963), *Biochemistry 2*, 798.
Englander, S. W. (1967), *in* Polyamino Acids as Protein Models, Fasman, G. D., Ed., New York, N. Y., Marcel Dekker, p 339.
Englander, S. W., and Crowe, D. (1965), *Anal. Biochem. 2*, 579.
Englander, S. W., and Englander, J. J. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 370.
Fresco, J. R., and Doty, P. (1957), *J. Amer. Chem. Soc. 79*, 3928.
Fresco, J. R., and Klemperer, E. (1959), *Ann. N. Y. Acad. Sci. 81*, 730.
Guschlbauer, W., and Vetterl, V. (1969), *FEBS Lett. 4*, 57.
Holcomb, D. N., and Timasheff, S. N. (1968), *Biopolymers 6*, 513.
Hvidt, A. (1964), *C. R. Trav. Lab. Carlsberg 34*, 306.
Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 287.
Klotz, I. M., and Frank, B. H. (1965), *J. Amer. Chem. Soc. 87*, 2721.
Laiken, S. L., and Printz, M. P. (1970), *Biochemistry 9*, 1547.
Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.
Maslova, R. N., Lesnik, E. A., and Varshavsky, Ya. M. (1969), *Biochem. Biophys. Res. Commun. 34*, 260.
Massoulie, J. (1965), *C. R. Acad. Sci. 260*, 5554.
McConnell, B., and von Hippel, P. H. (1970), *J. Mol. Biol.*, in press.
Neuman, R. C., Jr., Hammond, G. S., and Dougherty, T. J. (1962), *J. Amer. Chem. Soc. 84*, 1586.
Printz, M. P., and Trout, J. (1968), *Biophys. J. 8, Soc. Abstr.* TA10.
Printz, M. P., and von Hippel, P. H. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 363.
Printz, M. P., and von Hippel, P. H. (1968), *Biochemistry 7*, 3194.
Rich, A., Davies, D. R., Crick, F. H. C., and Watson, J. D. (1961), *J. Mol. Biol. 3*, 71.

Rose, M. C., and Stuehr, J. (1968), *J. Amer. Chem. Soc. 90*, 26.
Shelton, K. R. (1968), Ph.D. Thesis, University of Illinois, Urbana, Ill.
Shelton, K. R., and Clark, J. M. (1967), *Biochemistry 6*, 2735.
Steiner, R. F., and Beers, R. F. (1957), *Biochim. Biophys. Acta 26*, 336.
Steiner, R. F., and Beers, R. F. (1959), *Biochim. Biophys. Acta 32*, 166.
Ts'o, P. O. P., Helkamp, G. K., and Sander, C. (1962a), *Biochim. Biophys. Acta 55*, 584.
Ts'o, P. O. P., Helkamp, G. K., and Sander, C. (1962b), *Proc. Nat. Acad. Sci. U. S. 48*, 686.
von Hippel, P. H., and Printz, M. P. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 1458.
Wetmur, J. G., and Davidson, N. (1968), *J. Mol. Biol. 31*, 349.

Glycogen Turnover in *Dictyostelium discoideum**

Rosemarie Marshall,† Donna Sargent, and Barbara E. Wright

ABSTRACT: The rates of soluble glycogen synthesis and turnover have been determined *in vivo* at two stages of differentiation in *Dictyostelium discoideum*. Calculation of the rate of soluble glycogen synthesis was based upon the specific radioactivity of intracellular uridine diphosphate [^{14}C]glucose ([^{14}C]UDPG) following exposure of the cells to [^{14}C]glucose, and upon the increase with time in radioactivity of the glycogen pool. Glycogen turnover was determined by the exposure of cells which were previously labeled with [^{14}C]-glucose to [^{12}C]glucose, in order to determine the change in glycogen specific radioactivity with time. The rate of glycogen synthesis was found to be 0.034 and 0.160 μmole per min per ml of packed cells at aggregation and culmination, respectively; for glycogen turnover, the comparable values were 0.046 and 0.130 μmole per min per ml of packed cells. These rates are in excellent agreement with each other and with the rate of UDPG synthesis determined previously, thus substantiating two predictions of a kinetic model simulating glycogen metabolism during differentiation in this system.

The organism *Dictyostelium discoideum* grows as individual amoebae by engulfing bacteria. When the food supply is exhausted, the cells aggregate into a multicellular unit which maintains itself in the absence of exogenous nutrients for the remainder of the life cycle. Differentiation is completed within 24 hr at 23°, and is characterized by an orderly sequence of morphological changes which ultimately results in the formation of a mature fruiting body, or sorocarp, during a process called culmination.

It has been shown that, prior to sorocarp construction, the soluble glycogen pool (100,000*g* pellet fraction) accounts for about 5% of the total dry weight from the beginning of the differentiation cycle (amoeba stage) to culmination (Wright and Dahlberg, 1967), after which insoluble glycogen accumulates complexed to cellulose (Wright, 1966). Although the relative rates of soluble glycogen synthesis and degradation at aggregation and culmination had not been studied directly in the laboratory, the rates *in vivo* were predicted by a kinetic model which simulates glycogen metabolism during differentiation (Wright *et al.*, 1968; Wright, 1968). Although the specific activity of glycogen synthetase, the enzyme which catalyzes glycogen synthesis from UDPG,[1] decreases about tenfold *in vitro* between the aggregation stage and culmination (Wright and Dahlberg, 1967), this information was not incorporated in the model. In fact, the model predicted a threefold increase in the rates of glycogen synthesis and degradation *in vivo* over this same time period.

The purpose of this investigation was to determine experimentally the rates *in vivo* of glycogen synthesis and degradation at aggregation and at culmination. The information gained has enabled us to determine the relevance *in vivo* of flux values predicted by the kinetic model.

Materials and Methods

Materials. Maltase (type III), β-amylase (type II-B), bovine albumin (fraction V powder), and MES were all purchased from the Sigma Chemical Co. [U-^{14}C]Glucose (1.8×10^7 dpm/μmole) was purchased from New England Nuclear and oyster glycogen from Calbiochem. Sterile disposable petri dishes (150 × 50 mm) were obtained from Fisher Scientific.

Preparation of Cells. D. discoideum strain NC-4 was grown as previously described by Liddel and Wright (1961). The washed cells, essentially free of bacteria, were spread onto 2%

* From the Boston Biomedical Research Institute, Retina Foundation, Boston, Massachusetts 02114, and Harvard Medical School, Boston, Massachusetts. *Received January 20, 1970.* Supported by the National Institutes of Health, Research Grant No. GM 15938.

† Postdoctoral fellow of the National Institute of General and Medical Sciences (No. 5 F02 GM 30480-02).

[1] Abbreviations used are: UDPG, uridine diphosphate glucose; MES, 2-(*N*-morpholino)ethanesulfonic acid; PEI-cellulose, polyethyleneamine cellulose.