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On the Kinetics of Hydrogen Exchange in Deoxyribonucleic Acid. pH and Salt Effects*

Morton P. Printz and Peter H. von Hippel

ABSTRACT: The hydrogen-exchange kinetics of native calf thymus deoxyribonucleic acid have been studied as a function of pH and salt concentration using a tritium–Sephadex method. Previous work had demonstrated that only hydrogens involved in interchain hydrogen bonds in deoxyribonucleic acid exchange sufficiently slowly ($t_{1/2}$ = >50 sec) to be visualized by such gel filtration techniques. It is shown in this study that deoxyribonucleic acid hydrogen exchange proceeds at a minimum rate at 0° and pH values close to neutrality (pH_{min}); increasing or decreasing the pH from pH_{min} increases the rate of exchange. Furthermore, the rate of exchange at any given pH, as well as the value of pH_{min}, depends upon the salt concentration. At pH values greater than pH_{min}, the rate of exchange increases directly with log [Na^+]; at pH values below pH_{min} the rate decreases with log [Na^+]. pH_{min} also shifts to lower pH values as the salt concentration is increased. These findings correlate directly with salt-induced changes in the pH–titration curve of DNA. It is shown that pH_{min} for hydrogen exchange corresponds closely at a given salt concentration to the midpoint of the plateau of the pH–titration curve, and the rate of exchange at a pH removed from pH_{min} is inversely related to the absolute value of the difference between the experimental pH and the pK_{app} of the nearer limb of the titration curve. The results of this study are discussed in terms of a simple two-step exchange model (see eq 4 of text) involving a structure-controlled opening–closing reaction characterized by forward and reverse rate constants, k_1 and k_2, and an over-all rate constant for the chemical exchange process, k_3. Attempts are made to separate pH- and salt-induced changes in exchange rate into structural (k_1 and k_2) and chemical (k_3) effects.

Hydrogen exchange as a potential method of macromolecular conformation analysis was introduced and first developed by Linderstrom-Lang and his colleagues (*e.g.*, see Linderstrom-Lang, 1955). In the intervening years, conceptual and methodological refinements have improved this approach to the point that several variants of it are now being extensively used in protein structure studies (for reviews see Hvidt and Nielsen (1966), Harrington *et al.* (1966), and Englander (1967)).

More recently, this approach has also been extended to an analysis of DNA structure (Printz and von Hippel, 1965; von Hippel and Printz, 1965), using the tritium–Sephadex method introduced by Englander (1963). It has been shown that the potentially exchangeable hydrogens of DNA (hydrogens attached to nitrogen and oxygen; carbon-bound hydrogens do not exchange with solvent tritium under the conditions used) can be fully labeled by brief incubation in tritiated water, and that after passage of the sample through a Sephadex column to remove most of the free tritium, the exchange-out of the hydrogens involved in interchain hydrogen bonding can be followed (after another gel filtration step) on a time scale calibrated in seconds. Similar findings have been obtained by Englander and Englander (1965) with tRNA.

In DNA, exchangeable hydrogens are generally calculated as hydrogens/nucleotide pair (H/np) and the data are presented as plots of log $(H/np)_t$ *vs.* t (exchange-out time). Under conditions where exchange is moderately slow, such graphs can be extrapolated to zero time of exchange to determine the total measurable hydrogens per nucleotide pair, $(H/np)_0$, originally present in the structure. It has been shown (at pH 7–8 in 0.1 M NaCl and at temperatures below 4°), that exchange-out data obtained with calf thymus and bacteriophage T4 DNA (Printz and von Hippel, 1965) and various bacterial DNAs ranging in base composition from 30 to

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75 mole % dG–dC (McConnell and von Hippel, 1968), extrapolate to zero-time values (±0.2 H/np) which are in accord with expectations

$$(\mathrm{H/np})_0 = 2[\mathrm{dA\text{-}T}] + 3[\mathrm{dG\text{-}dC}) \qquad (1)$$

where [dA-T] and [dG-dC] represent the calculated mole fractions (from composition data) of each of the two conventional types of nucleotide pairs in the DNA. This result shows that only the hydrogens involved in interchain hydrogen bonds are measured by these techniques and furthermore that the determination of DNA hydrogens by tritium-labeling techniques involves no measurable equilibrium isotope effects. Thus extrapolation of hydrogen-exchange data to equilibrium values can, in favorable cases, be used to measure the extent of denaturation of DNA (Printz and von Hippel, 1965), the extent of hydrogen-bonded structure in complex systems such as tRNA (Englander and Englander, 1965), and hydrogen-bonding patterns in ordered synthetic polynucleotide systems (von Hippel and Printz, 1965; Printz and Trout, 1968).

Beyond its utility as a measure of equilibrium structure, study of the kinetics of the hydrogen-exchange-out (or -exchange-in) process in macromolecules appears to afford a unique opportunity to study the conformational motility of these structures, since the rate of release of tritium from potentially exchangeable loci within the structure should be related to the rate or extent to which these loci are exposed to the solvent environment as a consequence of conformational motility. Furthermore, for relatively simple (*i.e.*, homogeneous or monotonic) structures such as DNA, these kinetics should be interpretable in structural terms. It seems not unreasonable to speculate that the rate and/or extent of local opening–closing reactions in specific base sequences of DNA at temperatures well below T_m may play an important role in controlling or directing the interactions of DNA with polymerases, repressors, complementary nucleic acids, and other biologically relevant compound (*i.e.*, in controlling and directing the expression of the genome), and thus experimental approaches to the measurement of such opening–closing reactions are of interest (for other approaches see, for example, Wingert and von Hippel, 1968). In order to assess the usefulness of measurements of hydrogen-exchange kinetics in studies of conformational motility, we have begun a study of the effects of a number of environmental variables on the hydrogen-exchange behavior of DNA. In this paper we report on the effects of changes in pH and salt concentration on the kinetics of exchange.

Models of the Hydrogen-Exchange Process. The rate at which hydrogens located in any particular environment exchange with solvent hydrogens can be written as

$$\mathrm{d}H_i/\mathrm{d}t = k_i H_i \qquad (2)$$

where H_i is the number of hydrogens remaining in all sites of type i at time t, and k_i is the characteristic first-order rate constant for exchange of these hydrogens under a particular set of environmental conditions. For a macromolecule containing a number, n, of kinetically distinct types of sites which exchange independently, the integrated rate equation becomes

$$H_t = \sum_{i=0}^{n} H_{0,i} e^{-k_i} \qquad (3)$$

where H_t = total number of hydrogen per molecule not exchanged at time t, and $H_{0,i}$ and k_i are, respectively, the number of hydrogens initially present and the observed exchange rate constant for each kinetic class.

Since the exchange of DNA hydrogens fully exposed to water has been shown to be much too rapid to be followed by gel filtration techniques, the involvement of potentially exchangeable hydrogens in structure (*i.e.*, interchain hydrogen bonds) must be responsible for slowing exchange to measurable rates. Thus structure must be introduced into any potential model of the exchange process. This can most simply be done by writing the exchange mechanism for a single exchange unit[1] of DNA as a two-step process (comparable formalisms have previously been used by Linderstrom-Lang (1955) and Hvidt (1964) to describe hydrogen exchange in proteins, and by Englander and Englander (1965) in dealing with tRNA)

$$\text{closed} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{open} \xrightarrow{k_3} \text{exchanged} \qquad (4)$$

where k_1/k_2 defines an equilibrium constant, K, which is the ratio of open to closed nucleotide pairs at any instant of time. Since the experimental temperatures, T, in these studies are far below T_m, $k_1 \ll k_2$, and the fraction of time a given nucleotide pair is open = $[k_1/(k_1 + k_2)] \simeq k_1/k_2 = K$. The rate constant k_3 defines the rate of chemical exchange of the hydrogens of the open structure with solvent hydrogens under the experimental conditions. It is clear that this formulation is over simplified, since it does not allow for exchange from partially open sites, etc., but it appears to provide an adequate framework for the interpretation of present results.

Two limiting cases for the kinetics of exchange of any given class of sites may be considered in such a two-step mechanism: (a) $k_3 \gg k_2$: the rate of exchange will depend only upon the rate of opening, and the observed rate constant of exchange, k_i, for these sites will be equal to the rate constant for opening, k_1; *i.e.*, $k_i = k_1$. (b) $k_3 \ll k_2$: the observed rate of exchange for this class will depend upon the *product* of the chemical exchange rate constant, k_3, and the fraction of total time the sites are open; *i.e.*, $k_i = Kk_3$.

One major distinction between these limiting cases is

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[1] An exchange unit of DNA is defined as a set of sites containing potentially exchangeable hydrogens which open and close (*i.e.*, undergo local strand separation and recombination) as a unit. For DNA the exchange unit could range in size from one or a very few nucleotide pairs at temperatures well below the helix–coil transition temperature, T_m, to a large part of the DNA molecule at temperatures close to T_m.

that in a the observed rate constant is completely independent of the chemical exchange rate constant of the open structure, k_3, while in b the observed rate is directly proportional to k_3. Thus one approach to differentiating between these cases is to alter the solvent environment (*e.g.*, by adding exchange catalysts) so as to change the rate of the chemical exchange process without altering k_1 or k_2. If then the over-all exchange rate is altered, we may surmise that exchange proceeds by some variant of mechanism b. However, the difficulty lies in being sure the observed effects are not related in some way to structural effects since we can only observe exchange from the predominantly closed (native) double-helical structure.

With proteins this problem does not arise, since chemical exchange of the amide hydrogens is anomolously slow, and it has been possible to follow the chemical exchange process of simple amides and unstructured polypeptides at pH values close to that at which the exchange rate is minimal, pH_{min}, by methods with time resolution comparable with the tritium–Sephadex technique. It has been shown in such systems that peptide hydrogen exchange is subject to direct chemical catalysis by acid and base (Berger *et al.*, 1959; Bryan and Nielsen, 1960; Klotz and Frank, 1965) and by certain other proton donors and acceptors functioning as general acid–base catalysts (Klotz and Frank, 1965; Leichtling and Klotz, 1966). The actual values of pH_{min} for these simple compounds, though not the minimum rates have been shown by Leichtling and Klotz (1966) to depend upon inductive effects of neighboring groups.

In DNA the situation is more complex. There are two chemically distinct types of measurable, exchangeable hydrogens (*i.e.*, the exocyclic amino and the endocyclic imino hydrogens) and, as indicated above, both types exchange very rapidly with solvent hydrogens in the open form. Thus, unlike in proteins, the tritium–Sephadex technique cannot be used to study exchange of open DNA sites, and we must examine the effects of pH, salt, and catalyst concentration on the fully structured DNA molecule. Some results of such studies are reported here; in the Discussion we attempt to separate the results into chemical and structural effects in terms of eq 4.

Materials and Methods

Hydrogen-Exchange Procedures. Hydrogen exchange in DNA was studied using the tritium–Sephadex procedure originally developed by Englander (1963) for protein studies. DNA was tritiated, the kinetics of exchange-out were followed by one- or two-column methods, and the results were calculated, essentially as previously described (Printz and von Hippel, 1965). Methodological details and comments which supplement our earlier description are presented here.

Many of the experimental problems of the method arise from the fact that the exchangeable hydrogens of DNA (approximately 5 μmoles/ml in a 1-mg/ml DNA solution) are in equilibrium with all the hydrogens of water (approximately 111 mmoles/ml in a dilute aqueous solution). Thus the ratio of water to DNA hydrogens is approximately 2×10^4, and in order to introduce appreciable quantities of tritium into the DNA the initial equilibration must be carried out at very high specific activities of tritiated water (5–10 mCi/ml). After equilibration, the free tritium must be reduced to 10^{-6}–10^{-7} of its initial concentration, in order to obtain DNA-bound tritium levels which are at least tenfold above background at 0.1–0.01 H/np. Because of the magnitude of the separation required, extreme care must be used in the gel filtration procedure to avoid contamination of the final samples with the very high levels of free tritium initially present.

Most of the data presented in this paper was obtained by the two-column procedure, in which exchange-out is initiated by reducing the free tritium concentration to 10^{-3}–10^{-4} of its initial concentration by quick passage through a short Sephadex column. This is operationally equivalent to making an infinite dilution of the free tritium, and no back-exchange into DNA subsequently occurs. Englander and Englander (1965) have shown that the corrected zero time for exchange-out corresponds to the time at which the sample has proceeded far enough down the column to reduce the contaminating tritium concentration to 37% of its initial value. For our columns and conditions this time was 40 ± 10 sec after layering the sample onto the first column, and all measured exchange-out times have been corrected for this factor.

The DNA emerging from the first column is pooled and incubated under controlled conditions. Passage of an aliquot of the pool through a second Sephadex column at various times removes the remainder of the free tritium, plus that which exchanged-out of the DNA during the incubation. Fractions of the effluent of the second column are then collected and analyzed for DNA and tritium concentration. Several tubes are collected from each peak, and in a satisfactory run these must show a constant ratio (±10%) of tritium/DNA (*i.e.*, no break through of free tritium; see Englander, 1963).

The runs were made in standard 2-cm glass chromatographic columns fitted with coarse sintered-glass disks The columns were siliconized (Siliclad, Clay-Adams, Inc.) to reduce wall effects and channeling during separation. This process was repeated as needed, taking care to avoid contact of the siliconizing fluid with the sintered-glass disk. The columns were packed with G-25 Sephadex (coarse beads) obtained from Pharmacia (Uppsala, Sweden) and prepared (washed, swollen, and fined) in the usual manner. The gel was packed into the column in layers; 10–15 ml of gel slurry at a time was added to a closed column (filled with water or buffer) and allowed to settle. The column was then briefly oscillated to stabilize and level the gel, flow was resumed briefly to pack it, another aliquot of slurry was then added, and the process was repeated. The number of layers per unit column length was not found to be critical; three layers per centimeter were quite adequate to achieve good separation. Column lengths of ∼11 cm were used in most of the runs. The gel was covered with a disk of sharkskin filter paper (Schleicher & Schuell, Keene, N. H.), to spread the sample and minimize gel disruption on sample and buffer application. After each run the top 3–4 cm of the gel was stirred with a soft Teflon rod (to avoid scratching the silicon coating of the column and consequent bubble formation), and the filter paper disk was replaced. The columns were run and stored at temperatures below 4°. After each experiment the columns were washed *immediately* with ten or more column volumes of distilled water, purged with 1 M NaCl to remove charged contaminants, and stored in 1 M NaCl to inhibit bacterial growth. Columns in which contaminating tritium could not be reduced to normal background levels by such washing were discarded.

Temperature control is a crucial aspect of hydrogen-exchange studies; ideally the columns, the buffer reservoir, and the exchanging pool after the first gel filtration must all be kept at the same (constant) temperature. Preliminary measurements have shown that the rate of exchange of DNA increases about 10–12%/°C, and the temperature which applies to a particular experiment is clearly the time average of the thermal experience of the sample during the entire exchange-out period (*i.e.*, samples exchanged for short times spend a proportionately larger fraction of the exchange-out period in the columns, and less in the pool). To minimize temperature fluctuations all exchange experiments were carried out in a specially constructed, insulated refrigerated box with access provided through small doors. The temperature of the box was continuously variable, with regulation of the entire internal environment to better than ±1.5°. During an experiment the doors of the cold box were open for extended periods of time, but the increase in the air temperature of the box was never greater than 3–5°. To compensate for this small warming, the temperature control during the run was set to ensure continuous operation of the refrigeration unit and minimize temperature fluctuations. The pool tube and the lines from the buffer reservoir were immersed in ice at all times. The buffer reservoir was large enough to damp out any moderate temperature fluctuations. The heat capacity of the columns was also quite large, and column temperatures have been shown by direct measurement to be quite insensitive to minor variations in air temperature (B. McConnell, private communication). Results presented in this paper were obtained over an extended period of time, during which various modifications were incorporated into our experimental procedures. The temperature within a single series of runs (*e.g.*, a pH series, presented as an individual graph in the Results section) was held constant (to ±1° unless otherwise stated), but the temperature variation *between* series was larger, runs having been made over the range from 0 to 4°.

For each run, the DNA solution (usually 2–5 ml containing 1–2 mg/ml of DNA) was adjusted to the pH and salt concentration of the experiment by dialysis against several liters of the relevant buffer. About 1 hr before a run, tritiated water (New England Nuclear Corp.) was added to the DNA sample to a final activity of 7–10 mCi/ml. Complete tritiation was achieved by incubation at room temperature for 30–60 min. Equilibration for much longer periods, or with an interim period of freezing, did not alter either the amount of tritium incorporated into the DNA, or the exchange kinetics, so we may assume that no additional slowly exchangeable class of hydrogens was present.

DNA. Native calf thymus DNA (Worthington Biochemical Corp.), dissolved, characterized, and sonicated as previously described (Printz and von Hippel, 1965), was used in all these studies. As pointed out previously, the DNA was sonicated to reduce viscosity effects and spreading of the DNA in the Sephadex columns. Control experiments have shown that sonicated and unsonicated DNA have essentially identical exchange kinetics (Printz and von Hippel, 1965). All preparations used were completely native and free of metal contamination as determined by melting criteria (Printz and von Hippel, 1965). DNA concentration was measured spectrophotometrically (at room temperature) by absorbance at 260 mμ, using 1.30×10^4 cm^{-1} as the molar extinction coefficient (per mole of nucleotide pairs) for native calf thymus DNA. The variation of extinction coefficient with salt or pH changes was not significant at Na^+ concentrations greater than 0.005 M.

Reagents and Solutions. Reagent grade or spectroscopically pure chemicals were used throughout, and all solutions were made up with double-distilled water. Solution pH values were adjusted at (or calculated to) the temperature of the experiment for buffers with appreciable enthalpies of ionization.

Melting Experiments. Hyperchromic DNA melting profiles were measured initially in a Zeiss PMQ II spectrophotometer and later in a Gilford Model 2000 automatic recording spectrophotometer. The procedures used in loading and sealing the cells, controlling and measuring the temperature, and analyzing the data have been described elsewhere (*e.g.*, see Olins *et al.*, 1967).

Titration Experiments. DNA titrations were performed using a Radiometer SBR2/SBU1/TTA2 automatic recording titrator equipped with separate Radiometer glass (G2023) and calomel (K401) electrodes. Titration was continuous; up to 0.5 ml of 0.02 M NaOH (or 0.02 M HCl) was added from an Agla precision syringe to raise (or lower) the pH. Titrations were run as a function of salt concentration with 10 mg of native or heat-denatured DNA dissolved in 14 ml of the relevant salt solution. Control titrations were carried out with the same volumes of salt solution in the absence of DNA, and substracted from the DNA runs. No corrections were made for the progressive change in salt concentration with added acid or base (less than 3% in the worst cases). The runs were made as a function of temperature in a water-jacketed titration vessel. The pH scale was calibrated with standard buffers at the temperature of the experiment. All titrations were carried out under water-saturated nitrogen (prepurified by bubbling through concentrated NaOH and H_2SO_4). Prior to each experiment the sample was bubbled with N_2 for 10 min to remove dissolved CO_2. Under these conditions there was no detectable uptake of acid or base at fixed pH in any of the titration experiments.

Sources of Experimental Error and Their Effects on the Accuracy of Hydrogen-Exchange Data. Both random and systematic errors enter into hydrogen-exchange experiments, and their magnitudes and effects on the

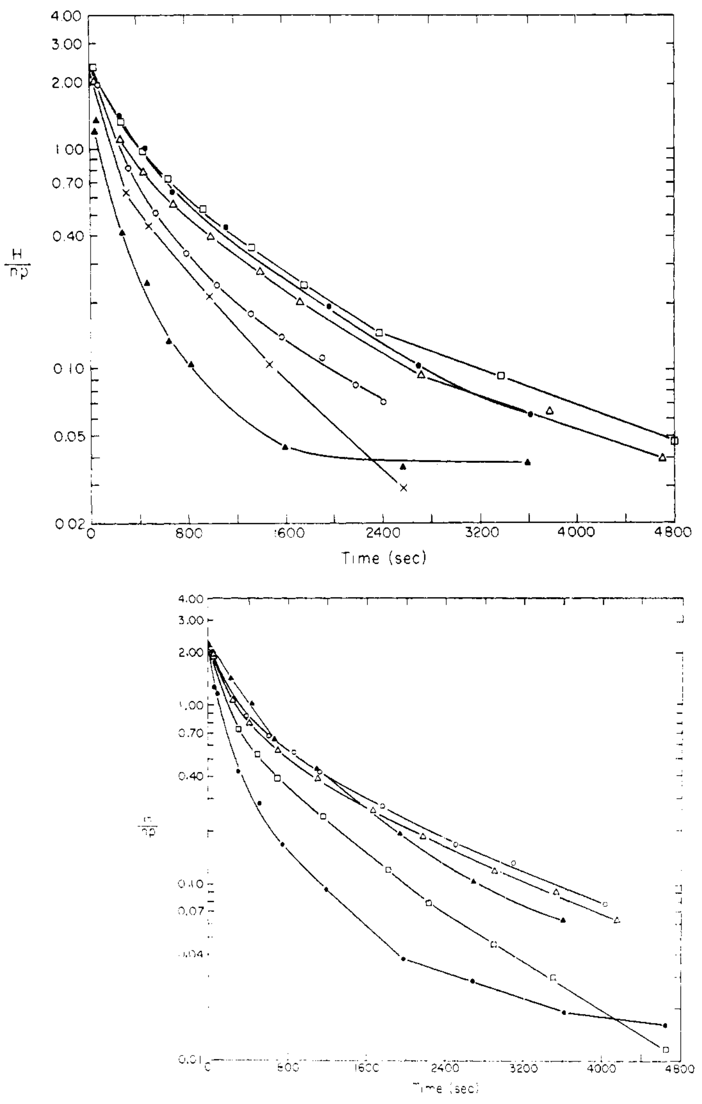

FIGURE 1: Hydrogen–tritium exchange-out curves for native calf thymus DNA at 0.5 ± 0.3° and various values of pH in 0.1 M NaCl–0.0001 M disodium EDTA. (a) 0.01 M sodium cacodylate. pH (measured at 23° directly after each experiment and corrected to 0.5°): (●) 7.8, (□) 7.37, (△) 7.15, (○) 6.75, (×) 6.35, and (▲) 5.95. (b) 0.01 M sodium borate. pH (measured at 1.0° after each experiment): (▲) 7.8, (○) 8.24, (△) 8.65, (□) 9.04, and (●) 9.31.

data must be evaluated prior to any attempt at serious analysis of the shapes of exchange-out curves. In general, the random error of an individual point on a hydrogen-exchange curve is affected by errors in tritium counting and in DNA concentration analysis. Counting errors are less than ±2% for early time points (*i.e.*, H/np ~1.0) where the counts are approximately 40 times background. The counting error approaches ±5–10% at H/np ~0.05 where the count rate is four to five times background. DNA concentrations were generally measured at optical densities in excess of 0.5, so the error in this measurement is probably less than ±1%, and pipetting errors were found to be less than ±2%. As mentioned above, each exchange point also represents the average of determinations on two to four different tubes. Error analysis suggests that the *random* error of *individual* hydrogen-exchange points ranges from about ±4% at the level of approximately 1 H/np, to about ±10% at approximately the 0.05-H/np level.

Superimposed on these errors are systematic errors which can effect the shape of entire exchange curves. The primary cause of these is temperature fluctuations and differences, which have been discussed above. Since time points on the exchange curves are derived from samples which have spent varying proportions of the total exchange time in columns and in the pool, differences in temperature between columns and pool can effect the shapes of the exchange-out curves. As a hypothetical (extreme) example to illustrate the possibilities, if the columns used in a particular run happened to be at 5° and the pool at 0°, a 200-sec sample will have spent almost the entire exchange-out period at 5° and thus measured value of H/np will be 5–6% lower than if the sample had been at 0° throughout. On the other hand, a 2000-sec sample obtained under the same conditions will have spent 90% of the exchange-out period at 0° (in the pool) and thus the measured value will only be about 0.5% lower than if the columns had been at 0° throughout. The net effect of this type of systematic error will be to make linear exchange curves appear slightly concave downward. (We should emphasize that this is an extreme example, since temperature differences between the pool and the columns were usually considerably less than this.)

Results

Effect of pH on Hydrogen-Exchange Kinetics. As reported previously (Printz and von Hippel, 1965; von Hippel and Printz, 1965) hydrogen exchange of native DNA proceeds most slowly at pH values near neutrality and at moderate salt concentrations. Thus such conditions (pH 7.6, 0.1 M NaCl) were used in our initial studies which were aimed primarily at obtaining a measure of $(H/np)_0$ (*e.g.*, Figure 1; Printz and von Hippel, 1965). In order to approach questions of mechanism, systematic data on the kinetics of hydrogen exchange as a function of pH and salt concentration are required. Such data are presented here. It will be apparent that as we move away from conditions of slow exchange, the exchange process is accelerated so that at extremes of pH and salt concentration we are only able to obtain experimental points for the last 30–50% of the reaction, and so lack direct information about the shape of the early part of the exchange curve. Nevertheless, considerable insight can be derived from the information which is experimentally attainable.

In Figure 1a, log (H/np) is plotted *vs.* exchange-out time for native calf thymus DNA (0.1 M NaCl, 0.5 ± 0.3°) at several pH values below pH 8.0. It is apparent that under these conditions the exchange rate increases and that the curves become progressively less linear (*i.e.*, deviate from simple first-order behavior) with decreasing pH. Figure 1b shows that similar effects occur on the alkaline side of neutrality at the same salt concentration and temperature (0.1 M NaCl, 0.5 ± 0.3°). As the pH is increased above pH 8, the rate of exchange

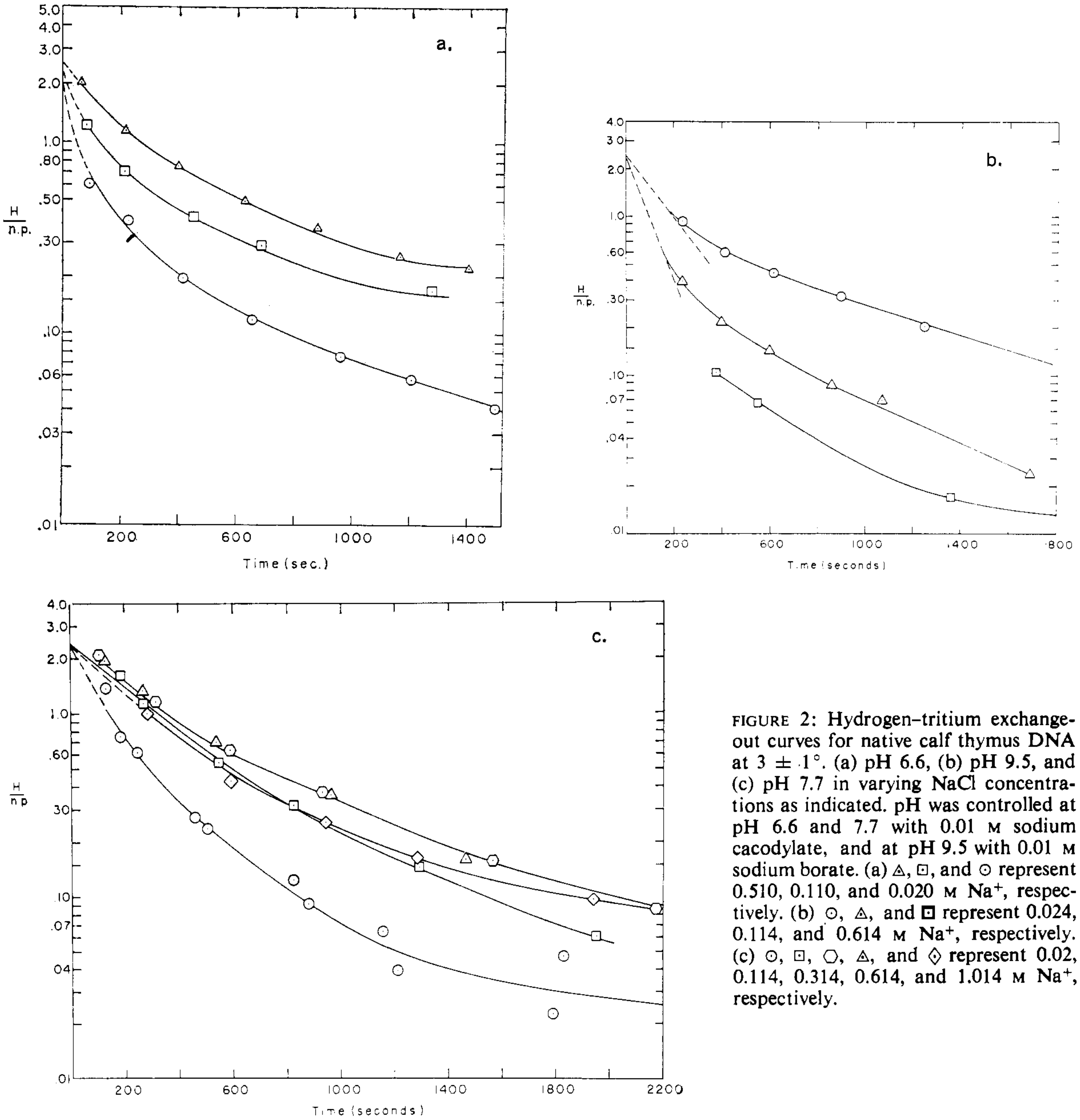

FIGURE 2: Hydrogen–tritium exchange-out curves for native calf thymus DNA at 3 ± 1°. (a) pH 6.6, (b) pH 9.5, and (c) pH 7.7 in varying NaCl concentrations as indicated. pH was controlled at pH 6.6 and 7.7 with 0.01 M sodium cacodylate, and at pH 9.5 with 0.01 M sodium borate. (a) △, ⊡, and ⊙ represent 0.510, 0.110, and 0.020 M Na$^+$, respectively. (b) ⊙, △, and ◻ represent 0.024, 0.114, and 0.614 M Na$^+$, respectively. (c) ⊙, ⊡, ○, △, and ◇ represent 0.02, 0.114, 0.314, 0.614, and 1.014 M Na$^+$, respectively.

again increases and the exchange curves again deviate progressively more from linearity.

Thus it appears that the exchange rate goes through a minimum in the vicinity of pH 7–8; either increasing or decreasing the pH from this region increases the rate of hydrogen exchange.

Effects of Salt Concentration on Hydrogen-Exchange Kinetics. Figure 2a shows that at pH 6.6, decreasing the salt concentration from 0.51 to 0.02 M NaCl results in a monotonic increase in exchange rate. Qualitatively it appears that decreasing salt concentration at pH values below the exchange minimum affects the exchange curve much like a progressive decrease in pH (Figure 1a). Figure 2b shows that at a pH (9.5) well above the pH range in which exchange is slowest, the low pH situation is exactly reversed; at this pH, as the salt concentration is decreased (from 0.61 to 0.02 M NaCl), the rate of exchange is markedly *decreased.* Furthermore, Figure 2c shows that at a pH (7.7) in the middle of the minimum exchange-rate region, a decrease in salt concentration (from 1.0 to 0.02 M NaCl) first decreases and then increases the rate of hydrogen exchange.

These pH–salt relationships may be compared in more revealing fashion by plotting the extent of reaction at constant exchange-out time, $(\mathrm{H/np})_t$, as a function of experimental variables such as pH or salt concentration. As eq 3 shows, for a first-order reaction log H_t at constant t is directly proportional to the first-order rate constant, k. Since the exchange-out plots obtained in this study are all curved, it is obvious that we are dealing with more than one class of exchangeable hydrogens (more than one concurrent first-order exchange reaction), and thus the extent of exchange at time t, (H_t) will be a complex function of a composite rate constant. Nevertheless, by examining the dependence of log (H/np) at various times upon pH and salt concentra-

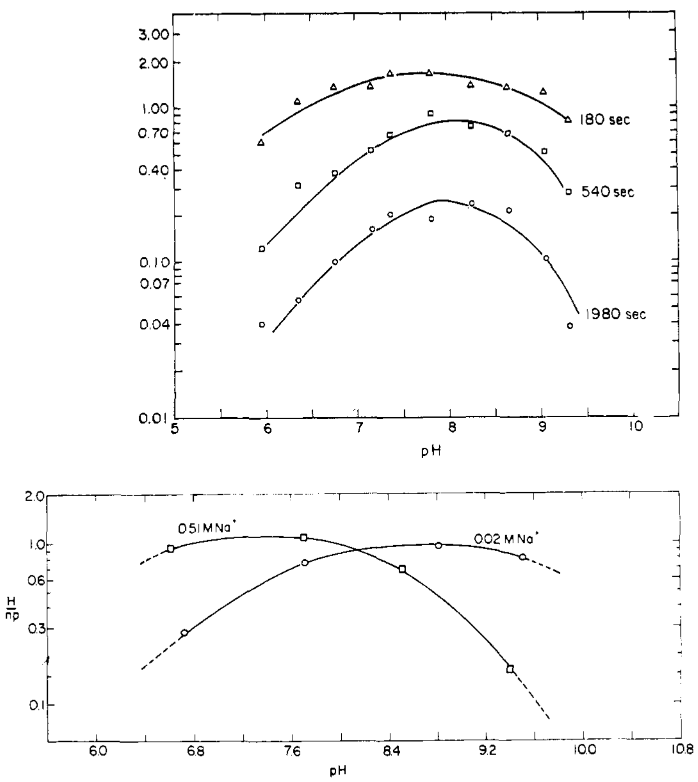

FIGURE 3: Extent of exchange $(H/np)_t$ curves at various exchange-out times plotted as a function of pH. The experimental points are derived, in large part, from the data of Figures 1 and 2. (a) 0.11 M Na^+ after 180, 540, and 1980 sec of exchange; (b) 0.51 and 0.20 M Na^+ after 300 sec of exchange.

tion, some semiquantitative assessment of the nature and trends of these complexities may be obtained.

Figure 3a displays the extent of reaction as a function of pH for the data of Figure 1a,b at three exchange-out times: 180, 540, and 1980 sec. It is clear that all these curves peak between pH 7.5 and 8.0; these data apply to 0.11 M NaCl. In Figure 3b we present similar plots using salt (Na^+) concentration as the independent variable, for data obtained after a 300-sec exchange in 0.02 and 0.51 M NaCl. As Figure 1a,b had shown, there is a minimum exchange rate region near neutrality, but in addition, Figure 3b shows that the apparent pH_{min} shifts markedly toward lower pH as the salt concentration is increased.

Outside the pH region of minimum exchange rate, it can be shown (Figure 4a,b) that log $(H/np)_t$ is approximately linearly related to log $[Na^+]$. Figure 4a presents data for pH 7.7 showing that $[Na^+]_{min}$ occurs between 0.1 and 0.8 M Na^+, with rates increasing (log $(H/np)_t$ decreasing) as the salt concentration is increased or decreased from this range (compare Figure 2a,b). Figure 4b presents data obtained at pH values above (9.5) and below (6.6) pH_{min}, and shows that the rate of exchange increases with increasing salt at high pH, and decreases with increasing salt at low pH. Note that the pH 6.6 and 9.5 family of curves cross one another at a salt concentration of ~0.05 M Na^+. Thus the rate of hydrogen exchange is the same at this salt concentration at both pH 6.6 and 9.5. At both of the pH values studied here, the logarithm of the exchange rate (as measured by extent of exchange at a given time) is approximately proportional to log $[Na^+]$; though this clearly is *not* the case for pH values in the exchange minimum region (Figure 4a). Thus at experimental pH values well above and below the minimum exchange "plateau" region we may write

$$\log (H/np)_t \simeq A \log [Na^+] + B \qquad (5)$$

where $(H/np)_t$ is the number of unexchanged hydrogens per nucleotide pair remaining at time t, at a particular pH, and A and B are constants which vary in magnitude with pH. The sign of A will also depend upon pH. At pH values well below the exchange minimum, A is large and positive, and decreases in size as the plateau region is approached. At alkaline values of pH, A is large and negative, again approaching zero as the experimental pH is decreased toward the plateau. The family of curves at any one pH valve all have roughly the same shape, suggesting that the factors responsible for the observed rate changes affect the entire *measurable* exchange curve about equally.

Effects of Salt Concentration on pH–Titration Curves of DNA. The major features of the above hydrogen-exchange behavior of DNA as a function of salt concentration and pH show significant correlations with the behavior of DNA pH–titration curves at various salt concentrations (*e.g.*, see Cox and Peacocke, 1956). These workers had early shown that the apparent pK's of the acid and alkaline "limbs" of the pH–titration curve for both native and denatured herring sperm DNA varied markedly with the salt concentration at which the titration was carried out. They found that pK_{app} for both the acid and the alkaline limbs of the DNA titration curve were shifted appreciably toward lower pH values as the salt concentration was increased; the effect depending primarily upon the cation used (*i.e.*, there was little or no dependence of pK_{app} upon the associated anion). To permit quantitative comparison of our hydrogen-exchange data with relevant pH–titration data, we have carried out a series of titrations of native and denatured calf thymus DNA in the presence of varying concentrations of NaCl, with results very similar to those obtained previously by other workers with other DNAs (*e.g.*, Cox and Peacocke, 1956–1958; Peacocke and Walker, 1962; Bunville *et al.*, 1965). The titration curves show an approximately parallel downward shift of the pK_{app} of both the acid and alkaline limbs of the titration curves for both native and denatured DNA with increasing salt concentration (*e.g.*, see Figure 5). As also previously observed by others, the native DNA titration curve is partially biphasic. This two-step titration results from the collapse of native double-helical structure as titration proceeds, accompanied by a transition to the less abrupt titration curve characteristic of denatured DNA (*e.g.*, see Peacocke and Walker, 1962).

To afford quantitative comparison of the effect of salt and pH on hydrogen-exchange curves and on DNA titration curves, pK_{app} has been measured for native and

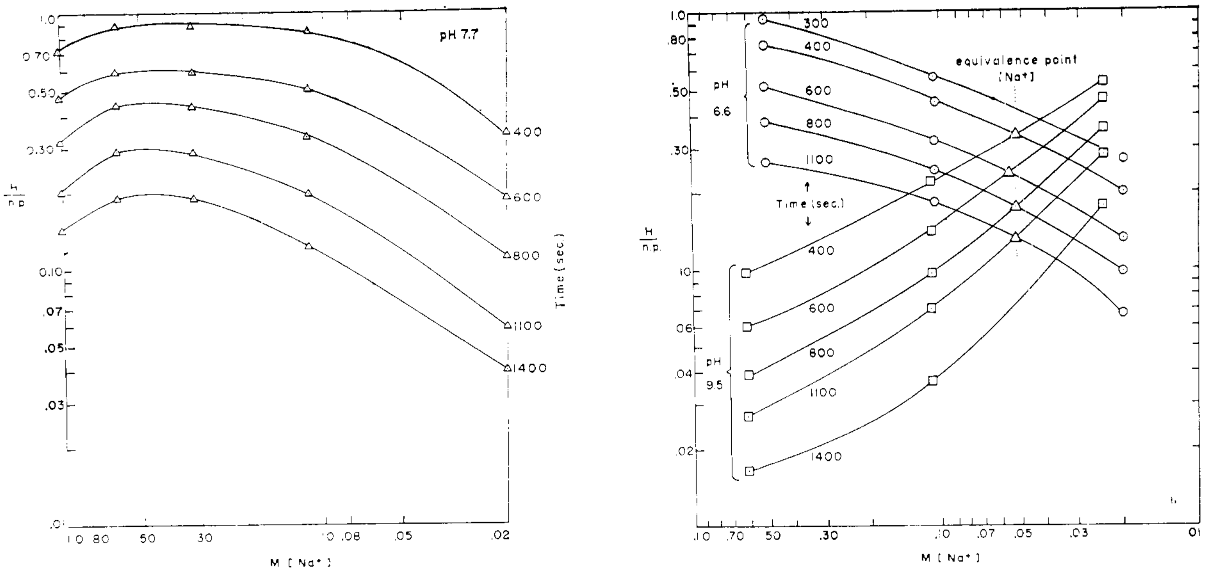

FIGURE 4: Extent of exchange $(H/np)_t$ curves at various exchange-out times plotted against log $[Na^+]$. The experimental points are obtained in part from Figure 2 and from other runs made at 3 ± 1°. (a) pH 7.7; (b) pH 6.6 and 9.5. The equivalence point marked in b (at $\sim$0.05 M Na^+) corresponds to the salt concentration at which exchange proceeds at equal rates at pH 6.6 and 9.5.

denatured DNA as a function of salt concentration. Starting from neutrality in each case, the completion of the acid titration of DNA (in the region of pH 2–4) involves the protonation of N_1 of adenine, N_3 of cytosine, and N_7 of guanine. Thus the acid pK_{app} for calf thymus DNA may be calculated from the DNA base composition to correspond to 0.71 equiv of H^+/np. The completion of the alkaline titration of DNA (in the region of pH 10–12) corresponds to a deprotonation of the N_1 of guanine and the N_3 of thymidine. Thus the alkaline pK_{app} falls at 0.5 equiv of OH^-/np for calf thymus DNA. Figure 6 shows that, at least on the acid side, the titration curves of native and denatured DNA merge at or near the pH at which pK_{app} is reached; so (pK_{app}) NDNA $\simeq$ (pK_{app}) DDNA.[2] (Note that denaturation is complete at a lower degree of protonation at low salt concentration, as has previously been shown by Bunville *et al.*, 1965.) Thus under the conditions examined, titration of the native structure to 0.71 equiv of H^+/np results in complete denaturation of the DNA structure. This conclusion is in reasonable accord with the findings of Bunville *et al.* (1965) who have shown that acid denaturation of a DNA with the base composition of calf thymus DNA (42 mole % G–C) is initiated at a degree of protonation of about 0.6 equiv of H^+/np, and is complete (all secondary structure lost as assessed by hypochromism) at about 1.0 equiv of H^+/np for measurements made at 25° and 0.1 M NaCl.

Figure 7 shows pK_{app} for both the acid and the alkaline limbs of the DNA titration curve as a function of log $[Na^+]$. The native and denatured DNA data on the acid side are superimposable (as indicated in Figure 6). No alkaline pK_{app} data were obtained with native DNA.[2] Clearly, at a given temperature, pK_{app} (on both the acid and the alkaline sides) is a linear function of log $[Na^+]$. Thus

$$(pK_{app})_T = C \log [Na^+] \tag{6}$$

where $(pK_{app})_T$ is the apparent pK at temperature T and $C \simeq -0.65$ pH units/tenfold increase in salt concentration.[3]

Correlation between DNA Hydrogen-Exchange Rates and pH-Titration Curves. These results, with their striking empirical correlations with the hydrogen-exchange results, suggest that the observed rate of exchange depends primarily upon the proximity of the experimental pH to the pK_{app} of the closest limb of the DNA titration curve. That is, the smaller ΔpH $(pH_{exp} - pK_{app})$, the

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[2] Accurate titration measurements for native DNA, especially on the alkaline side at low salt, are very difficult to obtain because the structural hypersharpening of the titration pushes the entire curve up to very high pH values where the corrections for the titration of the blank approach the magnitude of the DNA contribution itself. The shift in pK_{app} with salt concentration for heat-denatured DNA is the same as for native DNA, though the shape of the titration curve itself is different (see Figure 6).

[3] Although the same type of chemical groups are being protonated and deprotonated on the acid and alkaline sides of the DNA titration curves (*i.e.*, the endocyclic imino group of either the purines or the pyrimidines), the acid limb shows no temperature dependence of pK_{app} over the temperature range 10–40°, while the temperature dependence of the alkaline limb is marked. This may be reconciled by noting that the species which is active in deprotonation at alkaline pH is the hydroxide ion. Thus pK_{app} should be defined on the alkaline side on a pOH scale, since K_w, the dissociation constant of water, is temperature dependent and pOH = pK_w − pH. K_w increases with temperature, and it can be calculated that at a fixed pH, pOH increases by about 0.3 pH unit/10° rise in temperature. Such a correction to the alkaline pK_{app} values of Figure 7 makes these values also essentially temperature independent. (We are grateful to Dr. S. W. Englander for suggesting this interpretation to us.)

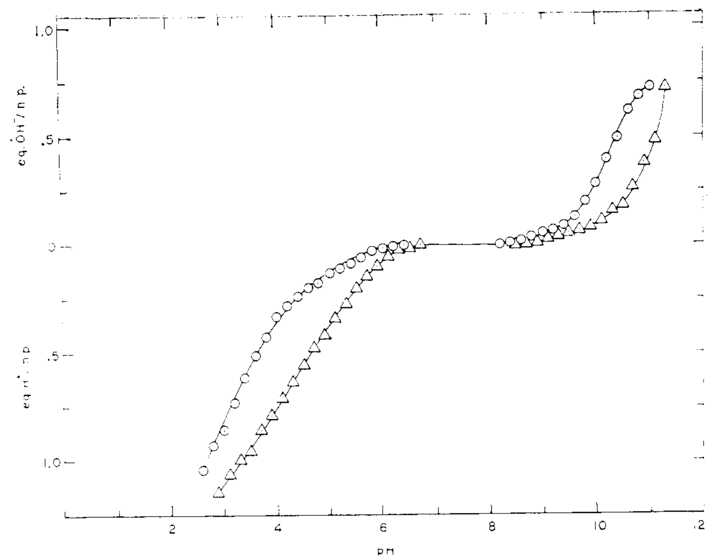

FIGURE 5: Continuous pH–titration curves of heat-denatured calf thymus DNA in 0.505 (⊙) and 0.01 M (◬) NaCl at 30°. For detailed titration conditions, see Materials and Methods section.

greater the rate of exchange. This expectation suggests that the rate of exchange should pass through a minimum when ($pK_{exp} - pK_{app}{}^a$) = ($pH_{exp} - pK_{app}{}^b$); *i.e.*, at the point at which the experimental pH is equidistant from $pK_{app}{}^a$ and $pK_{app}{}^b$ (the acid and alkaline apparent pK's for the DNA titration curve).

This proposal can be subjected to semiquantitative test, as follows. (a) Figure 3a shows that in 0.11 M Na^+, the pH region of minimum exchange is centered around pH 7.5–8.0. Figure 7 places the midpoint of the titration curve (extrapolated to 0° and 0.11 M Na^+) at pH ~7.7 ± 0.3. (b) Figure 3b suggests that in 0.51 M Na^+, the minimum exchange region is centered at ~7.3 ± 0.5 and in 0.02 M Na^+ at ~8.6 ± 0.5. Figure 7 places the midpoint of the titration curve at 0.51 M Na^+ (and 0°) at about pH 7.2 ± 0.3, and at 0.02 M Na^+ (and 0°) at about pH 8.2 ± 0.3. (c) Figure 4b shows that the exchange rates of DNA at pH 9.5 and 6.6 are equal at 0.05 M Na^+. In terms of the correlations we have developed here this suggests that the midpoint of the pH titration curve at 0.05 M Na^+ (and 0°) should fall midway between these pH values, or at pH ~8.0. Figure 7 gives a value of pH ~7.9 ± 0.3 for this point.

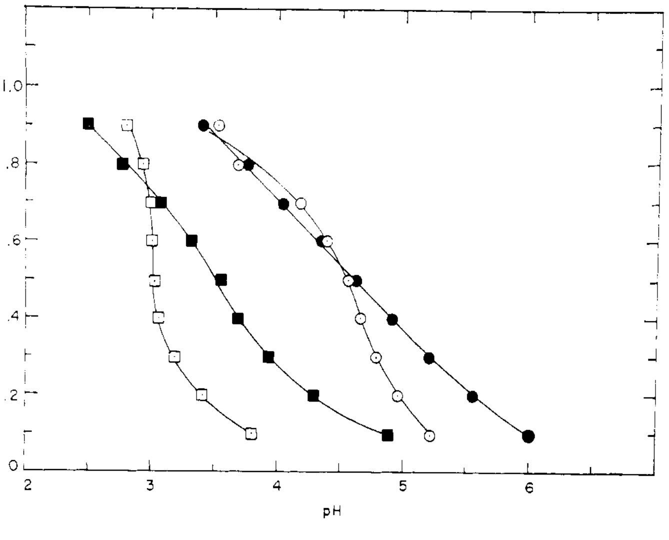

FIGURE 6: Continuous acid pH–titration curves of native and denatured calf thymus DNA at 40° and two sets of salt concentrations. (⊙) 0.005 M and (⊡) 0.505 M Na^+ and native DNA. (●) 0.005 M and (■) 0.505 M Na^+ and denatured DNA.

Thus these sets of data show that, within the limits of error of the measurements, the suggested correlations between the pH–titration curves and the effects of pH and salt on the hydrogen-exchange curves are indeed valid, and for pH values well removed from the pH of minimum exchange we may write

$$\log (H/np)_t \simeq C|pH_{exp} - pK_{app}| \qquad (7)$$

where $(H/np)_t$ is the number of unexchanged hydrogens remaining at time *t*, *C* is a proportionality constant, and $|pH_{exp} - pK_{app}|$ is the absolute value of the difference in pH between the experimental pH and the pK_{app} of the nearer limb of the titration curve; and

$$pH_{min} = (pK_{app}{}^b - pK_{app}{}^a)/2 \qquad (8)$$

where pH_{min} is the center of the minimum exchange rate plateau region, and $pK_{app}{}^a$ and $pK_{app}{}^b$ are the pK_{app} values relevant to the alkaline and acid limbs of the DNA titration curve, respectively.

We will consider mechanisms which may account for these effects in the Discussion.

Effects of Mg^{2+}. In order to test whether the correlations outlined above could be extended from sodium ion to tightly bound divalent ions such as Mg^{2+}, hydrogen-exchange experiments were carried out with DNA at high and low pH valve in the presence and absence of Mg^{2+}. The results are shown in Figure 8, in which exchange rates for native DNA in 0.005 M Na^+, with and without 0.001 M Mg^{2+}, are compared at pH 6.4 (well below the pH region of minimum exchange rate for 0.005 M Na^+) and pH 8.6 (close to pH_{min} for 0.005 M Na^+). The results are clear-cut, and show that while 10^{-3} M Mg^{2+} moderately decreases the exchange rate at pH 6.4, it markedly increases the rate of exchange at pH 8.6. These results suggest that Mg^{2+} operates similarly, but much more effectively, than Na^+ in affecting exchange rates. This is borne out by some preliminary DNA titrations carried out in the presence of increasing quantities of Mg^{2+} (precipitation occurred in some of these experiments; see also Lyons and Kotin, 1965). These experiments showed that increasing (small) quantities of Mg^{2+} displace both the acid and alkaline pK_{app} values for DNA titration curves toward lower pH, and that the pK_{app} is displaced by Mg^{2+} with 30–50 times the effectiveness of the displacement with Na^+. For example, we found that in the presence of 0.005 M Na^+, 0.001 M Mg^{2+} displaced pK_{app} about 0.5 pH unit toward the acid side. At this ionic strength 0.05 M Na^+ would be required to bring about a comparable change in pK_{app}.

Effects of Tris. In order to demonstrate directly that a

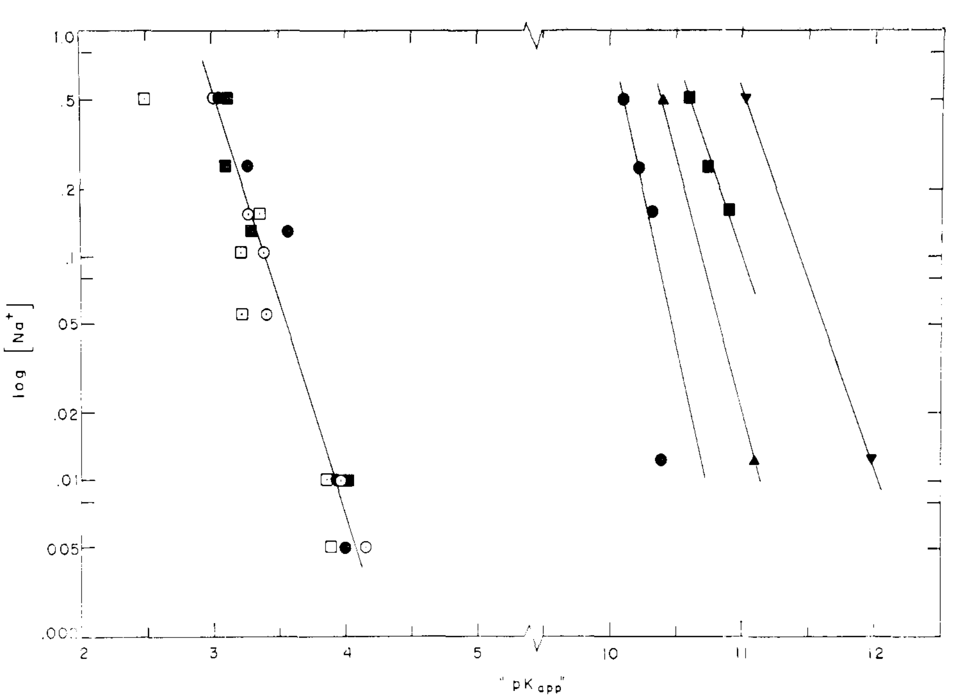

FIGURE 7: Plot of pK_{app} vs. log [Na^+] at various temperatures, where pK_{app} is defined as the pH at which 0.71 equiv of H^+/np has been added (acid side) or 0.50 equiv of OH^-/np (alkaline side). See text. At 20 and 40° native DNA is represented by ⊡ and ⊙, respectively. At 10, 20, 30, and 40° denatured DNA is represented by ▼, ■, ▲, and ●, respectively.

potential exchange catalyst (*i.e.*, a proton donor or acceptor other than $H_9O_4^+$ or $H_7O_4^-$; Eigen, 1964) can affect hydrogen-exchange rates in DNA, we have carried out experiments in which 0.2 M Tris, adjusted to pH 7.7, was added at two separate times to the pool in a hydrogen-exchange experiment proceeding under slow-exchange conditions (pH 7.7, 0.1 M NaCl, $\sim$3°). The results are shown in Figure 9, and demonstrate that this concentration of Tris is markedly catalytically effective, both early and late in the exchange process. Relevant control experiments (*e.g.*, Figure 2c) show that a comparable increase in the concentration of monovalent cation at this pH ($\sim$0.3 M Na^+, since Tris exists primarily as the protonated species under these experimental conditions) would result in a slight *decrease* in exchange rate. Thus this is not a salt effect on the exchange rate. In control melting experiments it was also shown that this concentration of Tris has no effect on the T_m of DNA.

Discussion

The following facts emerge from this study and earlier ones, and must be accounted for in formulating a mechanism of hydrogen-exchange for DNA.

The Role of Structure. Only hydrogens involved in interchain hydrogen-bonding exchange slowly enough to be detected by these (relatively) slow techniques, meaning that structure must somehow be implicated in limiting the rate of exchange (eq 4).

Chemistry of the Exchanging Groups. DNA contains two chemically distinct classes of hydrogens involved in hydrogen bonding. Each nucleotide pair contains one hydrogen attached directly to an endocyclic nitrogen of the purine or pyrimidine (the N_1 of guanine and the N_3 of thymine) and one (for an A–T pair) or two (for a G–C pair) hydrogens attached to exocyclic amino nitrogens (the 6-amino groups of adenine, the 2-amino of guanine, and the 4-amino of cytosine). These nitrogens differ widely in basicity, *i.e.*, in the ease with which they add or lose a proton, and thus if the observed exchange rate of these groups were to depend upon their acid strengths we would expect to see two kinetic classes of exchangeable hydrogens for calf thymus DNA, one containing 1.42 and the other 1.0 H/np, which would exchange at rates differing by several orders of magnitude. This is not the case. Under most conditions all (>90%) of the interchain hydrogens exchange at comparable rates; *i.e.*, as members of kinetic classes which differ in rate constant by at most a factor of 10 (and usually less).

Size of the Exchange Unit. The exchange unit for DNA appears to be small, and exchange quite noncooperative, under the experimental conditions used here. Perhaps the most direct evidence for this is the demonstration that short, interchain hydrogen-bonded helical regions re-formed upon cooling heat-denatured calf thymus DNA exchange at rates comparable with fully native DNA (Printz and von Hippel, 1965).

Correlation with T_m. The effect of various solvent additives on the melting temperature, T_m, of the DNA helix provides one criterion of the effect of these changes on the structural stability of the DNA helix. No correlation between T_m changes and changes in the rate of hydrogen exchange is evident for the variables studied here. Thus the T_m of DNA is essentially pH independent between pH 5.5 and 9.5 (*e.g.*, Lewin and Pepper, 1965). At more extreme values of pH T_m declines slowly.

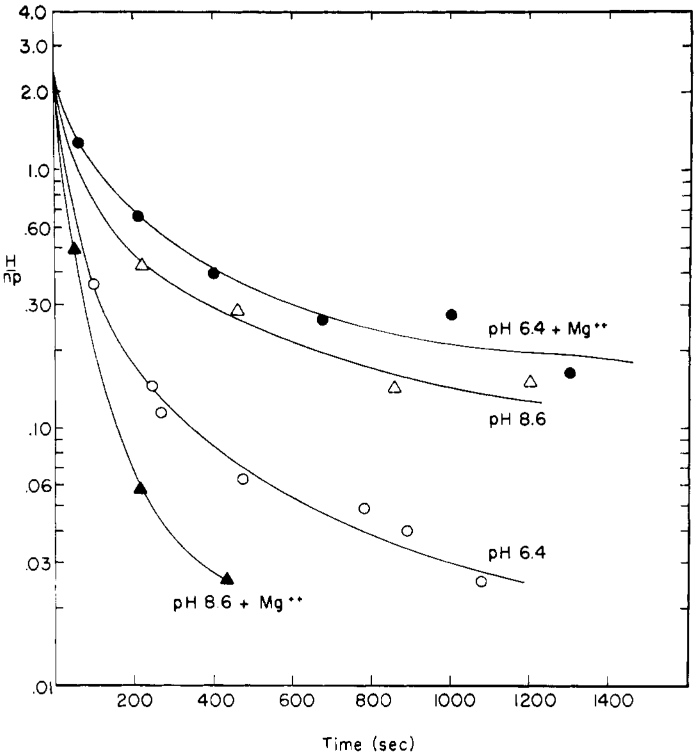

FIGURE 8: Hydrogen–tritium exchange-out curves showing the effect of added Mg^{2+} on the exchange-rate pH 6.4 (sodium cacodylate buffer) and 8.6 (sodium borate buffer). Control runs were made in 0.005 M Na^+. The Mg^{2+} runs contained 0.001 M $MgCl_2$ and 0.005 M Na^+ at the same pH values as the controls.

On the other hand, we have shown that the rate of hydrogen exchange is markedly dependent upon pH between pH 5.5 and 9.5. Furthermore, T_m increases directly with log [Na^+], up to about 1 M NaCl, at all pH values (*e.g.*, Dove and Davidson, 1962; Schildkraut and Lifson, 1965), and while the rate of exchange decreases directly with log [Na^+] at acid values of pH, the inverse is true on the alkaline side of neutrality.

In addition, McConnell and von Hippel (1968) have recently shown that the rate of hydrogen exchange is *not* affected by helix-(T_m) destabilizing salts until the experimental temperature actually gets into the helix–coil transition region.

Chemical Catalysis. We may ask whether the effects of pH and salt concentration on DNA hydrogen exchange are compatible with a direct effect on the chemical exchange rate, k_3. No simple correlation has been found. In amides and peptides hydrogen exchange is catalyzed by both acid and base (as well as by other acid–base catalysts), and the exchange rate is relatively independent of salt concentration. Furthermore, at values of pH more than 1 pH unit removed from pH_{min}, the exchange rate increases by a factor of 10/pH unit (*e.g.*, see Berger *et al.*, 1959; Klotz and Frank, 1965; Leichtling and Klotz, 1966). On the other hand in DNA the rate of exchange is markedly salt dependent, the value of pH_{min} is also salt dependent and dependent upon the pK_{app} of the exchanging groups (even though exchange of at least the hydrogens attached to the endocyclic nitrogens of the bases should be diffusion controlled and thus independent of the p*K* of the donor groups; Eigen, 1964) and nowhere, at least near-neutral pH, do we observed a tenfold increase in rate with a one unit change in pH.

However Tris (this study) and other proton donors and acceptors (McConnell and von Hippel, 1968) markedly increase the exchange rate of DNA (at constant pH and salt concentration) over the entire measurable exchange curve. The mechanism of the effects of general acid–base catalysts on DNA hydrogen-exchange rates will be discussed elsewhere (B. McConnell and P. H. von Hippel, in preparation).

Direct water catalysis might provide a partial explanation of the relative apparent pH independence of hydrogen exchange at pH values near neutrality in DNA. Whereas direct transfer to water has been shown to be quite ineffective relative to acid or base or general acid–base catalysts in accelerating the hydrogen exchange of simple amides (Berger *et al.*, 1959; Klotz and Frank, 1965), the rate of exchange of such groups is markedly modified when they occur in a "hydrophobic" environment (*e.g.*, see Grunwald *et al.*, 1960; Eigen, 1964; Scarpa *et al.*, 1967). Thus water catalysis might be appreciably more effective in the vicinity of the DNA helix for hydrogens attached to the DNA structure than for hydrogens attached to nucleotides free in solution.

pH-Titration Curve. In contrast to the factors discussed above, the exchange data presented in this paper show striking correlations with the behavior of the pH-titration curve of DNA. We have shown that the pH at which the exchange rate is minimal, pH_{min}), corresponds closely to the pH at the midpoint of the plateau region located between the two arms of the DNA titration curve, pH_{mid}. Furthermore, changes in salt concentration shift pH_{min} and pH_{mid} in parallel, and at pH values well removed from pH_{min} the rate of hydrogen exchange seems to be inversely proportional to the absolute value of the difference between the experimental pH and the pK_{app} of the nearest limb of the titration curve.

The initial effect of lowering the solution pH on the internucleotide hydrogens of DNA is the protonation of the N_1 of adenine and the N_3 of cytosine (as well as the N_7 of guanine). The pK_{app} for this protonation ranges from 3 to 5, depending upon temperature and salt concentration. This protonation leads to a disruption of the hydrogen-bonding patterns of the base pairs, and is responsible for the onset of acid denaturation of DNA at about this pH (*e.g.*, see Bunville *et al.*, 1965). Raising the pH leads to an initial deprotonation of N_1 of guanine and N_3 of thymine, with a pK_{app} of 10–12, resulting also in disruption of the hydrogen bonding of the base pairs and alkaline denaturation of the DNA. The values of pK_{app} for the amino groups fall well outside of the pH range accessible in aqueous solution.

We may ask how the above protonation or deprotonation events can so markedly affect hydrogen-exchange rates at pH values several pH units removed from pK_{app} (*i.e.*, when only 1 or less nucleotide/1000 are protonated or deprotonated) especially if the exchange units are very small. Obviously there must be a considerable amplification of the effect of these small degrees of protonation or deprotonation in their effect on hydrogen-exchange rates, and this amplification must be introduced through effects on structure. We may speculate that if exchange proceeds by a two-step mechanism such as shown in eq 4, and if, while open, an occasional nucleotide pair were protonated or deprotonated as indicated above, the associated exchange unit would not close properly (decreased k_2), or would open again much more easily (increased k_1) because it now contains a nucleotide pair which does not possess the correct pattern of protonation for proper interchain hydrogen bonding. Thus effectively (transiently) this site is "propped open," or reopens more easily; in either case the locally relevant K is transiently greatly increased and exchange proceeds in this region at disproportionately greater rates. We shall use the generic term "propping" to represent such structure-based effects, to distinguish them from direct effects on the chemical exchange process itself.

Such propping effects may also be produced by other agents which interfere with internucleotide hydrogen bonding. Thus Printz (1967) has shown that small quantities of Hg^{2+} considerably speed up the hydrogen exchange of DNA, and has speculated that this may also be a manifestation of internucleotide propping.

Conclusion. In this paper we have documented the effect of pH and salt on DNA hydrogen exchange and, by coupling these effects to the properties of the DNA pH–titration curve, have begun to formulate a mechanism for the hydrogen-exchange process in DNA. It has been demonstrated that hydrogen exchange measures a special type of local transconformation reaction which seems to consist of a relatively noncooperative local opening–closing process in which internucleotide hydrogen bonds are broken. Such a process may involve little unstacking (*e.g.*, see von Hippel and Printz, 1965) and thus would be expected to differ from the cooperative local melting processes observed by other methods at temperatures somewhat below T_m. Subsequent papers will deal with the effects of DNA base composition, helix destabilizers, and other potential acid–base catalysts on the DNA-exchange process, and with the analysis of the shapes of the exchange-out curves, as well as examine further the exchange behavior of denatured DNA and synthetic polynucleotides. These studies will provide a background against which one can use DNA hydrogen exchange to study various aspects of the conformational motility of the molecule, and more importantly, to study the effects on this motility of interactions with other biologically relevant molecules (*e.g.*, Lees and von Hippel, 1968).

Acknowledgment

We gratefully acknowledge the skilled technical assistance of Mrs. Janice Printz, Mrs. Priscilla Cebrick, and Miss Judith Trout in carrying out many of the hydrogen-exchange experiments reported here. Mr. Kwok-Ying Wong performed a number of the pH titrations of DNA. Mr. Gerald Coleman participated in some of the early phases of this work as a summer medical research student. We are also grateful to Drs. S. Walter Englander and Bruce McConnell for useful discussions on several aspects of this work.

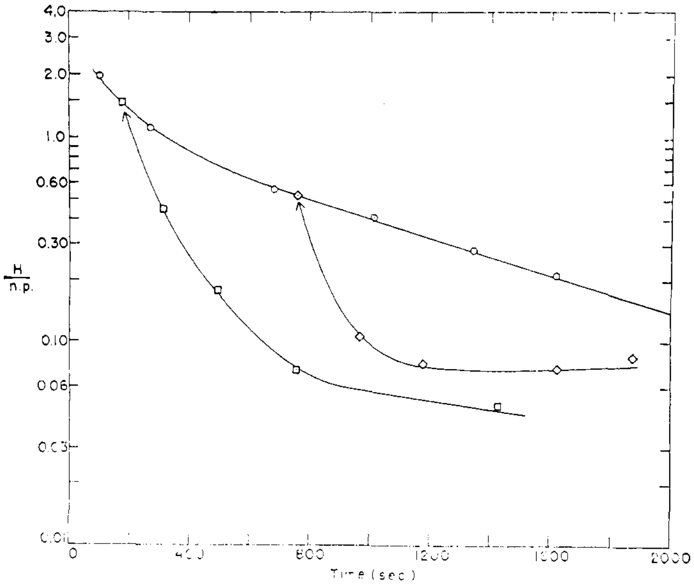

FIGURE 9: The effect of Tris in catalyzing hydrogen exchange. The upper curve is a control run at pH 7.7, 1.0 ± 1.0°, in 0.1 M NaCl–0.01 M sodium cacodylate. At the points indicated (175 and 780 sec) aliquots of the pool were made 0.2 M in Tris, and exchange was continued under otherwise identical conditions. (⊙) Control (0.1 M NaCl–0.01 M sodium cacodylate buffer at pH 7.7). (⊡) Control plus 0.2 M Tris-HCl (pH 7.7) at 175 sec. (◇) Control plus 0.2 M Tris-HCl (pH 7.7) at 780 sec.

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The Binding of Adenosine Triphosphate to Myosin*

L. H. Schliselfeld and M. Bárány

ABSTRACT: NaCl is a noncompetitive inhibitor of the Mg^{2+}–adenosine triphosphate phosphohydrolase activities of myosin and heavy meromyosin. By employing gel filtration chromatography in the presence of [γ-^{32}P]adenosine triphosphate and 1.50 M NaCl, both myosin and heavy meromyosin were observed to bind 1.6 and 1.7 moles of adenosine triphosphate per mole of protein, respectively. The binding of adenosine triphosphate by myosin required Mg^{2+}, was inhibited by adenosine diphosphate and inorganic pyrophosphate but was unaffected by inorganic phosphate. Both myosin and heavy meromyosin appeared to be labeled when precipitated in the presence of [^{14}C]- or [^{32}P]adenosine triphosphate by rapid addition of neutral $(NH_4)_2SO_4$.

The labeling of myosin with radioactive adenosine triphosphate required Mg^{2+} and showed a typical adenosine triphosphate saturation curve. It was also inhibited by adenosine diphosphate. When myosin labeled with [γ-^{32}P]adenosine triphosphate was dissolved in 10 M urea or washed with cold 0.020 M acetate and 1.50 M NaCl (pH 4.5), the radioactivity was released as inorganic phosphate.

The ATP-binding sites of rabbit skeletal muscle myosin and heavy meromyosin (EC 3.6.1.3, ATP phosphohydrolase) have been investigated by various methods. A number of investigations on the binding of ADP and PP_i to myosin and heavy meromyosin suggested two or three sites per protein molecule (Nauss and Gergely, 1967; Young, 1967; Luck and Lowey, 1968; Kiely and Martonosi, 1968). Nanninga and Mommaerts (1960) determined the ATP–myosin complex concentration by the decrease in free ATP resulting from the addition of myosin. They concluded that there was one ATP binding site per molecule. Burton and Lowenstein (1964) showed that when a mixture of heavy meromyosin with Mg^{2+} and ATP was passed rapidly through a Sephadex G-25 column at 2°, a significant quantity of ATP appeared in the protein peak. This last procedure showed that ATP was bound to myosin, but it did not provide quantitative data. Finally, the perturbation of the spectra of myosin and heavy meromyosin at 280 and 288 mμ caused by the binding of ATP and ADP to myosin and heavy meromyosin was employed to demonstrate that the dissociation constants of ATP for myosin and heavy meromyosin essentially were identical with their K_m values (Morita and Yagi, 1966; Sekiya and Tonomura, 1967).

Hummel and Dreyer (1962) have shown that passing a protein solution through a Sephadex column equilibrated with an ion which binds to that protein results

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