Hydrogen-Exchange Studies of Deoxyribonucleic Acid–Protein Complexes. Development of a Filtration Method and Application to the Deoxyribonucleic Acid–Polylysine System*

Charles W. Lees† and Peter H. von Hippel‡

ABSTRACT: A membrane filtration method has been developed for studying hydrogen-exchange kinetics in suspensions of insoluble macromolecular complexes. The method has been applied in a hydrogen-exchange study of the DNA–poly-L-lysine interaction. Because of the different pH dependence of the chemical exchange rates of the peptide hydrogens of poly-L-lysine and of the hydrogens involved in interchain hydrogen bonding in deoxyribonucleic acid (DNA), we have been able to study the effect of complex formation on the kinetics of exchange of both of these classes of hydrogens: the peptide hydrogens at pH 2–4, and the DNA hydrogens near neutrality. The results show that about one-fourth of the interchain DNA hydrogens are shifted into an "instantaneously" exchanging class on interaction with poly-L-lysine, though the exchange rate of the remaining hydrogens is essentially unaffected. Various artifactual explanations of these results have been tested and rejected, and we conclude that this change in the exchange behavior of DNA is probably due to a conformational change in the DNA molecule which results from complex formation. The peptide hydrogens exchange as a single kinetic class which extrapolates to 1.0 hydrogen/lysyl residue.

The rate of exchange of these hydrogens was studied as a function of pH in both the free and the complexed form (as well as in complex with denatured DNA and apurinic acid) and it is shown that the peptide hydrogens exchange appreciably more slowly in the native DNA–poly-L-lysine complex, suggesting that these hydrogens are partially shielded from the aqueous environment in this system.

The hydrogens involved in internucleotide hydrogen bonding in native DNA can be specifically "labeled" with tritium by brief incubation in tritiated water, and the subsequent "exchange-out" of these hydrogens into the aqueous environment followed by gel filtration techniques (Printz and von Hippel, 1965; von Hippel and Printz, 1965). Exchange data on DNA can provide a measure of both the number of slowly exchangeable hydrogens and of the rate (and/or extent) of the exposure of these hydrogens to the solvent enivronment. Hydrogen exchange is also being used to obtain comparable information about proteins (for recent reviews, see Hvidt and Nielsen, 1966; Englander, 1967).

There is considerable, though mostly indirect, evidence which suggests that the interaction of proteins of biological interest (*e.g.*, histones, protamines, repressors, and polymerases) with DNA may induce specific changes in the static or dynamic conformational properties of one or both partners in the complex. Furthermore, it seems reasonable to speculate that such changes might be revealed as altered patterns of hydrogen exchange from the constituent macromolecules. To examine the potential of this approach, we have initiated hydrogen-exchange studies of DNA–protein complexes.

The first problem with which one is faced in such a study is the relative insolubility of complexes in which the DNA phosphates have been largely neutralized by cationic groups on the protein. This makes gel filtration measurements difficult or impossible, and has led us to develop a filtration method for studying the hydrogen-exchange behavior of insoluble macromolecular systems. In this paper we report on the development and testing of an appropriate filtration method, and its application to the study of DNA–poly-L-lysine complexes. The results obtained show that the interactions involved in the formation of the complexes modify the hydrogen-exchange behavior of both the DNA and poly-L-lysine.

Experimental Procedures

Materials and Reagents. Highly polymerized calf thymus DNA (lot 634, Worthington Biochemical Corp.) was used in all these experiments. It was dissolved by gentle stirring overnight at room temperature and shown to be fully native by melting criteria. The characterization of various lots of this DNA has been described previously (von Hippel and Felsenfeld, 1964; Printz and von Hippel, 1965; Olins *et al.*, 1967). Poly-L-lysine hydrogen bromide (mol wt ~75,000, lot LY55) was syn-

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† Present address: Department of Biochemistry, Boston University School of Medicine, Boston, Mass. 02118.

‡ Present address: Department of Chemistry and Institute of Molecular Biology, University of Oregon, Eugene, Ore. 97403.

thesized by Yeda (Rehovath, Israel). Deuterated water, ^2H_2O (99.84 mole %), was purchased from Bio-Rad, and tritiated water, ^3H_2O (1 Ci/ml), from New England Nuclear Corp. The borosilicate glass-paper filters (Grade GF/C) were obtained from Whatman. Buffered solutions were made up as follows and are referred to in the subsequent text as pH 7.6 cacodylate buffer, etc.: 0.10 M NaCl–0.02 M sodium cacodylate (pH 7.6) and 0.10 M NaCl–0.02 M sodium phosphate (pH 7.6), 0.10 M NaCl–0.02 M sodium cacodylate (pH 6.5), 0.10 M NaCl–0.02 M sodium acetate (pH 4.6, 4.1, and 3.6), 0.10 M NaCl–0.02 M sodium formate (pH 3.1 and 2.6), and 0.10 M NaCl–0.02 M HCl (pH 1.9). In all cases pH was measured at, or calculated to, 0°.

Denatured DNA and sonicated DNA were prepared as described by Printz and von Hippel (1965). Apurinic acid was prepared according to the method of Tamm *et al.* (1952) and concentrations were determined using the molar (phosphate) extinction coefficient ($\epsilon_{268\ m\mu}$ 4600) reported by these authors. Hydrogen-exchange measurements were carried out on apurinic acid by the filtration method described in this paper, and showed (as might be expected for this "open" structure; Printz and von Hippel, 1965) less than 0.01 exchangeable hydrogen/nucleotide pair remaining after 150 sec of exchange-out in pH 7.6 phosphate buffer at 0°. Annealed DNA–poly-L-lysine complexes with input ratios (lysine/DNA phosphate) of unity were prepared by salt gradient dialysis as described by Olins *et al.* (1967). It was determined analytically, by the methods described below, that these complexes actually contained 0.89 lysyl residue/DNA phosphate.

Concentration Analyses. The concentrations of DNA stock solutions were determined spectrophotometrically, based on a molar (phosphate) extinction coefficient ($\epsilon_{260\ m\mu}$) of 6500. Polylysine concentrations were determined interferometrically on a Zeiss laboratory interferometer, using a value of $(dn/dc)_{550\ m\mu} = 1.79 \times 10^{-1}$ ml/g for polylysine (based on Kjeldahl nitrogen analysis). The apparent absorbance at 260 mμ cannot be used as an accurate measure of DNA concentration in DNA–polylysine complexes because of light-scattering contributions (Olins *et al.*, 1967). To determine DNA concentration in such samples, aliquots containing the complexes were diluted into 4 M NaCl–0.02 M sodium cacodylate (pH 7.6) and allowed to stand for 1 hr prior to reading. This treatment resulted in quantitative dissociation of the complexes, as judged by the total absence of turbidity at 320 mμ and a complete recovery of the initial 260-mμ absorbance from control complexes. Poly-L-lysine does not absorb at 260 mμ, and the salt-induced hyperchromicity of native calf thymus DNA in 4 M NaCl was found to be less than 3%. The polylysine content of the complexes was determined by chromatography on the short column of a Spinco Model 120-C amino acid analyzer after 72-hr hydrolysis in 6 N HCl, 110° (in unevacuated vials). No losses of lysine were observed in this procedure.

Filtration and Washing Procedures. Neither DNA nor polylysine alone adhere to the glass-paper filters to any appreciable extent, but we have found that DNA–polylysine complexes can be quantitatively retained on these filters. The following procedure was routinely used for trapping the tritiated complexes and washing them free of background tritium.

Glass Buchler funnels of 20-ml capacity fitted with 2-cm diameter fritted glass disks (porosity A, ASTM 145–175 μ) were obtained from Ace Glass, Inc., and used as filter holders. The glass-paper filters were placed on the fritted glass disks and the filter holder attached to a suction flask was connected to an aspirator. The GF/C filters used were 2.1 cm in diameter, and so were bent up slightly around the edges when placed in the holder. However, trimming to 2 cm was found to have no effect and was not routinely carried out. (Filter holders in which the filters are clamped at the edges between ground-glass surfaces were not used since free tritium can be trapped in these spaces and is then very difficult to wash out.) Prior to an experiment, the filter holder was jacketed with ice water, and the entire apparatus was cooled to 0°. Buffer for washing the filter (generally the same buffer used in the tritiation and exchange-out procedures) was dispensed from a 0° reservoir with a 10-ml syringe equipped with a Cornwall adapter to permit rapid refilling. The filter and funnel were prerinsed with a 10-ml aliquot of buffer, which was allowed to drain without suction. The aspirator was then adjusted to give a flow rate through the filter of about 0.5 ml/sec, and the solution containing the tritiated complex was poured into the filter holder. A 10-ml aliquot of buffer was immediately pumped into the mixing tube, and then poured into the filter holder as soon as all the liquid had disappeared from the filter surface. This rinsing process was repeated with a second aliquot of wash buffer. The third through sixth aliquots of wash buffer were dispensed directly into the filter holder. After the sixth wash the suction was increased to the maximum and the filter became whiter in color within 5 sec, indicating that it had become relatively dry. (The filter was found to retain 0.05 ml of water by weight under these conditions.) The filter was then quickly transferred to a liquid scintillation vial. The entire filtration procedure, as described, could be carried out in about 60–80 sec.

Filtration Efficiency. The amount of DNA trapped on a filter as DNA–polylysine complex was measured as follows. The filter was placed in a screw-cap tube (150 × 25 mm) containing 5 ml of 4 M NaCl–cacodylate buffer (pH 7.6), and agitated vigorously on a Vortex-type mixer until the glass fibers had been completely dispersed. The resulting solution was allowed to stand for 1 hr at room temperature, and then transferred quantitatively to a 15-ml conical centrifuge tube. The glass fibers were removed by centrifugation at 900*g* for 15 min, and the DNA content of the supernatant fraction was determined spectrophotometrically at 260 mμ. The trapped polylysine was determined by rolling the filter into a tight cylinder and inserting it into a 10-ml glass ampoule. After addition of 2 ml of 6 N HCl, the ampoule was sealed (unevacuated) and subjected to hydrolysis and amino acid (lysine) analysis as described above. Control experiments were run in parallel on solutions containing only blank filters or known amounts of DNA and polylysine in complex (plus filters added separately).

Counting Procedure. A dioxane-based scintillation

medium was made up as follows and used in these studies: PPO (diphenyloxazole), 6 g; POPOP (1,4-bis[2-(5-phenyloxazolyl)]benzene), 0.275 g; napthalene, 168 g; and dioxane, 1 l. The filter to be counted was added to 10 ml of this medium, agitated vigorously for 1 min on a Vortex-type mixer, and placed in the refrigerated (3°) sample chamber of a Packard liquid scintillation spectrometer. If counted within 15 min, the efficiency of this system for tritium (aqueous volume, 0.2%) was about 30%. The solution froze in about 1 hr, and the counting efficiency then increased to about 37%. Toluene-based systems and the usual Bray's solution (Bray, 1960) showed appreciably lower efficiencies. The glass filter itself quenches about 4% in the dioxane system, and so blank filters were routinely added to all standards. Nitrocellulose filters (Millipore) which dissolve in dioxane-based scintillation fluids quench about 10% in this system.

Hydrogen-Exchange of DNA. The exchange-out kinetics of DNA were studied as follows. A small volume (5–20 μl) of tritiated water was added to a solution of DNA (2 mg/ml) in pH 7.6 cacodylate buffer to give a final specific activity of 10–40 mCi/ml. Equilibration (exchange-in) was allowed to proceed overnight at 5°, and then aliquots of the tritiated solution, containing about 0.1 μmole of DNA phosphate (0.025 ml) were transferred to individual test tubes in an ice bucket. Exchange-out was initiated by the rapid addition to one of these tubes of 10 ml of cold (0°) pH 7.6 cacodylate buffer (this represents a 400-fold dilution), and simultaneously a timer was started.

To stop the exchange process, a small volume (0.01–0.05 ml) of concentrated polylysine (2 mg/ml) was added to the DNA solution to achieve a lysine/DNA phosphate input ratio of unity. The solution was mixed for 10 sec and then the DNA–polylysine complex was filtered, washed six times with iced buffer, and the filter was partially dried and counted as previously described. Exchange was considered to be terminated, and thus the timer was stopped, when all of the sixth wash had disappeared from the top of the filter. Average retention of the initial DNA on the filters by this procedure was 97% for 6 washes, 96% for 14 washes, and 98% for 15 washes.

Larger polylysine/DNA ratios, higher DNA concentrations, different orders of mixing (*i.e.*, dilute polylysine into concentrated DNA), and longer mixing times all resulted in the production of visible precipitates or "clots." These often stuck to the walls of the tubes or filter holder, resulting in poor recoveries. The routine procedure described here avoids visible precipitates.

Hydrogen Exchange of Polylysine. Small volumes (5–20 μl) of tritiated water were added to 2-mg/ml solutions of polylysine to give a final specific tritium activity of 10–20 mCi/ml. The solutions were equilibrated overnight at 5°, and aliquots representing about 0.25 μmole of lysyl residues (0.025 ml) were diluted, filtered, and washed as described above for DNA, except that in this case a small volume of concentrated DNA solution was added at the appropriate times to provide a lysine/DNA phosphate input ratio of unity. Control recoveries of polylysine from the filter were $\geq 95\%$.

Hydrogen Exchange of Annealed DNA–Polylysine Complexes. A 20-μl aliquot of tritiated water was added to 0.5-ml samples of annealed DNA–polylysine complex (about 2 mg/ml in DNA), suspended in pH 7.6 cacodylate buffer, to give a final specific tritium activity of 40 mCi/ml. The solution was equilibrated overnight at 5°, and aliquots (0.025 ml) containing about 0.1 μmole of DNA phosphate and 0.1 μmole of lysyl residues were diluted as above to start exchange-out. It was found unnecessary to add additional polylysine or DNA to achieve quantitative recovery of the complex on filtration. The filters were washed and counted as described above.

In annealed complexes where both the DNA and the polylysine had been equilibrated with tritiated water, the exchange behavior of both components must be evaluated in interpreting the data. At pH 7.6 (phosphate buffer) it was observed that polylysine retains essentially no exchangeable hydrogens after 225 sec. At pH 4.6 (acetate buffer) DNA retains 0.13 hydrogen/nucleotide pair (H/np) at 305 sec. This is equivalent to 0.08 hydrogen/mole of lysyl residue (H/Lys) in these (0.89 Lys/phosphate) complexes. At pH 4.1 (acetate buffer), DNA showed 0.10 H/np at 125 sec. This is equivalent to 0.06 H/Lys. At longer times and lower values of pH, DNA showed essentially no exchangeable hydrogens. The small contributions of the DNA hydrogens (always less than 10%) have been subtracted from the exchange data reported for the annealed complexes at pH 4.6 and 4.1.

Hydrogen-Exchange Calculations. The hydrogen-exchange data were calculated to hydrogens per nucleotide pair (or hydrogens per lysyl residue) using the following formulas (Englander, 1963; Printz and von Hippel, 1965)

$$\text{H/np} = [(1.11 \times 10^2)/M_{\text{np}}](C/C_0) \qquad (1a)$$

$$\text{H/Lys} = [(1.11 \times 10^2)/M_{\text{Lys}}](C/C_0) \qquad (1b)$$

where 1.11×10^2 is the molar concentration of hydrogen in water, M_{np} and M_{Lys} are the molar concentrations of DNA nucleotide pairs or lysyl residues applied to the filter, and C_0 and C are the corrected tritium activities in counts per minute applied to, and recovered from, the filters.

Results

Exchange of DNA in Free Solution and in DNA–Polylysine Complexes. In Figure 1 we plot log H/np *vs.* exchange-out time for native calf thymus DNA at pH 7.6 and 0°, exchanging either free in solution or as a complex with polylysine. The points represent data collected on several DNA and DNA–polylysine solutions on different days over a period of 6 months. As Figure 1 shows, the points (covering exchange-out times of 100–600 sec) may all be fitted (by a least-squares procedure) to a straight line, and follow a first-order course for at least 2–3 half-times (~250 sec). Thus the DNA hydrogens involved in interchain hydrogen bonding seem to undergo exchange with solvent hydrogens at the same constant rate whether the DNA molecule is free in solution or in-

volved in a DNA–polylysine complex. On the other hand, linear extrapolation of the data back to the ordinate gives an equilibrium value, $(H/np)_0$, of 1.8 ± 0.2 H/np (Figure 1). Using the Sephadex gel filtration method (Englander, 1963), Printz and von Hippel (1965) had found, for DNA free in solution, an extrapolated value of 2.4 ± 0.1 H/np (with a major class exchanging with a half-time of approximately 300 sec) which is the value of $(H/np)_0$ expected for calf thymus DNA on the basis of two interchain hydrogen bonds per A–T pair and three per G–C pair. The difference between 1.8 and 2.4 H per np is well beyond the limits of error of the methods involved, and thus might suggest that DNA undergoes a significant conformational change as a consequence of complex formation with polylysine. However, before accepting such a conclusion, we undertook to eliminate experimentally as many as possible of the potential artifacts which might account for the observed difference of about 0.6 H/np in $(H/np)_0$. The following possibilities were tested explicitly.

(1) Incomplete trapping of DNA on the filter: As described in Experimental Procedures, within the limits of error all the DNA applied to the filter was retained.

(2) Possible equilibrium isotope effects: In our calculations it is assumed that at equilibrium the ratio of tritium to hydrogen on exchangeable macromolecular sites is the same as the ratio in the solvent. We tested this assumption by equilibrating some systems with tritiated water in 50% (v/v) D_2O. The results were identical with those obtained in H_2O, suggesting that there is no significant equilibrium isotope effect in this system, and confirming earlier similar findings with DNA (Printz and von Hippel, 1965) and tRNA (Englander and Englander, 1965).

(3) Possible differences in counting efficiency for the sample and the tritiated stock solution: In applying eq 1, it is assumed that the counting efficiency for C and C_0 are the same. In determining C_0, the sample counted was 0.025 ml of an aqueous solution containing some DNA (and/or lysine) plus a blank filter, while determinations of C were carried out on a macromolecular complex (plus 0.05 ml of wash solution) adhering to the filter in some undefined way. Thus it could be argued that tritium adhering to the complex on the filter might not be counted as efficiently as tritium free in solution.

Routinely the filters were mechanically agitated in the scintillation fluid for 1 min prior to counting. If this were not sufficient to liberate the counts from the filter, one would expect techniques aiding the solution of the complex to result in higher counts. Therefore, the following experiment was performed. Hydrogen exchange on DNA was carried out as previously described, except that the filters containing the complex were transferred to empty scintillation vials. One milliliter of pH 7.6, 4 M NaCl cacodylate buffer was then added, and the vials were allowed to stand at room temperature overnight. It is known that such treatment results in complete solubilization of all the DNA from the filters (Experimental Procedures). The usual scintillation fluid (10 ml) was then added and the vials were counted. (Aliquots of tritiated solution for C_0 determination were also made 4 M in NaCl.) The experiment was also repeated with Bray's scintillation fluid. The results obtained were the same as those shown in Figure 1, suggesting that all counts are being released from the filters by the routine procedures.

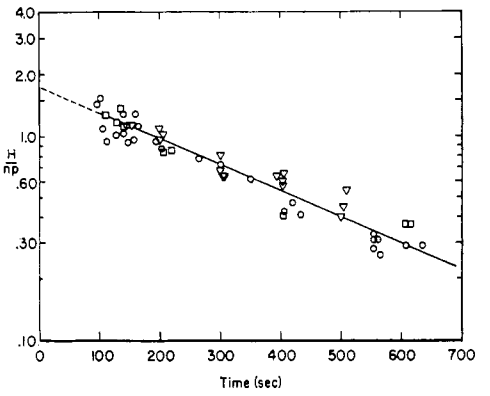

FIGURE 1: Hydrogen–tritium exchange of native calf thymus DNA (○), sonicated DNA (□), and annealed DNA–polylysine complexes (▽) at 0° in 0.10 M NaCl–0.02 M sodium cacodylate buffer (pH 7.6).

(4) Possible trapping of solvent tritium: Estimates of the extent of trapping of solvent tritium, either by the filter or within the complex, were obtained from experiments in which tritiated polylysine was permitted to exchange out in pH 7.6 cacodylate buffer for times in excess of 300 sec. The polylysine was then trapped by adding DNA, and the resulting complex was washed and counted as previously described. Control experiments (Experimental Procedures) had shown that essentially no real polylysine counts should be obtained under these conditions. The measured background was 300 cpm for a C_0 of 1.5×10^8 cpm, and 600 cpm for a C_0 of 1.5×10^9 cpm. This background was not very sensitive to the total counts applied, and was not reduced by a greater number of washes, a greater wash volume, slower filtration rates, more extensive preequilibration of the filter with buffer, or by substituting a polyethylene filter holder. We have tentatively concluded that this background must be due to some essentially irreversible (over the time course of the experiment) uptake of tritium by the glass filter. Nitrocellulose filters were observed to give about twice as many such counts. All experiments were corrected for these background counts, and concentrations of DNA and polylysine were always maintained sufficiently high to limit this correction to appreciably less than 20% of the observed counts. At exchange-out times of 100–200 sec, this background correction was less than 3% of the total observed experimental counts, and so cannot possibly account for the observed discrepancy in $(H/np)_0$.

(5) Brief exposure to conditions which might favor more rapid exchange-out: A brief exposure of the complex early in the exchange-out period (at times less than 100 sec) to conditions favoring more rapid exchange could provide an artifactual explanation of the results obtained. One possibility might be a brief, inadvertent exposure to an increased temperature, since this has been shown to increase the rate of exchange of DNA hydrogens by 10–12%/deg (M. P. Printz and P. H. von Hippel, unpublished). In the course of routine manipu-

lations, the (cold) solutions are exposed to room temperature for about 10–20 sec before pouring onto the ice-jacketed filter, but running the experiment in a 3° cold box, on the one hand, and exposing the solutions to room temperature for as much as 35 sec, on the other hand, both gave results identical with those shown in Figure 1.

During a typical hydrogen-exchange measurement by the filtration technique, the DNA is actually in four different physical states: *i.e.*, free in solution before filtration, complexed with polylysine in suspension, complexed and adsorbed on a glass filter under suction, and complexed and absorbed on a glass filter without suction. It could be hypothesized that in one of these states the exchange rate of DNA is anomalously fast. This possibility was ruled out by performing several exchange-out experiments with a 600-sec total exchange time, but varying the time spent in each of the four states described above. In all cases the values obtained were identical within experimental error. The experiment in which the complex was under suction much longer than usual (350 sec, 15 washes), also further demonstrates that recovery of DNA from the filters is indeed essentially quantitative.

(6) A possible transient conformational change in DNA on forming the complex: It might be proposed that a *transient* conformational change in DNA on *forming* the complex could result in the rapid loss of about 0.6 H/np from the DNA. This possibility is ruled out by the data on annealed complexes, where the formation of the complexes *preceded* tritiation. Figure 1 shows that the exchange-out data for these complexes fall on the same line as those in which the complex is formed *during* the exchange-out period.

The above control experiments seem to have eliminated most of the obvious artifactual explanations of the change of $(H/np)_0$ from 2.4 to 1.8 H/np.[1] Since all the measurements we have carried out by this procedure do involve the formation of a DNA–polylysine complex *sometime* prior to filtration, the most obvious explanation remaining is that formation of a DNA–polylysine complex brings about a permanent conformational change in the DNA molecules (there are other, more indirect types of evidence which also are consistent with the occurrence of a conformational change in DNA on reacting with polylysine, see Discussion). The effect of this conformational change on the hydrogen-exchange properties of DNA must then be to alter the exposure to solvent of about one-fourth of the interchain hydrogens in a way which results in their essentially instantaneous ($t_{1/2}$ = <10 sec) exchange, without affecting the exchange rate of the remainder of the DNA hydrogens.

These effects seem to be independent (Figure 1) of whether the DNA–polylysine complexes are formed by gradient dialysis from high salt (see Olins *et al.*, 1967), or by direct mixing at low ionic strength, and whether sonicated or unsonicated DNA is used.

Exchange of Polylysine in Free Solution and in DNA–Polylysine Complexes. At pH values of 4.6 or below, hydrogen exchange from polylysine proceeds at a rate which is slow enough to measure accurately by filtration techniques. The exchange-out kinetics of polylysine complexed with DNA were followed by diluting aliquots of tritiated polylysine with buffer containing DNA, so that the complexes were formed at the instant that unidirectional exchange-out was initiated. Hydrogen-exchange data for such complexes at pH 4.1 are shown in Figure 2. Clearly exchange under these conditions is very slow and the initial part of the exchange-out curve can be represented by a kinetic class of hydrogens characterized by a half-time of ~1500 sec. Previously annealed (by salt gradient dialysis) DNA–polylysine complexes were also followed at this pH, and were shown to exchange at the same rate as the unannealed complexes formed at the instant of dilution (Figure 2). Both types of complexes extrapolate to an equilibrium value, $(H/Lys)_0$, of 1.0 ± 0.05 H/Lys, after appropriate correction for DNA hydrogens (see Experimental Procedures). Figure 2 also shows exchange-out data for free (uncomplexed) polylysine at pH 4.1, corrected for the small amount of exchange which occurred during the complexing and filtering operations. The rate of exchange of polylysine in the free form is clearly much faster ($t_{1/2}$ ≃ 100 sec) than in the complex, and the data extrapolate to $(H/Lys)_0 = 0.85 \pm 0.1$ H/Lys.

These $(H/Lys)_0$ values of about one hydrogen per lysyl residue suggest that exchange of the peptide (amide) hydrogen of the polypeptide backbone is being followed in these experiments, and that the ε-amino hydrogens (three per lysyl residue) exchange too rapidly to be observed by these techniques. This conclusion is in keeping with the results of others, which also show that the side-chain hydrogens of proteins and polypeptides exchange much more rapidly than the peptide (and amide) hydrogens (for recent summaries of polypeptide hydrogen-exchange results, see Hvidt and Nielsen (1966) and Englander (1967)).

Thus Figure 2 suggests that the exchange rate of the peptide hydrogens of polylysine are strikingly reduced as a consequence of complex formation with DNA. However, in addition to possible structural differences, the peptide hydrogens of free polylysine and polylysine complexed with DNA also differ markedly in their electrostatic environment. To evaluate and separate structural and electrostatic factors (see Discussion) we have studied the exchange kinetics of polylysine, both free in solution and in complex with DNA, at pH values between 1.9 and 4.6.

In all cases the data obtained could be represented by a single kinetic class of hydrogens extrapolating to a value of $(H/Lys)_0 = 1.0$ (± 0.1) H/Lys. Apparent first-order rate constants were calculated from such data and are plotted in Figure 3 as a function of pH. This figure shows that the pH at which exchange is slowest (pH_{min})

[1] Since all our experiments involve adsorption of the complex onto the glass membrane filter, we cannot rule out the possibility that a particular group of exchangeable hydrogens are labilized as a consequence of this interaction. However this explanation requires that only a particular class of the DNA hydrogens (and none of the poly-L-lysine peptide hydrogens, see below) be labilized, and that the size of this class be independent of both the gross ("clot" or suspension) or fine (annealed or non-annealed) structure of the complex, as well as of the length of time the complex spends absorbed onto the filter.

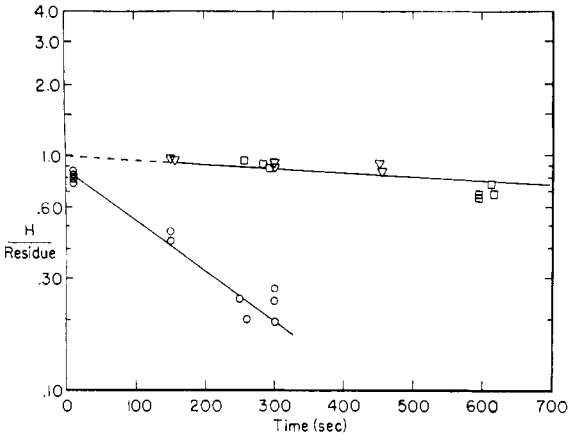

FIGURE 2: Hydrogen–tritium exchange of free polylysine (○), DNA–poly-L-lysine complex (□), and annealed DNA–poly-L-lysine complex (▽) at 0° in 0.10 M NaCl–0.02 M sodium acetate buffer (pH 4.1).

shifts down from about pH 3.6 for complexed polylysine to pH 3.0 or below for the free material. This shift is in qualitative accord with expectations for an acid–base-catalyzed exchange reaction in a highly charged system, since the large positive charge density of the free polylysine molecule would be expected to facilitate the approach of hydroxide ions while repelling protons, thus favoring basic over acidic catalysis and shifting pH_{min} toward lower pH values. These effects should be largely abolished in the complex as a result of the stoichiometric neutralization of the ε-amino charge by the DNA phosphates. The solid lines in Figure 3 are drawn through the data points assuming full acid–base catalysis. That is, at pH values above or below pH_{min} the catalyst concentration (and thus presumably the exchange rate of an unstructured system) increases by a factor 10/pH unit (see Leichtling and Klotz, 1966). It is clear that the data are in reasonable accord with this assumption. Runs were not made below pH 1.9, so the low-pH side of the exchange curve for free polylysine has not been defined.

Though differences in the charge state of polylysine in the free and in the complexed form can account for the shift of pH_{min}, these differences would not be expected to result in major differences in exchange *rate* of the two systems at their respective pH_{min} (see Discussion). As Figure 3 shows, at pH_{min} free polylysine exchanges with a first-order rate constant of about 2×10^{-3} sec^{-1} ($t_{1/2} \simeq 350$ sec) while complexed polylysine exchanges much more slowly, with $k \simeq 1.5 \times 10^{-4}$ sec^{-1} ($t_{1/2} \simeq 5000$ sec) at pH_{min}; *i.e.*, exchange of the peptide hydrogen of polylysine in the complex is slowed by more than an order of magnitude relative to exchange in the free state.

Since it had been shown (Figure 2) that the exchange behavior of polylysine in annealed and unannealed complexes is the same, even though it is known on other grounds that a complete equilibrium structure is not attained in nonannealed DNA–polylysine complexes (*e.g.*, see Olins *et al.*, 1967, 1968), we have attempted to define further the structural features of DNA which are required for the observed decrease in polylysine peptide

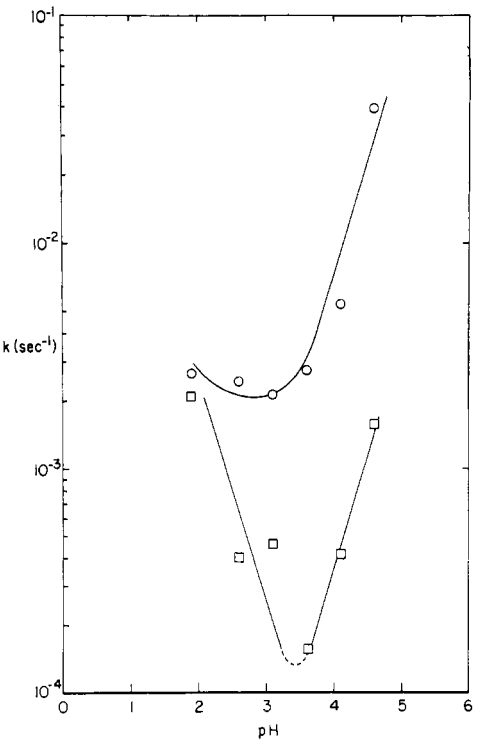

FIGURE 3: Hydrogen-exchange rate constants for free poly-L-lysine (○) and poly-L-lysine complexed with DNA (□) at 0° in 0.10 M NaCl–0.02 M HCl (pH 1.9), 0.10 M NaCl–0.02 M sodium formate (pH 2.6 and 3.1), and 0.10 M NaCl–0.02 M sodium acetate (pH 3.6, 4.1, and 4.6).

hydrogen-exchange rate in the complex. To this end, 1:1 (lysyl cation–DNA phosphate) complexes were formed between polylysine and denatured DNA and between polylysine and apurinic acid. Exchange data obtained on these systems at pH 4.6 are plotted in Figure 4, and show that both of these types of complexes exchange under these conditions as a single kinetic class with $(H/Lys)_0 = 1.0$ (±0.1) H/Lys and $t_{1/2} \simeq 250$ sec. The data points shown were obtained on several different days with different preparations. The exchange rate of these complexes may be compared with half-times of about 20 sec for free polylysine at this pH, and 550 sec for annealed complexes of polylysine with native DNA (see Figure 4). Complete pH-exchange profiles were not carried out for the polylysine–apurinic acid and the polylysine–denatured DNA complexes, but if the reasonable assumption is made that the pH dependence of exchange of these complexes is much like that of the native DNA–polylysine system, we would expect that $t_{1/2}$ for these complexes at pH_{min} should be approximately 1200 sec. Thus complex formation with these partially stacked (at 0°) but quite disorganized polynucleotide structures also slows the rate of exchange of the peptide hydrogens of polylysine relative to exchange at pH_{min} in the free form, though not as much as complex formation with native DNA.

Discussion

The results presented in this paper show that hydrogen-exchange studies can be conducted with rather good

accuracy by filtration methods on suspensions of insoluble macromolecular complexes, with results which are comparable with those obtained in free solution. In general, however, it is apparent that if this method is to be applied to soluble systems, the effect of the "trapping" (insolubilizing) agent on the hydrogen-exchange rate of the macromolecule of interest must be determined in each case, and it must be shown that exchange is not limited by the mechanical properties of the insoluble system. The most straight-forward approach to these questions is probably to conduct exchange for a fixed total time, varying the fraction of time spent by the macromolecule in solution and in the insoluble complex. If the total exchange in this period is independent of time spent in the two states, it is reasonable to assume that access of the macromolecular hydrogens to the solvent is not limited by the aggregation involved in forming the complex. Furthermore, if the rate of exchange does not vary with fraction of time spent in complex in such an experiment, one may conclude that the trapping agent does not affect the exchange rate. However one must still look for the instantaneous (on the time scale of this method) labilization of a particular class of hydrogens as a consequence of interaction with the trapping agent, as appears to occur with some of the DNA hydrogens as a consequence of interaction with polylysine. Such effects are best detected by comparing the $(H/molecule)_0$ values obtained by trapping with the results of a non-trapping method such as gel filtration.[2]

The major advantage of membrane filter over gel filtration techniques is that the sample may be a suspension of insoluble material, which might not pass through the gel in a reproducible manner, though the commercial availability of gels with a very low degree of cross-linking may reduce this problem. Furthermore, it is very difficult to measure accurately the concentration of an aliquot of insoluble suspension. Another advantage of a filter trapping method over gel filtration is that exchange-out can be initiated merely by dilution, leading to a more unequivocal definition of zero time for the exchange curve.

In general, hydrogen exchange offers a particularly favorable approach to the study of nucleic acid–protein complexes because of the different pH dependence of the exchange rates of the hydrogens of the two types of macromolecules. Thus the exchange of DNA hydrogens is too rapid to follow in the pH range 2–4, where the peptide hydrogens can be demonstrated. Conversely, all the protein hydrogens exchange "instantaneously" at neutral pH, where the exchange of the DNA hydrogens is sufficiently slow to be studied by our methods.

Most types of possible artifactual explanations of the apparent decrease of 0.6 H/np in $(H/np)_0$ for DNA as a result of complex formation with poly-L-lysine have been ruled out by the control experiments described in the Results section. Thus we conclude that this effect is probably due to a conformational change in the DNA which occurs as a consequence of complex formation. Leng and Felsenfeld (1966) and Olins *et al.* (1967, 1968) have observed a cooperative effect in the binding of poly-L-lysine and other polypeptides to DNA, and have also suggested that a conformational change in DNA would afford the most likely explanation of this phenomenon.

The nature of this hypothetical conformational change is not clear. To fix ideas, we present the following as an *illustration* of the types of changes which might account for our observations. If we speculate that poly-L-lysine reacts with DNA in only one groove (as X-ray evidence suggests for DNA–protamine complexes; Feughelman *et al.*, 1955) then the groove might change size and shape slightly to accommodate the poly-L-lysine in a way which results in the most favorable electrostatic, hydrophobic, and hydrogen-bonding interactions. Such changes in groove geometry can be brought about without unstacking the DNA bases by a slight alteration of the angle of tilt of the bases relative to the DNA axis. This would result in a concomitant change in the geometry of the other groove. (Consider the alterations in groove geometry which accompany the conversion of DNA from the B into the A form with changes in humidity.) Such an alteration of the angle of tilt of the bases, in turn, might change the environment of the edge of the stacked DNA bases sufficiently to trigger the rapid exchange of the group of hydrogen-bonded hydrogens located next to the "free" groove (for example, the hydrogens involved in the hydrogen bond between the 2-amino group of guanine, and the 2-keto group of cytosine, which are located next to the small groove in the native DNA structure). A change of this nature is consistent with our observations, though we reemphasize that this mechanism is presented as an illustration of the possibilities available rather than as a definitive proposal at this point.

It seems surprising that the rate of exchange of the remaining DNA hydrogens is not affected by the postulated conformational change in the DNA, though recent studies (B. McConnell and P. H. von Hippel, in preparation) suggest that the hydrogen-exchange kinetics of DNA are quite insensitive to environmental changes which markedly alter the conformational free energy of the native structure (*e.g.*, reducing T_m to within 20° of the experimental temperature by adding large quantities of destabilizing salts). Our present model for the mechanism of hydrogen exchange of DNA (von Hippel and Printz, 1965; Printz and von Hippel, 1968) relates the rate of exchange directly to two parameters. One is K, the equilibrium constant for the opening $\rightleftarrows$ closing process which is a measure of the fraction of time a given "exchange unit" (probably a stretch of DNA helix a few nucleotide pairs long) is "open" (*i.e.*, not internally hydrogen bonded and therefore available for chemical exchange). The other is k_{chem}, the first-order rate constant for the chemical exchange process itself. The observed first-order exchange rate constant, k_{obsd}, is simply viewed as the product of K and k_{chem}.

[2] Various combinations of gel filtration and "trapping" on membrane filters are also possible with soluble systems. For example, Buckingham and Stocken (1966) have published a preliminary account of hydrogen-exchange experiments on γ-irradiated DNA, in which the tritiated DNA was first separated from free tritium by gel filtration, and then precipitated and counted on membrane filters as a DNA–protamine complex.

It has been shown that the exchanging class we are considering in DNA is composed of hydrogens involved in interchain hydrogen bonding of complementary base pairs (Printz and von Hippel, 1965). This is supported by our observation in this study that apurinic acid, which should be incapable of such hydrogen bonding, exchanges its hydrogens with solvent too rapidly to be detected by our methods. One might initially predict that complexing of DNA with polylysine would limit the opening of the cooperative exchange units of the DNA, or cause them to close more rapidly. Such changes would decrease K, and result in a decreased exchange rate. However this is not observed. Therefore one must either argue that K does decrease, and is balanced by a compensatory increase in k_{chem} (which seems most unlikely) or that the effective K for the remaining DNA hydrogens is not much affected by the postulated conformational change of the DNA which accompanies complex formation.

The latter conclusion is not inconsistent with hypotheses which suggest that polylysine binds in (or over) only one groove of the DNA, thus permitting exchange to continue relatively unimpeded through the other groove. Because the energy barrier to even local unstacking in the presence of poly-L-lysine at 0° should be formidable, this conclusion is also most consistent with "untwisting" models for the exchange process which permit separation of the hydrogen-bonded nucleotide pairs without appreciable unstacking (von Hippel and Printz, 1965).

Between pH 1.9 and 4.6, polylysine has been shown to exchange as a single kinetic class consisting of one exchangeable hydrogen per lysyl residue. This stoichiometry, as well as the fact that ε-amino hydrogens should exchange much more rapidly than peptide hydrogens, suggests that this class represents the peptide hydrogens of poly-L-lysine. The results presented in Figure 3 show that pH_{min} for free polylysine falls at or below pH 3.0, and shifts to about pH 3.6 for poly-L-lysine complexed with charge-equivalent quantities of DNA. This is consistent with the results of Leichtling and Klotz (1966), who showed in an infrared study of the hydrogen exchange of peptides and polypeptides in D_2O, that pD_{min} for poly-L-lysine is about 0.7 pD unit below pD_{min} for uncharged poly-L-glutamic acid. However, Leichtling and Klotz (1966) have also shown that the rate constant for the exchange of the amide hydrogen of a number of charged and uncharged polypeptides and peptide models is quite constant, though pH_{min} (or pD_{min}) can vary appreciably. Thus k_{min} (the first-order rate constant for exchange at pD_{min}) for acetylglycine ethyl ester, chloroacetylglycine ethyl ester, and poly-L-lysine at 15° in D_2O–dioxane (1:1) are all 0.015 ± 0.002 min^{-1}, while pD_{min} falls, respectively, at 5.00, 3.60, and 2.55 for these compounds. These results suggest that charge neutralization *per se* should shift pH_{min} (as observed) but not have a major effect on k_{min}.

In contrast, we observe that k_{min} for the peptide hydrogens of poly-L-lysine involved in an annealed stoichiometric complex (lysyl cation/DNA phosphate) with native DNA is at least tenfold smaller than k_{min} for the peptide hydrogens of free polylysine. Thus the mode of

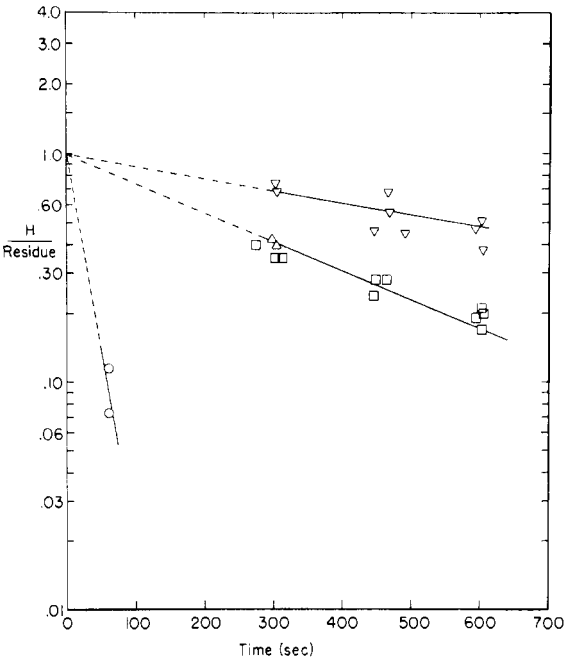

FIGURE 4: Hydrogen–tritium exchange of free poly-L-lysine (○), poly-L-lysine annealed with native DNA (▽), poly-L-lysine complexed with denatured DNA (△), and poly-L-lysine complexed with apurinic acid (□) at 0° in 0.10 M NaCl–0.02 M sodium acetate (pH 4.6).

interaction of DNA with poly-L-lysine to form the complex must involve sufficiently intimate contact with the peptide hydrogen to severely limit its access to the aqueous environment. In this connection we may note that Bradbury *et al.* (1967) have studied the rate of deuteration (in thin films) of peptide hydrogens of histones involved in DNA–histone complexes, and find that some of these peptide hydrogens exchange anomalously slowly. They also attribute this decrease in the exchange rate to a steric shielding of the peptide hydrogens as a consequence of complex formation.

To test the sensitivity of this structural slowing of the exchange of the peptide hydrogens to the details of the structure of the complex, we have examined the effect on the exchange rate of forming a complex with two partially disorganized DNA derivates: denatured calf thymus DNA and apurinic acid. Denatured calf thymus DNA consists of short double-stranded hydrogen-bonded segments interspersed with single-stranded regions (Felsenfeld and Sandeen, 1962). Apurinic acid can form no hydrogen-bonded base pairs of the Watson–Crick type. It was observed (Figure 4) that complex formation of poly-L-lysine with charge-equivalent quantities of either of these DNA derivatives did slow exchange appreciably relative to free poly-L-lysine, even after taking into account the shift in pH_{min}. Furthermore, both derivatives slowed exchange to the same extent, though considerably less than seen in the native DNA–poly-L-lysine complexes. Thus even if we take the extreme position of attributing the entire effect on the peptide hydrogen-exchange rate of these degraded DNA derivatives to neutralization of the lysyl ε-amino groups by a high molecular weight polyanion, there is

still an appreciable additional slowing of peptide hydrogen exchange in the native DNA–poly-L-lysine complex. We conclude that in postulated models of the DNA–poly-L-lysine complex, the polypeptide backbone should be positioned to provide at least a partially nonaqueous environment for the peptide hydrogens.

References

Bradbury, E. M., Crane-Robinson, C., Rattle, H. W. E., and Stephens, R. M. (1967), *in* Conformation of Biopolymers, Vol. 2, Ramachandran, G. N., Ed., New York, N. Y., Academic, p 583.

Bray, G. A. (1960), *Anal. Biochem. 1*, 279.

Buckingham, R. H., and Stocken, L. A. (1966), *Biochem. J. 101*, 33P.

Englander, S. W. (1963), *Biochemistry 2*, 798.

Englander, S. W. (1967), *in* Poly-α-amino Acids as Protein Models, Fasman, G., Ed., New York, N. Y., Marcel Dekker, p 339.

Englander, S. W., and Englander, J. J. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 370.

Felsenfeld, G., and Sandeen, G. (1962), *J. Mol. Biol. 5*, 587.

Feughelman, M., Langridge, R., Seeds, W. E., Stokes, A. R., Wilson, H. R., Hooper, C. W., Wilkins, M. H. F., Barclay, R. K., and Hamilton, L. D. (1955), *Nature 175*, 834.

Hvidt, A., and Nielsen, S. O. (1966), *Advan. Protein Chem. 21*, 288.

Leichtling, B. H., and Klotz, I. M. (1966), *Biochemistry 5*, 4026.

Leng, M., and Felsenfeld, G. (1966), *Proc. Natl. Acad. Sci. U. S. 56*, 1325.

Olins, D. E., Olins, A. L., and von Hippel, P. H. (1967), *J. Mol. Biol. 24*, 151.

Olins, D. E., Olins, A. L., and von Hippel, P. H. (1968), *J. Mol. Biol. 33*, 265.

Printz, M. P., and von Hippel, P. H. (1965), *Proc. Natl. Acad. Sci. U. S. 53*, 363.

Tamm, C., Hodes, M. E., and Chargaff, E. (1952), *J. Biol. Chem. 195*, 49.

von Hippel, P. H., and Felsenfeld, G. (1964), *Biochemistry 3*, 27.

von Hippel, P. H., and Printz, M. P. (1965), *Federation Proc. 24*, 1458.

The Luminescence of Tryptophan and Phenylalanine Derivatives*

Ira Weinryb† and Robert F. Steiner

ABSTRACT: The fluorescence and phosphorescence of a series of tryptophan and phenylalanine derivatives have been examined. For tryptophan compounds, the luminescence is much more sensitive to chemical modification at 25° than at liquid nitrogen temperatures. In particular, the observed phosphorescence lifetimes at 91°K were invariant within experimental uncertainty. The kinetic processes responsible for the variation in yield and lifetime at 25° were found to be first order with respect to the excited state. Proton transfer appears to be a dominant influence in some cases. Luminescence studies of the phenylalanine peptides revealed differences between peptides which persisted at liquid nitrogen temperatures.

During the last few years the luminescence properties of the aromatic amino acids have attracted considerable interest. Numerous investigations have been made of the free amino acids, their derivatives, and related model compounds (Teale and Weber, 1957; White, 1959; Cowgill, 1963a,b, 1967; Nag-Chaudhuri and Augenstein, 1964; Longworth, 1966; Bishai *et al.*, 1967). Synthetic polymers and copolymers containing tryptophan, tyrosine, and phenylalanine have been examined (Fasman *et al.*, 1964; Pesce *et al.*, 1964; Wada and Ueno, 1964; Weber and Rosenheck, 1964; Fasman *et al.*, 1966; Longworth, 1966; Lehrer and Fasman, 1967). The emission properties of these amino acids, when incorporated into proteins have also received considerable attention (Chen *et al.*, 1968, and references therein).

The interpretation of protein luminescence has been hampered by the multiplicity of environments in which the aromatic amino acids may be found, as a consequence of the wide variations in primary, secondary, and tertiary structure for different proteins. The systematic exploration of the behavior of simple peptide

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† National Academy of Sciences, National Research Council postdoctoral resident research associate.