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High Resolution Proton Magnetic Resonance Spectroscopy of Histones and Histone–Histone Complexes in Aqueous Solution†

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ABSTRACT: Low molecular weight histone complexes of H2A (≃dimer), H2B (≃tetramer), H3-H4 (≃tetramer), H2A-H2B (≃dimer), and H2B-H4 (≃dimer) have been prepared in 2 M NaCl and neutral pH at 4 °C. These materials are free of nonspecific aggregate and are suitable for study by high resolution proton magnetic resonance spectroscopy. Such spectra have been recorded in aqueous solution under conditions allowing a study of the exchangeable proton resonances of histone complexes for the first time and indicate that the structured regions are rich in hydrophobic amino acids, as well as arginine and some acidic amino acids. Most of the lysine and probably alanine residues remain in a motile, random coil-like state after formation of the complexes. It is suggested that arginine residues may be important in inter- and/or intra-subunit interactions in histone complexes.

Since the demonstration by Hewish & Burgoyne in 1973 that eukaryotic chromatin consists of subunits, there has been considerable interest in the structure of this subunit. It now appears that the repeating subunit (nucleosome or ν body) consists of ~140-200 base pairs of DNA double helix wound around the outside of an octameric histone core of two each of the histones H2A, H2B, H3, H4. (For recent reviews, see Lewin, 1975; Weintraub et al., 1976; Kornberg, 1977).

Recent work has also shown that, under appropriate conditions, acid extracted isolated histones can form secondary structures (see, e.g., Bradbury & Rattle, 1972; Bradbury et al., 1975; Pekary et al., 1975; Shih & Fasman, 1971; Adler et al., 1975a,b), relatively specific heterotypic histone-histone complexes (see, e.g., D'Anna & Isenberg, 1973, 1974a,b), and/or very high molecular weight aggregates (see, e.g., Sperling & Bustin, 1975; above references; as well as a review by Van Holde & Isenberg, 1975).

Assessment of the biological relevance of the various renatured forms of histones is a difficult task in the absence of specific enzyme-like functional assays. The only valid criterion for functional renaturation is the formation of nucleosomal structures when bound to DNA. Recent studies of Felsenfeld and co-workers (Camerini-Otero et al., 1976) have shown that acid extracted histones may renature and reassociate with DNA to form a nucleosome-like core.

Evidence is accumulating in support of specific small molecular weight histone complexes stable at high ionic strength and low temperature as the kinetic units responsible for nucleosome structure (Pardon et al., 1978; Weintraub et al., 1975). These histone complexes may reassociate with DNA to form chromatin-like material (Oudet et al., 1975; Fulmer & Fasman, results in progress).

Since it is probable that, to a large extent, nucleosome structure is determined by the structure of low molecular weight histone complexes rather than high molecular weight histone aggregates, it is of considerable importance to prepare homogeneous renatured complexes for physical studies. It has been found that the direct mixing of salt to histones in low ionic strength buffer, at ambient temperature, resulted in considerable aggregation (~30%) even at intermediate final con-

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centrations of NaCl (0.27 M). These irreversibly formed aggregates are large enough to elute in the void volume of Bio-Gel A-0.5m but do not appear turbid to the eye. They are *not* ^{1}H NMR[1] invisible, as might be expected for locally mobile histone segments attached to a large slowly tumbling protein matrix. Quantitative conclusions drawn from studies on complexes prepared in such a manner may require reconsideration. In this paper we report proton magnetic resonance (^{1}H NMR) studies on complementary suboctameric histone–histone complexes in 2 M NaCl, pH 7, in predominantly ^1H_2O solution. Particular attention is given to the problem of obtaining these complexes in an aggregate-free form and in studying the exchangeable proton resonances of these complexes in aqueous solution.

Materials and Methods

Histones. Histones were isolated from chicken erythrocytes. Blood was collected from chickens immediately after exsanguination into a flask containing 100 mL of 10% (w/w) sodium citrate/L of blood. Nuclei preparations and histone extractions were performed as described by Murray et al. (1968) with extractions at pH 1.90 and pH 0.99 using titration with 0.2 and 3.5 N HCl, respectively. The histones were then fractionated into their individual components using the selective extraction schemes of Oliver et al. (1972) and Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, Calif.) chromatography as described by Van der Westhuyzen et al. (1974). Purity of individual histone fractions was assessed by acid–urea polyacrylamide (20%) gel electrophoresis (Panyim & Chalkley, 1969) which showed single bands for all histone fractions except H3 which contained 3–5% of H2A as contaminant.

Poly(L-lysine) (mol wt 32 000) (CH-1-95) was synthesized as previously described (Fasman et al., 1961) and poly(L-arginine) was lot AR-34 from Miles.

Histone–Histone Complexes. Purified histone fractions in 10^{-3} N HCl were mixed in equimolar proportions for heterotypic complexes at a total concentration of 10–15 mg/mL ($\simeq$0.7–1.0 mM). Homotypic complexes were prepared from stock solutions in 10^{-3} N HCl at a concentration of 10–15 mg/mL. All solutions were adjusted to 8 M urea, 1% dithiothreitol, 0.1 M Tris-HCl buffer, pH 8.8. These solutions were incubated at 40 °C for 1 h to reduce H3 and then dialyzed against 1000 volumes of 10^{-2} N HCl at 4 °C for 14 h. The solution was then mixed with an equal volume of 4 M NaCl, 0.1 M sodium phosphate buffer (pH 7) with magnetic stirring at 4 °C and immediately applied to a Bio-Gel A-0.5m column (180 × 1 cm) at 4 °C and eluted with 2 M NaCl, 10 mM sodium phosphate buffer (pH 7) at a flow rate of 17 mL/h. Usually, an aggregate peak eluted at the void volume of the column and a second major peak eluted at larger volumes corresponding to specific, small molecular weight complexes of histones (Figure 1). The fractions corresponding to the small molecular weight complex were pooled, concentrated in dialysis tubing (spectrapor membrane tubing type 3) by submersion in dry Sephadex G-100 or G-200, and dialyzed against elution buffer, all at 4 °C. An aliquot was passed through a 20-mL column of Biol-Gel A-0.5m to demonstrate the absence of aggregation during concentration. Concentrations of histones were determined by using the following molar extinction coefficients at 275.5 nm: H3, 5.5×10^3; H4, 5.4×10^3; H2A, 4.0×10^3; and H2B, 6.7×10^3 (D'Anna & Isenberg, 1974b). The ratio of aggregate to complex for any histone or histone pair is variable and depends on handling but H3, H4, and H3-H4 always showed a greater tendency to aggregate than the other histones.

Proton Magnetic Resonance Spectra. ^{1}H NMR spectra were taken on a 270-MHz Fourier transform spectrometer designed and built by Dr. A. Redfield at Brandeis University. This spectrometer is capable of measuring weak proton resonances in predominately aqueous solutions. A radiofrequency "2-1-4" observation pulse is chosen such that its power spectrum nearly vanishes at the frequency of the water proton resonance so that the contribution of water protons to the free induction decay is minimal (Redfield et al., 1975; Redfield, 1977). Approximately 0.2–0.1 volume of 99.8% D_2O (ICN) was added to the concentrated histone solution to result in a final concentration of 0.7–1.0 mM in each histone species. Solutions of histones were thus in aqueous buffers which contained 10–20% D_2O required for the internal lock of the spectrometer.

Since this observation method does not saturate or otherwise perturb the water proton resonance, histone protons which are exchanging with water protons do not have their ^{1}H NMR intensities perturbed so that the exchange process can be studied. This is done by applying a long (usually 200 ms), selective preirradiation pulse to saturate the water proton resonance and observing the decay of other resonances as this saturation is transferred by chemical exchange. In principle, one can obtain chemical exchange rates for individual classes of exchanging protons in the protein by this method.

Such saturating preirradiation experiments were checked for artifacts in several ways. First, the saturation prepulse frequency was moved away from the water proton resonance and toward the proton resonance of interest in small steps. If the resonance of interest grew in intensity, this was taken as evidence of saturation transfer by chemical exchange rather than saturation caused by the use of a too broad or too powerful saturation prepulse. Second, the power attenuation on the preirradiation pulse was increased or decreased to ensure that saturation of the water resonance was nearly complete yet not so powerful that it directly affected nearby resonances. Finally, the delay time between the preirradiation and observation pulses, as well as the length of the pulses, were varied to ensure against relaxation of the saturated protons.

All data are reported as nominal intensity of resonances vs. hertz from the internal standard, H_2O. The frequency of the water protons is sensitive to pH so that resonances of other protons appear to have shifted relative to water protons at different pHs. Between pH 3 and pH 7 the water proton frequency shifts 50 Hz upfield.

Fluorescence Studies. Fluorescence anisotropy measurements were made using an instrument built according to Weber & Bablouzian (1966). One-centimeter cuvette cells were used, which were thermostated at 4 °C. The concentration of the samples was $\simeq$0.3 mg/mL ($A_{275} \simeq 0.1$), made by diluting the column fractions with elution buffer. Anisotropy is defined as $r = (E - B)/(E + 2B)$; E is the fluorescence component polarized parallel to the vertically polarized exciting light, and B is the horizontally polarized fluorescence component.

Results

Histone–Histone Complexes. Fractionation of renatured histone complexes on Bio-Gel A-0.5m usually resulted in two well-resolved peaks. The elution profiles in Figure 1 show that the high molecular weight aggregates eluting at V_0 are not in

[1] Abbreviations used: the histone nomenclature used was accepted at the CIBA Foundation Symposium on Structure and Function of Chromatin, April 3–5, 1974; H2A = F2A2, IIb1, ALK; H2B = F2B, IIb2, KSA; H3 = F3, III, ARK; H4 = F2a1, IV, GRK; ^{1}H NMR, proton magnetic resonance; T_1, spin-lattice relaxation time in the absence of chemical exchange; τ_1, the observed apparent relaxation time; τ, the chemical exchange time.

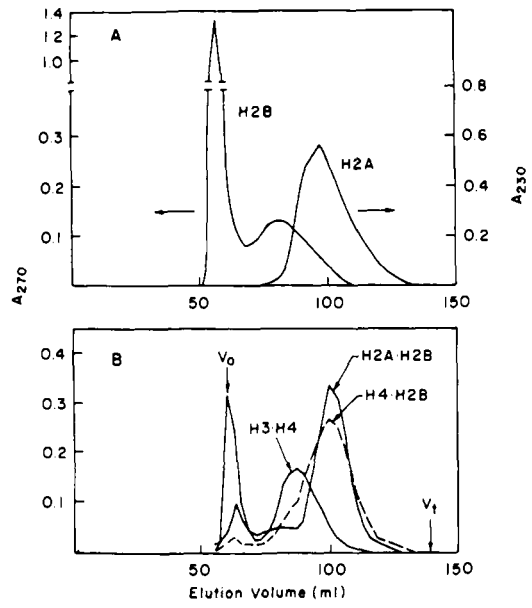

FIGURE 1: Chromatography of isolated histones (A) or equimolar mixtures of histone pairs (B) from chicken erythrocytes on a Bio-Gel A-0.5m column (180 × 1 cm). Elution was with 2 M NaCl, 10 mM sodium phosphate (pH 7.0) at 4 °C at a flow rate of 17 mL/h. Total histone applied was usually 10–15 mg in 2 mL. V_0 is the void volume of the column and V_t = total bed volume of the column.

rapid equilibrium with the smaller molecular weight complexes. The absence of slow reequilibration between the two states of association was shown by two criteria. First, pooled fractions from the small molecular weight peak were concentrated and refractionated at 4 °C. The material eluted at the same position giving no high molecular weight aggregate peak. Second, no reequilibration was detected by fluorescence anisotropy. Material from corresponding high and low molecular weight peaks was diluted with buffer to identical concentrations ($A_{275} \approx 0.1$). Fluorescence anisotropy of the intrinsic tyrosine fluorophores was monitored at 4 °C over a period of 5–7 days with storage between measurements at 4 °C. High molecular weight aggregates displayed a fluorescence anisotropy (r) of about 0.4 while the corresponding values for low molecular weight complexes ranged between 0.13 and 0.17. Nonconvergence of the high and low molecular weight data show that the two forms of aggregation are not in equilibrium for most practical purposes (unpublished results). Thus, it appears that the irreversible formation of a high molecular weight histone aggregate represents an alternate kinetic pathway to the formation of a small molecular weight histone complex upon renaturation. The molar stoichiometry of all heterotypic histone complexes was 1:1 as determined by gel electrophoresis. Renaturation of either H3 or H4 alone by the above pathway results in ~100% aggregate.

The degree of association of the small molecular weight complexes may be qualitatively assessed by gel filtration chromatography on Bio-Gel A0.5m relative to H3–H4. H3–H4 exists predominately as tetramer under these conditions when examined by meniscus depletion sedimentation equilibrium (unpublished results). This is in agreement with similar studies on H3–H4 under other solution conditions (Roark et al., 1976; D'Anna & Isenberg, 1974c). Complexes of H2B with either H2A or H4 appear to form predominately a dimer. Self-association of H2A also results in material eluting in the dimer region but may represent a distribution of rapidly equilibrating species (see Roark et al., 1976). H2B self-associated into complexes of at least a tetramer. The data to be presented are based on pooled fractions from the major small molecular weight peaks. It should be noted here that the relative magnitudes of peaks in Figure 1, although representative, were not completely consistent from experiment to experiment and depended on exact handling.

Proton Magnetic Resonance (^1H *NMR*). Our upfield ^{1}H NMR spectra for purified individual histone fractions in 10^{-3} N HCl are in general agreement with those published by others in D_2O solutions (Bradbury et al., 1975; Pekary et al., 1975) and are in good agreement with spectra reconstructed from the known amino acid composition and chemical shifts for various proton types (modified from McDonald & Phillips, 1969) so that they represent unstructured proteins approximating random coils under these conditions. However, our spectra are taken with the histones in 80–90% H_2O so that the downfield spectra contain additional resonances from chemically exchangeable protons (mainly amide, lysine amino and arginine guanidino protons). Since histones are noted for their exceptionally high content of lysine and arginine residues, these exchangeable resonances may be of interest as additional probes of histone structure. Interpretation of the downfield spectra of the histone complexes is based on appropriate random coil synthetic polypeptides.

When a proton is exchanging between two magnetically nonequivalent sites (in our case between a protein site and bulk water) then, under appropriate conditions (Forsén & Hoffman, 1963), the measured intensity of the proton resonance signal on the protein site is given by

$$M_z(t \rightarrow \infty) = M_0(\tau_1/T_1) \quad (1)$$

where $M_z(t \rightarrow \infty)$ is proportional to the signal intensity of the proton at equilibrium after saturation of the water resonance and M_0 is proportional to the signal intensity in the absence of water saturation. τ_1 is the observed relaxation time for the protein proton signal and T_1 is its spin–lattice relaxation time in the absence of chemical exchange. They are related by the following equation:

$$1/\tau_1 = 1/\tau + 1/T_1 \quad (2)$$

where $1/\tau$ is the chemical exchange rate. It follows that the fractional reduction in signal intensity of an exchanging proton signal caused by saturating preirradiation of water protons is $[\tau/(\tau + T_1)]$. If the chemical exchange rate is slow in comparison with the spin–lattice relaxation rate ($\tau \gg T_1$), then little signal reduction will be seen but if it is relatively very fast ($\tau \ll T_1$) the signal reduction will be large.

A proton signal can apparently disappear by a different mechanism also. If the proton is in rapid chemical exchange between two sites and the exchange rate is much faster than the frequency separation of two resonances, the proton will resonate at a weighted average of the two frequencies. In the case of exchange with water, the signal due to the proton on the protein site will become lost in the larger water signal.

These properties are shown for poly(L-lysine) and poly(L-arginine) in aqueous solution in Figures 2 and 3. Figure 2 shows the downfield ^{1}H NMR spectra of poly(L-lysine) at pH 3 and pH 7, with and without presaturation of the water protons. From the relative peak areas of the resonances in Figure 2A, it is clear that the resonance near 1000 Hz from water arises from the amide protons while the resonance near 750 Hz from water arises from the ϵ-NH_3^+ protons of the lysine side chains. The application of a selective preirradiation pulse (200 ms) at the water proton frequency (causing 90–95% saturation of these protons) decreases the amino proton resonance by about 90%, while hardly affecting the amide proton resonance.

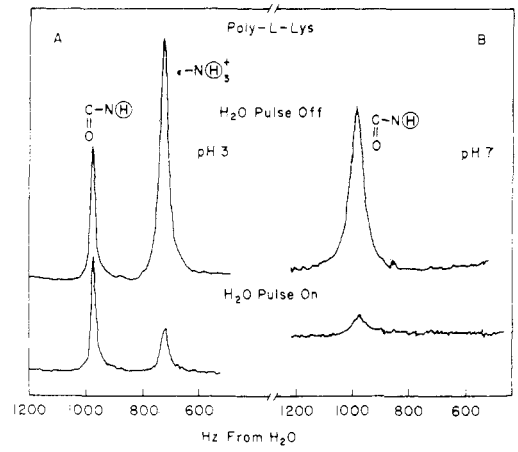

FIGURE 2: The 270-MHz ^{1}H NMR spectra of poly(L-lysine) in the downfield region at pH 3.3 (A) and pH 7.0 (B) (20 °C). For pH 3.3 poly(L-lysine) hydrochloride was dissolved in 1 mM HCl (85% H_2O, 15% D_2O) at 15 mg/mL and this same solution was titrated to pH 7.0 with 1 N NaOH. The lower spectrum in both cases was taken on the same sample and with the same instrumental settings as the upper spectrum except that a preirradiation pulse of 200-ms duration, centered at the water proton frequency, was applied 100 ms before the observation "2-1-4" pulse. Free induction decays, 120-320, were accumulated.

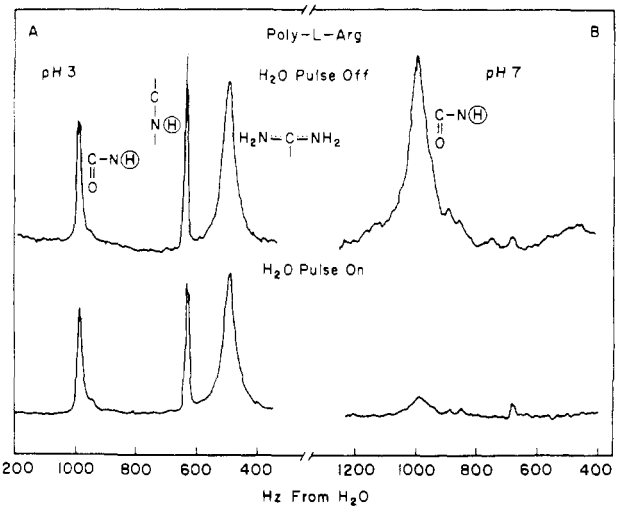

FIGURE 3: The ^{1}H NMR spectra of poly(L-arginine) in the downfield region at pH 3.5 (A) and pH 7.0 (B) (20 °C). Concentrations and conditions as for Figure 2.

This indicates that, at pH 3, the ϵ-NH_3^+ protons of a lysine residue in a random coil structure are in rapid chemical exchange ($\tau \ll T_1$) with water while amide proton exchange is slow ($\tau \gg T_1$). At pH 7 (Figure 2B) only the amide proton resonance remains and now a water preirradiation pulse causes about a 90% reduction in signal intensity. This shows that chemical exchange rates with water are much faster at pH 7 than at pH 3. For the amide protons at pH 7, τ is now much less than T_1. For the amino protons the exchange rate is now very fast even in comparison with the frequency separation of the amino proton and water proton resonances so that the amino proton resonance has merged into the larger water resonance.

The equivalent experiments for random coil poly(L-arginine) at pH 3 and pH 7 are shown in Figure 3. It can be seen from Figure 3A that the ϵ-NH and guanidino protons of arginine, in contrast to the ϵ-amino protons of lysine at pH 3, are only slightly affected by saturation of water protons indicating a relatively slower exchange rate ($\tau \gg T_1$). At pH 7 (Figure 3B),

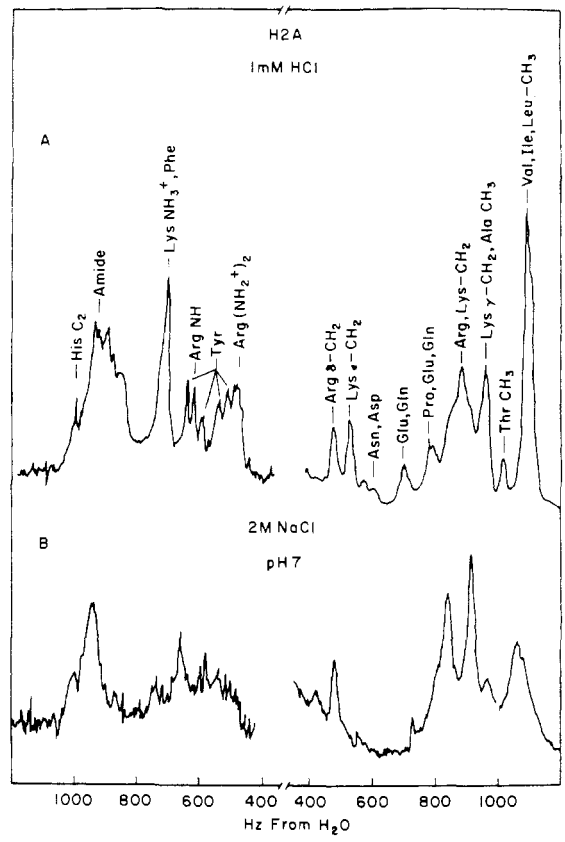

FIGURE 4: ^{1}H NMR spectra of chicken erythrocyte H2A in both upfield and downfield regions in 1 mM HCl at 20 °C (A) and as a small molecular weight complex in 2 M NaCl, 10 mM sodium phosphate, pH 7 at 5 °C (B). All solutions contained 10% D_2O and the concentration of H2a was 15 mg/mL.

however, the exchange rate is again very rapid and both of these resonances have apparently disappeared. In all cases the amide protons of poly(L-arginine) behave in the same way as those of poly(L-lysine).

Similar behavior was observed for denatured histones in acid. The downfield ^{1}H NMR spectra of a lysine rich (H2A) and an arginine rich (H4) histone in 1 mM HCl (pH 3) with and without preirradiation of water protons were obtained (not shown). The amide peak now shows a well-defined fine structure presumably corresponding to classes of amides adjacent to different amino acid residues (vicinal effect) since there is no evidence for secondary structure of histones under these conditions (Fasman et al., 1976, and references therein). Cross-saturation with water protons by application of a preirradiation pulse was demonstrated for the ϵ-amino protons of the lysines though, as expected, not for arginine guanidino protons or amide protons. Phenylalanine aromatic proton resonances were hidden under the lysine amino peak and tyrosine protons were spaced near the arginine resonances. The assignments for the above mentioned resonances can be found in Figure 4.

The ^{1}H NMR spectra of H2A and H2B separately in 1 mM HCl at 20 °C and of their small molecular weight complexes in 2 M NaCl, pH 7 at 5 °C are compared in Figures 4 and 5. It is clear from Figure 4 (for H2A) that the salt and higher pH, that results in the formation of an H2A dimer (Figure 1), have caused a dramatic change in the ^{1}H NMR spectrum relative to the random state. In particular, the upfield region shows that the methyl protons of valine, isoleucine, leucine, and threonine as well as the protons of proline, glutamate, glutamine, aspartate, and asparagine have strongly reduced resonance in-

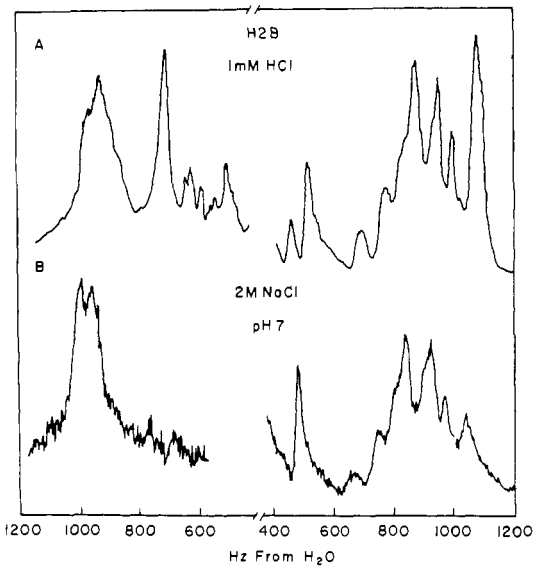

FIGURE 5: ^{1}H NMR spectra of chicken erythrocyte H2B under similar conditions as those for Figure 5.

tensities (presumably due to extensive line broadening) relative to the lysine and alanine proton resonances. In contrast to all the lysine resonances, the arginine proton resonances (see especially the δ_{CH_2} peak) also show a loss of intensity and broadening. These results are in general agreement with the changes observed upon addition of salt to H2A and H2B solutions in D_2O at 20 °C (Bradbury et al., 1975; Bradbury & Rattle, 1972). In the downfield region, much of the resonance intensity due to tyrosine and phenylalanine has also disappeared due to broadening effects. The lysine ϵ-NH_3^+ peak has also disappeared but for a different reason. Since the methylene proton resonances for lysine residues in the upfield region remain sharp and intense, the loss in intensity of ϵ-NH_3^+ proton resonances must be due to rapid chemical exchange with water at this pH as also observed for random-coil poly(L-lysine) (Figure 2). On the other hand, much of the arginine NH proton resonance remains at pH 7 in contrast to the behavior of random-coil poly(L-arginine) at this pH. This indicates a slowed chemical exchange rate of these protons with water and along with the broadening of arginine methylene proton resonances in the upfield region suggests that arginine residues are involved in structured regions of the histone complex.

The equivalent data for H2B (tetramer vs. random-coil monomer), is seen in Figure 5, and it is sufficient to point out that the same trends as seen for H2A in Figure 4 are also observed for H2B. When a water saturation prepulse was applied (not shown), the amide peaks in both cases (H2A and H2B) decreased in intensity only by about 50% (compare Figures 2 and 3) indicating that a significant number of amide protons were in relatively slow exchange and possibly involved in maintaining secondary structures.

Figures 6-8 show the upfield and downfield ^{1}H NMR spectra for the histone complexes H2A-H2B, H2B-H4, and H3-H4, respectively, in 2 M NaCl, pH 7 at 6 °C. All three sets of spectra show marked similarities with each other and with the individual histones under the same conditions (Figures 4 and 5). In particular, we again point out that the valine, isoleucine, leucine, proline, acidic and aromatic residue resonances are broadened much more than the lysine and alanine resonances, when compared with the sum of the spectra for the individual random-coil (pH 3) histones (not shown). In all cases the arginine δ_{CH_2} peak is greatly reduced relative to the lysine ϵ-$_{CH_2}$ peak and this is all the more remarkable for the

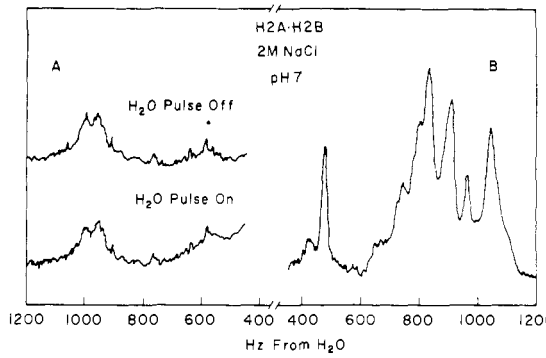

FIGURE 6: ^{1}H NMR spectra of the small molecular weight complex of chicken erythrocyte H2A-H2B in 2 M NaCl, 10 mM sodium phosphate, pH 7 at 5 °C. The upper spectrum in A is the downfield region and the lower spectrum is with a water proton irradiation pulse (200-ms duration, 100 ms delay before observation pulse). Spectrum B is the upfield region.

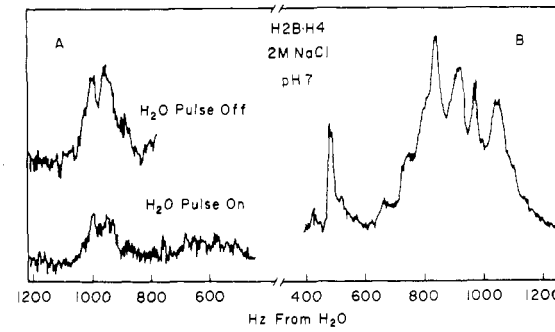

FIGURE 7: ^{1}H NMR spectra of the small molecular weight complex of chicken erythrocyte H2B-H4 under the same conditions as Figure 7.

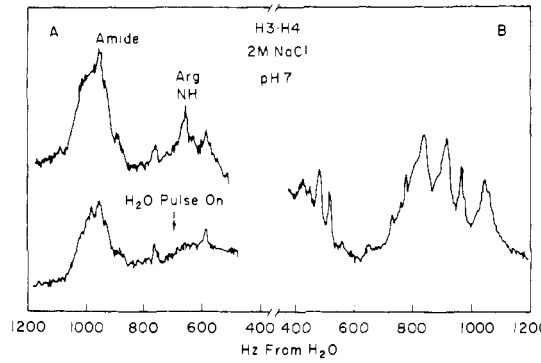

FIGURE 8: ^{1}H NMR spectra of the small molecular weight complex of chicken erythrocyte H3-H4 under the same conditions as Figure 7.

H3-H4 complex (Figure 8) since the arginine/lysine ratio overall is about 1.32:1. In all the complexes the lysine ϵ-NH_3^+ protons are in very rapid chemical exchange with water protons and their resonances are not visible, but at least in the case of H3-H4 where it is easiest to see because of the relatively high arginine content, the arginine guanidino protons are in slowed chemical exchange with water relative to the random coil. Their chemical exchange rate is, however, fast enough to show cross-saturability with water protons (Figure 8) (i.e., $\tau \ll T_1$). In all cases the amide proton peak also shows reduced cross-saturability with water protons relative to the random coil. When water protons are preirradiated, the amide proton peak is decreased in area only by about 40-50% indicating that about half of the peptide bonds in these complexes are involved in secondary structure or shielded to some extent from bulk

water solvent. The additional resonance seen for H3–H4 at about 520 Hz upfield from water (Figure 8) is probably due to protons from methylated or acetylated derivatives since it is also seen for individual histones H3 and H4 at pH 3 but not for the other histones (not shown).

The above results are in general agreement with the ^1H NMR spectra recently published for H3–H4 and H2A–H2B (Moss et al., 1976a,b) which had been extracted by salt from chromatin. These spectra were taken in D_2O (so the downfield regions are quite different from those observed herein) and, at least in the case of H3–H4, a considerable amount of the protein was in a high molecular weight aggregate.

Discussion

We have presented ^1H NMR spectra in aqueous solution for specific histone–histone complexes which are free of high molecular weight nonspecific aggregate. Since this aggregate is not in equilibrium with the smaller molecular weight complexes, it does not appear to represent a higher order structure of the small molecular weight complexes and may not be directly relevant to the biological situation. The smaller molecular weight complexes, on the other hand, are of the right size to be involved in forming the histone core of the nucleosome. These complexes are the H2A dimer, H2B tetramer, H3–H4 tetramer, H2A–H2B dimer, and H2B–H4 dimer. It is easy to construct a closed nucleosome core of two each of all four histones using the above specific complexes. The only problem is in utilizing the H2B tetramer since only a dimer would give a closed set of interactions. From Figure 1A it is possible that H2B may be in a dimer–tetramer equilibrium. It may be that H2B forms weaker contacts *between* nucleosomes helping to maintain the condensed state of chromatin (along with H1 and H5), or that interaction of H2B with H4 in the nucleosome precludes further interaction with another H2B molecule.

All of these histone complexes at high salt and neutral pH show remarkably similar structures as judged by ^1H NMR spectroscopy and also utilizing qualitative measures of chemical exchange rates. Unfortunately, it is not possible from our present data to decide whether these structures are formed in individual histone molecules under these conditions or only as a consequence of complex formation.

In all cases the formation of structured regions in the histones, as judged by selective broadening of proton resonances and reduced chemical exchange rates relative to the random coil, involves the immobilization of nonpolar and aromatic amino acid residues (valine, leucine, isoleucine, tyrosine, phenylalanine), glutamate and glutamine, aspartate and asparagine and possibly some threonine and proline residues. Of special interest because of their high content in histones is the behavior of lysine and arginine. Under all conditions the ϵ-NH_3^+ protons of the lysines are in rapid chemical exchange as expected for a random-coil structure and the CH_2 resonances remain sharp. This is in contrast to the behavior of arginine in the H3–H4 tetrameric complex in high salt where the chemical exchange rate of the guanidino protons is slowed relative to that for a random-coil. The amide protons also show a slowed chemical exchange rate relative to the random-coil involving up to 50–60% of the total amides which are probably involved in hydrogen bonding for the maintenance of secondary structures.

A schematic diagram of the histone sequences has been published in Fasman et al. (1976; Figure 5). It is apparent that the N-terminal tails are rich in lysine and arginine with few hydrophobic amino acids (usually alanine and glycine occur at a high frequency here). The central regions of the sequence contain most of the hydrophobic amino acids and in H3 and H4 are relatively rich in arginine rather than lysine. The C-terminal tails are lysine rich in H2A and H2B but not in H3 and H4. Our data are best fitted by a model where, in high salt or under neutralizing conditions with DNA, the central and possibly C-terminal ends fold up to form a structured globule with the N-terminal ends random and free to direct the folding of the DNA. For H2A and H2B at least the C-terminal ends may also be free. The structured globule consists of a hydrophobic core and probably dipolar interactions between arginine and acidic or hydrogen bonding side chains. Recent NMR studies on Cys-^{19}F-labeled H3 by Puigdomènech et al. (1977) support extensive structure formation in the central portion of the H3 sequence. These hydrophobic globules are the obvious candidates for histone–histone interactions, and it may be that arginine residues are utilized mainly for intra- and intersubunit interactions among histones while the lysine residues are used for interaction with the DNA.

This would be consistent with the observations that H3–H4 (the arginine-rich histones) form the strongest histone–histone complex (D'Anna & Isenberg, 1974b) and that this complex is the only one that alone gives a nucleosome-like structure when complexed to DNA (Camerini-Otero et al., 1976). It may be that the H3–H4 complex can cross-link the DNA and cause it to coil because of the strong intersubunit interactions between histones whereas other histone pairs alone can be pulled apart by the stronger electrostatic interactions with DNA. We would suggest that arginine residues might be involved in some of the interactions essential for coiling the DNA in a nucleosome through histone–histone interactions.

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Identification of Phyllostine as an Intermediate of the Patulin Pathway in *Penicillium urticae*†

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ABSTRACT: A patulin negative mutant (J1) of *Penicillium urticae* (NRRL 2159A) was found to accumulate large quantities (>128 mg/L culture) of a reactive, photosensitive compound, which was isolated and identified as (−)-phyllostine (5,6-epoxygentisylquinone). This epoxyquinone possessed an antibiotic activity against *Bacillus subtilis* which was ~80% of that exhibited by patulin. In separate in vivo feeding experiments, [2-^{14}C]acetate and [G-^{3}H]gentisaldehyde were readily incorporated into phyllostine by mutant J1 and [^{14}C]phyllostine was incorporated into patulin by the parent strain (NRRL 2159A). When fed to a washed-cell suspension of a second patulin negative mutant (J2) which produced gentisaldehyde but not phyllostine, unlabeled phyllostine was efficiently converted to patulin in yields of 33, 56, and 92% after 30 min, 1 and 5 h, respectively. The role of phyllostine as an intermediate of a new post-gentisaldehyde portion of the patulin biosynthetic pathway is discussed.

Patulin was first discovered as a potent antibiotic produced by *Penicillium expansum* (Van Luijk, 1938), *Aspergillus clavatus* (Wiesner, 1942), and by *P. claviforme* (Chain et al., 1942). Its present name derives from its subsequent isolation from the culture medium of *P. patulum* syn. *P. urticae* (Birkinshaw et al., 1943a). The determination of its structure (Woodward & Singh, 1949) and the identification of gentisyl alcohol (Birkinshaw et al., 1943b) and gentisic acid (Brack, 1947) as additional products of *P. urticae* provided the basis for the first speculation concerning the biosynthesis of patulin (Birkinshaw, 1953). This suggestion that ring cleavage of the then unknown metabolite, gentisaldehyde, led directly to patulin was supported by the subsequent isolation of gentisaldehyde and by the radiolabeling pattern observed for patulin which was derived from ^{14}C-labeled 6-methylsalicylic acid (Bu'Lock & Ryan, 1958; Tanenbaum & Bassett, 1959). Although various routes from 6-methylsalicylic acid to gentisaldehyde have been proposed, the most probable route is via *m*-cresol, *m*-hydroxybenzyl alcohol, and *m*-hydroxybenzaldehyde (Bassett & Tanenbaum, 1958; Bu'Lock et al., 1965; Scott & Yalpani, 1967; Forrester & Gaucher, 1972; Scott et al., 1973; Scott & Beadling, 1974; Murphy et al., 1974; Murphy & Lynen, 1975).

Although it is not obvious from its structure (see Table I), patulin is a classic example of the large group of polyacetate-derived secondary metabolites known as "polyketides" (Turner, 1971). We have chosen patulin biosynthesis in *P. urticae* (NRRL 2159A) as a model system for polyketide biosynthesis in particular and for secondary metabolite biosynthesis in general. In a continuing examination of the function as well as the regulation of patulin biosynthesis, we have recently shown that this pathway does not appear to be

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