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Association Products and Conformations of Salt-Dissociated and Acid-Extracted Histones. A Two-Phase Procedure for Isolating Salt-Dissociated Histones†

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ABSTRACT: We present an extremely rapid and efficient method for the separation of salt-dissociated histones from DNA in which the macromolecular components of chicken erythrocyte chromatin are partitioned in a two-phase system of the water-soluble, nonionic polymers, poly(ethylene glycol) and dextran. We have compared the association products and conformations of salt-dissociated histones purified with the two-phase procedure and histones that had been extracted with 0.4 M H_2SO_4. In the gel chromatography system of D. R. van der Westhuyzen and C. von Holt [(1971), *FEBS Lett. 14,* 333-337] the association products of salt-dissociated and acid-extracted histones are indistinguishable. Furthermore, the circular dichroism spectra of histones prepared with the two methods are identical within experimental error. These results indicate that histones extracted with sulfuric acid can adopt conformations at least very similar to those of salt-dissociated histones. In addition, we have found that the circular dichroism properties of total erythrocyte histones are the same in 2 M NaCl as those of these histones bound to DNA in chromatin in 1 mM Tris-Cl (pH 7.5). This result and the studies of Weintraub et al. [Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85-110] on the patterns of tryptic digest products of histones strongly suggest that in 2 M NaCl the histones exist in conformations very similar to their conformations when bound to DNA. The concept of native histone conformations is discussed in light of our results.

Proteins are usually isolated with considerable care to avoid denaturation, but this precaution has until recently been ignored in the purification of histones, perhaps because they lack any readily monitored activity that can be used to assess whether they are in their native conformation. In the absence of a clear cut probe of conformational integrity and therefore of the ability to determine if the native state has been regained after probable denaturation under harsh isolation conditions, the safest approach is to avoid overt denaturing conditions in the isolation procedure. A widely used method for the isolation of histones that was developed with the expressed intent of avoiding denaturing conditions is that of van der Westhuyzen and von Holt (1971), who used 3 M NaCl and protamines to displace histones from DNA rather than the much harsher standard conditions of relatively concentrated mineral acids. Their procedure and the gel chromatography system they introduced to fractionate the isolated histones have had a major impact on studies of histones in recent years, especially after Kornberg and Thomas (1974) demonstrated that specific oligomers of histones are obtained in the gel chromatography separation.

Further study of histone association reactions will require substantial quantities of histones that either have been isolated without exposure to denaturing conditions or have been demonstrated to have returned to their native conformations after an isolation that used denaturing conditions. We present here an extremely efficient new procedure for isolating salt-dissociated histones that is considerably faster and more convenient than previously published methods. We have used histones prepared by this procedure for two types of studies. First, by examining association products and circular dichroism properties, we have demonstrated that acid-extracted histones can very likely assume the same conformations as salt-dissociated histones. Secondly, we have found that in 2 M NaCl histones in solution have the same helix content as histones that are bound to DNA in chromatin. This provides quantitative corroborative support for the suggestion of Weintraub et al. (1975) that in 2 M NaCl histones adopt the same conformations as histones that are bound to DNA.

Materials and Methods

Chicken blood (with heparin) was purchased from Pel-Freeze Biologics. Poly(ethylene glycol) 6000 was a product of Union Carbide and Dextran 500 was obtained from Sigma.

All preparative and chromatographic steps were carried out at 4 °C.

Preparation of Chromatin. Chicken blood was suspended in 10 vol of 0.15 M NaCl and centrifuged at 200*g* for 10 min. After removing the layer of white cells with an aspirator, the

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erythrocytes were homogenized 3 times in 1% Triton X-100–0.25 M sucrose–3 mM $CaCl_2$–0.01 M Tris-Cl (pH 7.5) using a Potter-Elvehjem homogenizer. Each homogenization was followed by centrifugation at 4000*g* for 10 min. The final pellet from this series was homogenized twice in the same buffer lacking Triton. The purified nuclei in the pellet from the final homogenization were stored at −20 °C.

Chromatin was isolated by homogenizing thawed nuclei in solutions of decreasing Tris-Cl concentrations (pH 7.5): 0.05, 0.01, 0.005, and 0.001 M. Each homogenization was followed by centrifugation at 4000*g* for 10 min. The gel obtained from this series of washes was sheared in a Waring Blendor at 90 V for 120 s and centrifuged at 16 000*g* for 15 min to pellet any unsheared material. The DNA concentration in the sheared chromatin was determined by absorbance measurements at 260 nm in 1% sodium dodecyl sulfate, assuming $A_{260}^{0.1\%} = 21$.

Two-Phase Separation of Histones and DNA. To 100 mL of sheared chromatin at a DNA concentration of up to 1 mg/mL were added 29.2 g of NaCl, 10 g of PEG,[1] 4 g of dextran, and 1 mL of 1 M Tris-Cl (pH 7.5). This mixture was shaken vigorously until the polymers were dissolved and the phases were then separated by centrifugation at 4000*g* for 10 min. The top phase, which constituted 80% of the total volume and contained the histones, was carefully removed with a pipet. The bottom phase and the small quantity of remaining top phase were then centrifuged again—this time in smaller tubes—and the remaining top phase withdrawn with care not to remove any bottom phase.

Polymer Removal and Histone Concentration. To lower the NaCl concentration, the top phase was diluted tenfold with 0.01 M Tris-Cl (pH 7.5) and applied to a Bio-Rex 70 column. The Bio-Rex had been previously washed in 1 M HCl and 1 M NaOH and equilibrated to 0.01 M Tris-Cl (pH 7.5). One milliliter of packed Bio-Rex will adsorb about 20 mg of histones. We used at least a twofold excess of resin, packed in a column having a 2.6 cm diameter, for adsorption of the histones. The sample was applied at 400 mL/h and the column then washed with 0.01 M Tris-Cl (pH 7.5). The protein was eluted with 3 M NaCl–0.01 M Tris-Cl (pH 7.5) and was detected in the effluent stream by absorbance measurements at 280 nm.

Gel Electrophoresis. Discontinuous electrophoresis in the presence of sodium dodecyl sulfate was conducted in 15% acrylamide gels using the methods of Laemmli except with half the amount of cross-linking reagent (Weintraub and van Lente, 1974). The gels were stained for 15 min in 0.5% amido black in 7% acetic acid–40% ethanol and destained in the same solvent in the presence of beads of Dowex-1. Densitometric scans were obtained with a Gilford 240 spectrophotometer.

Determination of Yield of Histones. Chicken erythrocyte histones that had been separated from DNA using the two-phase procedure and concentrated on a Bio-Rex column were dialyzed exhaustively against 1 mM HCl and lyophilized to dryness. The protein was then weighed and brought to a final volume of 10.0 mL in 1 mM HCl. This sample served as the standard in later measurements of histone concentration.

To determine the recovery of histones in a typical preparation, we first determined the histone content of erythrocyte chromatin. A sample of chromatin was extracted with cold 0.4 M H_2SO_4 for 1.5 h and then subjected to centrifugation at 12 000*g* to remove the precipitated DNA. The supernatant was dialyzed against a large excess of 1 mM HCl. The amount of histone in this dialyzed sample was determined by the Lowry procedure with histones as the standard. The histone/DNA mass ratio was found to be 1.12.

The amount of histone recovered from the Bio-Rex chromatography was determined by the Lowry method to allow calculation of percentage yield from a known amount of chromatin used as starting material.

Partitioning of Histones. To a sample of histones that had been eluted from Bio-Rex were added sufficient solid NaCl, PEG, and dextran to bring the sample to the solute concentrations used in partitioning dissociated chromatin. The mixture was shaken vigorously to dissolve all components and the phases were separated by centrifugation. The relative concentrations of histones in the two phases were determined by absorbance measurements at 220 nm. For these measurements, the top phase was diluted 1:50 and the bottom phase 1:10 with 3 M NaCl–0.01 M Tris-Cl (pH 7.5). Appropriately diluted top and bottom phases from a two-phase system lacking protein were used as blanks for these measurements.

Gel Chromatography. To 4-mL samples of histones eluted from Bio-Rex columns was added 200 μL of 1 M sodium acetate (pH 5.0). These samples were applied to a 2.6 × 83 cm column packed with Sephadex G-100 and equilibrated to 0.05 M sodium acetate (pH 5.0). The column was developed at 15 mL/h with the same buffer.

Circular Dichroism Measurements. All CD spectra were obtained on a Cary 60 spectropolarimeter with a Model 6001 CD attachment, using a 1-cm cell. Ellipticities are reported in deg-cm^2/dmol of amino acid residues and were calculated using 110 as the mean molecular weight of the amino acid residues. For the spectrum of chromatin, the protein concentration was calculated from the DNA concentration, using our experimentally determined value of 1.12 g of histone/g of DNA for chicken erythrocyte chromatin. For the measurements on isolated histones, 200 μL of stock histone solutions at concentrations of about 1 mg/mL was diluted with 5.0 mL of buffer. The concentrations of the stock solutions were determined by the Lowry method, with histones as the standard.

Results

Partitioning of Chromatin Components in the PEG–Dextran System. Albertsson (1971) found that each of several proteins he studied partitioned preferentially into the top phase of poly(ethylene glycol)–dextran systems in 5 M NaCl, whereas DNA very strongly favors the bottom phase in the presence of NaCl at concentrations of 0.01 M or higher (Albertsson, 1965). Since high concentrations of NaCl dissociate histones from DNA (Ohlenbusch et al., 1967) a PEG–dextran two-phase system could provide an efficient means of physically separating salt-dissociated histones from DNA.

We have found the particular system described under Materials and Methods to be very suitable for this purpose. After chicken erythrocyte chromatin is subjected to partitioning as described under Materials and Methods, essentially all of the proteins must be in the top, PEG-rich phase, since absorbance measurements of the bottom phase give $A_{260}/A_{220} = 1.35$, the same value that we obtain for purified chicken erythrocyte DNA. Reliable absorbance measurements are impossible to obtain in the top phase because of the turbidity of the PEG and dextran and because of the low absorbance of chromatin components in the top phase (approximately 0.4 at 260 nm when a chromatin solution having $A_{260} = 22$ was partitioned). This very low absorbance is itself an indication that very little

[1] Abbreviations used are: PEG, poly(ethylene glycol); CD, circular dichroism.

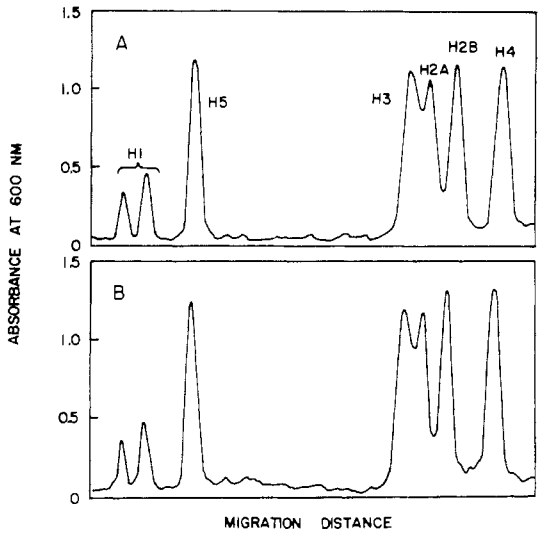

FIGURE 1: Densitometric scans of gels of acid-extracted (A) and salt-dissociated (B) histones. Only those portions of the gels containing proteins are shown. The gel system is described under Materials and Methods.

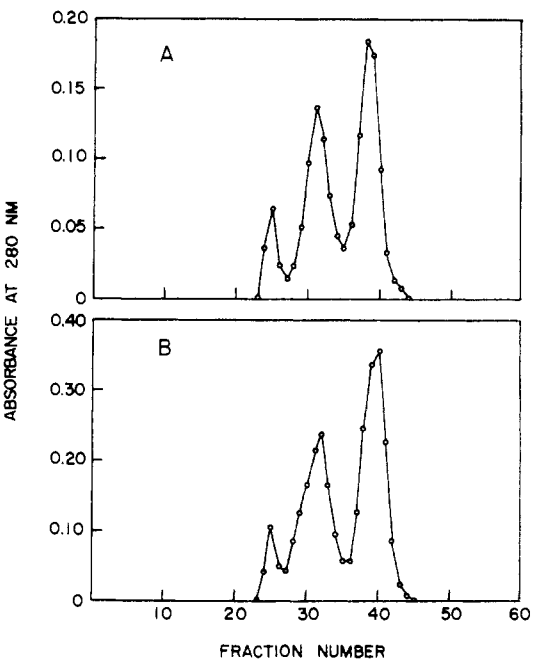

FIGURE 2: Gel chromatography of acid-extracted (A) and salt-dissociated (B) histones. Chromatography was performed at a flow rate of 15 mL/h using a column of Sephadex G-100 with bed dimensions of 2.6 × 83 cm. The solvent was 0.05 M sodium acetate (pH 5.0) and the fraction volume was 5.6 mL.

DNA can be present in the top phase and therefore that the partitioning of DNA into the bottom phase must be nearly complete.

We have examined the extent to which histones favor the top phase by partitioning purified histones as described under Materials and Methods. Using A_{220} as a measure of protein concentration, we found that the concentration of histones in the top phase was 20 times the histone concentration in the bottom phase. Thus, one would expect that only about 1% of the histones would be present in the bottom phase when chromatin is partitioned, since the volume of the top phase is about four times that of the bottom phase.

Isolation of Histones. After partitioning erythrocyte chromatin, the top phase was removed and diluted tenfold with 0.01 M Tris-Cl (pH 7.5). The histones were then adsorbed by Bio-Rex 70 and eluted with 3 M NaCl. Using the procedures described under Materials and Methods we have determined that, in a preparation in which care was taken to maximize yield, we recovered in the protein eluted from Bio-Rex 93% of the histones from the chromatin we started with. The densitometric scans shown in Figure 1 demonstrate that the individual histones are obtained in this isolation procedure in the same relative proportions as from extraction of chromatin with 0.4 M H_2SO_4. This result implies either that the individual histones have very similar partition coefficients in the two-phase system or that all the partition coefficients are sufficiently large that the partitioning of all the histones into the top phase is for practical purposes complete.

Oligomers of Salt-Dissociated and of Acid-Extracted Histones. Kornberg and Thomas (1974) presented evidence that two specific histone complexes are obtained when histones dissociated from DNA with NaCl and protamine are chromatographed on Sephadex G-100 in 0.05 M sodium acetate (pH 5.0)–0.05 M $NaHSO_3$, conditions introduced by van der Westhuyzen and von Holt (1971). We have chromatographed the histones isolated using the two-phase procedure outlined in this paper in 0.05 M sodium acetate (pH 5.0) to compare their behavior with that observed with histones prepared by the NaCl–protamine dissociation. (The inclusion of $NaHSO_3$ is not necessary here since erythrocyte chromatin lacks the protease present in chromatin from most tissues; Carter and Chae, 1976). The elution profile (Figure 2B) is essentially identical with that presented by van der Westhuyzen and von Holt. Figure 2A shows the result of gel chromatography of chicken erythrocyte histones that had been extracted with 0.4 M H_2SO_4, precipitated with 2 vol of ethanol, dissolved in 0.05 M Tris-Cl, adjusted to pH 7.5, and concentrated by adsorption to Bio-Rex 70 and elution with 3 M NaCl. The elution profile from the gel chromatography of the acid-extracted histones is, in its essential aspects, identical with that obtained with salt-dissociated histones.

The first peak to emerge was also observed by van der Westhuyzen and von Holt (1971) and is quite clearly a complex of DNA and proteins (almost exclusively H3 and H4, gel not shown). It is a substantial peak in terms of absorbance but is very small on a mass basis. To obtain an upper estimate of the DNA contamination we have assumed that all of the A_{260}-absorbing material in the peak is DNA, and calculated that in the sample for chromatography in Figure 2B, DNA was present at only 0.1% of the level of histones on a mass basis. This indicates that the two-phase system is very efficient in removing DNA from salt-dissociated histones. Note that the level of DNA contamination in acid-extracted histones is comparable to that of the histones obtained in the two-phase procedure.

The distribution of the four small histones within the next two peaks is in essential agreement with the results of van der Westhuyzen and von Holt (1971), and is shown by the gels in Figure 3. Peak two contains H3 and H4, whereas H2A and H2B are found in the third peak. Note, however, that small but significant quantities of H4 are found consistently in the third peak. The erythrocyte-specific histone H5 was not, of course, present in the histone samples from calf thymus used by van der Westhuyzen and von Holt. In our preparations of erythrocyte histones, H5 chromatographs similarly to H1 of calf thymus.

Circular Dichroism Studies of Acid-Extracted and Salt-Dissociated Histones. CD measurements provide a powerful

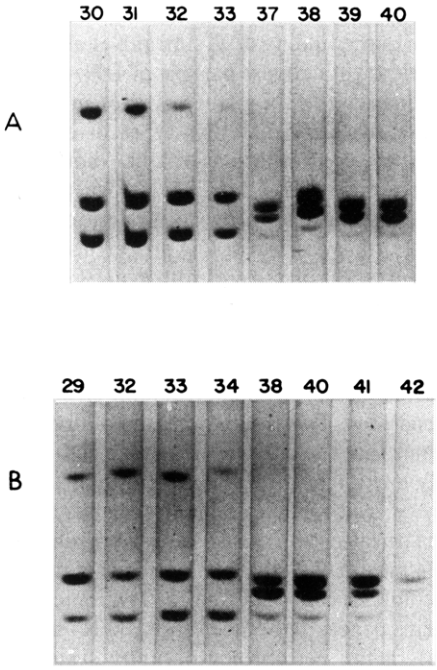

FIGURE 3: Sodium dodecyl sulfate gels of proteins from the chromatograms in Figure 2. Samples for the gels in sections A and B were taken from the profiles of Figure 2A and 2B, respectively. The number above each gel indicates the fraction number of the appropriate chromatogram the sample was taken from. The small amount of H1 in the sample eluted in the second peak (with H3, H4, and H5) but had diffused out of the gel before photography.

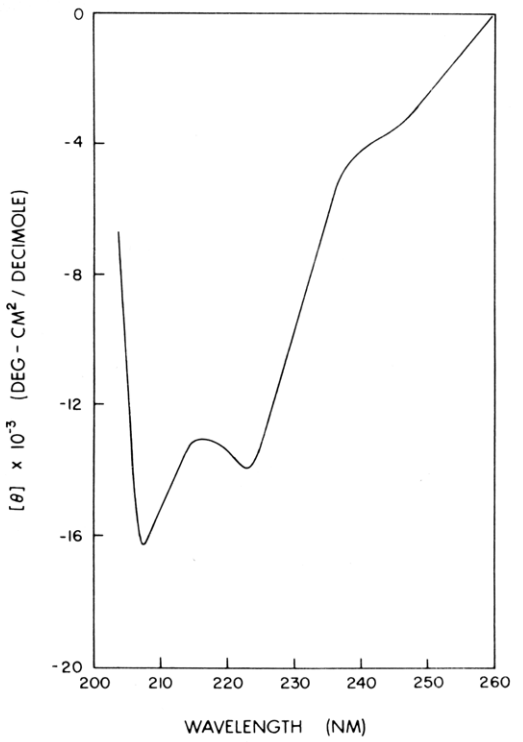

FIGURE 4: Circular dichroism spectrum of chicken erythrocyte chromatin in 1 mM Tris-Cl (pH 7.5).

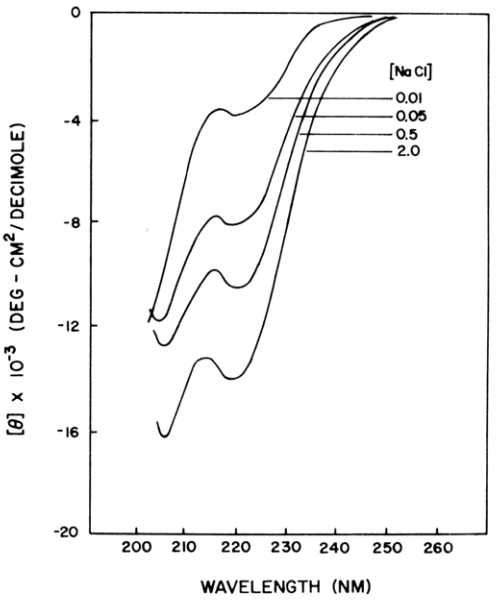

FIGURE 5: Circular dichroism spectra of chicken erythrocyte histones in solutions of 1 mM Tris-Cl (pH 7.5) and several concentrations of NaCl.

means of examining the conformations of proteins since the spectra of polypeptides in irregular conformations, the α helix, and the pleated sheet are readily distinguishable (Greenfield and Fasman, 1969). We have obtained CD spectra of histones prepared by the two-phase procedure and by acid extraction (along with the subsequent steps given in the previous section) in order to determine if any differences in conformations can be detected by this technique. The CD spectra of the two samples of histones were identical within experimental error in 0.5 M NaCl–0.05 M Tris-Cl (pH 7.5) (result not shown). Because of the very low level of DNA contamination in the two histone preparations (see above), DNA did not make a significant contribution to these CD spectra.

Conformations of Histones in Solution and Histones Bound to DNA. We have used histones prepared by the two-phase procedure to explore whether these proteins can, when separated from DNA, assume the same conformations they maintain when bound to DNA. Chicken erythrocyte chromatin is particularly well suited for this purpose since it contains very little nonhistone chromatin protein, and thus the proteins contributing to its CD spectrum from 210 to 260 nm (Figure 4) are almost entirely histones. (A clear indication of the low nonhistone content of our preparations of chicken erythrocyte chromatin is provided by the fact that the A_{260}/A_{220} ratio of the residue from acid extraction of erythrocyte chromatin is 1.3, which is nearly the same as the value of 1.35 that we obtain for purified erythrocyte DNA.) The ellipticities in this region are almost entirely due to the dichroism of the chromatin proteins although DNA contributes significantly from 230 to 260 nm (Simpson and Sober, 1970). With this spectrum as a reference point, we have asked whether purified histones can exhibit the same CD properties as histones that are bound to DNA in chromatin.

In Figure 5 are shown the CD spectra of total histones at several ionic strengths. The conformations of histones are extremely dependent upon the ionic strength of the solvent in a manner that is consistent with earlier studies by other investigators on individual histones (Bradbury et al., 1975) and pairs of histones (D'Anna and Isenberg, 1974b; Moss et al., 1976). The spectra indicate that in 1 mM Tris-Cl–0.01 M NaCl the histones exist in predominantly irregular conformations and that at increasing ionic strengths substantial portions of the polypeptide chains assume helical conformations. At 2 M NaCl, the CD spectrum of the histones corresponds closely to the CD spectrum of the proteins attached to erythrocyte chromatin (Figure 4), suggesting that at this high ionic

strength the proteins assume conformations similar to those they have when bound to DNA.

Discussion

Since NaCl, even at high concentrations, neither stabilizes nor destabilizes the native conformations of proteins (von Hippel and Schleich, 1969), it seems safe to assume that the dissociation of histones from DNA by NaCl and the profound effects of NaCl on the histones' conformations are simply due to weakening of electrostatic interactions and not to alterations in the strength of hydrophobic interactions. It is therefore reasonable to term salt-dissociated histones "native" in the restricted sense of not having been exposed to a denaturant.

Because of the widespread use of acid-extracted histones in many types of studies and the suggestions of some (Kornberg and Thomas, 1974; Roark et al., 1974) that acid extraction might irreversibly denature these proteins, we have looked for differences in physical properties between acid-extracted and salt-dissociated histones. The fact that histones prepared by the two techniques have indistinguishable CD properties (see Results) is consistent with the notion that acid-extracted histones can assume the same conformations as the salt-dissociated proteins but falls well short of proof since CD spectra reflect the averaged properties of all the proteins in a sample.

We believe that gel chromatography provides a rather stringent test of renaturation of acid-extracted histones, because the behavior of of individual proteins can be assessed and because the association reactions among histones should be sensitive to conformational differences. A variety of pairwise associations are possible among histones (D'Anna and Isenberg, 1974b) and the fact that only the H3–H4 tetramer and the H2A–H2B dimer are observed in 0.05 M sodium acetate (pH 5) most likely indicates that these are the most stable species under these conditions. If the conformation of the globular portion of a histone were irreversibly altered by acid extraction, the interactions of this protein with other histones would almost certainly be affected. Our observation that acid-extracted histones form the same oligomers as salt-dissociated histones (Figures 2 and 3) therefore provides strong evidence that each individual protein has a similar conformation in the two samples and that acid extraction and subsequent ethanol precipitation do not cause large irreversible changes in histone conformations. A similar conclusion could be reached for H3 and H4 from the work of D'Anna and Isenberg (1974a), who demonstrated that acid-extracted H3 and H4 can combine to form a tetramer that is very similar to that obtained from salt dissociation. Our results permit a more general conclusion, since we have shown that both the H3–H4 tetramer and H2A–H2B dimer form when all histones are present, conditions that could potentially lead to formation of many possible oligomers (D'Anna and Isenberg, 1974b).

Our studies on physical properties of histones extracted with sulfuric acid are consistent with the finding of Weintraub et al. (1975) that acid-extracted histones that have been dialyzed against 2 M NaCl–0.2 mM EDTA (pH 7.1) exhibit the same pattern of tryptic digest products that is found with salt-dissociated histones. Together, the data strongly suggest that at least the globular portions of the histones can be fully renatured after extraction with sulfuric acid.

Our CD measurements on the conformations of the histones in erythrocyte chromatin (which in our hands is very low in nonhistone content) and of total, DNA-free erythrocyte histones demonstrate that in 2 M NaCl the CD spectrum of the dissociated proteins corresponds closely to the CD spectrum of histones when bound to DNA in chromatin. This result is consistent with the finding of Weintraub et al. (1975) that the characteristic tryptic digest pattern of DNA-bound histones is also obtained with DNA-free histones in 2 M NaCl. Together, these two very different types of evidence suggest strongly that the conformations of DNA-bound histones and histones in solution in 2 M NaCl are at least very similar.

Histones in solutions of low to moderate ionic strengths exhibit substantially different CD properties and susceptibilities to tryptic digestion (Weintraub et al., 1975) than do histones bound to DNA. This has led Weintraub et al. (1975) and Martinson et al. (1976) to refer to the conformations of DNA-bound histones as "native". This by inference labels DNA-free histones at physiological ionic strengths as not native, i.e., denatured. This is somewhat discomforting since there is no evidence that the changes in histone conformations that accompany lowering of the ionic strength of the solvent lead to exposure of hydrophobic residues, which is the hallmark of protein denaturation (Brandts, 1969). Histones H2A, H2B, H3, and H4 must have substantial portions of their polypeptide chains in compact conformations at low ionic strength since these proteins penetrate Sephadex G-100 (even as oligomeric complexes) in the gel chromatography system of van der Westhuyzen and von Holt. Furthermore, for the brief time following their cytoplasmic synthesis until their deposition of DNA, the histones occur in vivo under conditions in which their conformations will differ from those they have in chromatin. It therefore seems more reasonable to consider the conformations of histones in low ionic strength solutions and when bound to DNA as different, native conformations. The simplest interpretation that is consistent with the available information (but not proved by it) is that histones in solution exist in two types of conformations—one largely irregular and the other having substantial helical content; the two are in equilibrium with each other at any salt concentration. At very low ionic strengths the irregular conformations are considerably more stable, but at high ionic strengths the helical conformations are favored. In this two-state model, which once again is not proved by currently available data, the two types of conformations would be present in approximately equal amounts at physiological ionic strengths (Figure 5), and the histones in the helical conformations would necessarily bind more tightly to DNA than histones in the irregular conformations.

We believe that the two-phase isolation procedure we have described is preferable to acid extraction at least under certain circumstances despite the fact that acid-extracted histones can be readily renatured. The two-phase procedure is particularly attractive in two respects: the rapidity with which it can be applied and the highly concentrated form in which the proteins are obtained. Starting with chromatin, the entire procedure requires only about 4 h to complete and the proteins are obtained at concentrations up to 15 mg/mL. The latter factor is especially significant if one intends to proceed with histone fractionation by gel chromatography. Although acid extraction is itself a rapid procedure, one obtains the histones in much more dilute solution, typically about 1 mg/mL. Concentration of the proteins, which is more readily accomplished by ethanol precipitation, requires considerable time, and in our experience the precipitated histones cannot be dissolved at concentrations greater than about 5 mg/mL. In addition, in our two-phase procedure, the DNA is readily recovered in solution in the bottom phase, whereas the DNA precipitate from acid extraction is dissolved only with considerable difficulty.

The two-phase procedure we have presented in this paper is a good deal simpler and more rapid than the NaCl–prota-

mine procedure introduced by van der Westhuyzen and von Holt (1971), and, finally, our procedure should be readily applicable to isolation of other DNA-binding proteins, including the nonhistone chromatin proteins that are dissociated by NaCl.

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Immunological Determination of the Order of Folding of Portions of the Molecule during Air Oxidation of Reduced Ribonuclease†

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ABSTRACT: An immunological method is used to follow the folding of different portions of the reduced bovine pancreatic ribonuclease molecule during air oxidation. Antibodies that react specifically with segments 1-13, 31-79, and 80-124 of native ribonuclease, as they are folded, were purified by affinity chromatography, using antiserum to native ribonuclease and columns to which the ribonuclease fragments were attached. The kinetics of reaction between these purified antibodies and refolded portions that are produced when reduced ribonuclease is oxidized by air demonstrate the presence of intermediate states of folding, and are consistent with folding of the antigenic determinants in the order 80-124, 1-13, and 31-79. The relative stabilities of each of these segments to thermal denaturation in the native protein provide additional evidence that the native conformation of region 80-124 is a very stable one in the intact molecule. On the basis of these two types of evidence, it appears that segment 80-124 contains a nucleation site for the folding of the protein molecule.

Several methods are being used to determine the pathway of folding of either thermally denatured or reduced bovine pancreatic ribonuclease A. These include calorimetric measurements (Tsong et al., 1970), kinetic measurements (Tsong et al., 1971; Hantgan et al., 1974; Garel and Baldwin, 1975; Garel et al., 1976), measurements of the sequence of formation of disulfide bonds (Hantgan et al., 1974), nuclear magnetic resonance (NMR) and proteolytic digestion studies (summarized by Burgess and Scheraga, 1975; Burgess et al., 1975; Benz and Roberts, 1973), laser Raman spectroscopy (Chen and Lord, 1976), flash photolytic labeling of exposed segments of the molecule (Matheson et al., 1977b), and measurement of correlation times of electron spin resonance (ESR) labels attached to various segments of the molecule (Matheson et al., 1977a). In this paper, we apply the immunological technique of Teale and Benjamin (1976a,b) and Chavez and Benjamin (manuscript in preparation) to determine the order in which the various portions of the molecule fold during air oxidation of reduced ribonuclease. Purified antibodies, which are specific for different antigenic sites of the native ribonuclease molecule, are used in kinetic experiments to detect the rates at which the

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