Hamel, E., Koka, M., and Nakamoto, T. (1972), *J. Biol. Chem. 247,* 805.
Miskin, R., Zamir, A., and Elson, D. (1968), *Biochem. Biophys. Res. Commun. 33,* 551.
Miskin, R., Zamir, A., and Elson, D. (1970), *J. Mol. Biol. 54,* 355.
Nakamoto, T., and Hamel, E. (1968), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2453.
Pestka, S. (1968), *J. Biol. Chem. 243,* 4038.
Traub, P., and Nomura, M. (1969), *J. Mol. Biol. 40,* 391.
Tsuda, Y., and Nakamoto, T. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1728.
Zamir, A., Miskin, R., and Elson, D. (1971), *J. Mol. Biol. 60,* 347.

Cross-Complexing Pattern of Plant Histones†

Steven Spiker and Irvin Isenberg*

ABSTRACT: Pea histones H2a, H2b, H3, and H4 have been isolated and their interactions studied by fluorescence anisotropy, light scatter, and circular dichroism. Histones H3 and H4 are almost identical in plants and animals, but plant histones H2a and H2b differ markedly from their mammalian counterparts. Pea H2b has a molecular weight approximately 20% greater than that of calf thymus H2b; the amino acid compositions of the two proteins are different. Calf thymus H2a exists as a single molecular weight species, while pea H2a exists as two species which differ by about 1500 daltons. The larger plant H2a is about 19% greater in molecular weight than calf thymus H2a. The smaller is about 8% greater. Despite these differences between calf and pea histones, the strong interactions between histone pairs H3 and H4, H2b and H4, and H2a and H2b, previously demonstrated for calf histones, also exist for pea histones. There are also weak interactions between pea H2a and H4 and between pea H2b and H3, and an interaction of intermediate strength between H2a and H3. The cross-complexing pattern of the plant histones is therefore the same as that reported for calf thymus histones [D'Anna, J. A., Jr., and Isenberg, I. (1974), *Biochemistry 13,* 4992], despite the dissimilarities of H2a and H2b.

The subunit structure of chromatin has been demonstrated by nuclease digestion and electron microscopy (Olins and Olins, 1973, 1974; Woodcock, 1973; Van Holde et al., 1974; Kornberg, 1974). A general picture has emerged in which two each of the histones H2a, H2b, H3, and H4 associate strongly with one another to form a globular histone core. The DNA is wrapped about the core (Pardon et al., 1975). The subunit structure of chromatin has now been found in a wide variety of organisms (Lohr and Van Holde, 1975; Griffith, 1975; McGhee and Engle, 1975; Gorovsky and Keevert, 1975; Jerzmanowski et al., 1976; Morris, 1976; Noll, 1976; Thomas and Furber, 1976; Nicolaieff et al., 1976).

Two of the histones, H3 and H4, have the most highly conserved primary structures of any proteins known (DeLange et al., 1969; Patthy et al., 1973). The classic comparison between calf thymus H4 and pea H4 demonstrated (DeLange et al., 1969) that these differ by only two conservative replacements.

Although we know the sequence of H3 and H4 from widely divergent species (DeLange et al., 1969; Patthy et al., 1973), such knowledge is not yet available for H2a and H2b. Indeed, H2a and H2b must have changed to a much greater degree throughout evolution. Their molecular weights are much greater in plants than in animals (Sommer and Chalkley, 1974) and, as we point out in this paper, based on amino acid content, H2a and H2b have evolved perhaps more like cytochrome *c* than like the highly conserved H3 and H4 histones.

Before the discovery of the subunit structure of chromatin, histones were defined operationally by their chemical composition, electrophoretic mobility, and solubility characteristics. However, the plant histones H2a and H2b have higher molecular weights and differing amino acid contents from their animal counterparts. These differences prevented a definitive identification of the plant histones by operational criteria (Oliver et al., 1972). This was emphasized by Nadeau et al. (1974) and Brandt and Von Holt (1975), who made no attempt at identifying plant histones H2a and H2b, but rather used the neutral designation "plant histones" (PH1 and PH2) for certain bands seen on gels. Others (Panyim et al., 1970; Spiker and Chalkley, 1971; Spiker and Krishnaswamy, 1973; Sommer and Chalkley, 1974; Spiker, 1975, 1976a,b) speculated on which bands might be H2a and H2b and made tentative identifications based on the pioneering work of Fambrough and Bonner (Fambrough and Bonner, 1966, 1969; Fambrough et al., 1968). More recently, Spiker et al. (1976) suggested that the identification could be made on the basis of electrophoretic mobility in Triton-containing gels, staining properties, and solubility characteristics. As the present paper shows, these suggestions were correct.

Calf thymus histones interact with one another in specific ways (Skandrani et al., 1972; D'Anna and Isenberg, 1973, 1974a,b; Kornberg and Thomas, 1974; Roark et al., 1974; Lewis, 1976; Van Holde and Isenberg, 1975; Isenberg, 1977; Weintraub et al., 1975; Sperling and Bustin, 1975). These interactions are responsible for maintaining the histone core structure. It is therefore important to ask: Will histone–histone interactions occur in organisms evolutionarily distant from vertebrates and, if so, will the interaction pattern be the same as that found for calf thymus histones? We will show here that

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pea histones have the same binding pattern as calf thymus histones (D'Anna and Isenberg, 1974b). This will suggest that the binding of the histones is evolutionarily conserved. In addition, the binding pattern itself will serve to identify unambiguously which pea proteins are H2a and H2b.

Materials and Methods

Histones were obtained by sulfuric acid extraction of purified pea chromatin (Spiker and Chalkley, 1971). Fractions H3 and H4 were obtained directly from a Bio-Gel P-60 column (Spiker et al., 1976) and the peak from the column which contains pea H2a and H2b histones was further fractionated by preparative electrophoresis. Polyacrylamide slabs 3 × 100 × 140 mm were used. They contained 2.5 M urea, as described by Panyim and Chalkley (1969), and 0.38 M Triton DF-16 as described by Alfageme et al. (1974). Fifteen milligrams of the H2a–H2b mixture was applied to the slabs and electrophoresis was carried out for 22 h at a constant 50 V. H2a and H2b were widely separated and were located by cutting the edges away from the slabs and staining them in Coomassie brilliant blue G-250 in 3.5% perchloric acid (Reisner et al., 1975). After cutting out the bands containing pea H2a and H2b, the remaining pieces of the slabs were stained and no cross-contamination of the histones was indicated. Proteins were recovered from the cut-out bands electrophoretically. Pea H2a and H2b, thus isolated, were dialyzed against 0.01 N HCl and lyophilized.

Analytical electrophoresis of whole histone and fractions was carried out in three systems. Sodium dodecyl sulfate electrophoresis was as described by Laemmli (1970) and modified by Bonner and Pollard (1975) and by Thomas and Kornberg (1975). Acetic acid–2.5 M urea gels were prepared according to Panyim and Chalkley (1969). Trition X-100 gels were identical with the acetic acid–urea gels, but also contained 1% w/v Triton X-100.

Amino acid analyses were carried out by utilizing a 0.3 × 30 cm column of Durrum DC-4A resin, an *o*-phthalaldehyde fluorometric reagent as described by Roth and Hampai (1973), and a detection system similar to that described by Ayres et al. (1974). Samples were hydrolyzed in 6 N HCl in vacuo for 20, 48, and 72 h. Proline was determined by the method of Spackman et al. (1958).

Histone H3 was reduced prior to measurement by treating samples dissolved in water with 1 mM dithiothreitol at 40 °C for 1 h. Aliquots were checked for complete reduction by electrophoresis (Spiker and Chalkley, 1971).

Alfageme et al. (1974) reported that methionine in *Drosophila* H2b could be oxidized during electrophoresis in Triton gels. For reasons that will become clear in the Results section, we believed that our pea H2b histone, so prepared, also contained methionine sulfoxide. Our preparations were therefore reduced by treating them with 40% thioglycolic acid in water for 40 h at 22 °C (Alfageme et al., 1974). Following this they were dialyzed against 0.01 N HCl.

Concentrations of stock solutions of histone fractions in water were determined from their absorbance at 275.5 nm (D'Anna and Isenberg, 1973). The number of tyrosines in pea H3 and H4 is known from sequence analysis to be 2 and 4, respectively (DeLange et al., 1969; Patthy et al., 1973). Extinction coefficients used were 2.7×10^3 L cm^{-1} mol^{-1} for H3 and 5.4×10^3 L cm^{-1} mol^{-1} for H4. Pea H2a and H2b have not been sequenced and no molecular weight determinations have been made except by sodium dodecyl sulfate electrophoresis (Sommer and Chalkley, 1974). For this work we have used the value of 16 500 daltons for pea H2b (Sommer and Chalkley, 1974), determined by sodium dodecyl sulfate electrophoresis using calf thymus histones as standards (Panyim and Chalkley, 1971). We have confirmed this value by the same method. For H2a we estimate that the molecular weights are slightly higher than those reported by Sommer and Chalkley, and have used 16 500 daltons for H2a form 1 and 15 000 for H2a form 2, yielding a weighted average of 15 900 for pea H2a. Using these values, we have estimated 150 amino acid residues for pea H2b and 145 residues for pea H2a. From amino acid analysis, we estimate 4 tyrosines per pea H2b molecule for an extinction coefficient of 5.4×10^3 L cm^{-1} mol^{-1} and 3 tyrosines per pea H2a molecule for an extinction coefficient of 4.0×10^3 L cm^{-1} mol^{-1}. These values are supported, at least approximately, by the stoichiometries of the complexes we have observed.

Fluorescence anisotropy and CD[1] were measured as described by D'Anna and Isenberg (1973) and light scattering at 365 nm as described by Smerdon and Isenberg (1973). CD measurements are reported as $\Delta\epsilon$ in units of L cm^{-1} mol^{-1} of amino acid residue. The α-helical content of individual histones and complexes was determined by the method of Baker and Isenberg (1976). We found that good sum tests and wavelength invariances were obtained when polylysine reference spectra were used for α helix and β sheet, and the spectra of histones in 0.01 N HCl were used for random coil references.

Solutions for continuous variation measurements were mixed as described by D'Anna and Isenberg (1974b). All solutions were diluted with phosphate buffer. Final concentrations were 16 mM sodium phosphate and pH was 7.0 unless otherwise stated.

The equations relating fluorescence continuous variation data to stoichiometry of the complexes have been given (D'Anna and Isenberg, 1973, 1974a). Let F be the fluorescence intensity and r the anisotropy. For a complex, $A_{n1}B_{n2}$, the variable $Fr - F_1r_1$ is directly proportional to the concentration of the complex. (The subscript 1 denotes values of the variables expected for noninteracting mixtures.)

Results

Figure 1 shows sodium dodecyl sulfate gels of whole pea histone, of whole calf thymus histone, and of isolated pea histones. Note that, as previously observed (Sommer and Chalkley, 1974; Nadeau et al., 1974; Spiker et al., 1976), the highly evolutionarily conserved histones H3 and H4 from pea have the same electrophoretic mobilities as the corresponding calf thymus histones. In sharp contrast, H2a and H2b histones from pea migrate much more slowly than those from calf, indicating a much greater molecular weight for the pea histones. Based on calf thymus histone standards, we estimate a molecular weight of 16 500 daltons for pea H2b, which is 20% greater than the 13 774 reported for calf thymus (Iwai et al., 1972). For pea H2a we estimate 16 500 daltons for the slower migrating form 1 and 15 000 daltons for form 2. These molecular weights are 19 and 8% greater than the 13 960 reported for calf thymus H2a (Yeoman et al., 1972).

We note that histones run anomolously on sodium dodecyl sulfate gels (Panyim and Chalkley, 1971). This was why we used calf thymus histones to measure the molecular weights of pea histones. Despite this it is, of course, possible that our values for plant histones would be in error.

Figure 2A shows Triton X-100 gels of whole pea histones and fractions as isolated. As previously demonstrated (Spiker et al., 1976), the mobility of pea H2a in Triton gels is much

[1] Abbreviations used: CD, circular dichroism; UV, ultraviolet.

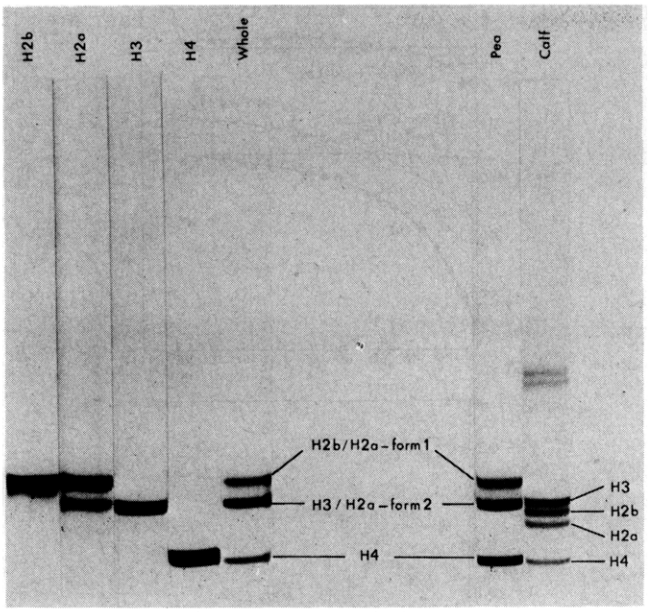

FIGURE 1: Sodium dodecyl sulfate gels of whole pea histone, whole calf thymus histone, and pea histone fractions. Direction of migration is from the top (−) toward the bottom (+).

TABLE I: Amino Acid Analysis of Pea and Calf H2a and H2b Histones.

Amino Acid	Mol %			
	H2a		H2b	
	Pea	Calf[a]	Pea	Calf[b]
Lys	11.2	10.9	17.8	16.0
His	1.2	3.1	1.2	2.4
Arg	7.5	9.3	3.7	6.4
Asx	6.2	6.2	5.7	4.8
Thr	4.0	3.9	6.4	6.4
Ser	6.3	3.1	9.3	11.2
Glx	7.0	9.3	9.9	8.0
Pro	7.6	3.9	5.4	4.8
Gly	11.0	10.9	6.3	5.6
Ala	13.9	13.2	10.0	10.4
Cys	0	0	0	0
Val	8.2	6.2	5.8	7.2
Met	0	0	0.7	1.6
Ile	2.6	4.7	7.3	4.8
Leu	10.1	12.4	5.4	4.8
Tyr	1.7	2.3	2.3	4.0
Phe	1.5	0.8	2.7	1.6
Lys/Arg	1.49	1.17	4.8	2.5
Basic/acidic	1.42	1.30	1.38	1.75

[a] Based on sequence analysis (Yeoman et al., 1972). [b] Based on sequence analysis (Iwai et al., 1972).

lower than it is in the same acetic acid–urea gels without Triton. This property of histone H2a was first noted by Alfageme et al. (1974) with *Drosophila* H2a.

Note that pea H2b *as isolated* (Figure 2A) has no corresponding band in the whole histone preparation. Alfageme et al. (1974) attributed a similar, rapidly moving H2b from *Drosophila* to an artifact of oxidation. They suggested that methionine was oxidized to methionine sulfoxide leading to diminished Triton binding, and thus a greater electrophoretic mobility. We therefore suspected that the same thing had occurred to our pea H2b during its isolation. Consequently, we treated the isolated pea H2b (denoted H2b oxidized) with thioglycolic acid (Alfageme et al., 1974), as outlined in the Materials and Methods section. After reduction (Figure 2B) the treated H2b (denoted H2b reduced) migrates much more slowly and appears in the position of pea H2b in unfractionated histone. This change in electrophoretic mobility can only be observed in Triton gels. In acetic acid–urea gels or sodium dodecyl sulfate gels, H2b oxidized and H2b reduced are indistinguishable. As seen below, the pea H2b must be present in its reduced form in order for it to complex with H2a.

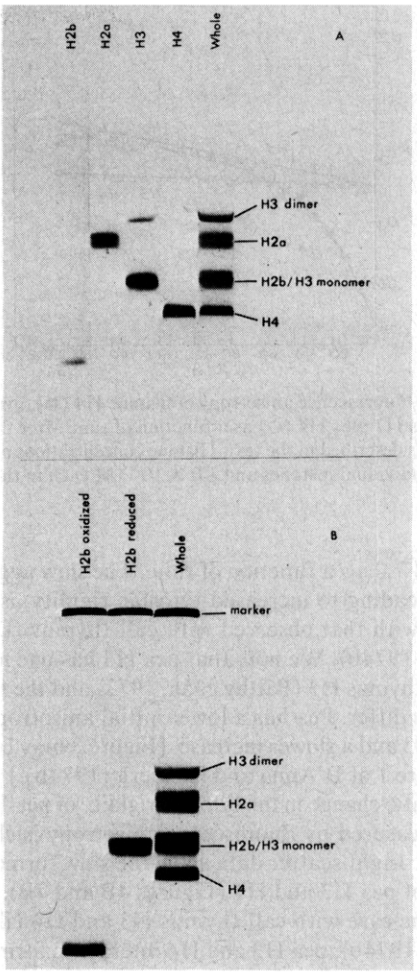

FIGURE 2: Triton X-100 gels of (A) whole pea histones and fractions as isolated and (B) whole pea histone, H2b as isolated (oxidized) and H2b treated with thioglycolic acid (reduced). Direction of migration is from the top (+) toward the bottom (−).

Table I gives the amino acid analyses of pea H2a and H2b and compares them with the corresponding calf thymus histones. The sum of the mole percent differences of the amino acids between pea and calf histones is 22% for H2a and 21% for H2b. This is in the same range as the 40% difference between wheat and calf cytochrome *c* molecules (Dayhoff, 1972). Thus the primary structure of H2a and H2b is much less conserved than that of H3 and H4.

Pairwise Interactions of Pea Histones. H3–H4. Since pea H3 and H4 histones have sequences essentially identical with those of the corresponding calf thymus histones, we expected to observe similar physical properties in these pea histones, including strong complex formation. This we found.

Figure 3 shows the fluorescence anisotropy of pea H3–H4 and a 1:1 mixture of these histones, in 16 mM sodium phos-

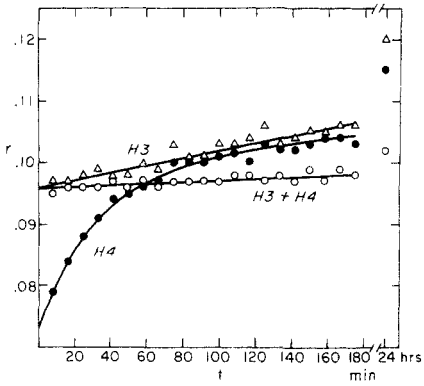

FIGURE 3: Fluorescence anisotropy of histone H4 (●), histone H3 (△), and histones H3 plus H4 (○) as a function of time after the addition of phosphate as described in the text. Histone concentrations are 1.0×10^{-5} M for the individual histones and 1.0×10^{-5} M each in the mixed solution.

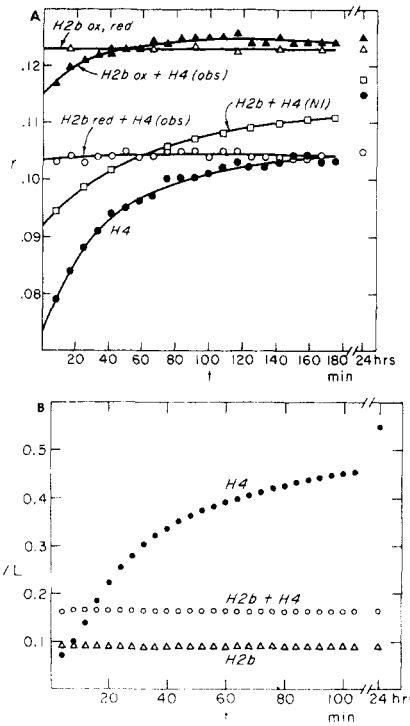

FIGURE 4: (A) Observed fluorescence anisotropy curves as functions of time, after adding phosphate, of histone H2b oxidized or reduced (△), histone H4 (●), histone H2b oxidized plus histone H4 (▲), histone H2b rereduced plus H4 (○) and the curve of histone H2b plus H4 calculated for mixtures of noninteracting (NI) proteins (□). (B) Light scattering of histone H4 (●), histone H2b reduced (△), and histone H4 plus histone H2b reduced (○) as a function of time after adding phosphate. For both A and B, concentrations of individual histones are 1×10^{-5} and 1×10^{-5} M each in the mixed solutions. Buffer conditions are as described in the text.

phate, pH 7.0, as a function of time. The slow aggregation of pea H4, leading to increased tyrosine rigidity, is essentially identical with that observed with calf thymus (D'Anna and Isenberg, 1974b). We note that pea H3 has one less tyrosine than calf thymus H3 (Patthy et al., 1973) and the fluorescence properties differ. Pea has a lower initial anisotropy than calf thymus H3 and a slower increase. [Figure 3 may be compared with Figure 1 of D'Anna and Isenberg (1974b).] Despite the less dramatic change in the tyrosine rigidity of pea H3, the slow step as measured by fluorescence anisotropy is clearly demonstrated. Light scatter data show the slow formation of aggregates of pea H3 and H4 (Figures 4B and 7B).

As is the case with calf thymus H3 and H4 (D'Anna and Isenberg, 1974b), pea H3 and H4 interact to form a complex that is stronger than the self-aggregation of these histones. Thus, as shown in Figure 3, when pea H3 and H4 histones are mixed in a 1:1 ratio a complex is formed which stops the slow aggregation of the individual histones.

H2b–H4. Figure 4A shows that pea H2b, in both the oxidized and the reduced form, interacts with H4 but the complexes have different properties. H2b in the reduced form stops the slow step of H4, indicating a strong interaction. The resulting complex has an anisotropy of 0.104, which does not change with time, and is higher than the anisotropy calculated for a mixture of noninteracting proteins according to the addition law of Weber (1952). This result is similar to that found with calf thymus H2b–H4 (D'Anna and Isenberg, 1973).

H2b in the oxidized form also interacts with H4. However, while the slow aggregation of H4 is greatly inhibited, it is not completely blocked. The anisotropy of the 1:1 H2b (oxidized)–H4 mixture is 0.125 as compared with the value of 0.104 measured for the H2b (reduced)–H4 complex.

Figure 4B shows light scattering data of H4 and H2b (reduced) alone and in a 1:1 mixture. The increase in scatter from H4 with time demonstrates the aggregation of H4 alone. H2b by itself does not aggregate. The slow aggregation of H4 is stopped by the presence of equimolar quantities of H2b.

Table II summarizes the CD data of H2b, H4, and the H2b–H4 complex, analyzed by the method of Baker and Isenberg (1976). The α-helical content of H2b is calculated to be 10%, or 15 residues. The α-helical content of the pea H2b–H4 complex is calculated to be 18%, or 45 residues. This is an increase of 14 residues in α-helical conformation over the 31 that are calculated for noninteracting mixtures of H2b and H4. The α-helical content of pea H4 was assumed to be the same as that of calf thymus H4—16 residues (Baker and Isenberg, 1976). The increase in α-helical content that occurs when pea H2b complexes with pea H4 is the same as the corresponding increase for calf thymus histones (Baker and Isenberg, 1976).

We have made high-speed equilibrium runs on the 1:1 mixture of H2b and H4 and have found results similar to those reported by D'Anna and Isenberg (1974a). Log plots of concentration vs. the radius squared were linear for a portion of the column yielding dimer values for the 1:1 complex. Toward the bottom of the cell, however, higher molecular weight aggregates were found.

H2a–H2b. Since neither pea H2a nor H2b at 10^{-5} M undergoes self-aggregation in 16 mM sodium phosphate, it is feasible to investigate their complex by the technique of continuous variation without the necessity of extrapolating values to zero time. The results are shown in Figure 5.

Different ratios of H2a and H2b were mixed together and diluted with sodium phosphate so that the final concentrations were 10^{-5} M in total protein. Figure 5A shows the fluorescence intensity and anisotropy of the mixture of H2a and H2b (reduced). Figure 5B is the plot of the product of the fluorescence intensity and anisotropy observed for each mixture minus the product calculated for noninteracting mixtures. H2b in the reduced form complexes strongly with H2a in a 1:1 ratio. We estimate the binding constant to be of the order of 2×10^6 M^{-1}. H2b in the oxidized form shows no detectable interaction with H2a.

Table II summarizes the CD data of H2a, H2b, and the H2a–H2b complex. Both pea H2a and H2b have an estimated α-helical content somewhat lower than their calf thymus

TABLE II: α-Helical Content of Individual Histones and Complexes.

Organism	Histone	No. of Residues	% α Helix	Residues α Helix	Sum Test
Pea	H2a	145	6	9	1.01
	H2b	150	10	15	0.95
	H2a-H2b	295	22	65	1.07
	H4-H2b	252	18	45	1.01
Calf[a]	H2a	129	11	14	1.10
	H2b	125	14	18	0.97
	H2a-H2b	254	23	58	0.84
	H4	102	16	16	0.94
	H2b-H4	227	21	48	0.93

[a] Data for calf from Baker and Isenberg (1976).

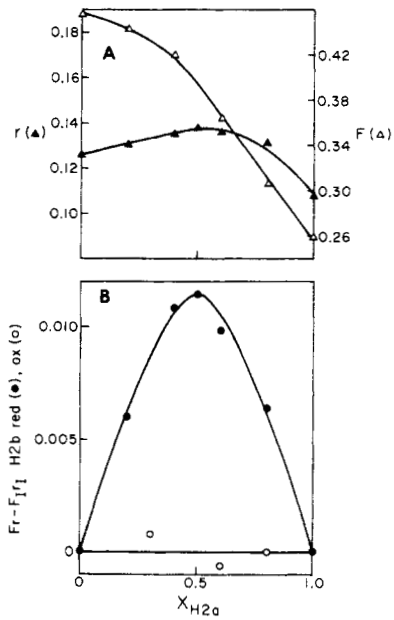

FIGURE 5: Fluorescence continuous variation curves of histone H2a plus H2b. (A) Anisotropy (▲) and intensity (△) of H2a, H2b reduced mixtures. (B) $Fr - F_1r_1$ for H2a plus H2b reduced (●) and H2a plus H2b oxidized (○). For all curves buffer conditions are as given in the text. $C_0 = 1 \times 10^{-5}$ M. The subscript I denotes values calculated for noninteracting mixtures.

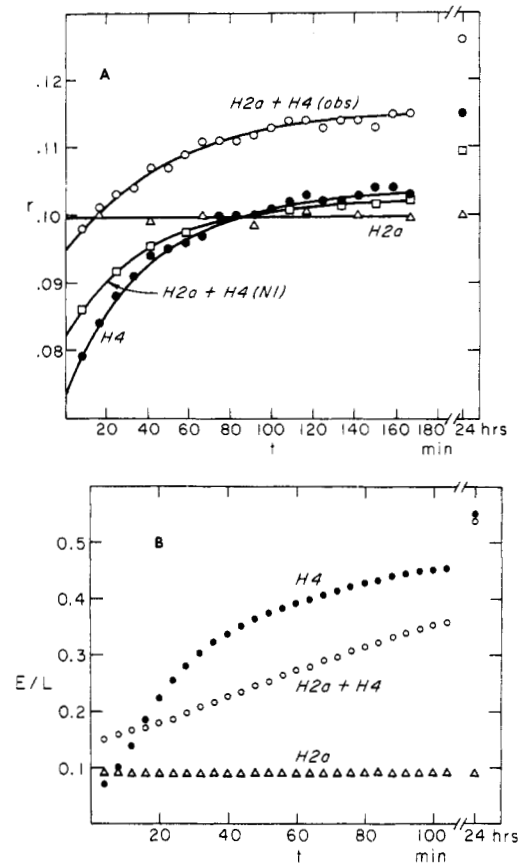

FIGURE 6: (A) Fluorescence anisotropy of H4 (●), H2a (△), and H2a plus H4 observed (○) and calculated for mixtures of noninteracting (NI) proteins (□) as a function of time after adding phosphate. (B) Light scatter of H4 (●), H2a (△), and H2a plus H4 (○) as a function of time after adding phosphate. Buffer conditions are as described in the text. Concentrations of individual histones are 1×10^{-5} and 1×10^{-5} M each in the mixed solutions.

counterparts. Pea H2a has 6% α helix, or 9 residues, and H2b has 10% or 15 residues. These compare with 11%, or 14 residues, for calf thymus H2a and 14%, or 18 residues, for calf thymus H2b (Baker and Isenberg, 1976). The α-helical content of pea H2a and H2b increases dramatically upon complexing. In the complex 22%, or 65 residues, are in α helices. This is an increase of 41 residues over the 24 that are calculated for noninteracting mixtures of H2a and H2b. This 41-residue increase is even larger than the 26-residue increase upon complexing of calf thymus H2a and H2b (Baker and Isenberg, 1976).

Ultracentrifuge data for 1:1 mixtures of H2a and H2b were similar to those obtained for H2b and H4. We interpret these to mean that a dimer of H2a and H2b is formed and is in equilibrium with higher molecular weight aggregates when the concentration is increased by sedimentation.

H2a-H4. Calf thymus H2a and H4 interact only weakly (D'Anna and Isenberg, 1974a). Pea H2a also shows only a weak interaction with pea H4. Figure 6A shows fluorescence anisotropy data of pea H2a, H4, and a 1:1 mixture of H2a and H4. Two things are immediately obvious. First, there is indeed an interaction since the observed anisotropy values are much greater than those calculated for a noninteracting mixture of H2a and H4. However, the interaction is weak and does not block the slow aggregation of H4.

The light scatter data in Figure 6B confirm the conclusion from the anisotropy data. The aggregation of H4 is only slightly inhibited by the presence of equimolar concentrations of H2a.

H2a-H3. Calf thymus H2a and H3 interact to form a complex of intermediate strength (D'Anna and Isenberg,

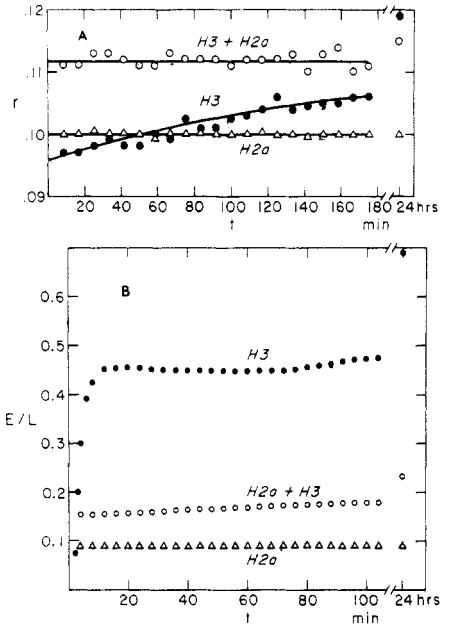

FIGURE 7: (A) Fluorescence anisotropy and (B) light scatter of histone H3 (●), H2a (△), and H3 plus H2a (○) as a function of time after adding phosphate as described in the text. Concentrations of individual histones are 1.0×10^{-5} and 1.0×10^{-5} M each in mixed solutions.

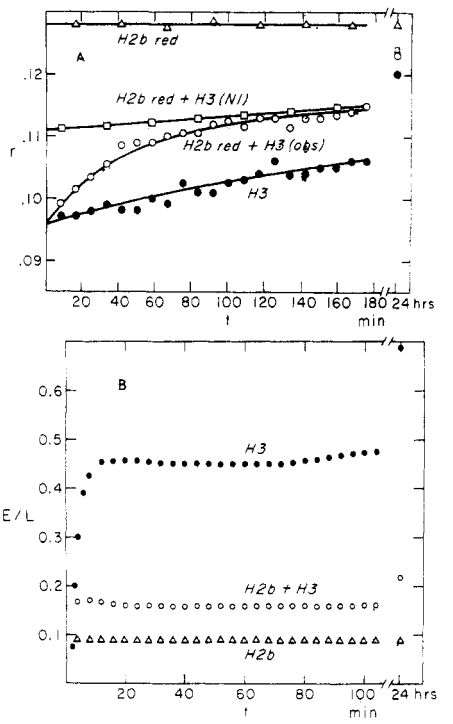

FIGURE 8: (A) Fluorescence anisotropy of H2b reduced (△), H3 (●), and H2b reduced plus H3 observed (○) and calculated for a mixture of noninteracting (NI) proteins (□), as a function of time after adding phosphate. (B) Light scatter of H2b reduced (△), H3 (●), and H2b reduced plus H3 (○) as a function of time after adding phosphate. Buffer conditions are as described in the text. Concentrations of individual proteins are 1.0×10^{-5} and 1.0×10^{-5} M each in the mixed solutions.

1974b), although the complexing is not strong enough to completely inhibit the aggregation of H3. In peas the H2a–H3 complex appears to be stronger. Figure 7A shows that pea H2a prevents the slow change in anisotropy of pea H3.

Light scatter data confirm the conclusions drawn from the anisotropy studies. Figure 7B shows that H2a present in equimolar concentrations effectively stops the slow aggregation of H3.

H2b–H3. The interaction between calf thymus H2b and H3 is so weak that no association constant could be estimated (D'Anna and Isenberg, 1974b). Calf thymus H2b did not stop the slow aggregation of H3 as measured by light scatter, and there was little or no change in CD or fluorescence anisotropy when the two were mixed together.

In contrast, it is clear that there is an interaction between pea H2b and H3. Figure 8A shows that immediately after mixing, the fluorescence anisotropy of the H2b–H3 mixture is well below that calculated for noninteracting mixtures of the two histones. However, the anisotropy observed for the H2b–H3 complex asymptotically approaches that calculated for a noninteracting mixture of the two proteins. The calculated and the observed values are very close after 24 h. Thus the interaction must be classed as a weak one.

Light scatter data (Figure 8B) indicate that the pea H2b–H3 interaction prevents formation of large aggregates of H3. The increase in light scatter of H3 is essentially stopped by the presence of equimolar concentrations of pea H2b.

Discussion

We have shown that pea histones complex with one another and that the cross-complexing pattern is the same as that of calf thymus histones. The similarity in physical properties of the complexes formed by the plant histones to those formed by animal histones is remarkable in light of the approximately 20% greater molecular weight of plant H2a and H2b and the differences in amino acid content between corresponding plant and animal proteins.

Neutron scattering studies (Pardon et al., 1975) have demonstrated that the DNA of the nucleosome is on or near the outside of the particles. The histones form a core which is held together by histone–histone interactions. Many laboratories have used cross-linking agents to study the relationship of the various histones to one another in chromatin. Thus H2b and H4 have been linked by tetranitromethane (Martinson and McCarthy, 1975), formaldehyde and glutaraldehyde (Van Lente et al., 1975), and dimethyl suberimidate (Thomas and Kornberg, 1975). H2a and H2b were linked by UV light (Martinson et al., 1976) and by formaldehyde, glutaraldehyde, and dimethyl suberimidate (Van Lente et al., 1975; Chalkley, 1975; Thomas and Kornberg, 1975).

H3 and H4 have been linked by carbodiimide (Bonner and Pollard, 1975), dimethyl suberimidate (Thomas and Kornberg, 1975), and glutaraldehyde and dimethyl adipimidate (Chalkley, 1975).

It must be emphasized that cross-linking and complexing measure two completely different properties (Isenberg, 1977). Cross-linking measures a geometrical property: How close are the proteins to one another, and can they be joined by a cross-linking agent of appropriate length? Complexing measures a thermodynamic property: Do the proteins interact with one another to any appreciable extent? It is clear that two histones in the nucleosome could be cross-linked by an agent of appropriate length even though they were not interacting at all.

From this it follows that cross-linkers of zero length occupy a special position. These link proteins that are in contact, and hence proteins that are probably interacting. It is noteworthy, therefore, that the partners of all the strong complexes (H2a–H2b, H2b–H4, and H3–H4) may be joined by zero-length cross-linkers (Martinson and McCarthy, 1975; Martinson et al., 1976; Bonner and Pollard, 1975).

It should be recognized that the structure of a histone complex in solution may not be the same as it is in chromatin. There may be structural alterations akin to allosteric effects when histones join to form nucleosomes. Along the same lines, we note that the structure of a histone complex is different from the structure of the individual folded forms. In fact, there is a remarkable increase in the α-helical content upon complex formation (Table II).

No one has reported the cross-linking of H3 and H2a in chromatin at physiological ionic strengths, although we note that these form a moderately strong complex, almost as strong as the strong ones. These histones may, of course, never interact in chromatin. On the other hand, they may interact in various states of development, or stages of the cell cycle which have not yet been studied by cross-linking techniques.

We find that the oxidation of H2b eliminates the interaction with H2a, but only weakens the interaction with H4. This, in itself, would not necessarily show that H2b had two binding sites, one for H2a and the other for H4, because it would always be possible that oxidized H2b could interact with H4 in a manner completely different from that of reduced H2b. However, Martinson and McCarthy (1976) have already presented strong evidence that H2b, at least in the mouse, has two such binding sites. The simplest interpretation of our results is that pea H2b does also, and our data may be considered as a verification of that of Martinson and McCarthy.

It is interesting that H3 and H4 are highly conserved, while H2a and H2b are not. Camerini-Otero et al. (1976) found that the H3–H4 complex, by itself, could form reconstituted nucleoprotein with DNA protected from staphylococcal nuclease digestion; the other complexes could not. Boseley et al. (1976) reported that H3 + H4 combined with DNA gave low-angle x-ray diffraction patterns, indicating the formation of a definite nucleohistone structure. Clearly H3–H4 plays a special role among the histone complexes. This special role may be related to their evolutionary conservatism.

In addition to demonstrating the cross-complexing pattern of the pea histones, the present work offers a definitive determination of which pea histones should be called H2a and H2b.

Acknowledgments

We thank Dr. Alfred Zweidler for advice on the reduction of H2b. Thanks also are due to Dr. Robert Becker for carrying out amino acid analyses.

References

Alfageme, C. R., Zweidler, A., Mahowald, A., and Cohen, L. H. (1974), *J. Biol. Chem. 249,* 3729.

Ayres, W. A., Small, E. W., and Isenberg, I. (1974), *Anal. Biochem. 58,* 361.

Baker, C. C., and Isenberg, I. (1976) *Biochemistry 15,* 629.

Bonner, W. M., and Pollard, H. B. (1975), *Biochem. Biophys. Res. Commun. 64,* 282.

Boseley, P. G., Bradbury, E. M., Butler-Browne, G. S., Carpenter, B. G., and Stephens, R. M. (1976), *Eur. J. Biochem. 62,* 21.

Brandt, W. F., and Von Holt, C. (1975), *FEBS Lett. 51,* 84.

Camerini-Otero, R. D., Sollner-Webb, B., and Felsenfeld, G. (1976), *Cell 8,* 333.

Chalkley, R. (1975), *Biochem. Biophys. Res. Commun. 64,* 587.

D'Anna, J. A., Jr., and Isenberg, I. (1973), *Biochemistry 12,* 1035.

D'Anna, J. A., Jr., and Isenberg, I. (1974a), *Biochemistry 13,* 2098.

D'Anna, J. A., Jr., and Isenberg, I. (1974b), *Biochemistry 13,* 4992.

Dayhoff, M., Ed. (1972), Atlas of Protein Sequence and Structure, Silver Spring, Md., National Biomedical Research Foundation.

DeLange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969), *J. Biol. Chem. 244,* 5669.

Fambrough, D. M., and Bonner, J. (1966), *Biochemistry 5,* 2563.

Fambrough, D. M., and Bonner, J. (1969), *Biochim. Biophys. Acta 175,* 113.

Fambrough, D. M., Fujimura, F., and Bonner, J. (1968), *Biochemistry 7,* 575.

Gorovsky, M. A., and Keevert, J. B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3536.

Griffth, J. D. (1975), *Science 187,* 1202.

Isenberg, I. (1977), in Search and Discovery—a Volume Dedicated to Albert Szent-Gyorgi, Kaminer, B., Ed., New York, N.Y., Academic Press (in press).

Iwai, K., Hayashi, H., and Ishikawa, K. (1972), *J. Biochem. (Tokyo) 72,* 357.

Jerzmanowski, A., Staron, K., Tyniec, B., Bernhardt-Smigielska, J., and Toczko, K. (1976), *FEBS Lett. 62,* 251.

Kornberg, R. D. (1974), *Science 184,* 868.

Kornberg, R. D., and Thomas, J. O. (1974), *Science 184,* 865.

Laemmli, U. K. (1970), *Nature (London) 227,* 680.

Lewis, P. N. (1976), *Biochem. Biophys. Res. Commun. 68,* 2.

Lohr, D., and Van Holde, K. E. (1975), *Science 188,* 165.

Martinson, H. G., and McCarthy, B. J. (1975), *Biochemistry 14,* 1073.

Martinson, H. G., and McCarthy, B. J. (1976), *Biochemistry 15,* 4126.

Martinson, H. G., Shetlar, M. D., and McCarthy, B. J. (1976), *Biochemistry 15,* 2002.

McGhee, J. D., and Engel, J. D. (1975), *Nature (London) 254,* 449.

Morris, N. R. (1976), *Cell 8,* 357.

Nadeau, P., Pallotta, D., and Lafontaine, J. (1974), *Arch. Biochem. Biophys. 161,* 171.

Nicolaieff, A., Philipps, G., Gigot, C., and Hirth, L. (1976), *J. Microsc. Biol. Cell. 26,* 1.

Noll, M. (1976), *Cell 8,* 349.

Olins, A. L., and Olins, D. E. (1973), *J. Cell Biol. 59,* 252a.

Olins, D. E., and Olins, A. L. (1974), *Science 183,* 330.

Oliver, D., Sommer, K., Panyim, S., Spiker, S., and Chalkley, R. (1972), *Biochem. J. 129,* 349.

Panyim, S., and Chalkley, R. (1969), *Arch. Biochem. Biophys. 130,* 337.

Panyim, S., and Chalkley, R. (1971), *J. Biol. Chem. 246,* 7557.

Panyim, S., Chalkley, R., Spiker, S., and Oliver, D. (1970), *Biochim. Biophys. Acta 214,* 216.

Pardon, J. F., Worcester, D. L., Wooley, J. C., Tatchell, K., Van Holde, K. E., and Richards, B. M. (1975), *Nucleic Acids Res. 2,* 2163.

Patthy, L., Smith, E. L., and Johnson, J. (1973), *J. Biol. Chem. 248,* 6834.

Reisner, A. H., Nemes, P., and Bucholtz, C. (1975), *Anal. Biochem. 64,* 509.

Roth, M., and Hampai, A. (1973), *J. Chromatogr. 83,* 353.

Roark, D. E., Geoghegan, T. E., and Keller, G. H. (1974), *Biochem. Biophys. Res. Commun. 59,* 542.
Skandrani, E., Mizon, J., Sautiere, P., and Biserte, G. (1972), *Biochimie 54,* 1267.
Smerdon, M. J., and Isenberg, I. (1973), *Biochem. Biophys. Res. Commun. 55,* 1029.
Sommer, K. R., and Chalkley, R. (1974), *Biochemistry 13,* 1022.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Sperling, R., and Bustin, M. (1975), *Biochemistry 14,* 3322.
Spiker, S. (1975), *Biochim. Biophys. Acta 400,* 461.
Spiker, S. (1976a), *J. Chromatogr. 128,* 244.
Spiker, S. (1976b), *Nature (London) 259,* 418.
Spiker, S., and Chalkley, R. (1971), *Plant Physiol. 47,* 342.
Spiker, S., Key J. L., and Wakim, B. (1976), *Arch. Biochem. Biophys. 176,* 510.
Spiker, S., and Krishnaswamy, L. (1973), *Planta 110,* 71.
Thomas, J. O., and Furber, V. (1976), *FEBS Lett. 66,* 274.
Thomas, J. O., and Kornberg, R. D. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2626.
Van Holde, K. E., and Isenberg, I. (1975), *Acc. Chem. Res. 8,* 327.
Van Holde, K. E., Sahasrabuddhe, C. G., Shaw, B. R., Van Bruggen, E. F. J., and Arnberg, A. (1974), *Biochem. Biophys. Res. Commun. 60,* 1365.
Van Lente, F., Jackson, J. F., and Weintraub, H. (1975), *Cell 5,* 45.
Weber, G. (1952), *Biochem. J. 51,* 145.
Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85.
Woodcock, C. L. F. (1973), *J. Cell. Biol. 59,* 368a.
Yeoman, L. C., Olson, M. O. J., Sugano, N., Jordon, J. J., Taylor, C. W., Starbuck, W. C., and Busch, H. (1972), *J. Biol. Chem. 247,* 6018.

Optical Activity and Conformation of Cobra Neurotoxin†

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ABSTRACT: Cobra neurotoxin from Formosan cobra (*Naja naja atra*) venom is a compact globular protein having an intrinsic viscosity of 4.5 mL/g. The protein is stable in 7.5 M urea but can be denatured in 4.1 M guanidine hydrochloride or at elevated temperature (above 70 °C). Its conformation remains virtually the same in solvents of lower polarity than water such as 1,2-ethanediol or a mixed solvent of 1-propanol–1,2-ethanediol–water (5:1:1 by volume). The circular dichroism spectrum is "atypical" in water in that the peptide chromophores show a small negative circular dichroic (CD) band at 215 nm, a large positive one at 199 nm, and another large negative one below 190 nm. The CD pattern resembles to some extent that of a β form but differs in both positions and magnitudes from the latter. It agrees qualitatively with the theoretical calculations of the reverse β bends, suggesting that cobra toxin contains a considerable amount of β turns and possibly a mixture of β form and β turns.

The neurotoxin of Formosan cobra (*Naja naja atra*) venom is a postsynaptic membrane binding protein (Chang and Lee, 1963, 1966). Its toxicity based on the 50% lethal dose (LD_{50}) in mice is most lethal in the venom of the snake *Naja naja atra* (Lo et al., 1966). Like α-bungarotoxin, it has also been used to characterize the biochemical preparations of acetylcholine receptor molecules (Changeux et al., 1970; Miledi and Potter, 1971; Raftery et al., 1972; Brockes and Hall, 1975). This toxin is an important subject of molecular neurobiology, the knowledge of which will help us understand other snake neurotoxins (see, for instance, Lee, 1972; Tu, 1973).

The cobra toxin molecule consists of a single polypeptide chain of 62 amino acid residues (Yang et al., 1969). Its 4 disulfide linkages divide this small molecule into 4 loops: (a) residues 3 to 24 (with no charged side groups except Glu-21); (b) residues 17 to 41 (having most of the charged groups); (c) residues 43 to 54; and (d) residues 55 to 60(Yang et al., 1970). Two loops share residues 17 to 24. The molecule contains 6 acidic residues and 11 basic residues (including 6 arginine), which accounts for its high isoelectric point (our preliminary study indicates a value of 9.1 to 9.2).

In this work we report the conformation of cobra toxin based on CD[1] and ORD. Unlike other globular proteins, this protein has no detectable CD bands that are characteristic of a helix or are associated with a β form. Instead, the CD spectrum resembles the calculated curves by Woody (1974) for Venkatachalam's β turns (1968), suggesting that this protein contains a considerable amount of reverse β turns and possibly a mixture of antiparallel β form and β turns, which is consistent with its compactness as inferred from viscosity measurements.

Experimental Section

Materials. Crude cobra neurotoxin of snake venom was prepared and repeatedly purified on CM-Sephadex C-50 or

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[1] Abbreviations used: CD, circular dichroism; ORD, optical rotatory dispersion; CM, carboxymethyl; UV, ultraviolet.