An Investigation of Specific Interactions of Deoxyribonucleic Acid and Lysine-Rich (F1) Histone Preparations†

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ABSTRACT: The interaction of F1 histones and DNA was studied by means of circular dichroism spectroscopy and enzymatic assays with DNase I and RNA polymerase. F1 histones prepared by trichloroacetic acid extraction from a given animal species were more effective in protecting DNA of that species from DNase hydrolysis, than a similar preparation from a foreign species. Analogous results have been obtained with RNA polymerase assays. Changes in the circular dichroism spectra of DNA–histone complexes were attained at lower histone:DNA ratios when both histone and DNA were from calf thymus than if calf thymus histones were used with DNA from other species. In contrast, when chromatographically purified F1 histones were used in these experiments, no evidence for species specificity was obtained. We conclude that some component present in the trichloroacetic acid extracts and absent from the purified fractions imparts species-specific properties on the nucleoprotein complex either by acting alone or in concert with the F1 histones.

The genetic material of higher organisms exists as a complex of nucleic acid (DNA and RNA) and chromosomal proteins (histone and nonhistone). When isolated from interphase nuclei, this complex (chromatin) is less active than deproteinized DNA, isolated from the same chromatin, in supporting DNA-dependent RNA synthesis (Huang and Bonner, 1962). The template activity of chromatin may be raised to that of DNA by the selective removal of chromatin-bound histones by techniques which leave nonhistone proteins bound to DNA (Marushige and Bonner, 1966). Evidence for the regulatory role of histones comes from the finding that the template activity of deproteinzed DNA may be reduced by reannealing DNA with histones (Barr and Butler, 1963); furthermore, histones alter the base composition and nearest-neighbor frequency of the transcribed RNA (Skalka *et al.*, 1966). Bonner *et al.* (1963) have elegantly demonstrated that, by removal of histones from pea bud chromatin, a pea cotelydon-specific RNA could be synthesized. The RNA so produced was found to be capable of directing the synthesis of a cotelydon-specific globulin in an *in vitro* protein synthesizing system.

If histones do indeed serve some regulatory role, and do not act solely as structural elements in the architecture of chromosomes, one might expect to see variations in the pattern of distribution of the classes and numbers of histones throughout nature. Several groups (*e.g.*, Crampton *et al.*, 1957; Fambrough and Bonner, 1966) have failed to detect such variations, while Bustin and Cole (1968) and Panyim *et al.* (1971) have reported differences in chromatographic and electrophorectic patterns of vertebrate histones. Evidence that histones may act as generalized regulators rather than as genetic repressors comes partly from the finding that poly-L-lysine can act as an inhibitor of DNA-directed RNA synthesis (Shih and Bonner, 1970). Huang and Bonner (1965) have advanced the view that histones act as specific genetic repressors only when they are complexed with a special class of RNA (chromosomal RNA). This RNA was reported to be of wide occurrence and organ-specific (Bonner and Widholm, 1967; Benjamin *et al.*, 1966; Huang, 1967; Shih and Bonner, 1969). Bekhor *et al.* (1969) have shown that chromosomal RNA is required for the sequence-specific interaction of DNA and chromosomal proteins. Recently, however, the unique nature of this RNA has come under question (Heyden and Zachau, 1971).

The conservation of the primary structure of the arginine-rich F2a1 histones throughout evolution (DeLange *et al.*, 1969) refutes the hypothesis that this histone component acts as an organ- or species-specific regulator of gene activity. Such a role, however, could be played by the lysine-rich F1 histone fraction. Rall (1970) has found that in the N-terminal third of three individual lysine-rich histones, one from calf thymus and two from rabbit thymus, there are between seven and fourteen amino acid replacements. Furthermore, the F1 histones of different vertebrate species vary in electrophoretic mobility and chromatographic properties.

In light of the similarity of F2a1 histones from calf and pea and the pronounced differences between F1 histones of calf and rabbit, it appeared interesting to test whether F1 histones of a given species might bind preferentially to DNA from that species. Over the past few years, a variety of methods have been used to probe the nature of the DNA–histone interaction: Bonner and his coworkers have pioneered the use of the RNA polymerase system with chromatin and reconstituted nucleohistones (*e.g.*, Bonner *et al.*, 1968); Clark and Felsenfeld (1971) have studied the action of nucleases on chromatin; many groups have investigated DNA–protein interactions by means of thermal denaturation studies (Olins and Olins, 1971) and by means of circular dichroism

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(Simpson and Sober, 1970; Fasman *et al.*, 1970; Shih and Fasman, 1971; Olins and Olins, 1971). We have used several of these methods in a search for species specificity in the interaction of the lysine-rich F1 histones with DNA.

Materials and Methods

Preparation of Lysine-Rich Histones. Thymus glands from calf were obtained from a local slaughterhouse and transported to the laboratory in wet ice. Rabbit thymus glands were obtained from the Pel-Freeze Biologicals Co., Rogers, Ark., and shipped to the laboratory on solid CO_2. Tissue was stored at $-20°$ until needed. Lysine-rich histones from these tissues were prepared by the method of DeNooij and Westenbrink (1962) as modified by Kinkade and Cole (1966). Thymus glands were homogenized in 10% (w/v) trichloroacetic acid and insoluble material was removed by centrifugation for 10 min at 1100*g*. The supernatant solution was made 20% (w/v) in trichloroacetic acid by the addition of 100% (w/v) trichloroacetic acid, and was stirred for 5 min. The precipitate was collected by centrifugation at 1100*g* for 10 min, and washed once with acidified acetone (475 ml of acetone plus 0.25 ml of concentrated HCl), and then repeatedly with neutral acetone. The final products were frozen at $-20°$ and then dried under reduced pressure over anhydrous P_2O_5 at room temperature. This preparation will be referred to as the acetone powder.

Column chromatography of the lysine-rich histones was carried out on Amberlite IRC-50 (Bio-Rad Laboratory, Richmond, Calif.) on a column measuring 2.5 × 36.5 cm, which had been prepared by the methods of Kinkade and Cole (1966). Acetone powder (100 mg) was dissolved in 12 ml of 0.1 M sodium phosphate buffer, pH 6.8, containing 7% (w/v) guanidine·HCl, by stirring overnight at room temperature. A small amount of insoluble material was removed by centrifugation, and the supernatant was applied to the column. The chromatogram was developed by elution with a linear gradient between 7 and 14% guanidine·HCl, in 0.1 M sodium phosphate buffer, pH 6.8. A flow rate of 9–10 ml/hr was maintained with a peristaltic pump. Fractions were collected every 30 min, and protein content was determined by turbidimetry. Each fraction (0.2 ml) was incubated for 30 min with 2.8 ml of 18% (w/v) trichloroacetic acid. The absorbancies were then measured at 400 nm with a Zeiss PM QII spectrophotometer, using cells of 1-cm path length. Guanidium chloride concentrations were determined by refractometry.

Amino acid composition analysis was performed on a Beckman/Spinco analyzer, according to the method of Spackman *et al.* (1958). The compositions presented are uncorrected for loss during 22-hr hydrolysis at 110°.

Protein determinations were performed routinely by the method of Lowry *et al.* (1951) using F1 histones purified by Amberlite chromatography as the standard.

Deoxyribonucleic acid was isolated from rabbit thymus and rat liver after the tissue had been treated in the following manner: frozen tissue was cut into small pieces and pressed through a sieve to remove connective tissue. This material was then homogenized in 200 ml of 0.32 M sucrose with a Teflon homogenizer. The homogenate was centrifuged at 1000*g* for 10 min at $-5°$, the supernatant was discarded, and the pellet (crude nuclei) was carried through the procedure of Marmur (1961) for the isolation of DNA. DNA prepared in this manner showed an A_{320}/A_{260} value of 0.03 and an $A_{260}/_{230}$ value of at least 2.0.

DNA was also obtained from commercial sources. Highly polymerized DNA from calf thymus was the product of the Sigma Chemical Co., St. Louis, Mo. (lot no. 60C-4800). DNA from salmon sperm was obtained from Calbiochem, Los Angeles, Calif. (lot no. 40039). Polydeoxyadenylate-thymidylate (poly[d(A-T)]) was a product of Miles Laboratories, Elkhart, Ind. (lot no. 27). DNA from phage T2 was a gift of Dr. M. Chamberlin, Department of Molecular Biology, University of California, Berkeley, Calif.

DNA concentrations were determined either spectrophotometrically, using an ϵ_{260} value of 6800, or by the diphenylamine procedure of Burton (1956), using 2-deoxy-D-ribose as the standard. Protein contamination of the various DNA samples was determined to be less than 0.5%.

RNA concentrations were determined by the orcinol reaction, as modified by Hatcher and Goldstein (1969) (without cadmium salt precipitation), using yeast RNA as the standard.

Sucrose gradient sedimentations were run on the DNA preparations to test for homogeneity with respect to molecular weight. A small volume (about 100 μl) of the DNA preparation to be tested was layered on a 5-ml gradient of 5–20% (w/v) sucrose in 0.01 M Tris-HCl, pH 7, in a centrifuge tube. Centrifugation was carried out in a Beckman Model L-2 Ultracentrifuge with a no. 39 rotor at 39,000 rpm at 0° for 2 hr. At the end of a run, the tubes were punctured and 0.2-ml fractions were collected with the aid of an apparatus previously described by Flamm *et al.* (1969). To each fraction was added 0.8 ml of water, and the absorbancies were read at 260 nm.

Complexes of DNA and lysine-rich histones were formed by three different methods. (1) Complexes were prepared by the urea–salt gradient dialysis procedure of Shih and Bonner (1970). Histone and DNA were mixed in 1.8 M NaCl–0.01 M Tris-HCl, pH 8.0, and dialyzed against a step gradient of 0.5, 0.3, and 0.14 M NaCl in 5 M urea–0.01 M Tris, pH 8.0. Each step was for a minimum of 3 hr at 4°. A final dialysis against 0.14 M NaCl–0.01 M Tris-HCl, pH 8.0, for the removal of urea was carried out for 16 hr. For enzymatic assays, the complexes were dialyzed against 1.5 mM NaCl–0.01 M Tris-HCl, pH 7, for at least 24 hr. Generally, 2 ml of sample was dialyzed against 2 l. of buffer at all stages of dialysis.

(2) The second method was based on the addition of excess histone to DNA in moderate salt concentration (0.23 M NaCl) and the removal of unbound histone from complexed DNA by means of chromatography on Bio-Gel P-30 (Bio-Rad Laboratories, control no. 56433). Histone was in a 2.5-fold excess of DNA (by weight). Histone–DNA solution (3 ml) was applied to a column measuring 2.4 × 20 cm, and was eluted with 0.01 M Tris-HCl, pH 7.5. Fractions of 3 ml (180 drops) were collected, and the A_{260} of each fraction was determined. A portion (0.25 ml) of each fraction was taken for protein determination.

(3) The final method was direct addition of DNA to histone solutions in low ionic strength buffer accompanied by vigorous agitation. Generally, 100–200 μl of DNA solution (about 1 mg/ml) was pipetted slowly into a histone solution of desired concentration (10–200 μg/ml) while the histone solution was being agitated on a Vortex mixer. Solutions of complexes formed in this manner are more turbid than those formed by either of the two methods above.

Ultraviolet absorption spectra were obtained with a Cary Model 14 recording spectrophotometer and with a Cary Model 14R spectrophotometer equipped with the Cary Model 1462 scattered transmission attachment. The path length for all absorption spectra was 1 cm. Spectra taken

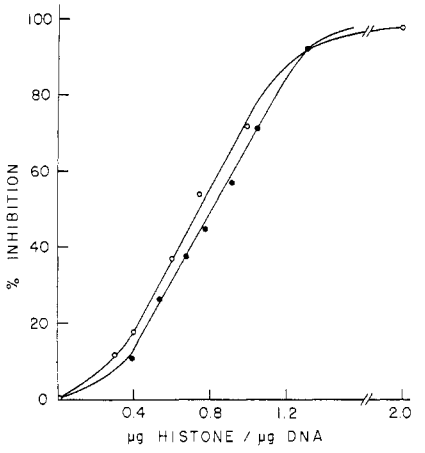

FIGURE 1: DNase assays on calf thymus DNA–calf thymus F1 histone (acetone powder) complexes formed by the direct addition method. Assays were carried out by the hyperchromic effect assay procedure (closed circles), and by the trichloroacetic acid soluble material assay procedure (open circles). For details, see Materials and Methods section.

with the Model 14R were obtained with a fluorscat cell (Dorman and Hearst, 1972[1]). All spectra were taken at room temperature.

Circular dichroism spectra were taken with a Cary Model 60 recording spectropolarimeter equipped with a Cary Model 6001 CD attachment. Spectra were recorded at room temperature with the sample cell (1-cm path length) in a nitrogen atmosphere. CD spectra are reported in terms of the difference in the extinction coefficients for light and right circularly polarized light respectively $[\epsilon_l - \epsilon_r]$ is defined as $\theta^\circ/33{,}050lc$, where θ° is the measured ellipticity in degrees, l is the path length of the sample in centimeters, and c is the concentration of the sample in moles per cubic centimeter. All spectra presented herein are reported in terms of molar concentration of DNA nucleotide residues.

Deoxyribonuclease assays were performed using three separate assay techniques. The spectrophotometric assay method of Kunitz (1950) was the procedure used most frequently in this work. DNase I (Worthington Biochemical Corp., Freehold, N. J., lot no D9AJ) was used at a final concentration of 0.25 μg/ml, unless otherwise stated. The assay buffer was 0.02 M Tris-HCl, pH 6.7, containing 8.0 mM $MgCl_2$ and 2.0 mM $CaCl_2$. Change in absorbance at 260 nm was followed on the Cary Model 14.

We have also measured the hydrolysis of DNA by the nuclease with a pH-Stat as described by Gottesfeld *et al.* (1971). The third assay technique was based on the measurement of trichloroacetic acid soluble material after hydrolysis of the DNA by the nuclease. To a DNA or DNA–histone complex solution (approximately 100 μg/ml in 3 ml of 0.02 M Tris-HCl, pH 6.7, 8.0 mM $MgCl_2$, 2.0 mM $CaCl_2$) was added 20 μl of DNase I (100 μg/ml) and hydrolysis was allowed to proceed for 5 min. To this solution was added 1 ml of cold 10% (w/v) trichloroacetic acid, and the solution stored in the cold for 15 min to allow a precipitate to form. At the end of this time, the solution was filtered through a Millipore (0.3 μ pore size) filter, and the A_{260} of the filtrate was measured on the Cary 14.

RNA polymerase assays were carried out in a final volume of 0.25 ml in a reaction mixture previously described by Marushige and Bonner (1966). [G-^{14}C]Uridine 5′-triphosphate (10 μCi/ml) was the labeled nucleotide (Schwarz/Mann, Orangeburg, N. Y.). RNA polymerase (*Escherichia coli* B) was purchased from Miles Laboratories (lot no. 31-1-642). A sample of RNA polymerase, known to contain the σ factor, was the generous gift of Dr. M. Chamberlin. Incubation was carried out at 37° for 30 min, and the reaction stopped by the addition of 3 ml of cold 10% (w/v) trichloroacetic acid. After incubation at 4° for 15 min to allow the precipitate to form, the acid-insoluble material was collected on a Millipore filter (0.3 μ pore size). The filter was washed twice with 3 ml of cold 10% trichloroacetic acid, and was sucked dry for 1–2 min before it was dissolved in 18 ml of a toluene–dioxane fluid previously described by Kinard (1957). Radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer.

Results and Discussion

Inhibition of DNase I by F1 Histones. Complexes of DNA and the very lysine-rich F1 histones (acetone powder and chromatographic fractions) prepared by the direct addition method (see Material and Methods) were resistant to the action of pancreatic deoxyribonuclease. We have measured the hydrolysis of DNA by DNase, and the inhibition of this hydrolysis by F1 histones, using three separate assay techniques: hyperchromic effect, pH-Stat, and trichloroacetic acid soluble material. Figure 1 illustrates the protection of calf thymus DNA by calf thymus F1 histones (acetone powder) as assayed separately by the hyperchromic effect and trichloroacetic acid soluble material assay procedures. Fifty per cent inhibition was attained, by both procedures at a histone:DNA ratio of 0.75 (w/w). The results of the pH-Stat assay procedure are also in good general agreement.

While the binding of F1 histones to DNA causes a hyperchromic shift, which is at least partly due to an increase in light scattering, this does not invalidate the use of the spectrophotometric assay procedure. The similarity of the results obtained with the hyperchromic effect assay procedure and those of the pH-Stat and the acid-soluble material assay procedures rules out the possibility that this initial hyperchromic shift on the binding of histones to DNA masks any subsequent hyperchromic shift on the addition of DNase (thereby causing an apparent inhibition of DNase hydrolysis). Due to the ease of hyperchromic effect assay technique, we usually used this procedure to explore the interaction between DNA and the lysine-rich F1 histones.

That inhibition arose from the action of the histones on the DNA, and not by direct action of the histones on the enzyme, was demonstrated by an experiment on the order of addition as reported in Table I. This series of assays was performed using calf thymus DNA and calf thymus F1 histones (acetone powder). Preincubation of DNA with histones completely inhibited hydrolysis on the subsequent addition of DNase (assay 2, compared with control, assay 1). However, preincubation of DNase with histones had no effect on the rate of hydrolysis when DNA was added later (assay 3). This indicated that inhibition arose from the interaction of histones and DNA; furthermore, the DNA–histone complexes were not formed fast enough to protect the DNA from hydrolysis by the nuclease. If an aliquot of DNA was added to a cuvet containing DNase and DNA which had been hydrolyzed by the enzyme, hydrolysis of the added DNA was observed (assay 5). It should be noted that the rate of

[1] Manuscript in preparation.

TABLE I: Effect of Order of Addition on the DNase Hydrolysis of DNA–Histone Mixtures.[a]

Mode of Assay	Initial Rate of Hydrolysis (ΔA_{260}/min)
1. DNA + DNase	0.27[b]
2. DNA + histone incubated 10 min; DNase then added	0.00
3. Histone + DNase incubated 10 min; DNA then added	0.28
4. DNA + histone incubated 10 min; DNase then added;	0.00
after 10 min, a second aliquot of DNA added	0.12
5. DNA + DNase;	0.25
after 10 min, a second aliquot of DNA added	0.09

[a] Assays based on the hyperchromic effect were performed on nucleohistone complexes formed by direct addition (see Materials and Methods) by the hyperchromic effect assay procedure. Final concentrations of calf thymus DNA and DNase were 62 μg/ml and 1.0 μg/ml, respectively. The ratio of calf thymus F1 histone (acetone powder) to DNA in all assays was 1.5 (w/w). In assays 4 and 5, the second aliquot of DNA was 50 μg in 1.0 ml added directly to the cuvet and mixed immediately. In assay 5, hydrolysis of the first aliquot of DNA was complete after 10 min, as determined by no further increase in A_{260}. [b] Precision of assays is ±0.01 (ΔA_{260} unit/min), average deviation.

hydrolysis of the second aliquot of DNA was lower than that of the first because (i) the concentration of DNA was 25 μg/ml in the second hydrolysis *vs.* 62 μg/ml in the first; (ii) DNase I is inhibited by the products of DNase hydrolysis (Privat de Garilhe, 1967).

When a second aliquot of DNA was added to a cuvet containing DNase and an enzyme-resistant DNA–histone complex, the second aliquot of DNA was hydrolyzed by the nuclease (assay 4). This hydrolysis was faster than that in assay 5 because in assay 4 there was no accumulation of hydrolysis products in the first incubation. We conclude from these experiments that DNase I is enzymatically active after exposure either to F1 histones or to DNA–F1 histone complexes.

Inhibition of Transcription by F1 Histones. The inhibitory action of histones on the transcription of DNA by RNA polymerase is well documented (Barr and Butler, 1963; Huang and Bonner, 1962; Allfrey *et al.*, 1963). Using RNA polymerase from *Escherichia coli* B, it was observed that inhibition by rabbit thymus F1 histones (acetone powder) of poly[d(A-T)] transcription parallels the inhibition of DNase I (Figure 2a); similarly, the inhibition by rabbit thymus F1 histones (acetone powder) of rabbit thymus DNA transcription roughly parallels the inhibition of DNase I (Figure 2b).

Circular Dichroism of DNA–F1 Histone Complexes. Fasman *et al.* (1970) have reported the CD spectra of DNA–F1 histone complexes formed by gradient dialysis in the absence of urea. They found that the relative magnitudes of the nucleic acid CD bands in the complexes were dependent both upon the histone:DNA ratio in the complex, and upon the

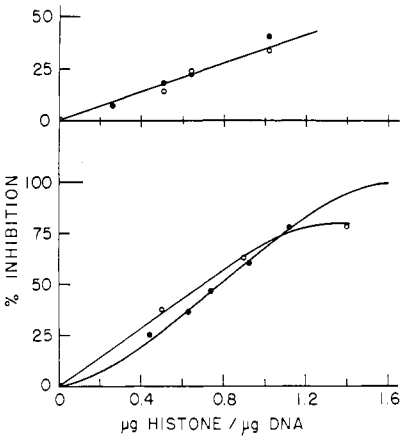

FIGURE 2: Inhibition of DNase I (closed circles) and RNA polymerase (open circles) by rabbit thymus F1 histones (acetone powder). Complexes of histones and DNA were formed by the direct addition method. (a) Upper graph: complexes of F1 histones and poly[d(A-T)]. (b) Lower graph: complexes of F1 histones and rabbit thymus DNA.

absolute concentration of complex when the spectra were taken. Furthermore, the CD spectrum of the complex was highly dependent on the salt concentration in the final step of the gradient; the CD spectrum of the complex formed by dialysis to 0.01 M NaCl was identical with the sum of the spectra of histones and uncomplexed DNA taken individually (Olins, 1969), and was different from that of the DNA–histone complex formed by dialysis to 0.14 M NaF (Fasman *et al.*, 1970).

We have prepared complexes by adding excess histone to DNA in 0.23 M NaCl, and isolated the complexed DNA by chromatography on Bio-Gel P-30. Figure 3 illustrates one such chromatogram for calf thymus DNA and calf thymus F1 histones (acetone powder). The ratio of histone to DNA in this complex was 2.2 (w/w) for fraction number 11. It was

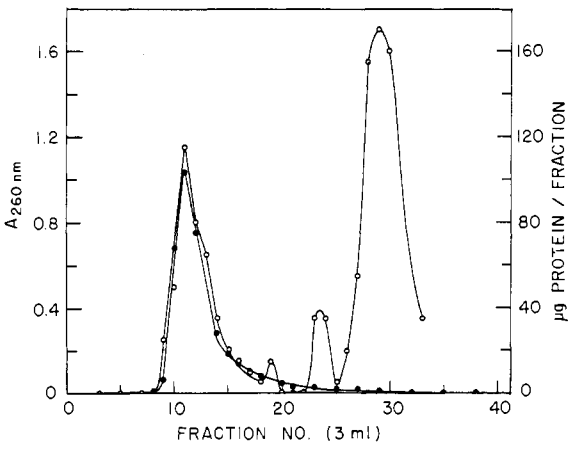

FIGURE 3: Isolation of calf thymus DNA–calf thymus F1 histone (acetone powder) complex by gel filtration chromatography on Bio-Gel P-30. DNA (1.5 mg) and histones (3.75 mg of acetone powder) were mixed in 0.23 M NaCl in a final volume of 3.0 ml. This mixture was applied to the column after a 5-min period of incubation at room temperature; chromatography was carried out as described in Materials and Methods. Protein concentrations (open circles) were determined by the method of Lowry *et al.* (1951) on 0.25 ml of each fraction; the absorbance of each fraction at 260 nm (closed circles) was taken as an indication of the presence of DNA.

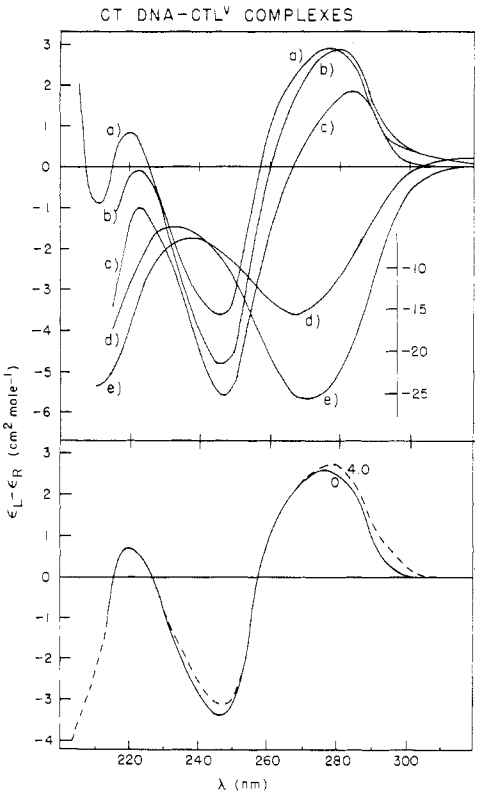

FIGURE 4: Circular dichroism spectra of calf thymus DNA–calf thymus F1 histone (acetone powder) complexes. Upper graph: complexes formed by direct addition method. The molar ratios of histone amino acids: DNA nucleotides were (a) 0, (b) 0.2, (c) 0.3, (d) 0.4, and (e) 0.75. Abscissa on right refers to spectra d and e which have been reduced in size by a factor of 5. Lower graph: complexes with excess histone formed in 0.23 M NaCl, and isolated by means of Bio-Gel P-30 chromatography. Solid line, uncomplexed DNA chromatographed on Bio-Gel P-30. Dashed line, DNA–histone complex with molar ratio of histone amino acids:DNA nucleotides = 4.0.

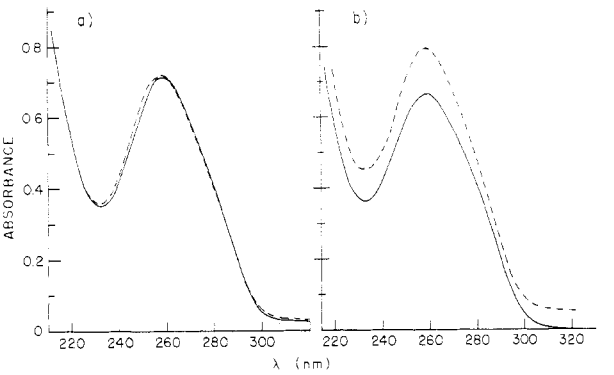

FIGURE 5: Ultraviolet absorption spectra of (a) calf thymus DNA and (b) calf thymus DNA–calf thymus F1 histone (Amberlite chromatogram peak 1) complex prepared by the urea–salt gradient dialysis procedure (histone to DNA ratio of 0.2 (w/w)). All spectra were taken in 0.14 M NaCl–0.01 M Tris-HCl, pH 8, at a DNA concentration of approximately 35 μg/ml. Spectra were taken with a Cary 14 (dashed line), and with a Cary 14R equipped with a Cary 1462 scattered transmission attachment and fluorscat cell (solid line).

demonstrated by means of thermal denaturation that complexes were indeed formed by the method of dialysis to 0.01 M NaCl (Olins, 1969; Olins and Olins, 1971), or by the Bio-Gel P-30 method (Gottesfeld and Schwab,[2] 1971). Figure 4a shows the CD spectra of calf thymus DNA–calf thymus F1 histone complexes formed by direct addition, and Figure 4b shows spectra of the similar complexes formed in moderate salt and isolated by Bio-Gel chromatography. It should be noted that there is a most dramatic spectral change as the histone:DNA ratio changes from 0.3 to 0.4 in Figure 4a. The molecular nature of this change is not clear. The comparison of Figure 4a with Figure 4b suggests that the CD changes accompanying DNA–F1 histone complex formation, while resembling the transformation of DNA into the "C" form, (Tunis-Schneider and Maestre, 1970), depend at least partly on aggregation of the DNA–histone complexes. It is well known that native nucleohistones are least soluble at 0.14 M NaCl (Zubay and Doty, 1959). These CD changes might have been partly the result of light scattering (Glaser and Singer, 1971). A comparison of the ultraviolet (uv) spectra of calf thymus DNA–F1 histone complex and uncomplexed calf thymus DNA, taken with a Cary 14 spectrophotometer, and with a Cary 14R spectrophotometer equipped with a scattered transmission attachment and fluorscat cell (Dorman and Hearst,[1] 1971), demonstrated that these complexes do scatter light (Figure 5). Although it is not yet known whether the CD of these DNA–F1 histone complexes arises from the differential scattering of left and right circularly polarized light (Dorman and Maestre,[2] 1971), investigation of the CD spectral changes upon complex formation nevertheless is a useful even though empirical technique in exploring species specificity of DNA–histone interactions.

Experiments with Acetone Powders of F1 Histones. SPECIES-SPECIFIC INTERACTION OF DNA AND F1 HISTONES AS DETECTED BY DNASE AND RNA POLYMERASE INHIBITIONS. Both calf and rabbit thymus F1 histones (acetone powders) protected their homologous DNAs more effectively than any foreign DNA tested from attack by DNase I. Figure 6 shows the inhibition of DNase hydrolysis of calf and rabbit thymus DNAs, salmon sperm DNA, and poly[d(A-T)], by calf and rabbit thymus F1 histones (acetone powders). All complexes assayed for species specificity were formed by the direct addition method.

It is interesting to note that calf thymus F1 histones (acetone powder) protected poly[d(A-T)] from DNase hydrolysis almost as effectively as they protected rabbit thymus DNA (Figure 6a); similarly, rabbit thymus F1 histones protected poly[d(A-T)] from DNase hydrolysis just as efficiently as they protected calf thymus DNA (Figure 6b). Barr and Butler (1963) have noted that F1 histones precipitate (A-T)-rich DNA preferentially to (G-C)-rich DNA. Our present data on the inhibition of DNase hydrolysis of poly[d(A-T)] by calf and rabbit histones might be a reflection of this base composition preference. The fact that F1 histones preferentially protect homologous DNA from nuclease action indicates that there is more than a base composition preference involved in the interaction of histones and DNA.

RNA polymerase and DNase assays showed a similar pattern of species specificity. Figure 7a shows the inhibition by rabbit and calf thymus F1 histones (acetone powders) of transcriptions of rabbit thymus DNA. Fifty percent inhibition was attained at histone:DNA ratios of 0.65 and 0.9 (w/w) with rabbit and calf thymus F1 histones, respectively. Rabbit

[2] Unpublished results.

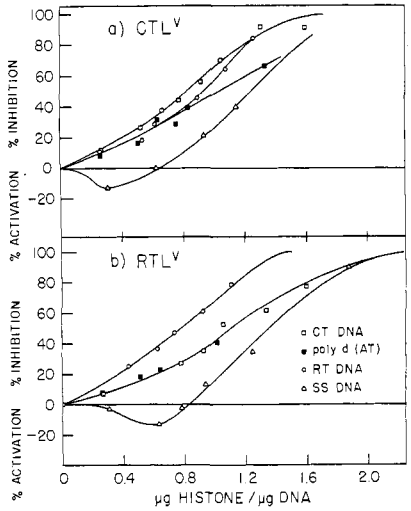

FIGURE 6: Inhibition of DNase I hydrolysis of calf thymus DNA (—□—), rabbit thymus DNA (—○—), salmon sperm DNA (—△—), and poly[d(A-T)] (—■—) by (a) calf thymus F1 histones (acetone powder) and (b) rabbit thymus F1 histones (acetone powder). Assays were performed by means of the hyperchromic effect procedure. Inhibition or activation was determined by comparing the change in absorbance 2 min after addition of the enzyme with a control sample of DNA in the absence of histone. Complexes were prepared by direct addition.

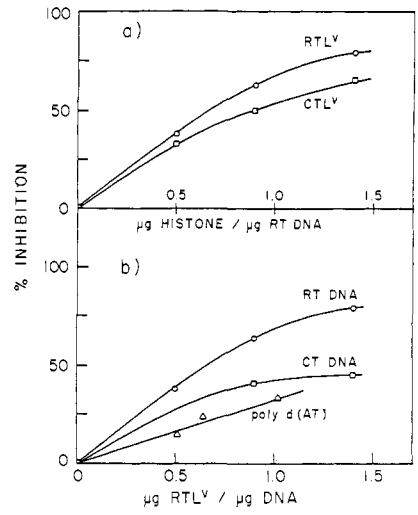

FIGURE 7: (a) Inhibition by rabbit (—○—) and calf (—□—) thymus F1 histones (acetone powder) of transcription of rabbit thymus DNA. (b) Rabbit thymus F1 histones (acetone powder) as an inhibitor of transcription of rabbit thymus DNA (—○—), calf thymus DNA (—□—), and poly[d(A-T] (—△—). RNA polymerase assays on complexes formed by the direct addition method were performed as described in the Materials and Methods section.

thymus F1 histones (acetone powder) restricted the transcription of rabbit thymus DNA more efficiently than it restricted the transcription of either calf thymus DNA or poly[d(A-T)] (Figure 7b).

Circular Dichroism of Histone–DNA Complexes. CD spectra of complexes between calf thymus F1 histones (acetone powder) and a variety of DNAs (complexes formed by the direct addition method) were obtained. Spectra for calf thymus–DNA–histone complexes have already been presented (Figure 4a); Figures 8 and 9 depict the CD spectra of complexes of calf thymus F1 histones with DNA from rat liver, salmon sperm, and T2 phage, respectively.

The binding of calf thymus F1 histones to rat liver DNA caused a change in the CD of the nucleic acid similar to that observed with the binding of calf thymus F2a1 histone (glycine and arginine-rich) to either calf thymus DNA (Shih and Fasman, 1971) or to T7 DNA (Olins and Olins, 1971). The 275-nm and 220-nm positive bands of rat liver DNA were increased in magnitude, and the 245-nm negative band decreased, upon complexing with F1 histones from calf. At a histone:DNA ratio of 1.75 (moles of amino acid residues/moles of nucleotide residues) the 275-nm positive band reached a maximum. The spectrum of rat liver DNA at this histone to DNA ratio was similar to either DNA in the "A" form (Tunis-Schneider and Maestre, 1970) or native RNA (Yang and Samejima, 1969). On further addition of F1 histones, the positive band decreased in magnitude, and shifted to longer wavelengths until, at a histone:DNA ratio of 3.0 (moles of amino acids/moles of nucleotides), the spectrum was similar to DNA in the "C" form (Tunis-Schneider and Maestre, 1970) or to a calf thymus DNA–calf thymus F1 histone complex at a histone:DNA ratio of 0.75 (moles amino acids/moles nucleotides). This pattern of transformation of the CD spectrum of DNA first to that of DNA in the "A" form, and then to that of DNA in the "C" form, has only been observed with rat liver DNA–calf thymus F1 histone complexes.

Addition of even very large amounts of calf thymus F1 histones to salmon sperm DNA caused only small changes in the CD spectrum of the DNA (Figure 9). Complexes of salmon sperm DNA and calf thymus F1 histone were nevertheless formed since the DNA became resistant to DNase hydrolysis at the ratios of histone:DNA shown on these spectra.

F1 histones from calf thymus caused only a slight change in the CD spectrum of T2 DNA (Figure 9). These data, however, should be viewed with caution since the cytosine residues of T2 DNA are glucosylated, and the presence of glucose residues in the major groove of DNA might interfere with the

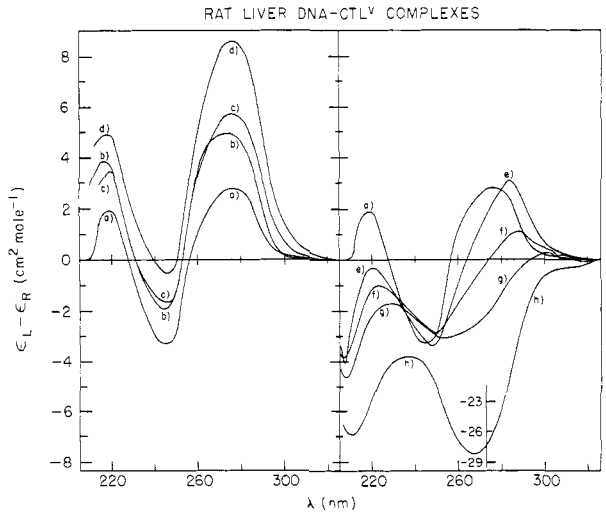

FIGURE 8: Circular dichroism spectra of rat liver DNA–calf thymus F1 histone (acetone powder) complexes prepared by the direct addition method. The molar ratios of histone amino acids:DNA nucleotides were (a) 0, (b) 1.0, (c) 1.5, (d) 1.75, (e) 2.0, (f) 2.1, (g) 2.2, and (h) 3.0. Spectra are not corrected for histone contribution. Abscissa insert on right refers to spectrum h, which has been reduced in size by a factor of 4.

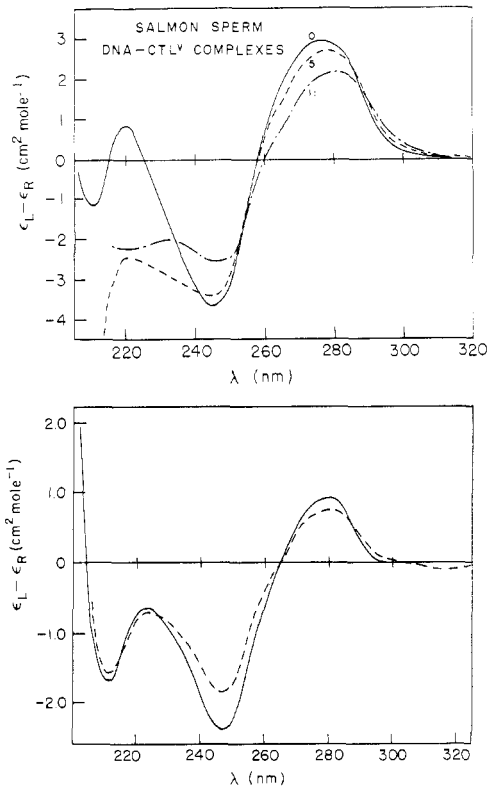

FIGURE 9: Upper part: circular dichroism spectra of salmon sperm DNA–calf thymus F1 histone (acetone powder) complexes prepared by the direct addition method. The molar ratio of histone amino acids:DNA nucleotides are given on the spectra. The data are not corrected for histone contribution. Lower part: circular dichroism spectra of T2 phage DNA–calf thymus F1 histone (acetone powder) complex (broken line) at a histone:DNA ratio of 3.0 (moles of histone amino acids:moles of DNA nucleotides), and of uncomplexed T2 phage DNA (solid line). DNA–histone complex was prepared by the the direct addition method.

TABLE II: Amino Acid Composition Analysis of Rabbit Thymus F1 Histone Acetone Powder and Chromatographically Purified Fractions.

Amino Acid	Mole Per Cent[a]				
	Acetone Powder	L1[b]	L2	L3	L4
Lysine	22.0	27.4	28.8	29.7	31.3
Histidine	2.2	0.2	0.0	0.2	0.0
Arginine	3.3	1.6	1.5	1.8	1.0
Aspartic acid	4.7	2.4	2.2	2.6	2.5
Threonine	4.4	4.2	4.5	3.8	4.1
Serine	6.1	7.4	6.9	6.4	7.5
Glutamic acid	7.5	4.7	3.9	4.2	4.6
Proline	7.9	10.1	9.5	9.6	9.2
Glycine	6.6	7.0	6.9	6.9	7.9
Alanine	18.2	23.3	24.8	24.7	23.1
Half-cystine	1.7	0.0	0.0	0.0	0.0
Valine	5.2	5.3	5.0	4.0	3.6
Methionine	0.0	0.0	0.0	0.0	0.0
Isoleucine	2.1	1.1	1.0	0.8	0.8
Leucine	5.5	4.3	4.3	4.0	3.5
Tyrosine	1.1	0.5	0.5	0.5	0.3
Phenylalanine	1.3	0.5	0.5	0.7	0.5
Total basic amino acids	27.5	29.2	30.3	31.7	32.3
Ratio of lysine to arginine	6.7	17.1	19.2	16.5	31.3

[a] Values are reported as moles of amino acids per 100 moles of all amino acids recovered after 22-hr hydrolysis at 110° in 6 N HCl. [b] Nomenclature of Bustin and Cole (1968).

formation of T2 DNA–histone complexes. Olins (1969) has reported that F1 histones interfere with the glucosylation of unglucosylated T2 DNA by glucosyl transferase when complexed with this DNA. In any case, calf thymus F1 is almost an order of magnitude more effective with calf thymus DNA than it is with heterologous DNAs.

Analysis of the Histone Preparations. F1 histones isolated from calf and rabbit, and purified by the procedure of Kinkade and Cole (1966), gave elution patterns on Amberlite IRC-50 cation-exchange columns very similar to those reported by Bustin and Cole (1968) for the same tissues, and their amino acid compositions as presented in Tables II and III are in good agreement with those presented previously. Unlike the earlier preparations of Bustin and Cole (1968) and Kinkade and Cole (1966) which showed no traces of histidine, the preparations used in the present case contain small amounts of histidine. As expected (Stellwagen and Cole, 1968) the unchromatographed F1 histones (acetone powders) contained considerably more histidine than any of the purified fractions obtained by chromatography. While the vast majority of published studies on lysine-rich histones are carried out with preparations containing traces of histidine residues, it is known (Stellwagen and Cole, 1968) that the histidine indicates contamination. This contamination probably occurs because the nuclei were not purified before trichloroacetic acid extraction.

The RNA composition of the acetone powders was determined to be 1.5% by weight, while the RNA compositions of the purified fractions were below the limit of detectability of the orcinol reaction.

Experiments with Amberlite Fractions of F1 Histones. CIRCULAR DICHROISM AND DNASE HYDROLYSIS OF COMPLEXES BETWEEN HOMOLOGOUS DNA AND F1 HISTONE SUBFRACTIONS. Complexes of DNA and F1 histone subfractions obtained by Amberlite cation-exchange chromatography were prepared by direct addition. The various fractions of calf and rabbit thymus F1 histones differed in their respective efficiencies in protecting their homologous DNAs from DNase I hydrolysis. The subfractions also differed in their ability to cause changes in their homologous DNAs as detected by CD. Table IV summarizes these results for complexes of calf thymus and rabbit thymus histone fractions with DNA isolated from the same tissue. The fractions from rabbit thymus were more effective inhibitors of DNase hydrolysis than were the subfractions from calf thymus. The order of effectiveness for inhibition of DNase hydrolysis was CTL1 > CTL3a $\simeq$ CTL2 > CTL3b, and RTL3 > RTL1 > RTL2 > RTL4. On the other hand, rabbit thymus histone fractions were less effective than calf thymus fractions in causing CD spectral changes. The order of effectiveness in inducing CD spectral changes was CTL3a > CTL1 > CTL2 > CTL3b, and RTL1 > RTL4 > RTL3 > RTL2. There appeared to be no obvious relationships between amino acid composition of the various

TABLE III: Amino Acid Composition Analysis of Calf Thymus F1 Histone Acetone Powder and Chromatographically Purified Fractions.

Amino Acid	Mole Per Cent[a]				
	Acetone Powder	L1[b]	L2	L3a	L3b
Lysine	20.7	28.8	30.5	28.5	28.9
Histidine	0.9	0.1	0.1	0.4	0.4
Arginine	3.5	2.7	1.0	1.5	1.5
Aspartic acid	6.0	1.7	2.6	2.3	2.7
Threonine	5.1	5.3	5.1	5.5	5.1
Serine	6.2	6.0	8.1	7.9	7.8
Glutamic acid	8.0	3.6	5.0	4.7	5.6
Proline	8.7	7.6	7.5	8.8	7.4
Glycine	7.1	6.6	7.8	7.6	8.2
Alanine	17.4	26.2	20.5	23.8	23.3
Half-cystine	1.2	0.0	0.0	0.0	0.0
Valine	4.8	5.4	4.8	4.2	4.0
Methionine	0.0	0.0	0.0	0.0	0.0
Isoleucine	1.9	0.8	0.9	0.7	0.5
Leucine	5.7	4.3	3.5	3.8	3.8
Tyrosine	1.1	0.5	0.5	0.4	0.5
Phenylalanine	1.6	0.6	0.5	0.5	0.5
Total basic amino acids	25.1	31.6	31.6	30.4	30.8
Ratio of lysine to arginine	5.9	10.7	30.5	19.0	19.3

[a] Values are reported as moles of amino acids per 100 moles of all amino acids recovered after 22-hr hydrolysis at 110° in 6 N HCl. [b] Nomenclature of Bustin and Cole (1968).

TABLE IV: Effect of Histone Subfractions on the Circular Dichroism and DNase Sensitivity of Homologous DNAs.

Subfraction		Histone:DNA Ratio (w/w)[a] Required for 50% Change in $[\epsilon_L - \epsilon_R]_{275\,nm}$	Histone:DNA Ratio (w/w)[a] Required for 50% Inhibition of DNase Hydrolysis[b]
Rabbit thymus	L1	0.75	0.3[c]
	L2	1.35	0.5
	L3	1.2	0.2[d]
	L4	0.85	0.7
Calf thymus	L1	0.15	0.6[e]
	L2	0.2	1.0
	L3a	0.1	1.0
	L3b	0.3	2.9

[a] All complexes prepared by direct addition. [b] Hyperchromic effect assays. [c] For heterologous DNAs the value was 0.27 ± 0.02. [d] For heterologous DNAs this value was 0.27 ± 0.04. [e] For heterologous DNAs this value was 0.51 ± 0.05.

histone fractions and the order of effectiveness in inducing CD changes or in protecting against DNase hydrolysis.

STUDIES ON HETEROLOGOUS COMPLEXES OF DNA AND F1 HISTONE SUBFRACTIONS. Complexes of the fractionated histones with a variety of DNAs were prepared by direct addition, and by urea–salt gradient dialysis. CD and ultraviolet absorption spectra were obtained, and these complexes were also assayed for resistance to DNase hydrolysis. Complexes of histone fractions and heterologous DNAs showed the same degree of resistance to DNase as did complexes of the same histone fractions with their homologous DNAs. No evidence for species specificity was obtained, even though from the data presented in Table IV, we may conclude that various histone subfractions differ in their respective abilities to protect DNA from DNase I hydrolysis; the amount of histone required to protect a given amount of DNA from hydrolysis by DNase I is affected neither by the base composition nor the biological source of the DNA. The results obtained with complexes prepared by dialysis were similar to those for complexes prepared by direct addition.

CD spectra were observed for complexes of calf thymus, rabbit thymus, and salmon sperm DNA, and poly[d(A-T)], with calf thymus histone fraction L3a and rabbit thymus histone fraction L3, which were prepared by urea–salt gradient dialysis. Calf thymus histone (CTL3a) and rabbit thymus histone (RTL3) showed the same order of preference for the various DNAs and only minor quantitative differences when the effects of the two histones were compared for any one DNA. Obviously, species specificity is lacking.

Since the experiments reported in this section indicate that there is no specificity directed toward the DNA in the binding of the individual purified F1 fractions to DNA, it appeared interesting to combine the individual fractions in the same relative proportions that they were found on chromatography of the acetone powders on Amberlite IRC-50, and test the mixtures for species specificity. The results of assays of complexes of rabbit and calf DNAs with the mixtures of rabbit thymus and calf thymus histone fractions were the same, however, as the results obtained with the individual fractions. Fifty per cent inhibition of hydrolysis of both calf and rabbit DNA was found at histone:DNA ratios of 0.9 (w/w) and 0.75 (w/w) for mixed histone fractions from calf thymus and rabbit thymus, respectively.

Concluding Remarks. We have shown that preparations of F1 histones (acetone powders) from a given species are more effective in protecting DNA of that species from DNase I hydrolysis, than a similar preparation of histones from a foreign species. Furthermore, F1 histones (acetone powders) isolated from a given species protect DNA from that species from hydrolysis by DNase I more effectively than foreign natural DNAs or a synthetic polydeoxyribonucleotide. Analogous results have been obtained using RNA polymerase assays. While each chromatographic fraction of F1 histones differs from others in the extent to which it protects DNA against DNase and RNA polymerase, it is indifferent to the nature of the DNA and thus does not exhibit any species specificity toward the nucleic acid. DNase assays on complexes formed between DNA and a combination of all F1 histone subfractions also failed to show species specificity. We therefore conclude that some component present in the F1 acetone powders, and absent from the Amberlite fractions, accounts for the species-specific recognition of DNA, either acting alone or in concert with the histone.

From the amino acid analyses of the acetone powders and

purified fractions we have concluded that the acetone powders contain certain protein components which are absent from the purified fractions. Furthermore, RNA determinations indicate the presence of significant amounts (1.5% w/w) of this nucleic acid in the acetone powders while the RNA composition of the purified fractions was found to be below the limit of detectability of the determination procedure used (less than 0.1% w/w). We have tested the possibility that the RNA component of the acetone powders imparts species specificity on the histone component of the acetone powders by treating the acetone powders with ribonuclease. Ribonuclease-treated acetone powders and untreated powders were then used in DNase assays on DNA of the same species and foreign species. No differences were found in the inhibitory effectiveness of ribonuclease treated and control acetone powders. These results, however, must be viewed with caution, since the RNA component of the acetone powders could exist in a complex with protein that renders this nucleic acid resistant to hydrolysis by ribonuclease. This is analogous to the fact that F1 histones in complex with DNA render DNA resistant to DNase hydrolysis.

At this time, we may conclude that *purified* F1 histones do not act as species-specific inhibitors of DNase hydrolysis. F1 histones in the presence of, or complexed with, either RNA, non-F1 histone proteins or nonhistone proteins, or some other unknown molecules, or with a combination of these molecular species, may act as species-specific inhibitors of the hydrolysis of DNA by DNase. If we may extend our results with DNase assays to RNA polymerase assays, we hypothesize that purified F1 histones do not act as species-specific regulators of gene activity unless perhaps in the presence of, or complexed with, some non-F1 histone component of the acetone powders.

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