# Transition from Noncooperative to Cooperative and Selective Binding of Histone H1 to DNA†

Manfred Renz* and Loren A. Day*

ABSTRACT: A transition from noncooperative to cooperative binding of DNA and histone H1 occurs between 20 and 40 mM NaCl in 5 mM Tris-HCl, pH 7.5. Below 20 mM NaCl in mixtures in H1 and excess DNA, H1 binds to all of the DNA molecules, causing them to sediment faster, and does not distinguish between DNA molecules that differ in size or base composition. However, at NaCl concentrations above the narrow transition range, H1 binds to only some of the DNA molecules and leaves the rest free. If the DNA molecules in a mixture are the same size, H1 selectively binds those that have the highest content of adenosine (A) + thymidine (T). By means of competition experiments at salt concentrations spanning the transition range, it is demonstrated that H1 selectivity requires cooperativity. A high degree of selectivity based on A + T content can be produced by cooperative binding; for average DNA sizes of $2 \times 10^6$ daltons, more than ten molecules of calf lymphocyte DNA (57% A + T) are chosen per molecule of *Escherichia coli* DNA (50% A + T).

The histones of eukaryotic chromosomes fall into five main fractions. Those in each of fractions H2a, H2b, H3, and H4 have amino acid sequences that vary only slightly from species to species (DeLange and Smith, 1975; Sautiere et al., 1975). These four histones are arranged with DNA in regular repeating units or nucleosomes to form the substructure of chromatin (Olins and Olins, 1974; Kornberg and Thomas, 1974; Noll, 1974; D'Anna and Isenberg, 1974; Sahasrabuddhe and Van Holde, 1974; Baldwin et al., 1975).

Histone H1, the lysine-rich histone fraction, is quite distinct from the other four. It is the fraction most easily removed from chromatin by salt extraction and is the fraction most responsible for the solubility properties of chromatin (Ohlenbusch et al., 1967). The total H1 fraction from a given tissue can be separated by column chromatography (Kinkade and Cole, 1966; Bustin and Cole, 1968) into subfractions that show amino acid sequence variability within certain regions of the H1 molecules (Rall and Cole, 1971). In contrast to other histones, H1 shows a definite preference for DNA sequences enriched in A + T content (Sponar and Sormova, 1972). Recently, it has been shown that H1 selectivity binds calf lymphocyte DNA in preference to *E. coli* DNA; the selectivity can exceed 15 molecules of lymphocyte DNA bound per molecule of *E. coli* DNA as shown by direct competition experiments (Renz, 1975). H1 selectivity, by which is meant the mole ratio of different types of DNA molecules bound by given amount of H1, can thus be large and the molecular basis for it is an interesting question.

It has been known for some time that, at suitable ionic strengths, DNA binding by H1 is a cooperative process (Touvet-Poliakow et al., 1970; Adler and Fasman, 1971), like DNA binding by other histones and basic polypeptides (Leng and Felsenfeld, 1966; Olins et al., 1967; Rubin and Moudrianakis, 1972). H1 cooperativity means that a given amount of H1 in the presence of more than its molecular equivalent of DNA will bind extensively to some of the DNA molecules and only partially or not at all to the rest. Studies by Shapiro et al. (1969) suggested a connection between the cooperativity in DNA precipitation by polylysine and the selectivity of polylysine for A + T rich DNA molecules (Leng and Felsenfeld, 1966). Relations between cooperativity and selectivity can be studied best in systems in which strong binding can be maintained, while cooperativity is reduced or eliminated and in which the complexes formed are soluble.

In the experiments described below a change from noncooperative to cooperative binding was observed to take place over a narrow range of ionic strength. It was also observed that a transition from nonselective to selective binding takes place over the same range. Investigation of the nature of the transition and the dependence of selectivity on A + T content has given some insight into the basis of H1 selectivity. It has also led to plausible explanations of previous observations on the nature of H1 binding to DNA.

## Experimental Procedures

*Materials.* Histones were removed from bovine lymphocyte chromatin that had been adsorbed to hydroxylapatite columns by elution with 2 M NaCl, 5 M urea, 10 mM sodium phosphate (pH 6.8) (Renz, 1975). H1 was separated from other histones and degradation products as the exclusion peak from Bio-Gel P10 column chromatography in $10^{-2}$ N HCl (Sung and Dixon, 1970). The exclusion peak was neutralized, made 1 M NaCl, 5 mM Tris-HCl (pH 7.5), and stored as stock solution at 4 or −20 °C. The preparations showed only one electrophoretic band on sodium dodecyl sulfate (Weber and Osborn, 1969) and acetic acid–urea (Panyim and Chalkley, 1969) polyacrylamide gels. Samples of H1 for extinction coefficient measurements were prepared by the trichloroacetic acid extraction procedure of De Nooij and Westenbrink (1962).

Radioactively labeled bovine lymphocyte DNA was obtained from lymphocytes that had been stimulated by phytohemagglutinin P(DIFCO) in medium containing [$^3$H]thymidine after 64-hr incubation. Cell nuclei were obtained by osmotic shock and differential centrifugation, then lysed by

† From the Max-Planck-Institut für Virusforschung, 74 Tübingen, Germany, and The Public Health Research Institute of the City of New York, New York, New York 10016. *Received January 22, 1976.* This work was supported by a Travel Fellowship from the European Molecular Biology Organization (M.R.) and by grants from the United States Public Health Service (AI 09049 and 1 K04 GM 70363) (L.A.D.).

‡ Abbreviations used are: CD, circular dichroism; OD, optical density.

pronase and RNase. Labeled *E. coli* DNA was isolated according to Thomas et al. (1967) from *E. coli* H 560 (Thy$^-$) that had grown in [$^{14}$C]thymidine medium. Bacterial and mammalian DNA were separately adsorbed onto an hydroxylapatite column in 2 M NaCl, 5 M urea, 0.15 M potassium phosphate (pH 6.8) and the column was washed with the same buffer. DNA was then eluted with 0.5 M potassium phosphate and freed from residual proteins by phenol extraction. The apparent specific radioactivities were $4.0 \times 10^3$ $^3$H cpm $\mu g^{-1}$ calf lymphocyte DNA and $1.0 \times 10^3$ $^{14}$C cpm $\mu g^{-1}$ or $6.0 \times 10^4$ $^{32}$P cpm $\mu g^{-1}$ *E. coli* DNA. Unlabeled DNA from bovine lymph nodes, mouse ascites cells, *B. subtilis, B. cereus,* and *E. coli* bacteria was also prepared by the hydroxylapatite–phenol method. Poly(dG:dC), poly(dA:dT), and poly(dAT) were purchased from Miles.

DNA fragments of various average size were obtained by preparative sucrose gradient sedimentation of sheared or sonicated DNA. DNA samples for the double-label competition experiments were made by mixing equal amounts of lymphocyte [$^3$H]DNA and *E. coli* [$^{14}$C]]DNA prior to shearing or sonication, and then carrying out sucrose gradient separation of the mixed fragments. Average sedimentation coefficients were determined in the analytical ultracentrifuge or in sucrose gradients. Radioactively labeled synthetically prepared relaxed covalently closed circular duplex fd virus DNA, which has $s_{20,w}$ equal to 17 S, was used as a sedimentation marker. It was the kind gift of Heinz Schaller. The average molecular weights of the DNA samples were taken as 10% less than those calculated from the relations of Studier (1965; Berkowitz and Day, 1975).

*Methods.* H1 concentrations were routinely obtained spectrophotometrically with an extinction coefficient of 2.0 mg$^{-1}$ cm$^2$ at 230 nm determined for H1 in 1.0 M NaCl, 5 mM Tris-HCl, pH 7.5. H1 concentrations of the solutions used to determine the extinction coefficient were obtained by dissolving weighed samples of lyophilized H1 into known volumes of buffer. We estimate the overall uncertainty in the extinction coefficient to be less than 10%, the principal source of error being the unknown contributions of moisture to the sample weights. In an earlier study (Renz, 1975), an extinction coefficient for whole histone was incorrectly assumed for H1 but the conclusions of that study were not affected by the actual value used. Very low H1 concentrations (ng ml$^{-1}$) were determined indirectly with highly labeled ($^{32}$P) *E. coli* DNA with the aid of the filter-binding technique as described in the two following paragraphs. DNA concentrations, whether determined by measurements of absorbance or of radioactivity, were based upon an assumed extinction coefficient at 260 nm of 20 mg$^{-1}$ cm$^2$ in 0.15 M NaCl, 5 mM Tris-HCl, pH 7.5.

The filter-binding assays were carried out as described earlier (Renz, 1975). In summary, stock solutions of H1 were diluted into 0.5-samples of 5 mM Tris-HCl (pH 7.5) buffer containing different concentrations of NaCl and an excess of labeled DNA. After incubation for 15 min at 20 °C, the reaction mixture was filtered through nitrocellulose membrane filters at flow rates of 0.1 ml sec$^{-1}$. The filters were washed three times with 0.7 ml of buffer, dried, and monitored for radioactivity. The values given are the means of three experiments; the standard deviations were less than 10% of the mean values.

Sucrose gradient analyses were made by layering H1–DNA mixtures onto preformed linear gradients from 20 to 5% sucrose in buffers containing 5 mM Tris-HCl, pH 7.5, and NaCl at the same concentration as in the mixtures. A Beckman SW41 rotor and 12.5-ml cellulose acetate tubes were used. Fractions of constant volume were collected from the bottom at the end of the runs by displacement of the sucrose column with paraffin oil. For the experiments shown in Figure 5, 0.7-ml cushions of 3 M CsCl in 50% sucrose were used and the fractions were counted for both $^3$H- and $^{14}$C-labeled DNA with established double-label procedures. For the experiment shown in Figure 6, the fractions were counted for $^{32}$P- and $^3$H-labeled DNA and assayed for histone H1 content by a new method based on the filter-binding technique. In this method $^{32}$P-labeled *E. coli* DNA of $6.6 \times 10^6$ daltons was added to a final concentration of 0.2 $\mu g$ ml$^{-1}$ to those fractions that did not already contain this DNA at this concentration. The fractions were then made 0.15 M NaCl, incubated at 23 °C for 20 min, and passed through membrane filters. This was done because the filter retention of DNA–H1 complexes depends on the size of the DNA used and histone H1 prefers large DNA fragments to small DNA fragments. About 80–95% of H1 that is bound to small DNA ($3 \times 10^5$ daltons) is transferred to the large *E. coli* DNA upon the addition of the latter to the mixture. Radioactivity from the DNA retained on the filter was then converted into the amount of H1 with the aid of a calibration curve relating counts of *E. coli* [$^{32}$P]DNA retained to input H1. The filter background of the *E. coli* [$^{32}$P]DNA in the absence of H1 was less than 0.3%.

Boundary sedimentation experiments were carried out in a Beckman Model E analytical ultracentrifuge equipped with an ultraviolet absorption scanner and multiplexer. The calibration of the instrument and rotors has been described by Berkowitz and Day, (1974). Runs were at ambient temperature 23 ± 1 °C. In order to avoid convection, the temperature control system was not used. Double-sector cells and an AN F rotor, which could accommodate three such cells simultaneously, were used. Speeds were changed during the course of the runs so that the sedimentation velocities of all the components in a mixture could be measured or estimated. The absorbance profiles showed the positions and plateau heights of free DNA and of DNA in complexes with histone H1; the absorbance at 270 nm due to H1 was below detectable levels. Sedimentation constants were obtained from linear least-square analyses of the logarithm of the radial distance of the boundary vs. time for five to seven time values. The relative amounts of different components were obtained from plateau heights that were measured at different times and extrapolated to zero time to correct for radial dilution. Any distortion in relative amounts by the Johnston–Ogston anomaly would be small because the DNA had an average mass of only $4.3 \times 10^6$ daltons and the concentrations were very low; Studier (1963) found small Johnston–Ogston effects in mixtures of DNA with eight times this molecular weight and at about five times the concentration of our experiment. To obtain the three dimensional representation of Figure 3, the plateau heights were combined with boundary shapes measured in the following approximate way. Three horizontal lines, one at the half plateau height and two that delimited the central 68% of the plateau height, were drawn for boundaries that were about halfway down the cell. The relative distances from the meniscus of the intercepts of these three lines with the boundary gave an indication of the distribution of sedimentation coefficients for a given component. This measure and the plateau height at zero time were used to sketch each peak in Figure 3. We tried to normalize the representation by making the sum of the peak areas at each salt concentrations equal to the peak area at 6.5 mM NaCl. We felt justified in using these methods since most of the boundaries, although sometimes very broad, had inflection points, and monodisperse samples of free DNA

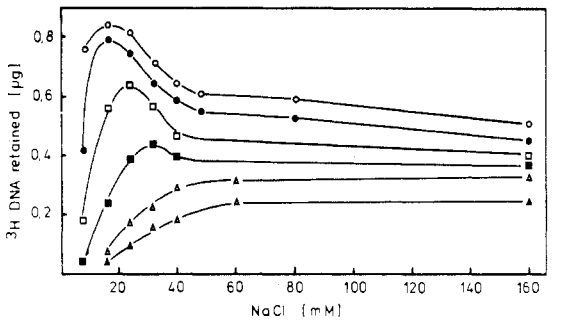


FIGURE 1: Nitrocellulose filter retention of calf lymphocyte DNA-H1 complexes. H1 (0.37 μg) was added to 0.5 ml of 5 mM Tris-HCl (pH 7.5) containing 1 μg of calf lymphocyte [$^3$H]DNA and different concentrations of NaCl. The average molecular weights of the DNA samples were, from the bottom to the top, 0.17, 0.45, 0.80, 1.5, 2.7, and 4.3 × $10^6$.

(4.3 × $10^6$ daltons) or of H1-DNA complexes would have had extremely sharp boundaries in comparison to those observed. Rigorous characterization of boundary shapes (see, for example, Schachman, 1959) did not seem to be necessary for the purposes of the study.

## Results

### *Transition from Noncooperative to Cooperative Binding*

*Nitrocellulose Filter Retention.* A change in the mechanism of H1-DNA binding at low ionic strength was indicated by the anomalous filter retention of DNA by H1. To describe the experiments it is necessary to point out that in the absence of DNA over 95% of H1 is retained on nitrocellulose filters, as shown by experiments with radioactive H1 over the NaCl and H1 concentrations used in the present experiments. Conversely, in the absence of H1, over 99% of the DNA passes through the filters (Renz, 1975). H1 causes DNA to be retained on nitrocellulose filters but the amount retained depends on the relative amounts of histone H1 and DNA, the size of the DNA, as well as the salt concentration.

The fractional retention of 1 μg of calf lymphocyte DNA of different sizes in the presence of 0.37 μg of H1 is shown in Figure 1. H1 is associated with the DNA that passes the filter at low NaCl concentrations, since this DNA can be retained on a second filter by increasing the NaCl concentration (Renz, 1975). These are the only conditions in the present experiments under which significant amounts of H1 pass the filter. As the salt concentration is increased, more and more DNA is retained on the filter by histone H1. For samples of large DNA (mol wt > 1 × $10^6$), maxima are reached near 20 mM NaCl. The larger the DNA, the lower the NaCl concentration for maximum retention. For the largest DNA used (4.3 × $10^6$ daltons), 0.37 μg of H1 retained 0.83 μg of DNA at 16 mM NaCl. As the salt concentration is increased beyond its value for maximum retention, more DNA passes through the filter and retention plateaus are reached by about 60 mM NaCl. At 160 mM NaCl, apparently no H1 is associated with the DNA in the filtrate regardless of the size of the DNA, since neither the filtrate DNA nor DNA added to the filtrate can be retained on a second filter. The amount of DNA of 1 × $10^6$ daltons retained by 0.37 μg of H1 corresponds approximately to 1 g of DNA/1 g of H1. This is the mass ratio for charge equivalence. Larger DNA requires less histone, and smaller DNA more histone, in order to be retained on the filter. This molecular weight dependence of filter retention efficiency is not understood.

The filter retention results suggest that at 160 mM NaCl the available H1 saturates an approximately equal mass of

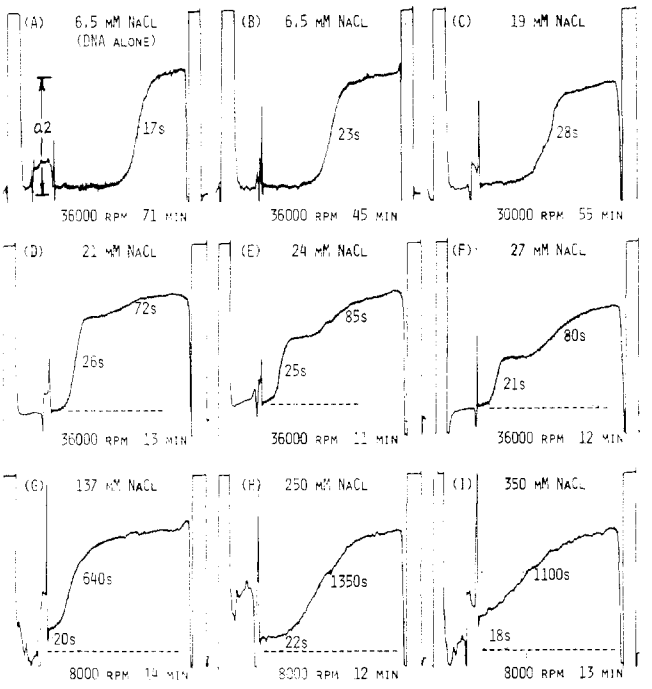


FIGURE 2: The effect of NaCl concentration on lymphocyte DNA sedimentation behavior in mixtures of 6.0 μg ml$^{-1}$ of histone H1 and 9.7 μg ml$^{-1}$ of DNA. The average mass of the DNA was 4.3 × $10^6$ daltons, and the buffer was 5 mM Tris-HCl, pH 7.5. Only 9 out of over 150 scanner tracings of absorbance (at 270 nm) vs. radial position are shown. The original traces were copied with a duplicating machine and the contrast of the traces were enhanced with india ink by hand. The pen deflection for OD = 0.2 indicated in (A) holds for all traces. With each trace are given the NaCl concentration, the rotor speed, the approximate time after reaching speed, the baselines (- - - - -) obtained after depletion of sedimenting species from the meniscus at high speeds, and the apparent sedimentation coefficients assigned to a given component. (A, B) At 6.5 mM NaCl, the mass of histone H1 bound to DNA increased the rate of DNA sedimentation. (C) At 19 mM, the boundary shape indicates the presence of more than one subpopulation. (D, E, F) At NaCl concentrations from 20 to 40 mM NaCl the distribution is bimodal; the slow subpopulations sediment at progressively slower rates and broader, faster populations sediment at rates 83 S. (G, H, I) The formation of very large aggregates ensues and only a small fraction of DNA sediments as free DNA. See also Table I, Figure 3, and the text.

DNA and leaves the rest free to pass the filter. This is characteristic of cooperative binding. At lower ionic strength the H1-DNA binding and filter retention mechanisms are quite different, DNA being retained maximally near 20 mM but passing through the filter with associated H1 at the very lowest salt concentrations.

*Analytical Ultracentrifugation.* Experiments were carried out to characterize the distribution of DNA-histone H1 complexes under the solution conditions used for the nitrocellulose filter assay. The most informative experiment was carried out with a sample of calf lymphocyte DNA of average molecular weight 4.3 × $10^6$ in mixtures containing 0.6 μg of H1/1.0 μg of DNA. A few of the scanner tracings of the boundary profiles are presented in Figure 2. We assigned apparent sedimentation coefficients to the boundaries as if stable distributions of material in different subpopulations were being separated. Sedimentation coefficients and the relative amounts of DNA of the different components, as well as the uncertainties in the coefficients from the least-square slopes, are given in Table I. Systematic deviations from linearity of log (radius position) vs. time were not evident, although the uncertainties were large for some components. Data given in Table I and the boundary shape analysis described under

TABLE I: Sedimentation Analysis of Mixtures Containing 6.0 $\mu$g ml$^{-1}$ of Histone H1 and 9.7 $\mu$g ml$^{-1}$ of Lymphocyte DNA at Different NaCl Concentrations.[a]

| | Sedimentation Coefficients and Amounts of Components | | |
|---|---|---|---|
| [NaCl](mM) | Slow | Fast[b] | Not Assigned (usually > 500 S) |
| 13 | 28 (99%) | | (1%) |
| 19[c] | 28 (95%) | | (5%) |
| 21 | 26 (68%) | 72 ± 5 (23%) | (9%) |
| 24 | 25 (48%) | 85 ± 2 (43%) | (9%) |
| 26 | 22 (50%) | 72 ± 1 (40%) | (10%) |
| 27 | 21 (42%) | 80 ± 4 (49%) | (9%) |
| 30 | 21 (33%) | 81 ± 5 (60%) | (7%) |
| 31.5 | 19 (35%) | 95 ± 2 (50%) | (15%) |
| 35 | 20 (33%) | 83 ± 7 (58%) | (9%) |
| 40 | 20 (31%) | 79 ± 5 (64%) | (7%) |
| 44 | 19 (25%) | 97 ± 8 (55%)[d] | (20%)[d] |
| 67 | 20 (18%) | 196 ± 10 (55%)[d] | (27%)[d] |
| 97[e] | 20 (15%) | 460 ± 10 (70%)[d] | (15%)[d] |
| 137 | 20 (18%) | 640 ± 20 (74%) | (8%) |
| 250 | 22 (6%) | 1350 ± 60 (90%) | (4%) |
| 350 | 18 (23%) | 1090 ± 50 (77%) | (0%) |

[a] All solutions were buffered with 5 mM Tris-HCl, pH 7.5, and the temperatures were 23 ± 1 °C. The sedimentation coefficients, in Svedbergs, obtained for DNA in the absence of added H1 were 20.1 S in 13 mM NaCl and 19.7 S in 40 mM NaCl. At 6.5 mM NaCl the DNA sedimented with 17.5 S in the absence of H1 and with 23 S in the presence of H1. [b] The uncertainties are the standard deviations in the mean sedimentation coefficients that were given by the least-squares analyses; they do not indicate the breadth of a given boundary. The uncertainties in average S values for free DNA and for the slow components in the mixtures were all under ±1 S except at 250 mM NaCl where the uncertainty was ±5 S. [c] The boundary at 19 mM NaCl was bimodal as seen in Figure 2C. [d] Designation of components as "fast" or "not assigned" at 44, 67, and 97 mM NaCl was more difficult than at other NaCl concentrations. [e] H1 concentration at 97 mM NaCl was 6.6 $\mu$g ml$^{-1}$ instead of 6.0 $\mu$g ml$^{-1}$.

Methods were used to obtain the representation given in Figure 3.

The results of this experiment might be summarized as follows. When H1 is added to DNA in 6.5 mM NaCl or 13 mM NaCl, the average sedimentation coefficient of the DNA increases because of the additional mass of bound protein, but the shape of the boundary does not change significantly. On addition of NaCl to approximately 20 mM, a separation into two subpopulations occurs. As the NaCl concentration is increased over the range from 20 to 40 mM, the sedimentation coefficients of the slow components drop to that of free DNA but those of the fast components remain near an average of 83 S. Further increases in NaCl lead to a slight reduction in the amount of free DNA and to large increases in the sedimentation rates of the fast components (to over 1000 S), as if free DNA were being drawn up into very large aggregates. As NaCl is increased beyond 0.25 M, the large aggregates sediment more slowly and free DNA is released.

The ultracentrifuge results are consistent with, and help to explain the ionic-strength dependence of the retention of DNA on the nitrocellulose filter. At the lowest NaCl concentrations (6.5, 13, and 19 mM NaCl) virtually all of the DNA sediments faster than free DNA; DNA that passes the filter at these salt concentrations is associated with H1. At 137 mM NaCl, if all H1 is sedimenting with the fast components (Table I) then they contain 1.3 $\mu$g of DNA/1 $\mu$g of H1; 1.4 $\mu$g of DNA/1 $\mu$g of H1 is retained on the filter at 160 mM NaCl (Figure 1, upper curve). The slow components present above 40 mM NaCl sediment as free DNA; the DNA that passes the filter at 160 mM NaCl is free of H1.

The combined results show that at NaCl concentrations below 20 mM, all of the DNA molecules are bound by H1, even if the DNA is present in much more than the equivalent amount. The binding by H1 is therefore noncooperative under these conditions. At NaCl concentrations above about 30 mM according to the ultracentrifuge results (Table I) or about 40 mM according to the filter retention results (Figure 1), H1 binds extensively to some of the DNA molecules but only partially or not at all to the rest. Binding under these conditions is therefore cooperative. An idea of the sharpness of the transition from noncooperative to cooperative binding can be obtained by considering, or actually plotting, the relative amounts of slow component (Table I) as a function of NaCl concentration. Most of the change has occurred over the narrow range of only 10 mM NaCl. Another aspect is seen by considering the average sedimentation coefficients of the fast components as a function of salt concentration. Intermediate values near an overall average of 83 S are obtained from 20 to 44 mM NaCl, then the values increase dramatically. It is as if two events occur, first the transition to cooperative formation of fairly well defined intermediates, possibly involving only one DNA molecule, then inclusion of some free DNA into very large aggregates above 44 mM NaCl.

*Kinetic Aspects of H1–DNA Interactions.* The times required for equilibrium to be established in mixtures of H1 and DNA were less than the times used for the experiments described above. No differences were noted in filter retention or centrifugation patterns after short vs. long incubation times, ranging from 10 min to 20 h. In general, most of the processes involved are too fast to be measured with the techniques used in this study. However, we have been able to obtain some kinetic information by comparing apparent overall rates at which unlabeled DNA replaces labeled DNA in preformed complexes.

Complexes between 1 $\mu$g of lymphocyte [$^3$H]DNA ($5 \times 10^5$ daltons) and 0.5 $\mu$g of H1 were allowed to form in 90 $\mu$l of 5 mM Tris-HCl, pH 7.5, containing either 5 mM NaCl or 150 mM NaCl. After 30 min, ten times as much unlabeled DNA was added. The amount of [$^3$H]DNA still in complexes with H1 at various times was measured by filter retention. The filter assay requires that a sample be at 150 mM NaCl. Therefore, the individual 90-$\mu$l samples reacting at 5mM NaCl were first made 150 mM NaCl by adding 900 $\mu$l of 167 mM NaCl (within 2 s) and then filtered (within 3 s). The reaction mixtures already at 150 mM NaCl were diluted with 900 $\mu$l of 150 mM NaCl buffer (2 s) and filtered (3 s). Before the addition of unlabeled DNA to the complexes, 0.35 $\mu$g of [$^3$H]DNA was retained for both 5 mM and 150 mM NaCl. Within 10 s after the addition of unlabeled DNA at 150 mM NaCl, the amount of [$^3$H]DNA retained had been reduced to 0.26 $\mu$g, and after 3 min to 0.13 $\mu$g, but no further reduction was observed up to 30 min. In contrast, within 10 s at 5 mM NaCl, the amount of [$^3$H]DNA had been reduced to 0.04 $\mu$g. This corresponds to complete exchange, and, since the rate at 150 mM NaCl is much slower, only a small part of it occurred during the 5-s exposure to 150 mM NaCl required by the assay. This experiment shows that a random distribution of H1 on an excess of DNA can be established very rapidly at low ionic strengths.

### *Transition from Nonselective to Selective Binding*

*Nitrocellulose Filter Binding Assay.* If H1 is added to a mixture of DNA in 5 mM NaCl, 5 mM Tris-HCl (pH 7.5)

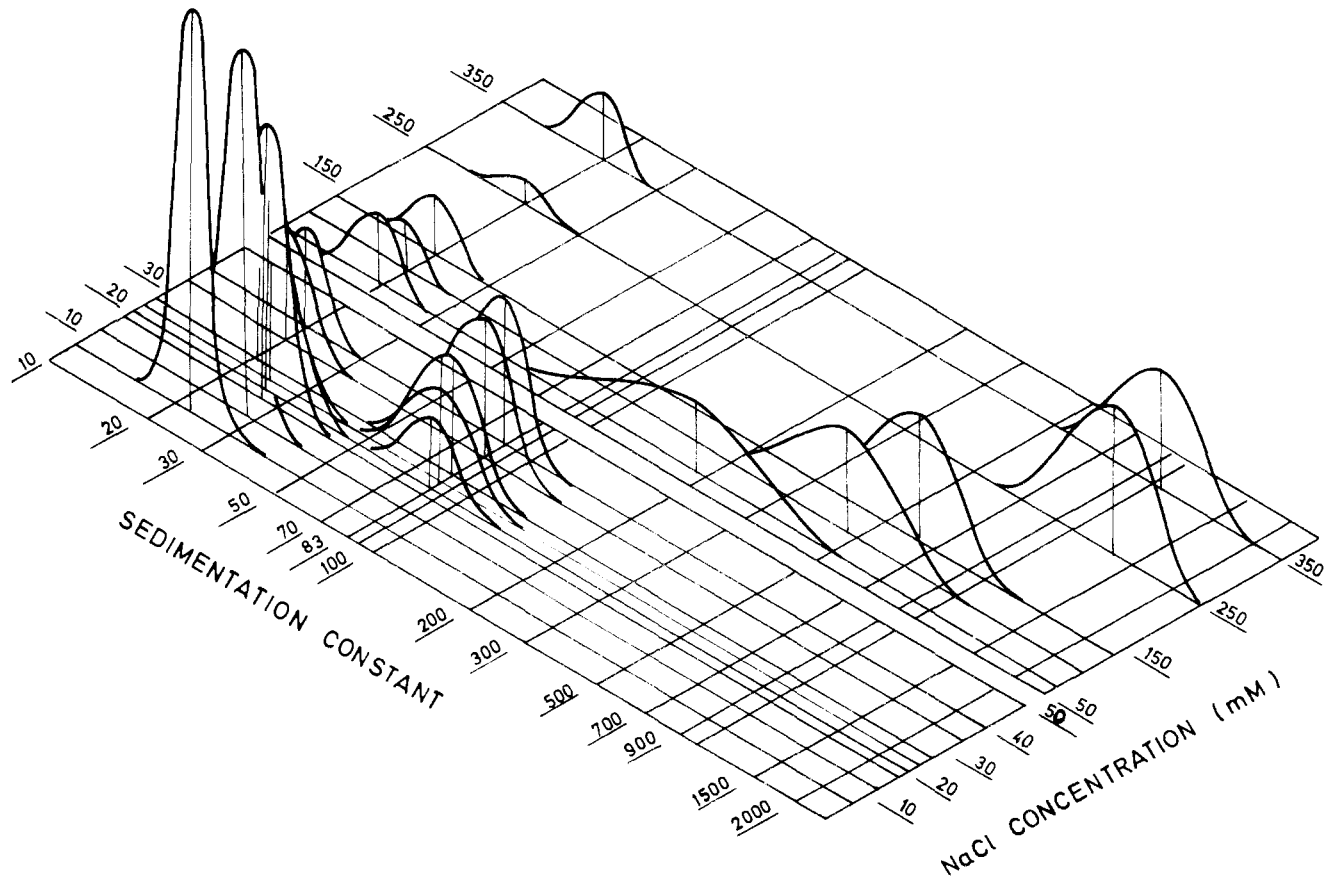


FIGURE 3: A representation of the sedimenting species and their distributions in mixtures containing 0.6 g of H1/g of lymphocyte DNA of average mass $4.3 \times 10^6$ daltons. This representation is intended as a conceptual aid, not as a quantitative presentation of data. The data of Table I together with analyses of boundary shapes, as described under Methods (see also Figure 2), were used to plot the average sedimentation coefficient vs. the NaCl concentration. The logarithmic plot, for which there is no theoretical basis, shows in one diagram species of broad distribution that differ in average S by almost two orders of magnitude. The areas under the peaks indicate the relative amounts of the species and are approximately normalized, the areas for complex at 6.5 mM NaCl being taken as unity; the material sedimenting with unassigned sedimentation coefficient (Table I) is not represented. All fast species at NaCl concentrations from 21 to 44 mM have been plotted at 83 S. All slow species for 27 mM NaCl and higher have been plotted at 20 S. The inking of the peak at 13 mM NaCl was interrupted to allow a view of the peak positions on the sedimentation coefficient axis. One should note that the increase from 23 to 28 S for the complexes between 6.5 and 13 mM NaCl is parallel to an increase from 17.5 to 20.1 S for free DNA between these two salt concentrations, the reasons for which are not understood.

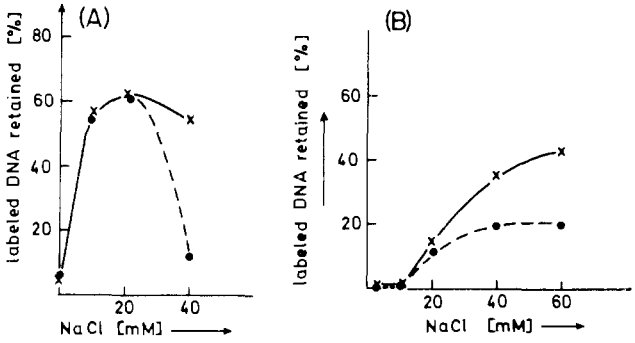


FIGURE 4: Nitrocellulose filter assay of the competition between fragments of bovine lymphocyte [$^3$H]DNA (x) and *E. coli* [$^{14}$C]DNA (O) for H1 at different concentrations of NaCl in 5 mM Tris-HCl (pH 7.5). (A) H1 (0.53 $\mu$g) was added to mixtures containing 1 $\mu$g of each DNA. The molecular weight of the DNA was $2.3 \times 10^6$. (B) H1 (0.72 $\mu$g) was added to mixtures containing 1 $\mu$g of each DNA. The molecular weight of the DNA was $0.49 \times 10^6$.

consisting of equal amounts of lymphocyte [$^3$H]DNA and *E. coli* [$^{14}$C]DNA of average molecular weight $2.3 \times 10^6$, very little DNA is retained on the filter (Figure 4A). There is no apparent preference for lymphocyte over *E. coli* DNA up to the salt concentration for maximum retention, about 20 mM NaCl. However, at 40 mM NaCl less than the maximum amount of total DNA is retained and the DNA that is retained is predominantly calf lymphocyte DNA. Thus, the salt-dependent separation of total DNA into H1-bound DNA and H1-free DNA described above is accompanied by the appearance of H1 selectivity for lymphocyte DNA.

The transition from nonselective to selective binding observed with small DNA fragments ($5 \times 10^5$ daltons) is not as prominent (Figure 4B).

*Sucrose Gradient Centrifugation Analysis.* Sedimentation experiments were carried out to confirm the existence of a transition from nonselective to selective binding at low ionic strength (Figure 5). Mixtures were made of equal amounts of lymphocyte [$^3$H]DNA and *E. coli* [$^{14}$C]DNA both of average molecular weight $2.3 \times 10^6$. With no added NaCl both DNA species sediment slightly faster in the presence of H1 than in its absence; a slight peak broadening occurs. At 10 mM NaCl both bands are asymmetrical and both sediment faster than free DNA. As was observed in the analytical ultracentrifuge and by the filter-binding assay, most if not all of the DNA is complexed by H1 at 10 mM NaCl. At 20 mM NaCl the band shapes for lymphocyte and *E. coli* DNA are different. At 40 mM NaCl, a large fraction of the lymphocyte DNA sediments fast (>55 S). The remaining lymphocyte and *E. coli* DNA seems to be free of histone H1, since the forms and positions of the peaks correspond to those of free DNA. The total

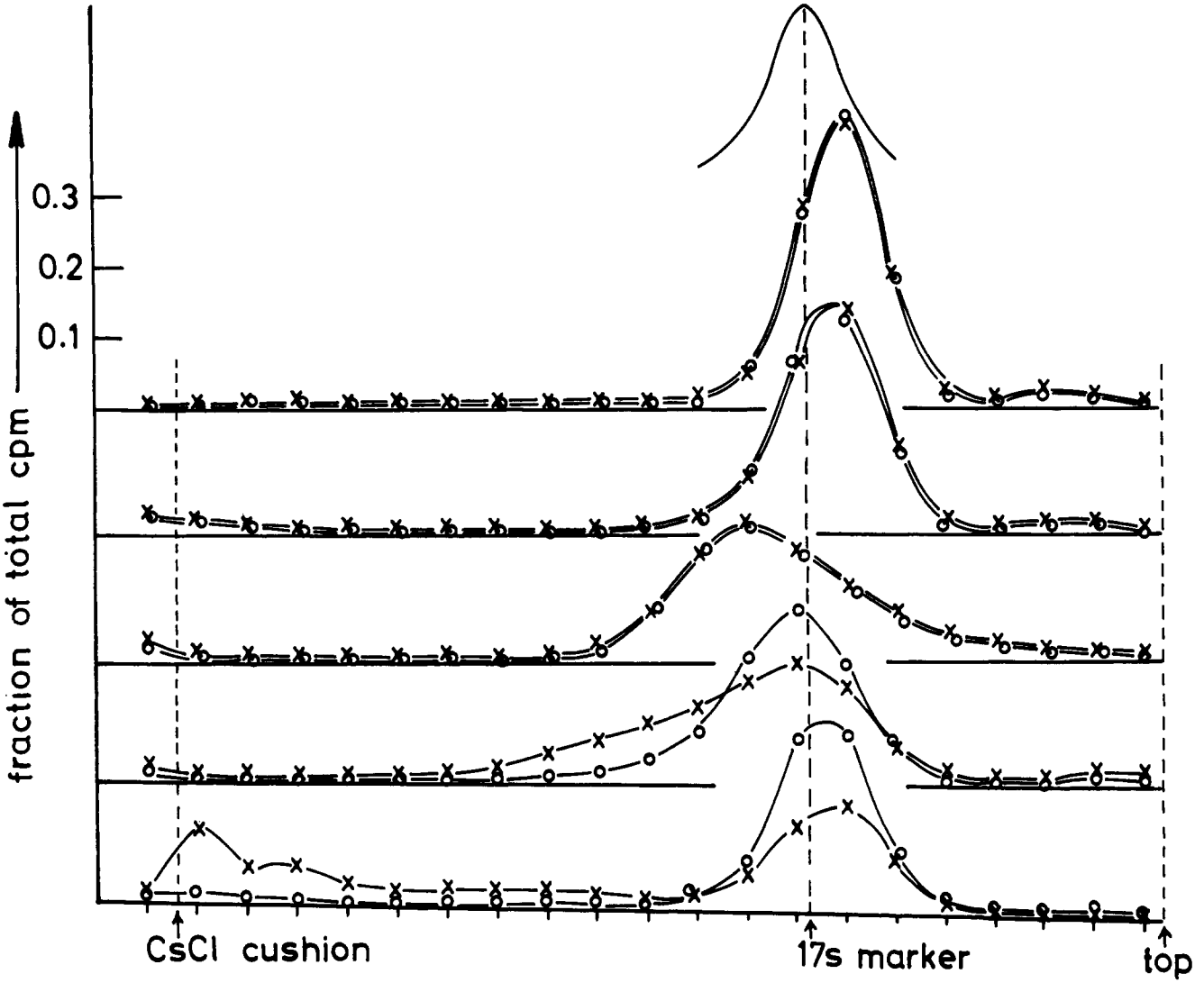


FIGURE 5: Sucrose gradient assay of the competition between 2.3 × $10^6$ dalton fragments bovine lymphocyte [$^3$H]DNA (x) and *E. coli* [$^{14}$C]DNA (o) for H1 at different concentrations of NaCl in 5 mM Tris-HCl (pH 7.5). DNA–H1 mixture contained 0.53 μg of H1 and 1 μg of each DNA. From the top to the bottom: 17S marker; the two DNA species with no added NaCl and no H1; DNA–H1 mixture with no added NaCl; DNA–H1 mixture in 10 mM NaCl; in 20 mM NaCl; and in 40 mM NaCl. The gradient was 20–5% sucrose, the cushion was 3 M CsCl in 50% sucrose, and centrifugation was at 33 000 rpm for 9 h at 4 °C.

amount of calf lymphocyte DNA sedimenting faster than free DNA is more than ten times the amount of *E. coli* DNA sedimenting faster than free DNA. The total amount of both DNA types sedimenting faster than free DNA at 40 mM NaCl is 0.5 ± 0.1 μg for a total input of 0.5 μg of histone H1. Thus, H1 binds as much DNA as it can at 40 mM NaCl and it chooses mostly calf lymphocyte DNA.

An additional sucrose gradient experiment was carried out to see if H1 exhibits any measurable selective interaction at salt concentrations below the transition concentration. Equal amounts of small lymphocyte [$^3$H]DNA and large *E. coli* [$^{32}$P]DNA were complexed with a very small amount of H1 in 5 mM Tris-HCl (pH 7.5). It was found that H1 binds to both DNA species to the same extent, as shown in Figure 6. *E. coli* DNA fragments (6.6 × $10^6$ daltons) bound an average of three H1 molecules and the small lymphocyte DNA fragments (3 × $10^5$ daltons) bound an average of 0.15 molecule of H1, which is the same H1/DNA mass ratio.

*Effects of DNA Base Composition and Molecular Weight on Selectivity.* The role of base composition in selective binding was investigated by allowing DNA fragments of the same molecular weight but with various average A + T contents to compete for a limited amount of H1 histone. This was done by comparing the efficiencies with which unlabeled DNA fragments from different species reduced the amount of lymphocyte [$^3$H]DNA retained on filters by H1. The results given in Figure 7 show that H1 cannot distinguish well between calf lymphocyte DNA, mouse ascites DNA, and *Bacillus subtilis* DNA, all of which contain 57% A + T. *E. coli* DNA, which is 50% A + T, is much less effective and *Bacillus cereus,* which is 67% A + T, is much more effective in reducing the amount of lymphocyte [$^3$H]DNA retained. Thus, filter competition assay shows that H1 prefers the DNA fragments rich in A + T. The synthetic species poly(dA:dT) and poly(dAT) were more effective competitors than all of the natural DNA fragments. Surprisingly, poly(dG:dC) is also a powerful competitor for H1.

To assess the importance of DNA size on H1–DNA interactions, H1 was added to equal amounts of $^3$H-labeled bovine lymphocyte DNA of 2.5 × $10^6$ daltons and unlabeled DNA of varying molecular weight and of varying base composition (Figure 8). There is a general preference of H1 for the larger

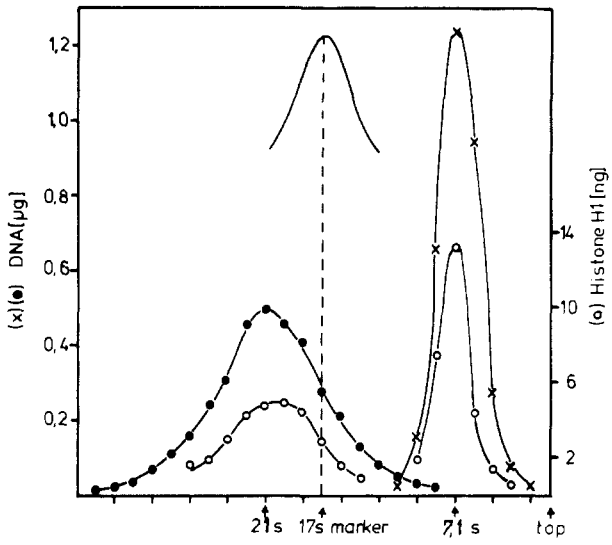


FIGURE 6: Sucrose gradient analysis of the competition between bovine lymphocyte [$^3$H]DNA of average mass $3 \times 10^5$ daltons (x) and *E. coli* [$^{32}$P]DNA of average mass $6.6 \times 10^6$ daltons (●) for a very small amount of H1 (○). There was only one H1 molecule per 3200 base pairs. The buffer was 1 mM EDTA, 5 mM Tris-HCl, pH 7.5, with no added NaCl. The distribution of H1 in the gradient was determined with the special filter binding technique for low H1 concentration described under Methods. Centrifugation was at 39 000 rpm for 5 h at 21 °C. The mixture applied contained 3.7 μg of each DNA and 74 ng of H1.

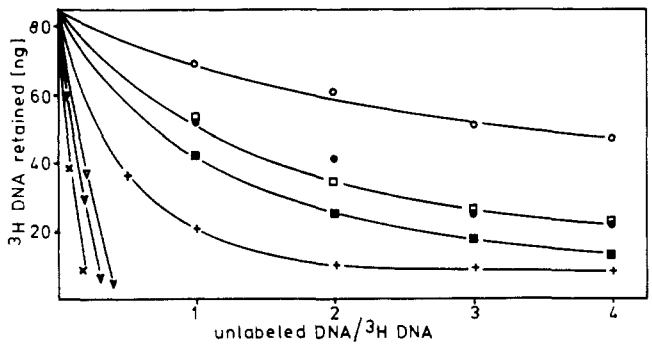


FIGURE 7: Nitrocellulose filter assay of the competition between bovine lymphocyte [$^3$H]DNA and unlabeled DNA of different origin and base composition. Histone H1 (35 ng) was added to 0.5 ml of 150 mM NaCl, 5 mM Tris-HCl, pH 7.5, containing 250 ng of lymphocyte [$^3$H]DNA and varying amount of the different unlabeled DNA. All DNA molecular weights were $4.5 \times 10^6$. (○) *E. coli*, 50% A + T; (□) bovine lymphocyte, 57% A + T; (●) *B. subtilis*, 57% A + T; (■) mouse ascites, 57% A + T; (+) *B. cereus*, 67% A + T; (▽) poly(d); (▼) poly(dG:dC); (×) poly(dA:dT).

DNA fragments, in agreement with earlier results (Renz, 1975). However, the DNA size range over which H1 prefers larger fragments depends on the base composition of the DNA. The size preference of H1 ceases for *E. coli* DNA (50% A + T) when the fragments exceed $15 \times 10^6$ daltons (results not shown) for bovine lymphocyte DNA (57% A + T) when the fragments are larger than about $4 \times 10^6$ daltons, and for *B. cereus* (67% A + T) when the fragments are larger than about $2 \times 10^6$ daltons.

## Discussion

*Cooperativity.* We have shown that a transition from noncooperative to cooperative binding of H1 and DNA occurs over a narrow salt concentration range. The transition is quite sharp in spite of the heterogeneity of the DNA and H1 samples used. The H1 samples were heterogeneous with respect to amino acid

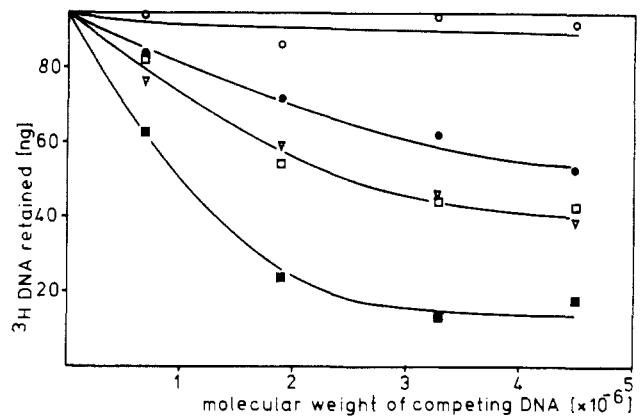


FIGURE 8: Nitrocellulose filter assay of the competition between small and large DNA fragments of different A + T content for H1. 40 ng of histone H1 was added to 0.5 ml of 150 mM NaCl, 5 mM Tris-HCl, pH 7.5, containing 250 ng of bovine lymphocyte [$^3$H]DNA ($2.5 \times 10^6$ daltons) and 250 ng of unlabeled DNA of different origin and different molecular weight. (○) *E. coli*, 50% A + T; (●) *B. subtilis*, 57% A + T; (□) lymphocyte, 57% A + T; (▽) mouse ascites, 57% A + T; (■) *B. cereus*, 67% A + T.

sequence, since our method of preparing the H1 did not include steps to separate it into its subfractions (Kinkade and Cole, 1966; Rall and Cole 1971). The DNA samples were heterogeneous with respect to base composition and sequence and with respect to size, although the sedimentation of boundaries of free DNA indicated only modest heterogeneity broadening. The sharp salt dependence of the transition in spite of the DNA and H1 heterogeneities seems to indicate a salt dependent change in the interactions between all H1 molecules and the DNA lattice.

We are not aware of sharp changes in DNA or H1 conformation near 20 mM NaCl that might initiate the transition. Nevertheless, gradual changes in DNA or protein secondary and tertiary structure could become sharply critical near 20 mM NaCl and initiate the transition. Conformation changes of the types observed by Olins and Olins (1971), Sponar and Fric (1972), and others that lead to large negative ellipticities near 270 nm for DNA in complexes with H1 do not seem to occur over the transition range within the times it takes for the "83S components" (Figure 3) to form, since preliminary experiments we have done have not shown significant changes in CD for complexes between 0 and 50 mM NaCl. We do not yet have molecular weight values or dimensions for the "83S components", but from the data presented here one might speculate on the possibility that the "83S components" contain only one molecule of DNA. Most of the complexes present at 40 mM NaCl are definitely soluble. The cooperative transition may well involve the formation of large intramolecular folds or hairpins by some of the DNA molecules that can then bind more H1.

Cooperativity is important in considerations of the results of Vogel and Singer (1975). They observed that the H1 filter retention of circular duplex DNA containing positive or negative superhelical turns was greater than the retention of nonsuperhelical DNA. If superhelicity were to restrict the cooperativity of the H1–DNA interaction, then the available H1 would be distributed over a larger fraction of the DNA molecules and more would be retained on the filter.

We think it noteworthy that the rate of replacement of DNA in preformed H1 complexes is very rapid below the transition range of salt concentration. The rapid rate does not imply weaker binding but does indicate that the distribution of H1

molecules on DNA can become random very quickly at low salt concentrations. This may be of importance in considering the results of Hayashi (1975) who examined the spatial distribution of H1 molecules on DNA after he had removed all other histones except H1 from chromatin with the sodium dodecyl sulfate elution method (Hayashi and Ohba, 1974). According to our results, the very low ionic strengths used in that study could have led to the random distribution of H1 on the DNA observed even if the distribution had been nonrandom in the original chromatin.

*Selectivity.* By means of competition experiments we have shown that a transition from nonselective to selective binding occurs over the same range of salt concentration as the transition from noncooperative to cooperative binding. At NaCl concentrations of 10 mM and below, no selectivity could be detected. Therefore, we conclude the observed selectivity requires cooperativity. It is notable that H1 selectivity is clearly expressed between 20 and 40 mM NaCl. This is within the first stage of the cooperativity transition when fast sedimenting soluble intermediates and free DNA are formed from slow sedimenting complexes (Figure 5), but without the aggregation to larger structure that takes place at higher salt concentrations. Therefore, the cooperativity-selectivity transition seems to be an intramolecular phenomenon.

Experiments presented above (Figures 7 and 8) show that H1 selectivity is based to a considerable extent on the A + T content and the size of the competing fragments. The A + T and size preference are interlinked in such a way that complexes between small fragments rich in A + T can be more stable than those between somewhat larger fragments richer in G + C. Also, for fragment containing 67% A + T there is no H1 preference for larger fragments when the molecular weights are greater than about $2 \times 10^6$ daltons, whereas for 50% A + T there is still a size effect up to $1.5 \times 10^7$ daltons. Size and base composition are not the only determinants of H1 selectivity, since single-stranded DNA (Renz, 1975) and poly(dGC), like poly(dAT), are strong competitors for H1 (Figure 7). Also, it might be significant that the curves for *B. subtilis* DNA, which like both calf lymphocyte and mouse ascites DNA contains 57% A + T, lie above the theoretical curves for self-competition in both Figure 7 and 8.

It has been known, since the studies of Sponar and Sormova (1972), that there is a general H1 preference for the DNA with higher A + T content, similar to the polylysine preference for the DNA with higher A + T observed earlier by Leng and Felsenfeld (1966). However, ratios of more than 15 molecules of lymphocyte DNA (57% A + T) per molecule of *E. coli* DNA (50% A + T) chosen by H1 at 150 mM NaCl, as shown by filter assay (Renz, 1975), and more than ten at 40 mM NaCl, as shown by centrifugation (Figure 5 above), are very large, perhaps too large to be explained in terms of A + T content alone. An explanation in terms of a distribution of rather specific H1 binding sites on calf lymphocyte DNA offered earlier (Renz, 1975) implied considerable H1 specificity for mammalian DNA.

The additional findings presented in Figures 7 and 8 do not support that explanation but indicate instead that H1 selectivity might be based largely on A + T content and size coupled with cooperativity. Nevertheless, the experimental findings of that study are fully confirmed by the present results. It should be pointed out that the competition experiments carried out so far have been done with unfractionated H1; the individual subfractions may show different degrees of selectivity.

It is clear that selectivity, as measured by centrifugation or filter assays, requires the amplification of a cooperative phenomenon to be measurable at all. Two possible initiating events that could lead to the accumulation of H1 on favored DNA molecules might be (1) the formation of a more suitable lattice for cooperative binding, either at the level of secondary structure, such as through a local change in the DNA helix structure, or at the level of tertiary structure, such as through coiling or folding of the whole DNA filament, or (2) binding of single H1 molecules at nucleation sties that have the highest affinities for H1. Thus, selectivity based on A + T content might derive from overall increased flexibility of the preferred molecules or of the presence of nucleation sites or sequences, presumably richer in A + T. Some aspects of these points have been discussed by Shapiro et al. (1969) in connection with polylysine binding to DNA, and by Sponar and Sormova (1972). Unfortunately, presently available data do not allow one to distinguish between these and other possible mechanisms. Whatever the actual mechanism might be, it is concluded in the present study that H1 selectivity requires cooperativity.

The experimental methods and conceptual framework of the studies of H1 binding to free DNA can be extended to questions concerning the roles of H1 selectivity and cooperativity in the coiling and folding of chromatin. One might ask such questions as whether H1 binding is cooperative and selective in chromatin, whether H1 binds to a polynucleosome lattice or to regions of DNA which are free of nucleosomes, and whether other proteins alter H1 binding patterns in chromatin.

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# Purification and Characterization of the Agglutinins from the Sponge *Axinella polypoides* and a Study of Their Combining Sites†

Hagen Bretting‡ and Elvin A. Kabat*

ABSTRACT: The hemagglutinins from the sponge *Axinella polypoides* were isolated by affinity chromatography using Sepharose 4B as an absorbent and eluting with DGal. Further separation on DEAE-cellulose and preparative disc electrophoresis on polyacrylamide and agarose gave three fractions. The physicochemical properties and binding specificities of the two main agglutinins were studied. Homogeneity was tested by polyacrylamide electrophoresis and immunoelectrophoresis and by sedimentation analysis. In isoelectric focusing, agglutinin I (mol wt 21 000) showed two bands at pH 3.8 and 3.9. Agglutinin II (mol wt 15 000) showed one band at pH 3.9. Both agglutinins have a carbohydrate content of about 0.5%, are immunochemically unrelated, and differ in amino acid composition. Both precipitate $A_1$, $A_2$, B, $Le^a$, and precursor I blood group substances but to different extents. Inhibition experiments revealed that both agglutinins are inhibited best by terminal nonreducing DGal glycosidically linked $\beta 1 \rightarrow 6$ or by *p*-nitrophenyl-$\beta$DGal. DGal and DFuc are equally active but about 20 and 12 times less active with agglutinin I and agglutinin II, respectively. DGalNAc and LFuc were inactive even at much higher concentrations. Both agglutinins have similar specificities and react with the immunodominant determinants of blood group B and $Le^a$ but not with A and H substances; in A and H substances, reactivity is with side chains in which $\beta$-linked DGal is unsubstituted at the nonreducing terminus. The *Axinella polypoides* lectins are compared with galactose-specific lectins of different origin and with the aggregation factor system in sponges.

The number of lectins which have been isolated from invertebrates is quite small as compared with the great variety of purified lectins of plant origin. Information about invertebrate agglutinins is mostly limited to those from molluscs and crustaceans (Hammarström and Kabat, 1969; Acton et al., 1969; Marchalonis and Edelman, 1968; Jenkin and Rowley, 1970). Agglutinations by these lectins and by those of plant origin are similar.

Since the discovery of hemagglutinins in sponges by Dodd et al. (1968), the serological and immunoelectrophoretic properties of several (*Axinella spec., Cliona celata, Aaptos papillata, and Axinella polypoides*) were investigated in detail by Khalap et al. (1970, 1971) and by Bretting (1973). Hemagglutination of human A, B, O, and AB erythrocytes by crude

† From the Departments of Microbiology, Human Genetics and Development, and Neurology, Columbia University, and the Neurological Institute, Presbyterian Hospital, New York, New York 10032. *Received January 20, 1976.* Aided by a grant from the National Science Foundation BMS-72-02219-A02 and A03 and by a General Research Support Grant from the United States Public Health Service.

‡ Fellow of the Deutsche Forschungsgemeinschaft on leave from the Zoological Institute and Museum, University of Hamburg (1974–1976).