J. Mol. Biol. (1972) **67**, 361–374

Co-operative Binding of Histones to DNA

ROBERT L. RUBIN AND E. N. MOUDRIANAKIS

Department of Biology, The Johns Hopkins University
Baltimore, Md. 21218, U.S.A.

(*Received 30 July 1971, and in revised form 13 January 1972*)

The interaction of certain components of chromatin was studied in a model system. It was found that when calf thymus histones were mixed with sonicated phage DNA and the macromolecules made to interact by lowering the ionic strength, a mixture of complexes was formed, each separable by velocity sedimentation and characterized by unique thermal stability properties and histone contents. The binding of histones to sonicated DNA was, therefore, co-operative. This co-operative binding also occurs when histones are made to interact with high molecular-weight DNA as manifested in the stoichiometry of binding and in the electron microscopic appearance of the complexes, which directly shows that histone is distributed non-uniformly when it binds to DNA. However, complexes of histones with high molecular-weight DNA can form structures that are not formed with low molecular-weight DNA.

The thermal stability, viscosity and electron microscopic appearance of these model DNA/histone complexes were compared to the same parameters already known in native chromatin. It was found that these characteristics are similar, supporting the validity of this model system for investigating chromatin structure. Furthermore, the data showing that histones bind co-operatively to DNA suggest how certain features of chromatin structure may be generated.

1. Introduction

The genetic material of the cell nucleus is present in the form of a huge macromolecular complex called chromatin. Disassembling purified chromatin has provided important information on the characteristics and quantities of many chromatin components (Zubay & Doty, 1959; Bonner *et al.*, 1968; Panyim & Chalkley, 1969*b*), but determining the role of each component in chromatin structure and function has been difficult due to the complexity of this system. Analyzing chromatin by assembling its components has proven to be a useful approach to understanding the physical chemistry of some of the components (Olins & Olins, 1971; Adler, Schaffhausen, Langan & Fasman, 1971; Li & Bonner, 1971), but the findings may have limited relevance to the structure of the natural product. However, both approaches are needed for a complete understanding of the mechanisms of gene regulation and the structural differentiation that takes place when chromatin condenses during the cell cycle. Insight into these problems may be gained by elucidating the molecular basis of the interaction of chromatin components under the closely controlled conditions of an *in vitro* system. This is the approach we have chosen in the present study. Using a model system designed for maximum simplicity and involving a number of components isolated from chromatin by mild procedures, our aim was to reveal how these

components interact and ultimately the significance of these interactions to the structure and biological properties of native chromatin.

It is well known that DNA and histone proteins, two of the major components of chromatin, can be separated and made to complex again by manipulating the ionic strength. Each class of histones is dissociated from DNA at a specific salt concentration (Ohlenbusch, Olivera, Tuan & Davidson, 1967), which suggests that the strength of ionic interactions that bind histones to DNA is not the same for all histones. Although the classes of histones differ in the number of basic amino acids, the histone class having the largest number of potential ionic binding sites is the first to be dissociated as the ionic strength is raised (Ohlenbusch *et al.*, 1967). Thus, forces other than ionic interactions between DNA phosphates and histone amino groups may be involved in the binding, such as histone/histone interactions or physical localization of specific histones within the chromatin. That this might indeed be the case is suggested by the recent results of Kleiman & Huang (1971) and Senshu (1971), who showed that the sequence of removal of the classes of histones from chromatin as the ionic strength is raised is changed by the presence of urea in the dissociation medium.

It is important to know, therefore, what kinds of interactions stabilize the chromatin structure. The results reported here show that when histones bind to DNA a co-operative interaction takes place among the proteins which results in complexes having unique structural features. The model system employed, in which phage DNA is made to associate with calf thymus histones, allows the formation of complexes having similar molecular weights. Thus, the complexes have a narrow dispersion in size, thereby permitting good separation during sedimentation and chromatography and accurate physical measurements. We find that the gross physical characteristics of the artificial DNA/histone complexes are similar to those of native chromatin, and our observations on co-operative binding may suggest how some known features of chromatin structure are generated.

2. Materials and Methods

(a) *Preparation of lambda DNA*

A λc phage was grown on *Escherichia coli* C600, and the phage were purified from the lysate by repeated low- and high-speed centrifugation. The purified phage were suspended in a medium consisting of 0·15 M-NaCl, 0·015 M-sodium citrate, 0·01 M-EDTA, pH 7·5, and were subjected to pronase digestion (100 μg/ml.) for 7 hr at 37°C, followed by 3 extractions with freshly distilled phenol (Smith, 1967). The phenol was removed by extensive dialysis, and the purified DNA solution was dialyzed against 2 changes of 100 vol. of 1·0 mM-EDTA, pH 7·5, followed by 20 vol. of 0·3 mM-EDTA, pH 7·5, for 12 hr per change to promote disaggregation of DNA. The DNA obtained had less than 1% contamination with protein. Its molecular weight was determined by viscometry (Crothers & Zimm, 1965) and by electron microscopic measurements and was found to have approximately the value of 31×10^6 daltons as obtained by Burgi & Hershey (1963).

(b) *Preparation of histone*

Calf thymus was frozen soon after slaughter and stored at −25°C. Chromatin was purified according to method (ii) of Maurer & Chalkley (1967), modified by the presence of 0·05 M-$NaHSO_3$ in the isolation media (Panyim & Chalkley, 1969*a*) and by 5 washings of the chromatin in 0·3 mM-EDTA, pH 7·5, by centrifuging in the SS34 rotor (Servall) at 10,000 rev./min for 10 min. The chromatin was then sheared in the Waring blender at top speed for 2 min, centrifuged at 10,000 rev./min for 15 min, and the supernatant was used as purified chromatin.

Chromatin was extracted twice with a solution containing 0·085 M-NaCl, 0·05 M-$NaHSO_3$, pH 7·5, (equivalent to 0·14 M-NaCl in conductance) by centrifuging at 7500 rev./min for 20 min. The pellet was extracted with 4·0 M-NaCl, 2 M-urea, 0·05 M-$NaHSO_3$ (pH 7·5) and the DNA was removed by centrifuging at 45,000 rev./min for 13 hr. The high salt extract was dialyzed against 0·4 N-acetic acid, lyophilized to dryness, and stored at −22°C. Its protein composition was analyzed electrophoretically in polyacrylamide gels according to the method of Panyim & Chalkley (1969*a*), and the gel pattern is shown in the inset of Fig. 1. Most of the protein in the high-salt extract appears to enter the gel, and 91% of this is resolved into the 5 major classes of calf thymus histones in the proportions shown by Panyim & Chalkley (1969*b*). Less than 1% is found in a minor band moving slower than F1 and the remaining 8% is found in a band between F2(a)2 and F2(a)1.

(c) *Complex formation and purification*

All operations were done at 0 to 4°C. The DNA was dialyzed against a solution consisting of 3·0 M-NaCl, 0·01 M-$NaHSO_3$, 1 mM-EDTA, pH 7·5; the dry histone was weighed, dissolved in 3·0 M-NaCl, 1 mM-EDTA, 2 M-urea, pH 7·5, at a concentration of 1 to 2 mg/ml., and then passed through a Millipore filter. Within each experiment different volumes of the histone solution were added to portions of the DNA solution whose volumes were first adjusted in such a way that all the DNA/histone mixtures had the same final DNA concentration. To each DNA solution, at a concentration of 100 to 150 μg/ml., was added histone solution dropwise with constant stirring on ice. This step allows complete mixing of DNA and histone without complex formation, thereby eliminating fast binding artifacts like those observed by Tsuboi, Matsuo & Ts'o (1966). The DNA/histone mixtures were dialyzed for 1 hr against DNA solvent to remove most of the urea, and then were gradient dialyzed for 6 hr against a total of 2 l. of 0·01 M-$NaHSO_3$, 1 mM-EDTA, pH 7·5, to give a final ionic strength equivalent to 0·1 to 0·2 M-NaCl. The complexes were then dialyzed for 6 hr against 100 vol. of 1 mM-EDTA, pH 7·5, and finally against 1 l. of 2 M-urea, 1 mM-EDTA, pH 7·5.

The complexes were purified by passing them through Biogel A50 columns (28 cm × 1·8 cm) pre-washed with 15 to 20 times the void volume with Millipore-filtered 2 M-urea, 1 mM-EDTA, pH 7·5. Urea was present to prevent some loss of complexes during chromatography due to aggregation. The excluded fractions were pooled and dialyzed against 0·3 mM-EDTA, pH 7·5.

(d) *Analytical determinations*

DNA concentration was determined either by absorbance at 260 nm using $E^{1\,mg/ml.}_{260\,nm} = 20$ at all histone/DNA ratios or by the diphenylamine method of Burton (1968). Protein was determined by the procedure of Lowry, Rosebrough, Farr & Randall (1951) modified as follows: up to 0·6 ml. of sample was reacted with 0·5 ml. of reagents whose concentrations were twice that reported in the original, unmodified procedure. Incubations were done at 37°C, and absorbance was measured at 500 nm using bovine serum albumin as the standard. At the protein/DNA ratios used, no inhibition of color development by protein was observed in the diphenylamine assay, and only a small inhibition of color development by DNA was observed at low histone/DNA ratios in the Lowry procedure.

(e) *Viscosity*

The viscosity of the complexes was analyzed in a low-shear viscometer constructed according to the design of Zimm & Crothers (1962), using a Teflon rotor. The shear gradient was calculated to be 0·4 sec^{-1} (Eigner, 1968); the solvent was 0·3 mM-EDTA, pH 7·5, at 25·00° ± 0·05 deg. C. For computations of reduced viscosity, the concentrations of complexes were based only on the DNA component, which varied from 20 to 25 μg/ml.

(f) *Thermal denaturation*

Thermal denaturation was performed on complexes dialyzed against 0·3 mM-EDTA, pH 7·5 (conductance of dialysate = 90 to 93 μmho using a Radiometer Conductivity meter type CDM 2d), in a Gilford Automatic Spectrophotometer model 2000 with a temperature increase of 0·4 deg. C/min.

(g) *Sucrose gradient sedimentation*

DNA was sonicated to an average size of 0·2 μm in a M.S.E. Ultrasonic Disintegrator (60-watt model) at maximum setting for four 2·5-min pulses at a DNA concentration of 100 to 150 μg/ml. in 1·0 mM-EDTA, pH 7·5. After complex formation and gradient dialysis to 0·3 mM-EDTA, 1·0 ml. of each solution was layered on a 5 to 20% sucrose gradient (in 0·3 mM-EDTA, pH 7·5) and centrifuged in the Spinco SW25·3 rotor at 24,000 rev./min for 38 hr at 25°C. Each gradient was collected from the bottom into 39, 0·44-ml. fractions.

(h) *Electron microscopy*

Complexes purified through Biogel were adsorbed (at a concentration of 0·05 O.D. $_{260\,nm}$ in 0·3 mM-EDTA, 2% formaldehyde, pH 7·5) to grids coated with a thick carbon film, made hydrophilic by floating on water for 1 to 2 days. After 10 to 20 sec the excess solution was removed. Details of this procedure will be published elsewhere (manuscript in preparation). Grids were placed 4 cm away from and 0·5 cm below a tungsten filament, and they were rotary shadowed with platinum wire and examined in a RCA-EMU 3F electron microscope at 50 keV using a 25-μm objective aperture.

3. Results

(a) *Stoichiometry of binding*

When a histone and lambda DNA mixture in a solvent containing 3·0 M-NaCl is made to form a complex by gradient dialysis to a solvent of low ionic strength, not all of the histone is complexed to the DNA. The amount of histone which chromatographs with the DNA over a Biogel A50 column is shown in Figure 1. At low input

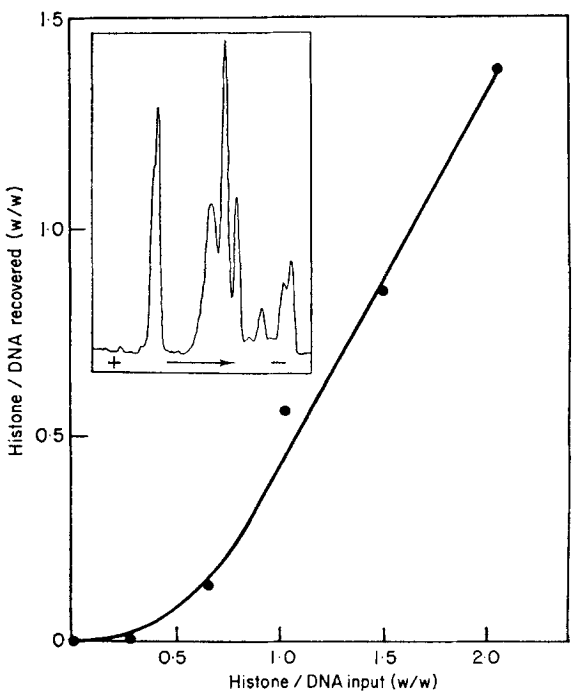

FIG. 1. Stoichiometry of histone binding to lambda DNA. Histone/DNA ratios before and after Biogel A50 chromatography. The DNA concentration during complex formation was 140 μg/ml. Inset: composition of the input histones as revealed by densitometer tracing of a polyacrylamide gel electrophoresis. Protein migration is indicated by the arrow.

ratios of histone to DNA, very little histone is bound. At higher input concentrations of histone, an increasingly larger percentage of the input histone complexes with DNA, reaching up to 70% of the input histone/DNA ratio. The purified complexes contain essentially all of the input DNA; unbound histone is found in the included volume of the column. The sigmoidal shape of the binding curve, showing the histone/DNA output ratio as a function of the input ratio, suggests that the affinity of histone for DNA increases as more histone is available for association. This increase in histone affinity may reflect a localized, histone/histone interaction taking place during complex formation (see Discussion section), which might be manifested in a non-random distribution of protein bound to DNA. To substantiate this we attempted to fractionate DNA/histone complexes into regions having different chemical and/or physical characteristics.

(b) *Hydrodynamics and fractionation of complexes*

When complexed to histones, DNA has different hydrodynamic properties than naked DNA. Figure 2 shows how the reduced viscosity of complexes decreases as the histone-to-DNA ratio increases. A quantitatively similar relationship between the reduced viscosity and the histone/DNA ratio as histone is removed from chick erythrocyte chromatin has been reported (Wilhelm, Champagne & Daune, 1970). Furthermore, the results of measurements using flow birefringence and flow dichroism (Ohba, 1966), circular dichroism (Shih & Fasman, 1971) and optical rotatory dispersion (Tuan & Bonner, 1969), indicate that a conformational change occurs in the DNA/protein complex as more histone is bound. However, little or no change in the secondary structure of DNA could be detected in the X-ray diffraction pattern of calf thymus chromatin as compared to that of free DNA (Garrett, 1971; Bram, 1971). The hydrodynamic and optical data strongly suggest, therefore, that the major structural change that occurs when DNA complexes with histones is a decrease in asymmetry, that is, the tertiary structure of DNA becomes more compact as the

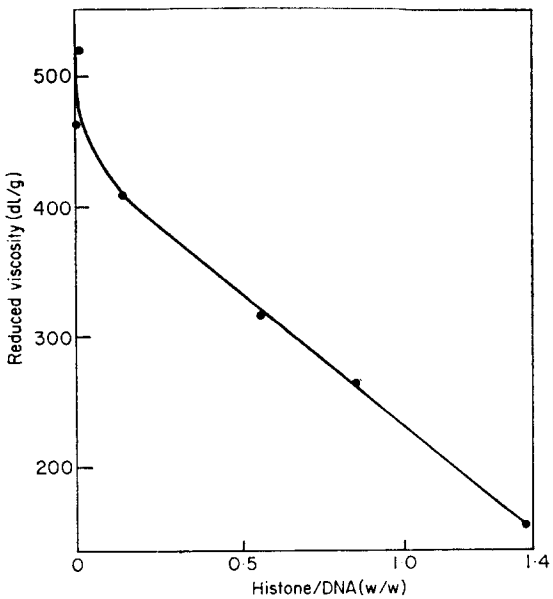

FIG. 2. Reduced viscosities of Biogel purified complexes of different histone/DNA ratios.

protein/DNA ratio increases. Bound histones, therefore, induce or stabilize the compaction of DNA.

A complex composed of a non-random distribution of histone would be expected to contain sections of DNA having different extents of compaction: regions of complexed DNA having a compact structure separated by regions of less complexed, relatively extended structure. To test this hypothesis, we used sonicated DNA to prepare complexes, so that, if histone is distributed non-uniformly on DNA, sections having different extents of compaction would not be covalently linked. The complexes formed were ultracentrifuged through sucrose gradients as described in Materials and Methods; the optical density profiles obtained are shown in Figure 3. Each complex has been separated into a number of components differing in S-value: a light, DNA-like fraction and one or more heavy fractions. Figure 4 shows that, as more histone is added to DNA, more of the DNA-like fraction is converted to material of high S-value. More than 95% of the DNA is recovered in each sucrose gradient.

We conclude that a complex between low molecular-weight DNA and histones is composed of a mixture of complexes differing in sedimentation characteristics. The formation of a nucleoprotein by the addition of histone to low molecular-weight DNA results in the conversion of only a portion of the DNA to material of higher S-value, rather than a gradual increase in the S-value of all the pieces of DNA. One explanation for the increase in S-value is that histones may cause the compaction of DNA, thereby decreasing its frictional coefficient (see Discussion section), an interpretation supported by Figure 2, which shows that histones decrease the reduced viscosity of

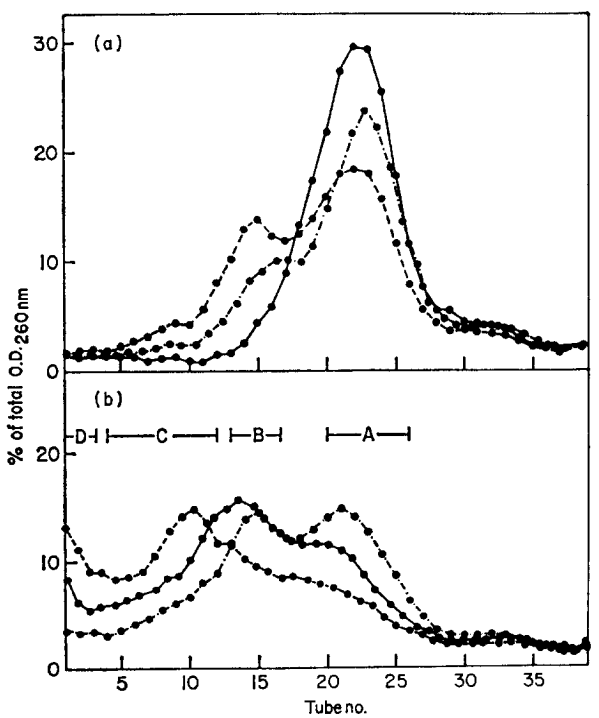

FIG. 3. Sucrose-gradient centrifugation profiles of complexes of histones with sonicated DNA. O.D.$_{260\,r.m}$ of each tube is expressed as % of total O.D.$_{260\,nm}$ recovered from each gradient. Histone/DNA input ratios are: (a), 0·0 (—●——●—), 0·63 (-·-●—·—●-·-), 0·91 (--●----●--); and (b), 1·18 (-·-●—·—●-·-), 1·54 (—●——●—), 1·90 (--●----●--).

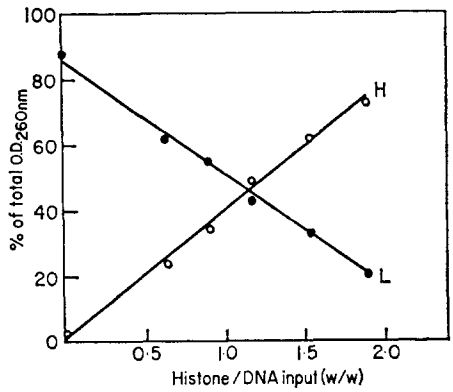

FIG. 4. Distribution of DNA in sucrose gradients: % of O.D. $_{260\,nm}$ from each gradient of light material (L = fraction A) and of heavy material (H = fractions B, C and D).

DNA. The data suggest, therefore, that these DNA/histone complexes contain components having different extents of compaction, and that as histones bind to low molecular-weight DNA, only a portion of the DNA is converted to a more compact structure.

(c) *Characterization of sucrose gradient fractions*

Fractions from each sucrose gradient were pooled into four groups, and the pooled fractions were concentrated and dialyzed against 0·3 mM-EDTA, pH 7·5. Some of the pooled fractions were re-run on sucrose gradients under conditions similar to the original analysis. Figure 5(a) and (b) shows that the pooled fractions have a profile whose peak is close to the middle fraction of the original analysis. We conclude that the components of each complex have discrete *S*-values and that one fraction cannot generate another fraction, due to equilibrium between them at low ionic strength.

The histone-to-DNA ratios of the pooled fractions are shown in Figure 6. The heavy peak from the complex which has an input histone/DNA ratio of 0·6 contains more than three times as much protein as the light peak. Complexes of higher input histone/DNA ratios have *increasingly greater* protein/DNA ratios of the light peak and *constant* protein/DNA ratios of the heavy components; all the fractions from complexes having input ratios greater than 1·2 show no significant differences in the amount of protein complexed to DNA. As the histone/DNA ratio increases, the *S*-value of the light peak increases slightly, while an increasingly greater proportion of the DNA in the heavy peaks appears in fractions of higher *S*-values. The generation of a discrete number of complexes differing in histone/DNA ratio from an originally homogeneous mixture of free histones and DNA suggests that histones bind co-operatively to DNA. The general phenomenon of co-operative binding of proteins to DNA is not unique to histones but has been observed with other basic proteins (Inoue & Ando, 1970) and basic polyamino acids (Wagner, 1969; Leng & Felsenfeld, 1966). These results are consistent with the binding curve shown above, although there may be important differences in the structures formed with each kind of protein/DNA complex.

A more sensitive technique for characterizing DNA/protein complexes is thermal denaturation. The meltings of unsonicated DNA/histone complexes are characterized by three-step transitions; derivative plots of melting profiles reveal a distinct shoulder between transitions 1 and 2. Table 1 shows the T_m and the proportion of each complex

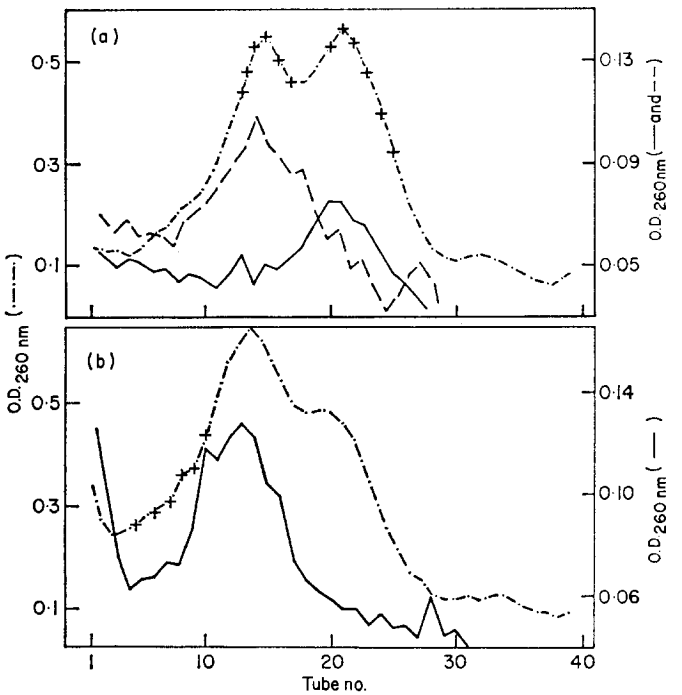

FIG. 5. Rerun of sucrose gradient fractions. (a) Histone/DNA input ratio = 1·18. Six fractions (+) from the left side of the original analysis (—·—·—·) were pooled and rerun (– – – – –), and six fractions (+) from the right side of the original analysis (—·—·—·) were pooled and rerun (———). (b) Histone/DNA input ratio = 1·54. Six fractions (+) from the left side of the original analysis (—·—·—·) were pooled and rerun (———).

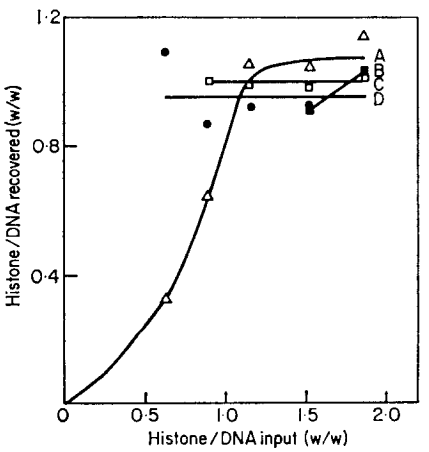

FIG. 6. Histone/DNA ratios before and after sucrose gradient sedimentation. A, B, C and D designate the pooled fractions shown in Fig. 3.

TABLE 1

Thermal denaturation characteristics of complexes

DNA	Input histone/ DNA	Transition 1 T_m (°C)	Transition 1 % of melt	Transition 2 T_m (°C)	Transition 2 % of melt	Transition 3 T_m (°C)	Transition 3 % of melt
Unsonicated	0·0	48·7	100				
	0·28	49·1	100				
	0·65	51·0	90	68	6	77	4
	1·02	53·0	76	76	18	86	6
	1·49	56·8	50	76	30	85	20
	2·05	66·6	36			83	64
Sonicated	0·0	47·3	100				
	0·63	47·7	85			72·2	15
	0·91	49·5	75			71·5	25
	1·18	53·3	69			74·3	31
	1·54	53·6	53			73·2	47
	1·90	57·6	38			74·2	62

melting at each transition. Triphasic melting curves have recently been reported by Li & Bonner (1971) for pea bud chromatin and have been observed in this laboratory for chromatin isolated from six different tissues of the chick embryo (Anderson & Moudrianakis, 1971). Steps in a melting curve of native DNA are thought to be the result of separate regions of DNA having differences in base composition (Mandel & Marmur, 1968) or differences in the extent of helix stabilization due to ligand binding. Since lambda, pea bud and chick embryo DNA have monophasic melting curves under the conditions used in these studies, the steps observed must be caused by different types of protein/DNA complexes present in each solution. Therefore, three-step melting curves presumably indicate the presence of DNA in three different extents of thermal stabilization: a relatively free DNA component and two components of complexed DNA, each having a unique thermal stability. The striking similarity between the melting of DNA/histone complexes and partially de-histonized pea bud chromatin (Li & Bonner, 1971) suggests that the structures formed when histones complex to DNA in a model system may be similar to the natural product.

Figure 7 shows that sonicated DNA/histone complexes have two-step melting curves (see Table 1). For complexes of histone with both sonicated and unsonicated DNA, as the histone/DNA ratio increases, the T_m of the first transition increases, while the T_m of the highest melting transition remains relatively constant. Biphasic melting curves have also been observed for complexes of DNA with basic homopolypeptides (Olins, Olins & von Hippel, 1967; Inoue & Ando, 1970; Matsuo & Tsuboi, 1969), clupeine (Inoue & Ando, 1970), protamine (Olins, Olins & von Hippel, 1968), and purified classes of histones (Shih & Bonner, 1970; Olins, 1969; Olins & Olins, 1971; Li & Bonner, 1971; Ansevin & Brown, 1971). In all examples of biphasic melting curves, as the protein/DNA ratio increases, the proportion of DNA which melts at transition 1 decreases, a fact which implies that the protein binds co-operatively to only a portion of the DNA and converts it to a more thermally stable structure. The melting profiles of the fractions from the sucrose gradients show that this interpretation is correct. Each pooled fraction (shown in Fig. 3) was subjected to thermal

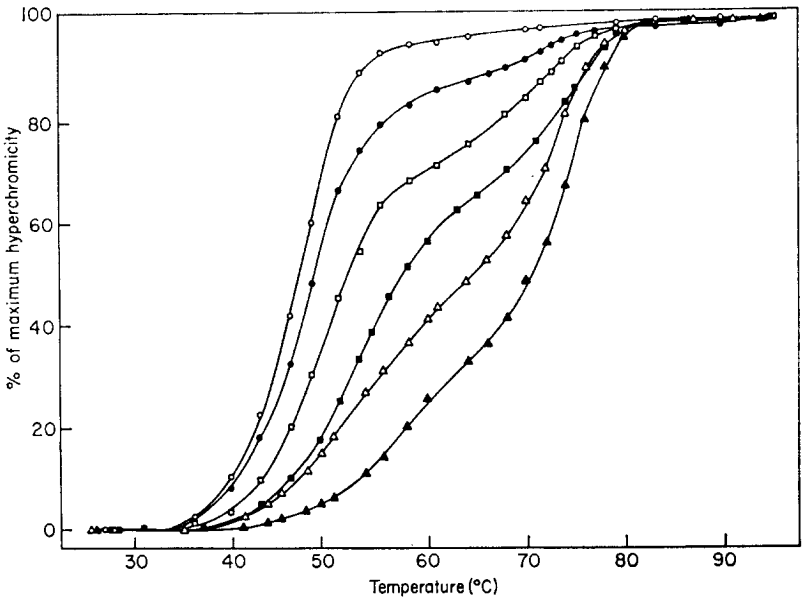

FIG. 7. Thermal denaturation of complexes of sonicated DNA with histones. Solvent is 0·3 mM-EDTA, pH 7·5. Histone/DNA input ratios are: 0 (—○—○—), 0·63 (—●—●—), 0·91 (—□—□—), 1·18 (—■—■—), 1·54 (—△—△—), 1·90 (—▲—▲—).

denaturation under identical conditions, and the melting curves are summarized in Figure 8, which shows the proportion of the DNA of each fraction which melts at transition 1. At all histone/DNA ratios the fraction which has an S-value close to that of naked DNA contains the greatest percentage of material which melts like naked DNA, while the fractions with higher S-values contain less material that melts like naked DNA. This demonstrates that sonicated DNA/histone complexes can be fractionated into components having major physical differences as revealed by velocity of sedimentation and by thermal stability. The results also show that the *second melting transition* of unfractionated DNA/histone complexes is caused by a unique, *separable* species of complexed DNA. The T_m values of each transition of the unfractionated complex are not significantly different from the T_m values of the transitions after fractionation; a similar observation was made by Ansevin & Brown (1971) using three bacterial DNA's differing in T_m.

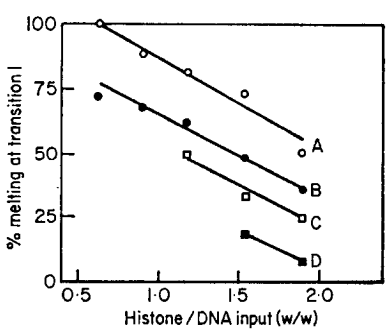

FIG. 8. Thermal denaturation of complexes after sucrose gradient sedimentation. Biphasic melting curves of each pooled fraction are presented as the % of DNA which melts at transition 1. A, B, C and D designate the pooled fractions shown in Fig. 3.

We have shown that DNA/histone complexes prepared from sonicated DNA by salt-gradient dialysis are composed of pieces that are tightly compacted, thermally stable structures as well as pieces that are more DNA-like in their sedimentation properties and thermal stability. Therefore, histones bind co-operatively to sonicated DNA; that is, histone/histone interaction takes place during binding in such a way as to result in a non-random distribution of protein complexed to DNA. Co-operative binding of all the individual histone classes has been suggested by the biphasic melting curves observed by Olins (1969), Shih & Bonner (1970), Ansevin & Brown (1971) and Olins & Olins (1971), and by the high sensitivity of histone binding at certain salt concentrations (Akinrimisi, Bonner & Ts'o, 1965). Our demonstration that a DNA/histone complex is a mixture of a unique number of separable components is in agreement with the results of the above authors and is the most definitive verification that histones can bind co-operatively to DNA. The inability to fractionate a complex into a naked DNA component and an entirely complexed component may be due to limitations in the ultracentrifugation technique or may reflect the true binding pattern.

(d) *Electron microscopy*

The stoichiometry data of Figure 1 indicate that a similar co-operative binding takes place with high molecular-weight DNA/histone complexes. To confirm this more directly, the electron microscopic appearance of the complexes was investigated, some examples being shown in Plates I and II. It was found that complexes are non-uniform in width—strands are composed of thick regions alternating with much thinner regions. At low histone/DNA ratios the width of most of each strand is similar to that of naked DNA, but numerous nodules are seen interspersed. As the histone/DNA ratio increases, the nodules become thicker, longer and less numerous, giving the appearance that separated nodules gradually increase in size and eventually fuse together. At a histone/DNA ratio of 0·6 only a small portion of each strand has a thickness like naked DNA. Intrastrand packing, characteristic of complexes of high histone/DNA ratios, often obscures thin regions of the strands, but DNA-like regions can always be seen on extended molecules whose histone/DNA ratio is 0·6 or less. At a ratio of 0·8 each strand has a more uniform width, which varies from three to six times as thick as naked DNA. At higher histone/DNA ratios, the complexes appear as compacted fibers having a width five to six times the thickness of DNA.

We interpret the nodules to be regions of high mass-to-length ratio (compaction) and conclude that histone is distributed non-uniformly when it forms a complex with high molecular-weight DNA. Electron microscopic studies have shown that chromatin fibers are relatively homogeneous in width, although the absolute diameter of the "unit" fiber has been estimated to be as low as 30 to 40 Å (Zubay & Doty, 1959; Solari, 1965; Anderson & Moudrianakis, 1969), 100 Å (Bram & Ris, 1971; Georgiev, Il'in, Tikhonenko, Dobbert & Anan'eva, 1967) and as high as 230 Å (DuPraw, 1965). Irregularities in the fiber thickness have been reported (DuPraw, 1965; Anderson & Moudrianakis, 1969; Bram & Ris, 1971), but these protuberances are generally interpreted as arising from localized "supercoiling" rather than localized higher histone concentrations. Our results show that, although high molecular-weight DNA/histone complexes have a structure similar to native chromatin based on thermal denaturation the relatively uniform width characteristic of complexes of high histone/DNA ratios (and similar to native chromatin) is actually generated from strands which originally

had a non-uniform thickness. It appears, therefore, that the chromatin fiber may have a heterogeneity not as yet detected by electron microscopic observations.

4. Discussion

The co-operative binding of histones to DNA has been demonstrated by the ability to fractionate a DNA/histone complex into components differing in certain physical and chemical properties. This separation can be achieved only if very *low molecular-weight DNA* is used—attempts to fractionate high molecular-weight DNA/histone complexes result in only broad sedimentation profiles (unpublished observations). However, electron microscopic evidence indicates that high-molecular-weight DNA/histone complexes also have a non-uniform distribution of histone complexed to DNA, as if they were composed of low molecular-weight complexes linked together. The mechanism for co-operative binding is not clear. Our working hypothesis is that regions of bound histone tend to accumulate more histone, presumably because the addition of more protein to a partially complexed molecule is energetically more favorable than the initiation of a DNA/histone complex. One explanation for this enhanced affinity is that histones complexed to DNA have previously unexposed functional groups which can bind to free histone or stabilize the binding of histone to DNA. This change in the reactivity of bound histone may be caused by a conformational change, a change in the extent of aggregation, or the presence of high concentrations of only one class of histones. Another explanation for co-operativeness might be that the affinity of histone for DNA increases as the tertiary structure of DNA becomes more compact, a change in the conformation of DNA which we have shown does, in fact, take place. Other explanations, such as a change in the secondary structure of DNA or an aggregation of non-covalently-linked strands, cannot be ruled out.

The increase in S-value *and* decrease in viscosity we observed as more histone is bound to DNA are, at least in part, caused by the reduction of intrastrand phosphate repulsion, similar to the well known effect of sodium ions on DNA. However, the magnitude of the hydrodynamic changes may suggest that intimate intrastrand interactions are induced by bound histones. Of course, other factors may be responsible for these changes (at least in part), such as charge effects (especially at low ionic strength), or changes in the extent of hydration of the DNA, or an increase in the extent of DNA aggregation. A more detailed analysis of this matter is currently under progress in this laboratory.

Although simple compared to studies using chromatin, the use of a model system has certain complicated features that lead to some interesting speculations. For example, the thermal stability of high molecular-weight DNA/histone complexes is considerably different from low molecular-weight complexes—the T_m of the most thermally stable DNA of the former is an average of 10 deg. C higher than that of the latter at the same histone/DNA ratio, and the *high* molecular-weight complexes undergo an *additional* intermediate transition. Complexes of sonicated DNA with basic polypeptides also have a lower T_m than unsonicated DNA/protein complexes (Olins, Olins & von Hippel, 1968). These physical differences suggest that high molecular-weight DNA/histone complexes can form structures that low molecular-weight complexes cannot. A comparison of high and low molecular-weight complexes by electron microscopy suggests that this difference may be manifested in the ability to form a supercoil.

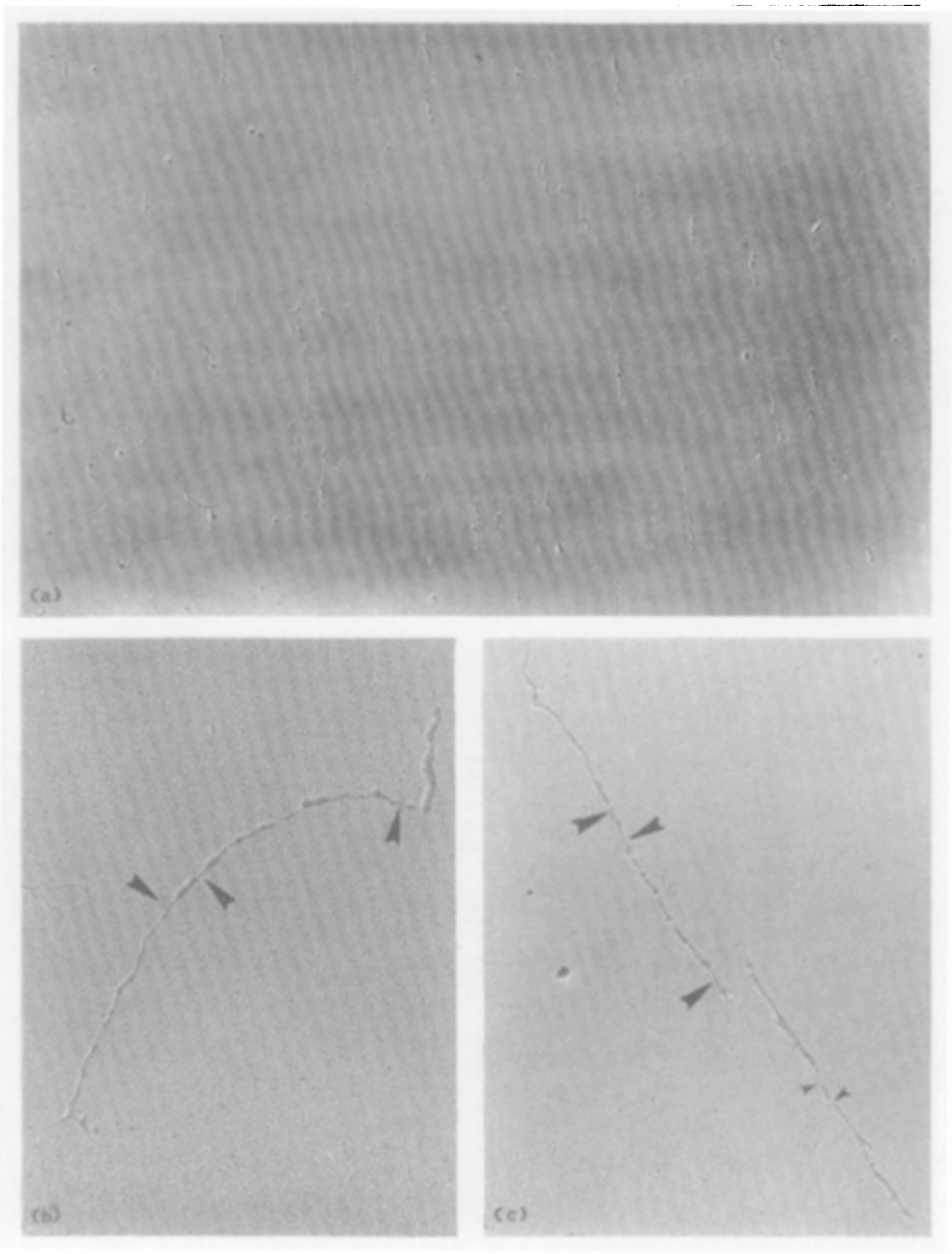

PLATE I. Electron micrographs of Biogel purified complexes having a histone/DNA ratio of 0·56.
(a) Magnification, 9,300; (b) magnification, 33,300; (c) magnification, 26,500. Arrows point to thin, DNA-like regions.

[facing p. 37

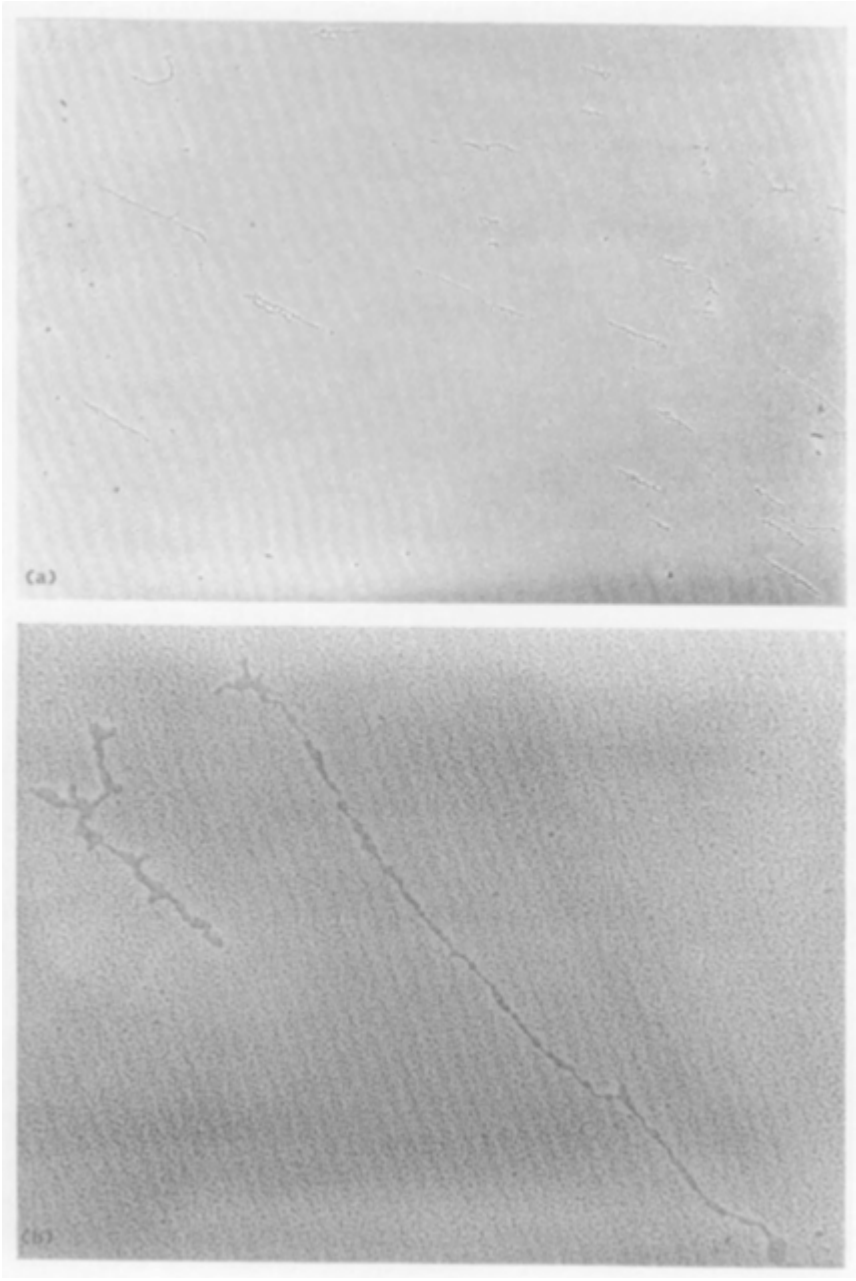

PLATE II. Electron micrographs of Biogel purified complexes having a histone/DNA ratio of 0·85. (a) Magnification, 10,300; (b) magnification, 41,200. Structures vary from highly compacted (upper left) to extended (right).

Another interesting complication is found in a comparison of the physical and chemical characteristics of the fractionated components of low molecular-weight DNA/histone complexes. It was observed that complexes of histone/DNA input ratios of 1·2 or greater are fractionated into components characterized by different *S*-values and thermal stabilities, but no differences in the histone/DNA ratios of these components could be detected. They may, however, have differences in the relative abundance of each histone class, which might result in the formation of unique DNA/histone structures having different physical properties. Considerable data now exist showing that the different histone classes display specificity with respect to the kinds of structures formed when complexed to DNA (Olins & Olins, 1971; Ansevin & Brown, 1971; Li & Bonner, 1971; Shih & Fasman, 1971; Adler *et al.*, 1971; Shih & Bonner, 1970; Akinrimisi *et al.*, 1965). Histone/histone interaction, which our data indicate plays an important role when histones bind to DNA, may involve associations of specific histone classes and may result in structures that depend on the juxtaposition of certain classes of histones.

The structure of chromatin has recently been analyzed by optical rotatory dispersion measurements and spectrophotometric titration of calf thymus chromatin using acridine orange (Permogorov, Sladkova, Debabov & Rebentish, 1970) and by nuclease digestion and polylysine binding (Clark & Felsenfeld, 1971). These data indicate that approximately one-third to one-half of chromatin has optical and chemical properties similar to naked DNA. Our results show that even in complexes of high histone/DNA ratio there is a significant proportion of physically separable DNA-like component. Furthermore, high molecular-weight DNA/histone complexes of high histone/DNA ratio are characterized by a large DNA-like thermal transition and have sections that appear in the electron microscope as free DNA. The non-random distribution of protein we observe in DNA/histone complexes has also been found in chromatin from Ehrlich ascites carcinoma cells and calf thymus; Ilyin & Georgiev (1969) showed that, after extraction with 0·6 M-NaCl and formaldehyde fixation, these chromatins can be ultracentrifuged in a urea/CsCl density gradient into a number of components differing in buoyant density and protein/DNA ratio. Our results suggest that this heterogeneity observed in chromatin may be generated by the co-operative binding of histones to DNA.

The model system we have described has proved useful in revealing how some components of chromatin interact, as well as the significance of this interaction to the structure of native chromatin. Further work may show how specific histone classes interact with each other to form a complex with DNA and how changes in histone/histone interactions might be responsible for the dynamic nature of chromatin.

This work was supported by grants GM-13518 and HD-326 from the National Institutes of Health (U.S.A.). One of us (R. L. R.) is supported by a National Institutes of Health Predoctoral Training grant, HD-139. This paper is contribution no. 683 from the Department of Biology, Johns Hopkins University.

REFERENCES

Adler, A. J., Schaffhausen, B., Langan, T. A. & Fasman, G. D. (1971). *Biochemistry*, **10**, 909.

Akinrimisi, E. O., Bonner, J. & Ts'o, P. O. P. (1965). *J. Mol. Biol.* **11**, 128.

Anderson, P. L. & Moudrianakis, E. N. (1969) Biophys. J. Soc. Abs. **9**, A-54.

Anderson, P. L. & Moudrianakis, E. N. (1971). Biophys. J. Soc. Abs. **11**, 152a.

Ansevin, A. T. & Brown, B. W. (1971). *Biochemistry*, **10**, 1139.
Bram, S. (1971). *J. Mol. Biol.* **58**, 277.
Bram, S. & Ris, H. (1971). *J. Mol. Biol.* **55**, 325.
Bonner, J., Dahmus, M. E., Fambrough, D., Huang, R. C. C., Marushige, K. & Tuan, D. Y. H. (1968). *Science*, **159**, 47.
Burgi, E. & Hershey, A. D. (1963). *Biophys. J.* **3**, 309.
Burton, K. (1968). In *Methods of Enzymology*, ed. by L. Grossman & K. Moldave, vol. 12, part B, p. 163. New York: Academic Press.
Clark, R. J. & Felsenfeld, G. (1971). *Nature New Biol.* **229**, 101.
Crothers, D. M. & Zimm, B. H. (1965). *J. Mol. Biol.* **12**, 525.
DuPraw, E. (1965). *Proc. Nat. Acad. Sci., Wash.* **53**, 161.
Eigner, J. (1968). In *Methods in Enzymology*, ed. by L. Grossman & K. Moldave, vol. 12, part B, p. 386. New York: Academic Press.
Garrett, R. A. (1971). *Biochemistry*, **10**, 2227.
Georgiev, G. P., Il'in, Y. V., Tikhonenko, A. S., Dobbert, N. N. & Anan'eva, L. N. (1967). *Molecular Biology* (Translated from Russian) **1**, 677.
Ilyin, Y. V. & Georgiev, G. P. (1969). *J. Mol. Biol.* **41**, 299.
Inoue, S. & Ando, T. (1970). *Biochemistry*, **9**, 388.
Kleiman, L. & Huang, R. C. C. (1971). *Proc. Fed. Amer. Soc. Exp. Biol.* **30**, 1084.
Leng, M. & Felsenfeld, G. (1966). *Proc. Nat. Acad. Sci., Wash.* **56**, 1325.
Li, H.-J. & Bonner, J. (1971). *Biochemistry*, **10**, 1461.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. Biol. Chem.* **193**, 265.
Mandel, M. & Marmur, J. (1968). In *Methods in Enzymology*, ed. by L. Grossman & K. Moldave, vol. 12, part B, p. 195. New York: Academic Press.
Matsuo, K. & Tsuboi, M. (1969). *Biopolymers*, **8**, 153.
Maurer, H. R. & Chalkley, G. R. (1967). *J. Mol. Biol.* **27**, 431.
Ohba, Y. (1966). *Biochim. biophys. Acta*, **123**, 76.
Ohlenbusch, H. H., Olivera, B. M., Tuan, D. & Davidson, N. (1967). *J. Mol. Biol.* **25**, 299.
Olins, D. E., Olins, A. L. & von Hippel, P. H. (1967). *J. Mol. Biol.* **24**, 151.
Olins, D. E., Olins, A. L. & von Hippel, P. H. (1968). *J. Mol. Biol.* **33**, 265.
Olins, D. E. (1969). *J. Mol. Biol.* **43**, 439.
Olins, D. E. & Olins, A. L. (1971). *J. Mol. Biol.* **57**, 437.
Panyim, S. & Chalkley, R. (1969*a*). *Arch. Biochem. Biophys.* **130**, 337.
Panyim, S. & Chalkley, R. (1969*b*). *Biochemistry*, **10**, 3972.
Permogorov, V. I., Sladkova, I. A., Debakov, V. G. & Rebentish, B. A. (1970). *Molecular Biology* (Translated from Russian), **4**, 285.
Senshu, T. (1971). *Biochim. biophys. Acta*, **236**, 349.
Shih, T. Y. & Bonner, J. (1970). *J. Mol. Biol.* **48**, 469.
Shih, T. Y. & Fasman, G. D. (1971). *Biochemistry*, **10**, 1675.
Smith, M. G. (1967). In *Methods in Enzymology*, ed. by L. Grossman & K. Moldave, vol. 12, part A, p. 545. New York: Academic Press.
Solari, A. J. (1965). *Proc. Nat. Acad. Sci., Wash.* **53**, 503.
Tsuboi, M., Matsuo, K. & Ts'o, P. O. P. (1966). *J. Mol. Biol.* **15**, 256.
Tuan, D. Y. H. & Bonner, J. (1969). *J. Mol. Biol.* **45**, 59.
Wagner, K. G. (1969). *Europ. J. Biochem.* **10**, 261.
Wilhelm, F. X., Champagne, M. H. & Daune, M. P. (1970). *Europ. J. Biochem.* **15**, 76.
Zimm, B. H. & Crothers, D. M. (1962). *Proc. Nat. Acad. Sci., Wash.* **48**, 905.
Zubay, G. & Doty, P. (1959). *J. Mol. Biol.* **1**, 1.