Volume 9 Number 2 1981 Nucleic Acids Research

Renaturation of DNA: a novel reaction of histones

Michael M.Cox and I.R.Lehman

Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305, USA

Received 21 October 1980

ABSTRACT

Histones isolated from several sources, either singly or in combination promote the renaturation of complementary single strands of DNA, as measured by the acquisition of resistance to S1 nuclease. The reaction is rapid ($T_{\frac{1}{2}}$ <1 min), and is stoichiometric rather than catalytic. Renaturation is stimulated by Mg^{2+}, Mn^{2+}, and Ca^{2+}, but is strongly inhibited by Zn^{2+}. Crude extracts of early embryos of Drosophila melanogaster possess renaturation activity which is protease sensitive, heat-stable, and acid-soluble, suggesting that most or all of it can be attributed to histones. This observation thus provides a functional assay for histones that should prove useful in studies of chromatin and histone-DNA interactions, as well as for the identification and isolation of histones and histone-like proteins in crude extracts.

INTRODUCTION

The recA protein of E. coli catalyzes the renaturation of complementary single strands of DNA coupled to the hydrolysis of ATP to ADP and Pi (1). In the course of a search for a comparable activity in extracts of early embryos of Drosophila melanogaster we observed a high level of ATP-independent renaturation activity. Characterization of this activity showed it to be heat-stable, protease-sensitive, acid soluble, and dependent on divalent cations. We subsequently found that purified histones can promote this reaction.

In this report we present a detailed characterization of the histone promoted renaturation activity. This activity has potential application to studies involving histones, chromatin, and histone-like proteins. It also provides a simple and rapid assay for the detection of histones and similar proteins in crude tissue extracts.

MATERIALS AND METHODS

PA solution contained 25 mM sodium acetate (pH 6.6), 20 mM NaCl, 40 mM sodium phosphate buffer (pH 6.6), 1 mM phenylmethylsulfonyl fluoride, and

© IRL Press Limited, 1 Falconberg Court, London W1V 5FG, U.K.

10 mM sodium bisulfite.

Hydroxylapatite (Bio-Gel HTP) and Bio-Rex 70 were purchased from Bio-Rad. Phenylmethylsulfonyl fluoride, purchased from Sigma, was dissolved in 2-propanol to make a 0.1 M stock solution. Baker Analytical Grade sodium sulfite was dissolved in H_2O, adjusted to pH 7.5 with HCl, and diluted to 1 M. Poly-L-lysines with average molecular weights of 3000, 13,000 and 30,000 were purchased from Sigma.

^{3}H-labeled phage P22 DNA was prepared as described (2). ^{3}H-labeled phage M13 single-stranded DNA was a gift from J. Kobori (Stanford).

Purified calf thymus histones H2A, H2B, H3, and H4 were donated by C. Glover (Stanford). Calf thymus and D. melanogaster histones H1 were provided by T. Hsieh (Stanford) . Aspergillus oryzae S1 nuclease was purchased from Sigma. Proteinase K was obtained from EM Laboratories. Bacteriophage T4 gene 32 protein was a gift from K. Arai (originally obtained from B. Alberts).

S1 Nuclease Assay for DNA Renaturation

DNA renaturation was measured at 30°C in 120-µl reaction mixtures containing 20 mM Tris·HCl (80% cation, pH 7.5), 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5% (v/v) glycerol, heat denatured P22 [^{3}H] DNA (0.9 µg, 4 x 10^4 cpm) or M13 [^{3}H] single-stranded DNA (0.9 µg, 1 x 10^5 cpm), and an appropriate amount of the indicated effector. The $MgCl_2$ was replaced or eliminated as indicated. Reactions were carried out in plastic microcentrifuge tubes with a 1.5-ml capacity. After preincubation for 5 min, reactions were started by addition of DNA and stopped by the addition of 0.6 ml of S1 buffer (150 mM NaCl, 50 mM sodium acetate pH 4.6, 1 mM zinc acetate, 0.1% sodium dodecyl sulfate), 20 µg of heat-denatured calf thymus DNA, and 100 units of S1 nuclease (1 unit = amount required to degrade 1 µg of DNA in 30 min at 37°C). The mixture was incubated at 37°C for 35 min at which time 100 µg of calf thymus DNA was added as a carrier together with 700 µl of cold 10% (w/v) trichloroacetic acid. Reactions were left on ice for 20 min and then filtered through Whatman GF/C filters. Acid-precipitable radioactivity was measured by liquid scintillation counting.

Controls to measure the S1 nuclease-resistance of native P22 [^{3}H] DNA in the absence of effector were incorporated into each experiment. This value was taken as 100% renaturation and variation in successive experiments did not exceed ±5%. Within an experiment, duplicate samples generally agreed to within 3%.

Preparation of histones from Drosophila cells grown in tissue culture. Eschalier's K_c line of D. melanogaster cells (3,4) was grown as previously described (5). The culture medium, designated D-22, was identical to D20-P medium (5) except for the addition of 3.5 mM sodium phosphate buffer (pH 6.7). Antibiotics were added to final concentrations of 330 units/ml of penicillin and 66 μg/ml of streptomycin sulfate.

Cells (1.5 liters) were harvested at a cell density of approximately 5×10^6 per ml and chromatin prepared from them by the method of Hancock et al. (6), with the modification that 1 mM phenylmethylsulfonyl fluoride and 10 mM sodium bisulfite were present to prevent proteolysis.

The procedure for purification of histones from the isolated chromatin was adapted from Germond et al. (7) as modified by R. Wiegand (personal communication). After sedimentation of the chromatin, it was resuspended in 100 ml PA solution containing 2 M NaCl with extensive sonication and vortexing. To the suspension was added 20 g hydroxylapatite (Bio-Gel HTP). The suspension was swirled gently for 15 min at room temperature, filtered, then washed with more PA containing 2 M NaCl. The filtrate and wash were collected (170 ml) and dialyzed extensively against a buffer containing 20 mM potassium phosphate (50% dianion, pH 6.8), 5 mM sodium bisulfite, and 0.1 mM phenylmethylsulfonylfluoride, then applied to a Bio-Rex 70 column (1.9 x 3.6 cm) equilibrated with the same buffer. After washing with 20 mM potassium phosphate (pH 6.8) containing 600 mM NaCl, the histones were eluted with the same phosphate buffer containing 2 M NaCl. Histone-containing fractions (as determined by OD_{230} measurements) were pooled, dialyzed against water, lyophilized, then dissolved in 2 ml water. The resulting preparation contained the four core histones plus H1 in approximately equivalent concentrations. Protein concentration, as measured by the Coomassie Blue staining method (8), was 1.6 mg/ml (bovine serum albumin standard), and the $O.D._{230}$ was 3.21.

Reaction products were visualized by electron microscopy by means of the formamide spreading method of Davis et al. (9).

Polyacrylamide gel electrophoresis was performed as described (10).

RESULTS

Renaturation of Complementary Single Strands of DNA by histones. Purified calf thymus histones can promote the renaturation of heat-denatured DNA as judged by its conversion to a form that is resistant to S1 nuclease. The reaction was strongly stimulated by divalent cations (Table

Reaction mixture	% Renaturation
Complete	69.0
- histone	3.5
- P22 DNA, + M13 single-stranded DNA	1.2
- $MgCl_2$	8.4
- $MgCl_2$ + 20 mM zinc acetate	3.2
+ $MgCl_2$ + 1 mM zinc acetate	4.7
- $MgCl_2$ + 10 mM $CaCl_2$	68.3
- $MgCl_2$ + 10 mM $MnCl_2$	63.8

TABLE 1: Requirements for histone promoted DNA renaturation

Reactions (120 µl) were performed as described in Methods and contained 2.4 µg of the K_c cell histones. Incubations were at 30°C for 10 min.

1). No S1 resistant material was formed with M13 single-stranded DNA which lacks a complementary single strand. Thus S1 resistance is a true measure of duplex formation. The maximum extent of renaturation was approximately 70%.

The efficiency of the reaction differed for the different histones. Calf thymus H1 was the most effective, with saturation occurring at a ratio of one molecule of histone H1 per 100 nucleotides. H2A, H2B, and H3 were approximately equivalent, but less effective than H1, saturating at about one molecule per 30 nucleotides. H4 was the least effective. The titration curve for each histone was sigmoidal, indicating some form of cooperative interaction (Fig. 1).

The enhanced response exhibited by calf thymus H1 is not necessarily an inherent property of H1. Purified H1 from K_c cells showed a response which was approximately equivalent to that of the calf thymus core histones. Upon polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, histone H1 from K_c cells migrated as a single band, while the calf thymus H1 preparation was heterogeneous, showing a group of closely migrating bands. Heterogeneity in histone H1 is commonly observed (11) and it is posssible that the enhanced reactivity is associated with one or more of the modified forms of this histone. Such an interpretation is consistent with the observation of Welch and Cole (12) that different subfractions of rabbit thymus H1 differ in their DNA binding efficiency.

Poly-L-lysines of molecular weight 13,000 and 30,000 promoted the renaturation reaction as effectively as calf thymus histone H1 (Fig. 1).

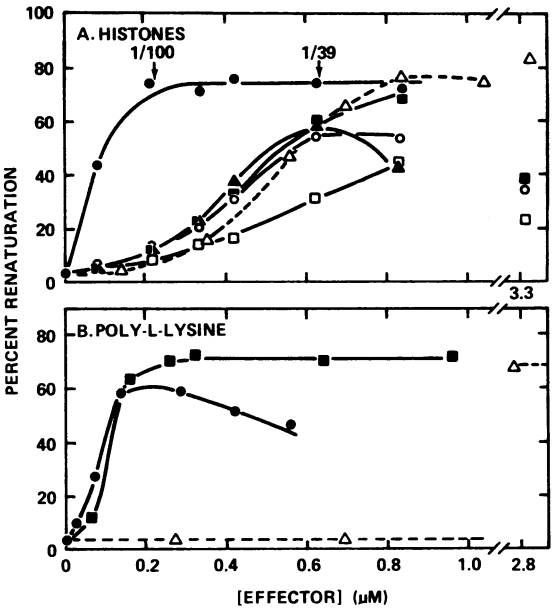

Fig. 1. Histone and polylysine promoted DNA renaturation. Reactions were carried out as described in Methods. Incubations were for 10 min at 30°C. A: Renaturation promoted by purified histones. The histones used were from calf thymus, with the exception of histone H1 from D. melanogaster. ●-● H1, ■-■ H2A, ▲-▲ H2B, o-o H3, □-□ H4, Δ-Δ D. melanogaster H1. B: Renaturation promoted by poly-L-lysine. ■-■ average molecular weight 13,000; ●-● average molecular weight 30,000; Δ-Δ average molecular weight 3,000. Polylysine concentration is given in moles of polymer. The designations 1/100 and 1/39 indicate the ratio of effector molecules to nucleotides.

Polylysines of lower molecular weight (3000) were much less active, saturating at approximately 1 molecule/3 nucleotides, although the maximum extent of renaturation was the same as for the larger polymers. The polylysine promoted renaturation reaction, like the histone dependent reaction, required complementary single strands and also showed a sigmoidal dependence on effector concentration.

The addition of histone in excess of that required to saturate the reaction partially inhibited renaturation. This effect was most pronounced for histones H2A and H2B although it occurred with the other core histones as well. When the histone H2A concentration was increased by a factor of 4 beyond the saturation point the extent of DNA renaturation was decreased by 50% (Fig. 1). The inhibition may be a result of the tendency of the core

histones to aggregate at neutral pH and low ionic strength, the conditions used in these experiments. In contrast, addition of excess histone H1, which does not normally form aggregates, did not inhibit renaturation. Among the polylysines, those of molecular weight 30,000 but not of molecular weight 13,000 showed a similar inhibition.

Renaturation was very rapid in all cases, with a half time of less than one min (Fig. 2). Within the limits of the sensitivity of the measurement, very little difference could be detected in rates of renaturation promoted by polylysine or histones from K_c cells. In both cases the reaction was approximately 75% complete within one minute.

The extent of the reaction was directly dependent on the concentration of effector so that the histones cannot be acting catalytically. As shown in Figure 2, subsaturating levels of K_c cell histones resulted in less than total renaturation, although the rate remained too rapid to be measured accurately.

As viewed in the electron microscope, the product of renaturation promoted by the K_c cell histones was a complex DNA aggregate involving hundreds of strands of phage P22 DNA (data not shown). These reactions were performed under conditions in which 70% of the DNA became resistant to

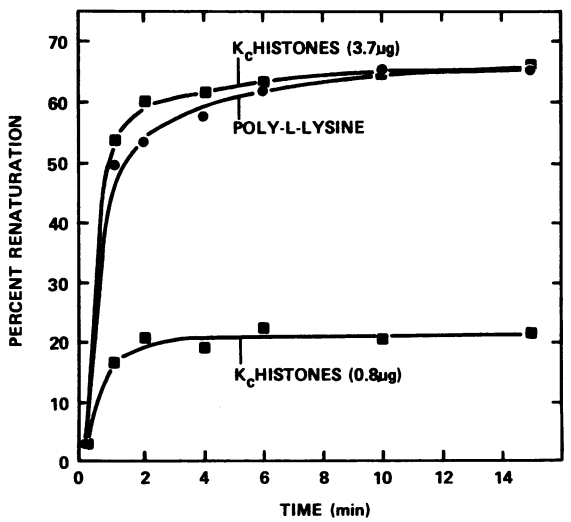

Fig. 2. Kinetics of histone and polylysine promoted DNA renaturation. Reactions were carried out as described in Methods with the indicated amounts of K_c cell histones or 0.5 µg of poly-L-lysine (average molecular weight, 30,000).

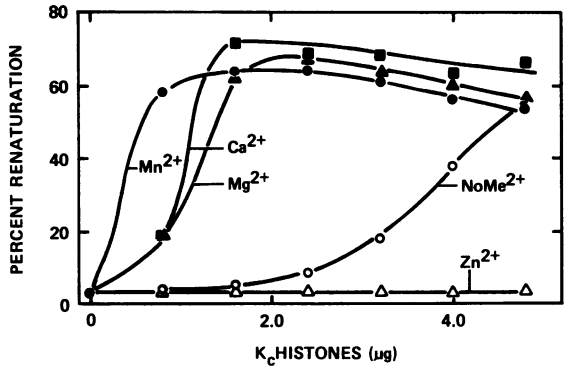

Fig. 3. Dependence of histone promoted DNA renaturation on divalent cations. Reactions were carried out as described in Methods except that $MgCl_2$ was replaced by $MnCl_2$, $CaCl_2$, or zinc acetate as indicated (10 mM). Incubations were for 10 minutes at 30°C.

S1 nuclease, indicating that extensive duplex regions were present within the aggregate. Simpler structures were not observed. Consistent with this finding is the observation that heat denatured P22 DNA was converted to a form that did not enter a 0.7% agarose gel. Similar results were obtained with polylysine. No aggregates were observed in samples incubated without histones or polylysine. P22 DNA is circularly permuted, so that a complex product and an inability to achieve 100% renaturation is not unexpected.

As shown in Figure 3, the histone-promoted renaturation reaction was strongly stimulated by Mg^{2+}, Ca^{2+}, or Mn^{2+} although the requirement was not absolute. The order of effectiveness appears to be Mn > Ca > Mg. Each cation decreased the amount of histone required to saturate the reaction by at least a factor of three.

In contrast, addition of zinc acetate at concentrations ranging from 1-10 mM resulted in inhibition which was not relieved by the addition of 10 mM $MgCl_2$. Thus, in the presence of 1 mM zinc and 10 mM magnesium the inhibition was complete under conditions where maximum renaturation was observed with magnesium alone. The inhibition was not the result of the activation of a contaminating, zinc dependent nuclease. Incubation of 10 mM Zn^{2+}, K_c histones and single-stranded DNA at 30°C for 20 min did not result in any loss of acid-precipitable DNA in samples not treated with

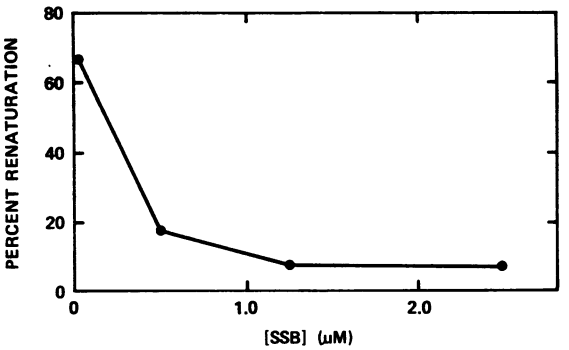

Fig. 4. Effect of E. coli single-stranded DNA binding protein on histone H1 promoted renaturation. Reactions were performed as described in Methods. Reaction mixtures contained 7 x 10^{-7} M D. melanogaster histone H1 and the indicated concentrations of SSB. Incubations were at 30°C for 10 min.

nuclease S1[1].

Renaturation of DNA by Other Proteins

Under certain conditions, the E. coli single-stranded DNA binding protein (SSB) and the gene 32 protein of phage T4 will promote DNA strand reassociation (13,14). This effect is attributed to their ability to melt out hairpin helices formed in DNA strands at low temperatures. In the case of SSB, the renaturation reaction shows a sharp pH optimum at pH 5.5, and little renaturation occurs above pH 7.0 unless spermine or spermidine is present. SSB did not promote renaturation under our conditions, and in fact acted as a potent inhibitor of the renaturation that occurred in the presence of D. melanogaster histone H1 (Fig. 4). Complete inhibition was observed at a stoichiometry of about 1 SSB per 20 nucleotides, and may result from competition between SSB and histones for the single-stranded DNA.

The gene 32 protein of phage T4 promotes DNA renaturation under the same conditions used to study the histone reaction (14, and Fig. 5). This can be distinguished from the histone-promoted reaction on the basis of two criteria. First, it is much slower. Using approximately saturating concentrations of gene 32 protein (1 gene 32 protein per 12 nucleotides), the reaction was 50% complete after 50 min. Increasing the temperature to 37°C

[1]Addition of zinc acetate in the renaturation reaction mixture lowered the pH of the mixture from 7.5 to 7.1 at 10 mM. At 1 mM the effect was negligible.

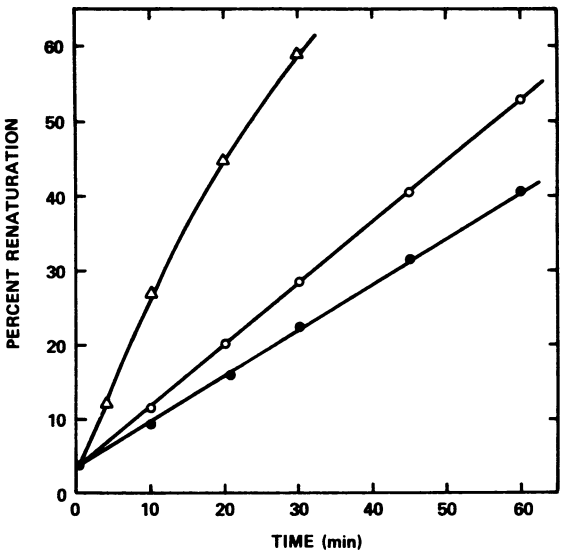

Fig 5. Phage T4 gene 32 promoted DNA renaturation. Reactions were performed as described in Methods and contained 1.7 x 10^{-6} M gene 32 protein. Δ-Δ 40 mM $MgCl_2$, 37°C; o-o 10 mM $MgCl_2$, 37°C; •-• 10 mM $MgCl_2$, 30°C.

resulted in a 25% increase in rate, and an increase in the Mg^{2+} concentration resulted in the large rate enhancement (~3 fold) observed previously by Alberts and coworkers (14). Even under optimal conditions the rate of gene 32-promoted renaturation was at least an order of magnitude slower than that of the histone-promoted reaction. Second, and more importantly, the extent of the renaturation reaction did not depend on the concentration of gene 32 protein. Increasing the concentration by a factor of 2 (30°C, 10 mM $MgCl_2$) resulted in a 2.5 fold increase in the rate of the reaction, but in both cases 70% of the DNA was renatured (data not shown).

Another E. coli protein with many of the properties of histone H2A has recently been isolated and characterized. Designated H protein, it renatures DNA efficiently as measured by this assay, and shares with histones the cooperative nature of the reaction, the requirement for complementary strands, and the rapid reaction rate (15).

DISCUSSION

The novel finding reported here is that purified histones can promote

the renaturation of DNA. The reaction requires complementary single strands and the product remains S1 resistant after removal of the histones. Thus, the reaction represents true hybridization, and not the simple binding and protection of single strands from the action of S1 nuclease. Formation of nucleosomes is not required since each of the histones can promote the reaction independently.

The mechanism of histone promoted renaturation cannot be the same as the mechanism proposed for gene 32 protein and E. coli SSB. Histones, as well as other basic molecules such as polylysine, raise the melting temperature of DNA (16), while single-stranded DNA binding proteins lower it. Histones should thus stabilize secondary structure in single-stranded DNA, rather than melt it out.

The similarities between the histone and polylysine reactions suggest a simple and plausible mechanism which can explain our findings. Interaction between the histones or polylysine and single-stranded DNA should act to collapse the DNA into a complex, so that the DNA is restricted to a fraction of the total volume. The resulting decrease in entropy allows rapid nucleations to occur between single strands that have been brought into proximity. Nucleation could also occur between several adjacent DNA molecules, leading to the complex aggregate seen in the electron microscope.

Consistent with this model is the observation that D. melanogaster histone H1, under these conditions, precipitates single-stranded M13 DNA in a reaction that closely parallels its ability to renature DNA. Similar results have been obtained with calf thymus histone H2A (data not shown).

An effector for this reaction must be able to interact with and bind together two single strands of DNA. It has recently been demonstrated that histones will bind to single-stranded DNA (17). Bringing several DNA molecules together may involve histone-histone interactions. Under the conditions of ionic strength used in these experiments, histones H2A and H2B exist predominantly as dimers, while histones H3 and H4 form large ordered fibers (18). Of interest is the observation that the ability of individual histones to promote renaturation does not coincide with their ability to form fibers at low ionic strength. Histone H4, which forms fibers readily, is the least efficient histone in the renaturation reaction. Thus, if histone-histone interactions are involved, histone dimers would appear to be sufficient.

In the absence of any effector the rate determining step in DNA

renaturation is the nucleation event, which is diffusion controlled (19). Decreasing the entropy requirements of the reaction will accelerate this step, possibly to the point where another step, perhaps complex formation, could become rate limiting. The methods employed in this work were not sensitive enough to test this possibility.

Of the divalent metal ions tested, Zn^{2+} was unique in its ability to inhibit the histone-promoted renaturation reaction. Inasmuch as zinc also inhibited the polylysine-promoted reaction, this effect is not caused by a specific interaction between histones and zinc, but rather is a result of an effect on the DNA. The interaction of zinc with DNA is quantitatively and qualitatively different from the other metals examined in that Zn^{2+} can bind not only to the phosphate backbone of DNA, but can also form coordination complexes with the bases which can cross-link denatured DNA strands (20,21). It is therefore possible that under the conditions of our experiments zinc might cross-link bases within the same DNA molecule, converting it to a form incapable of annealing to a complementary strand and thus inhibiting renaturation.

The finding that histones can promote the renaturation of complementary single strands of DNA provides a functional assay for histones that should be useful in studies involving these and related proteins. As a method for detecting histones in crude extracts it provides a simple and rapid screen for potential histone containing fractions. The assay will probably detect not only histones, but a number of related proteins as well, and should be useful in the isolation of histone-like proteins. The method also has the potential to detect newly synthesized or stored histones which have not yet associated with DNA.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (GM 06196) and the National Science Foundation (PCM 74-00865). M. M. C. is a Postdoctoral Fellow of the American Cancer Society.

REFERENCES

1. Weinstock, G. M., McEntee, K., and Lehman, I. R. (1979) Proc. Nat. Acad. Sci. USA 76, 126-130.
2. Botstein, D. (1968) J. Mol. Biol. 34, 621-641.
3. Eschalier, G., and Ohanessian, A. (1970) In Vitro 6, 162-172.
4. Dolfini, S. (1971) Chromosoma 33, 196-208.
5. Rubin, G. M., and Hogness, D. S. (1975) Cell 6, 207-213.

6. Hancock, R., Faber, A. J., and Fakan, S., in Methods in Cell Biol., D. M. Prescott, ed (1977) XV, 127-147, Academic Press.
7. Germond, J.-E., Bellard, M., Oudet, P., and Chambon, P. (1976) Nuc. Acids Res. 3, 3173-3192.
8. Bradford, M. M. (1976) Anal. Biochem. 72, 248-254.
9. Davis. R. W., Simon, M., and Davidson, N. (1971) Methods Enzym. 21, 413-428.
10. Laemmli, J. K., and Favre, M. (1973) J. Mol. Biol. 80, 575-599.
11. Isenberg, I. (1979) Ann. Rev. Biochem. 48, 148-191.
12. Welch, S. L., and Cole, D. R. (1980) J. Biol. Chem. 255, 4516-4518.
13. Christiansen, C., and Baldwin, R. W. (1977) J. Mol. Biol. 115, 441-454.
14. Alberts, B., and Frey, L. (1970) Nature (London) 227, 1313-1318.
15. Hübscher, U., and Kornberg, A. (1980) Proc. Natl. Acad. Sci. USA, in press.
16. Huberman, J. A. (1973) Ann. Rev. Biochem. 72, 355-378.
17. Palter, K. B., and Alberts, B. M. (1979) J. Biol. Chem. 254, 11160-11169.
18. Sperling, R., and Amos, L. A. (1977) Proc. Natl. Acad. Sci. USA 74, 3772-3776, and references cited therein.
19. Wetmur, J., and Davidson, N. (1968) J. Mol. Biol. 15, 111-123.
20. Venner, H., and Zimmer, C. Biopolymers 4, 321-335.
21. Shin, Y. A., and Eichhorn, G. L. (1968) Biochem. 7, 1026-1032.