Elion, G. B. (1967), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26,* 898.
Greer, S. B. (1958), *J. Gen. Microbiol. 18,* 543.
Hall, Z. W., and Lehman, I. R. (1968), *J. Mol. Biol. 36,* 321.
Hershfield, M. S., and Nossal, N. G. (1972), *J. Biol. Chem. 247,* 3393.
Huberman, J. A., and Kornberg, A. (1970), *J. Biol. Chem. 245,* 5326.
Hurlbert, R. B., and Furlong, N. B. (1967), *Methods Enzymol. 12A,* 193.
Jovin, T. M., England, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244,* 2996.
Lehman, I. R., and Richardson, C. C. (1964), *J. Biol. Chem. 239,* 233.
Loeb, L. A. (1974), *Enzymes, 3rd Ed., 10,* 173.
Mildvan, A. S. (1974), *Annu. Rev. Biochem. 43,* 357.
Miller, E. C., and Miller, J. A. (1971), in Chemical Mutagens, Vol. 1, Hollaender, A., Ed., New York, N.Y., Plenum Press, p 83.
Mitzutani, S., and Temin, H. M. (1976), *Biochemistry 15,* 1510.
Moore, E. C., and LePage, G. A. (1958), *Cancer Res. 18,* 1075.
Muzcyzka, N., Poland, R. L., and Bessman, M. J. (1972), *J. Biol. Chem. 247,* 7116.
Orgel, A., and Orgel, L. E. (1965), *J. Mol. Biol. 14,* 453.
Paterson, A. R. P. (1959), *Can. J. Biochem. Physiol. 37,* 1011–1023.
Penn, I., and Starzl, T. E. (1972), *Transplantation 14,* 407.
Schachman, H. K., Adler, J., Radding, C. M., Lehman, I. R., and Kornberg, A. (1960), *J. Biol. Chem. 235,* 3242.
Schein, P. S., and Winokur, S. H. (1975), *Ann. Intern. Med. 82,* 84.
Sedwick, W. D., Wang, T. S.-F., and Korn, D. (1975), *J. Biol. Chem. 250,* 7045.
Setlow, P. (1974), *Methods Enzymol. 29,* 3.
Speck, W. T., and Rosenkranz, H. S. (1976), *Cancer Res. 36,* 108.
Speyer, J. F. (1965), *Biochem. Biophys. Res. Commun. 21,* 6.
Springgate, C., and Loeb, L. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 245.
Timmis, G. M., and Williams, D. C. (1967), in Chemotherapy of Cancer—the Antimetabolic Approach, London, Butterworths.
Trautner, T. A., Swartz, M. N., and Kornberg, A. (1962), *Proc. Natl. Acad. Sci. U.S.A. 48,* 449.
Wang, T. S.-F., Sedwick, W. D., and Korn, D. (1974), *J. Biol. Chem. 249,* 841.

Characterization of Chromatin Modified with Ethyl Acetimidate†

Lois O. Tack and Robert T. Simpson*

ABSTRACT: Thymus chromatin was extensively modified with ethyl acetimidate, substituting up to 90% of the lysyl residues of the histones while retaining the positive charge of the basic amino acid. Physicochemical and immunochemical characterization of this derivative chromatin indicates a high degree of retention of the native structure of the nucleoprotein even after extensive modification. The alterations which are detected are most simply interpreted as resulting from a weakening of the interactions of histone H1 with DNA in the modified chromatin. The near-native character of amidinated chromatin contrasts with the more extensive structural alterations observed in acetylated chromatin. Our data demonstrate the suitability of this reagent for mapping available lysyl residues in this and other nucleoproteins and suggest that the related bisimido esters may be reagents of choice for cross-linking of chromatin histones.

Selective chemical modification of proteins has been one of the most useful approaches for identification of functional amino acid residues in enzyme active sites, delineation of surface topography, and determination of residues involved in subunit interactions. More recently, this experimental approach has been utilized for study of the topography of nucleic acid-protein complexes, with its most notable success in terms of chromatin structure thus far being the detection of histone octamers in nucleosome core particles and the definition of specific histone-histone interactions using cross-linking reagents (Hyde and Walker, 1975; Chalkley, 1975; Chalkley and Hunter, 1975; Thomas and Kornberg, 1975a,b; Martinson and McCarthy, 1975; Bonner and Pollard, 1975; Van Lente et al., 1975; Martinson et al., 1976). In both cross-linking studies and in studies of the relative reactivities of lysyl residues of histones when in the chromatin complex, the structural integrity of the modified species is of primary importance. Alterations in protein-protein or protein-nucleic acid interactions consequent to chemical modification may alter the reactivity of portions of the protein affected by the conformational changes. This is of particular concern, since many of the cross-linking reactions modify lysyl residues, which are expected to be a stabilizing force in histone-DNA interactions. Many of the chemical probes used in the past to study the structure of histones alone or in chromatin have resulted in the size, polarity, and/or charge of the modified residue being drastically altered, making it likely that extensive modification resulted in altered interactions among the constituents of chromatin. In the one case where structural characterization of a modified chromatin was performed, acetylation of chromatin lysyl residues led to definite changes in melting profile, consistent with destabili-

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zation of the complex (Simpson, 1971). Acetylation slightly increases the size of lysine, but more importantly removes the positive charge of the ϵ-amino group. Far more striking effects on chromatin structure are produced by other modifications. Thus, when chromatin core particles are cross-linked with dithiobis(succinimidyl)propionate, the melting profile of the modified nucleoprotein shows no stabilization of DNA structure by the modified histones (A. Stein, unpublished observations). We show here that ethyl acetimidate can modify 90% of the lysyl residues in chromatin while at the same time producing only minor changes in structural properties of the nucleoprotein.

Experimental Section

Preparation of Chromatin and Histones. Chromatin was isolated from frozen calf or rabbit thymus (Pel-Freez Biologicals) using Na_2EDTA[1]-saline buffers (Zubay and Doty, 1959). The protein to DNA mass ratio of these preparations was 1.35–1.65:1. Chromatin was also prepared from Triton X-100 washed nuclei from rabbit thymus or liver as previously described (Simpson and Sober, 1970). DNA concentrations were estimated using $A_{260}^{0.1\%} = 20$ and protein concentration was determined by the Lowry assay (Layne, 1957). Moles of lysine and arginine per A_{260} unit were calculated from amino acid analyses. H1-depleted chromatin was prepared by the method of Oudet et al. (1975) using 0.7 M NaCl extraction and centrifugation through a glycerol step gradient. All buffers used for this procedure contained 5 mM $NaHSO_3$ to inhibit proteolysis.

Total histones were isolated from calf thymus chromatin by extraction with 0.4 N H_2SO_4 at 4 °C, dialysis against water, and lyophilization. The lysine-rich histone, H1, was isolated at >95% purity (as measured by gel electrophoresis) by 5% trichloroacetic acid extraction of chromatin (Denooij and Westenbrink, 1962).

Chemical Modification of Chromatin and Histones. After dialysis against 0.25 mM EDTA, pH 7.0, for 48 h, chromatin was suspended at 1 mg of DNA/mL in 0.05 M sodium borate (pH 8–9) with stirring on ice. Ethyl acetimidate hydrochloride (Eastman) was partially neutralized with 1 N KOH and then quickly added to the solution with vigorous stirring (Wofsy and Singer, 1963). The final concentrations of reagent used were varying molar excesses of reagent to total lysine content. The reaction was allowed to proceed for 2 h and then dialyzed against 0.25 mM Na_2EDTA at 4 °C with several changes. The extent of amidination was determined by the TNBS assay and amino acid analysis. Amidination of histones was performed identically to that of chromatin, except for slight differences as noted in Figure 1.

Acetylation of chromatin was performed by addition of a fourfold excess of acetic anhydride (Ac_2O to DNA base pairs) to chromatin at a DNA concentration of 1 mg/mL in 0.05 M borate, pH 9.0, with vigorous stirring for 30 min at 4 °C. The reaction mixture was dialyzed against 0.25 mM Na_2EDTA and assayed for extent of modification using the TNBS procedure. Modification of 75–80% of the total chromatin lysines was consistently found at this molar excess.

Analytical Methods. Prior to gel electrophoresis, samples were solubilized in 2% $NaDodSO_4$ and dialyzed overnight into sample buffer. Discontinuous $NaDodSO_4$ slab gel electrophoresis (LeStourgeon and Rusch, 1973) was performed using a 15% polyacrylamide running gel (acrylamide–bisacrylamide ratio 45:0.4) with a stacking gel of 3% acrylamide–0.08% bisacrylamide. Gels were stained in 0.25% Coomassie blue (Sigma) in 50% methanol–7% acetic acid for 1 h and destained in 20% methanol–7% acetic acid. Samples were analyzed throughout the isolation and modification procedures to monitor the integrity of the chromatin preparations.

To study the extraction of modified histones by various NaCl solutions, amidinated chromatin (30, 80, and 95% amidinated) was extracted with 0.15 and 0.35 M NaCl. The suspensions were spun at 15 000 rpm for 15 min and the supernatants (after dialysis and concentration) and pellets were analyzed by $NaDodSO_4$ gel electrophoresis. Unmodified chromatin was treated in the same manner for comparison.

The TNBS assay for unmodified lysyl residues was performed using TNBS from Pierce Chemical Co. following the method of Habeeb (1966), using a molar extinction coefficient of 1.1×10^4 M^{-1} cm^{-1} at 345 nm. Unmodified chromatin or histone was included as a control in all assays and the results for all amidinated samples are expressed relative to the control. These values were compared to those calculated using a theoretical molar lysine value from sequence data and were found to be within 90 to 95% agreement. Amino acid analyses were also used to measure lysine and amidinated lysine content and compared to TNBS values. The assay is linear to 1 mg of total sample with $A_{345} = 1.6$.

To determine the amount of ϵ-amidinated lysine, quantitative amino acid analysis was performed using an *o*-phthalaldehyde fluorescent detection system on a modified JEOL 5AH analyzer. Hydrolyses were performed under nitrogen in 6 N HCl, 0.1% phenol, 0.1% β-mercaptoethanol at 110 °C. Amidinated lysine is relatively stable to acid hydrolysis with a first-order hydrolysis rate (similar to tyrosine, threonine, serine, and cysteine destruction) resulting in the generation of free lysine (Reynolds, 1968; Plapp and Kim, 1974). Samples were hydrolyzed for different times (usually 20 and 70 h) and the amount of amidinated lysine at zero time was determined by extrapolation. The color value for arginine was used to quantitate the amount of ϵ-acetimidyllysine (Plapp and Kim, 1974). A Durrum single-column resin system was used with standard buffers, resulting in ϵ-modified lysine eluting between ammonia and arginine. The sum of lysine and ϵ-acetimidyllysine in the different hydrolysates equaled the number of lysines in unmodified chromatin, also quantitated by amino acid analyses.

Physical Methods. Thermal melting curves were made on samples in 0.25 mM Na_2EDTA with $A_{260} = 0.25$ using an Acta III spectrophotometer over a temperature range of 35–95 °C at an increment of +0.33 °C/min. A solvent baseline was subtracted. Circular dichroism was measured on a Cary 61 polarimeter using samples with $A_{260} = 0.9$ in 0.25 mM Na_2EDTA in a 1-cm cell. Samples were spun at 10 000 rpm to remove any aggregated material (<3%).

Viscosity measurements were made using both a modified three-bulb low-shear Ostwald capillary viscometer (Union Carbide Corp., Nuclear Division) and a Cannon-Ubbelholde four-bulb shear-dilution viscometer (Cannon Instrument Co.) as described by Eigner (1968) with shear gradients of 26, 68, and 120 s^{-1} for the first and a range of 40–300 s^{-1} for the second. Since the Cannon viscometer was independent of sample volume and gave slightly more reproducible values with greater precision than the Ostwald type, all data given are from its use, although both viscometers gave results with ±10%. Measurements were made on four separate sheared-chromatin preparations at 20.0 °C in 0.25 mM Na_2EDTA. Each preparation was amidinated to the extent of 50 and 90% with un-

[1] Abbreviations used are: Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; TNBS, 2,4,6-trinitrobenzenesulfonic acid; $NaDodSO_4$, sodium dodecyl sulfate; Tris-Cl, 2-amino-2-hydroxymethyl-1,3-propanediol chloride; BSA, bovine serum albumin.

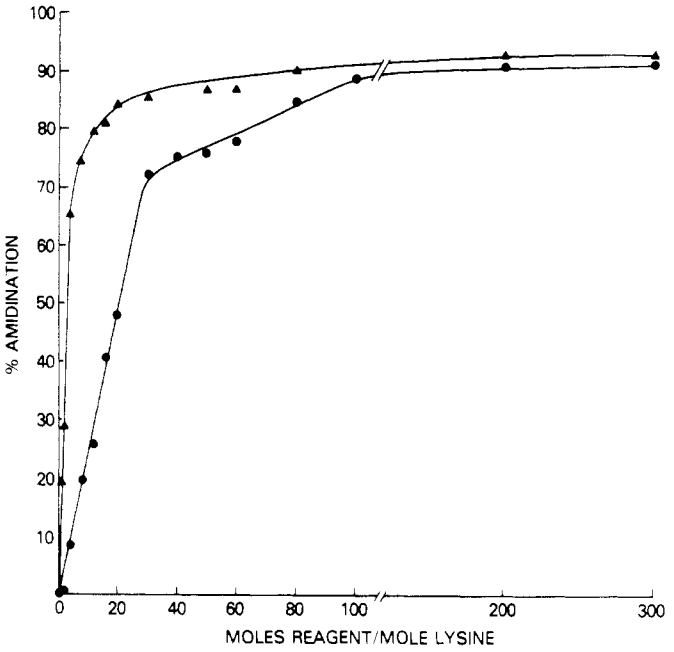

FIGURE 1: Measurement of the reactivity of histone lysyl residues with increasing molar excesses of ethyl acetimidate. Calf thymus histones (▲) at 1 mg/mL protein and thymus chromatin (●) at 1 mg/mL DNA in 0.05 M borate, pH 9.0, were reacted with the imido ester at 4 °C. Unreacted lysyl residues were measured using the TNBS assay.

modified chromatin as a control. The concentration range was from 0.01 to 0.1 mg of DNA/mL and all calculations are in terms of DNA mass only.

Nuclease Digestion. Micrococcal nuclease (Worthington) digestion of amidinated chromatin was carried out using 200 units of nuclease/mL in 1 mM Tris-Cl, pH 8, 2 mM $CaCl_2$ at 37 °C at a DNA concentration of 10 A_{260}/mL (Whitlock and Simpson, 1976). Acetylated chromatin and unmodified chromatin were used as controls. The increase in 0.6 N perchloric acid soluble A_{260} units with time was measured. DNase I (Worthington) digests of amidinated and control chromatin were carried out in 5 mM Tris-Cl, pH 8, 10 mM $MgCl_2$ at 37 °C with 200 units/mL. Aliquots of 100 μg of DNA were removed at 1, 2, 4, 8, and 16 min and made to 1% $NaDodSO_4$, 10 mM Na_2EDTA, 0.1 M Tris-Cl, pH 8. Following extraction at room temperature with an equal volume of buffer-saturated phenol for 30 min, the aqueous phase was adjusted with NaCl to 0.1 M and precipitated by adding 2 volumes of ethanol and storing at −20 °C overnight. The DNA was spun down and dissolved in electrophoresis buffer. The DNA was analyzed on a 6% slab gel containing 6 M urea according to the method of Peacock and Dingman (1967). After staining overnight in 0.005% stains-all (Eastman) in 50% (v/v) formamide, the gels were destained in running water and photographed.

Immunospecificity of Amidinated Chromatin. The antigenicity of amidinated and control H1 was compared using quantitative microcomplement fixation (Wasserman and Levine, 1961; Stollar and Ward, 1970). H1 was obtained from calf thymus chromatin as described earlier. H1 was amidinated using a 200-fold excess of ethyl acetimidate in 0.05 M sodium borate, pH 9, stirring for 2 h at 4 °C. The reaction mixture was dialyzed against water and lyophilized. Anti-H1 was a gift of Dr. M. Bustin.

Specificity of binding of anti-H1 sera to amidinated chromatin was tested by using the modified chromatin as an immunoadsorbant (Bustin, 1973; Goldblatt and Bustin, 1975). After incubating the control or modified chromatin with anti-H1 sera, the chromatin-bound antibody was precipitated with 0.14 M NaCl. The remaining unadsorbed antibody was tested against unmodified H1, the homologous antigen. Specifically, the immunoadsorption assay was performed in the following manner: to 50 μg of chromatin in 50 μL of 0.25 mM Na_2EDTA is added antisera at a 240-fold higher concentration than that of the final assay mixture. Sufficient 1 mM Tris-Cl, pH 8, is added to make a final volume of 0.25 mL. The reaction mixture is incubated at 37 °C for 30 min and at 4 °C with gentle shaking for 4 h. Concentrated NaCl (5 M) is added to make the final concentration 0.14 M and the precipitate is spun down. The supernatant is diluted 200-fold with iso-BSA (0.01 M Tris-Cl, pH 7.45, 0.14 M NaCl, 0.5 mM $MgSO_4$, 0.15 mM $CaCl_2$, and 1 mg/mL bovine serum albumin) containing the appropriate amount of complement. The microcomplement fixation assay is performed after adding 1 mL of diluted supernatant to 0.2 mL of antigen.

Results

Amidination of Histones and Chromatin. Calf and rabbit thymus were chosen as sources for chromatin because of their low content of nonhistone chromosomal proteins, ensuring that the structural effects of the modification are due primarily to histone modification. In limited studies with chromatin from rabbit liver, results similar to those detailed here for thymus chromatin were obtained. Chromatin preparations were made by the Zubay and Doty (1959) procedure, where all isolation steps are carried out in buffers of ionic strength 0.15, since shearing precipitated chromatin preparations at this ionic strength does not lead to redistribution of nucleosome cores (Woodhead and Johns, 1976), in contrast to shear-induced damage at low ionic strength (Noll et al., 1975).

Isolated histones and chromatin in solution were reacted with various molar excesses (1- to 300-fold) of the imido ester, ethyl acetimidate (Figure 1). The modification of isolated histones has an essentially monophasic character, with over 80% of the total lysyl residues modified at low excesses (<20-fold) of reagent, and a remaining 10% of residues modified only at much higher excesses. A small fraction (<10%) of the lysyl residues of histones are not reactive with ethyl acetimidate in aqueous solutions even at high excesses. Unsheared chromatin was used for the amidination shown in Figure 1; the chromatin was gently suspended in water and dialyzed into 0.05 M sodium borate, pH 9.0, for modification. Qualitatively, the amidination of chromatin resembles that of isolated histones, but with more of a biphasic curve of degree of modification vs. reagent excess. Also, the reactivity of lysyl residues of histones in chromatin is quantitatively different, the curve being shifted to higher molar excesses. Approximately 70% of histone lysyl residues were modified at a 30- to 40-fold molar excess of ethyl acetimidate, while maximal amidination (90–95% of total lysine) was achieved at 100- to 300-fold molar excess.

The difference in reactivity between chromatin and histones might be due to the viscosity of the unsheared chromatin solution, slowing the reaction by decreasing diffusion of the reactants. Sheared chromatin (Waring blender, 60 V, 60 s, DNA concentration of 1 mg/mL in 1 mM Tris-Cl, pH 8.0, 4 °C) was amidinated and gave an identical curve of degree of modification as a function of reagent excess to that shown for unsheared chromatin in Figure 1. This suggests that viscosity effects on the modification are not being detected; rather, the differences in reactivity for free histones and chromatin histones are related to histone–histone or histone–DNA interactions in the complex.

The quantitation of chromatin modification (Figure 1) was substantiated by TNBS assay on histones extracted by 0.4 N

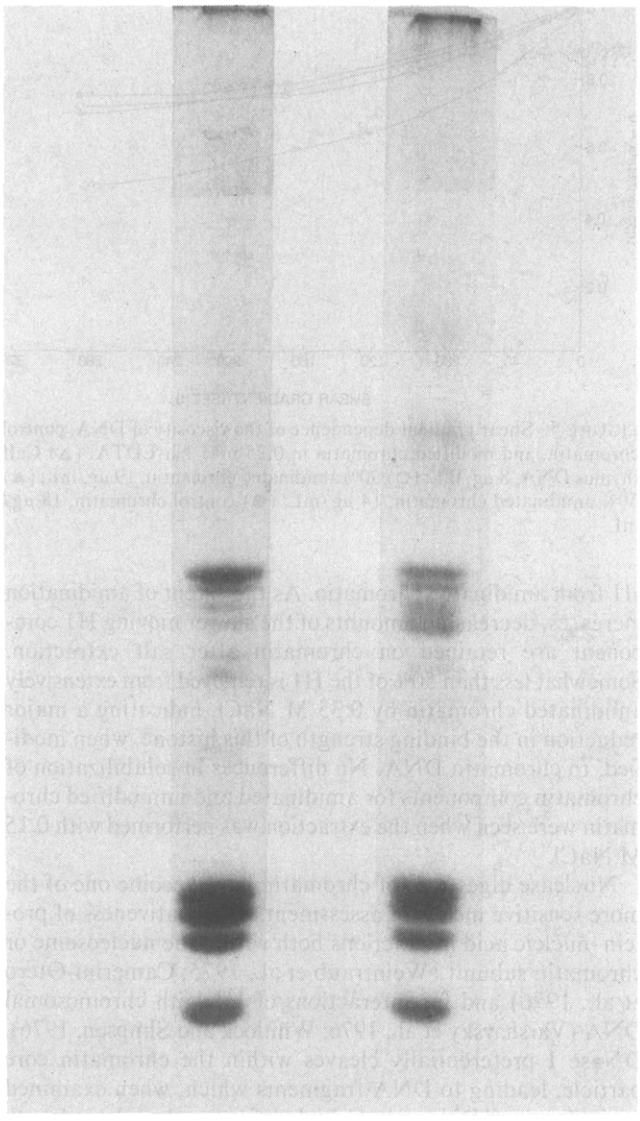

FIGURE 2: Histones of unmodified (left track) and 90% amidinated (right track) chromatins. $NaDodSO_4$ gel electrophoresis was carried out as described in the Experimental Section. From the bottom, histones are H4, H2A, H2B, H3, and H1.

H_2SO_4 from amidinated chromatin. Amino acid analysis of samples hydrolyzed for different times and extrapolated to zero time of hydrolysis provided for direct quantitation of ϵ-amidinated lysine content in modified chromatin. The degree of modification determined by amino acid analysis agreed well with that determined by colorimetric assay. The time course of loss of ϵ-acetimidyllysine on acid hydrolysis was determined on three separate chromatin samples amidinated at 300-fold excess. The loss is first order with a half-life of about 80 h and allows extrapolation to zero time of hydrolysis for quantitation of the degree of modification.

Amidinated chromatin histones generally have the same relative mobility on sodium dodecyl sulfate gel electrophoresis as unmodified histones (Figure 2). Only the lysine-rich histone H1 is altered—a doublet being seen for the amidinated species where there was a single major band for the parent species. In some preparations low levels of putative cross-linked products (less than 5% of total protein) are seen, corresponding to possible dimers, trimers, and tetramers of one or more histone species. A side reaction observed by Browne and Kent (1975a) during amidination reactions at pHs of 9 or less is probably responsible for these higher-order products.

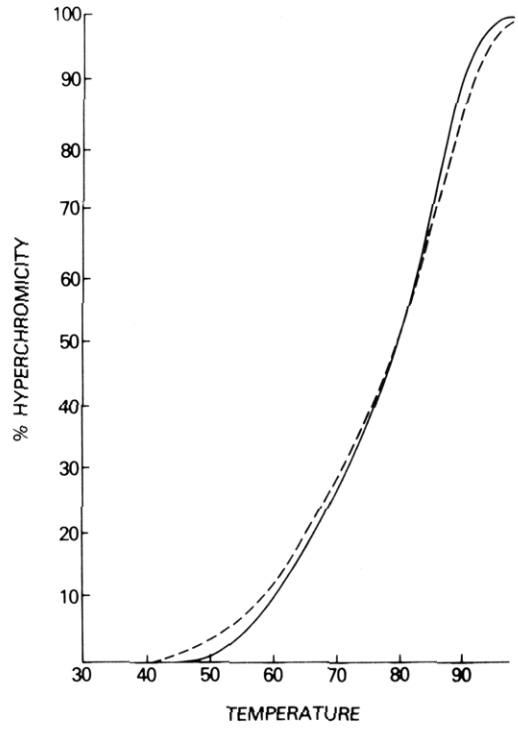

FIGURE 3: Thermal denaturation of unmodified chromatin (—) and 90% amidinated chromatin (- - -). Measurements were made in 0.25 mM Na_2EDTA at about 15 μg/mL of DNA.

Physiochemical Properties of Amidinated Chromatin. Figure 3 shows the thermal melting curve for control and amidinated chromatin samples. Compared to control samples, chromatin amidinated up to 80% of total lysines modified (not shown) had an identical melting profile at lower temperatures but showed an increase in melting temperature of about 1 °C on the average at greater than 80 °C. Samples amidinated to 90–95% modification had a slight destabilization of about 1–2 °C for the early phase of the thermal transition and the same stabilization seen in less extensively amidinated samples at higher temperatures; the differences were slight but reproducible. Comparison of these data with the thermal melting profile of acetylated chromatin (Simpson, 1971), where the overall melting curve was destabilized approximately 4–5 °C, demonstrates that extensive amidination of chromatin leads to minimal alterations in the stabilization of DNA structure by histones.

Figure 4 shows circular dichroism spectra which again demonstrate only minor differences between control chromatin, 50% amidinated chromatin, and 90% amidinated chromatin. The circular dichroism spectra of all the chromatin samples were similar to those previously reported (Shih and Fasman, 1970; Simpson and Sober, 1970) with a split positive maximum in the 260–300 nm region, molar ellipticity, $[\theta]_{275}$, of +4500°, and $[\theta]_{222}$ of −37 000°, based on DNA nucleotide residue concentration. It is known that the lysine-rich histone, H1, may be removed without affecting the circular dichroism spectrum of chromatin above 260 nm (Simpson and Sober, 1970; Hjelm and Huang, 1974) so it is possible that H1–DNA interactions might be altered without being detectable by this technique. No evidence is seen for the generation of free DNA stretches.

The viscometric properties of control and various amidinated chromatins were compared to evaluate possible shape changes in the nucleoprotein complex following chemical modification. The viscosity of chromatin is shear dependent and thus all

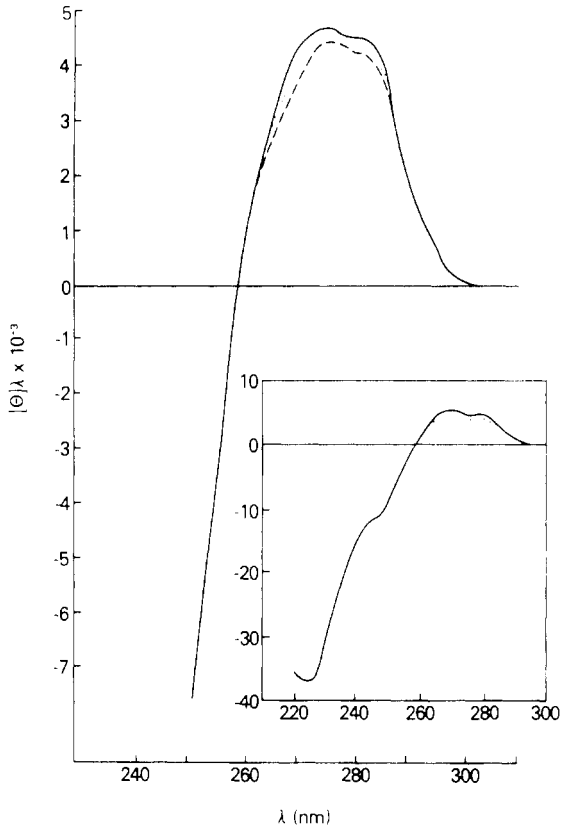

FIGURE 4: Circular dichroism spectra of control chromatin (···), 50% amidinated chromatin (—), and 90% amidinated chromatin (- - -).

specific viscosities were extrapolated to zero shear. Free DNA has a much higher dependence of viscosity on shear rate than chromatin and amidinated chromatin was found to have a sensitivity to shear rate similar to that of unmodified chromatin (Figure 5).

We determined the dependence of relative viscosity on concentration for unmodified and amidinated chromatins; the intrinsic viscosity, $[\eta]$, of unmodified chromatin is 19 dL/g, in good agreement with Hensen and Walker (1970) and slightly higher than that of Simpson (1972) of 13 dL/g for sheared chromatin. The intrinsic viscosity for 50% amidinated chromatin was found to be 25 dL/g (not shown) and that for 90% amidinated chromatin was 31 dL/g. Since extrapolation to zero shear gave specific viscosity values calculated in the region most sensitive to shear, comparisons were also made at different shear values; the same relative differences were always found. The viscosity of amidinated chromatin shows the same concentration dependence as that of control chromatin.

Thus, amidination of chromatin alters the shape of the nucleoprotein, evidenced by an increase in specific viscosity from 19 dL/g for control samples to 31 dL/g at maximum modification. This difference is comparable to that found on removing H1 from chromatin (Hensen and Walker, 1970) or after mild tryptic digestion of chromatin (Simpson, 1972). Electrophoresis of the proteins of amidinated chromatin (Figure 2) demonstrates that H1 is still present; the possibility remains that altered binding of this lysine-rich histone to DNA may be the cause of the increased specific viscosity of amidinated compared to unmodified chromatin.

Protein–Nucleic Acid Interactions in Amidinated Chromatin. The stability of H1 binding to DNA in amidinated chromatin was assessed by salt extraction. NaCl concentrations of 0.35 M remove no histone from control chromatin. In contrast, such salt concentrations remove significant portions of

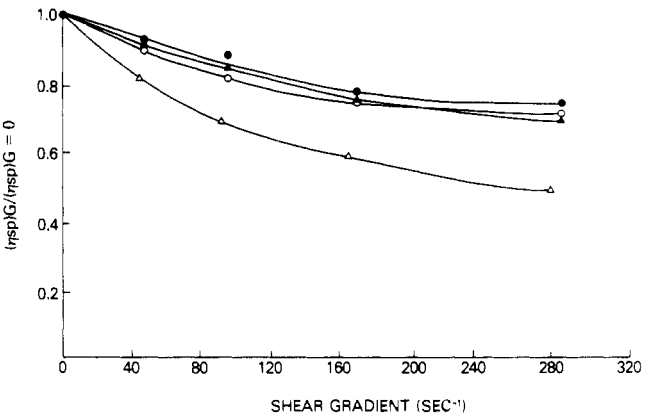

FIGURE 5: Shear gradient dependence of the viscosity of DNA, control chromatin, and modified chromatin in 0.25 mM Na_2EDTA. (▲) Calf thymus DNA, 8 μg/mL; (○) 90% amidinated chromatin, 19 μg/mL; (▲) 50% amidinated chromatin, 14 μg/mL; (●) control chromatin, 18 μg/mL.

H1 from amidinated chromatin. As the extent of amidination increases, decreasing amounts of the slower moving H1 component are retained on chromatin after salt extraction. Somewhat less than 50% of the H1 is removed from extensively amidinated chromatin by 0.35 M NaCl, indicating a major reduction in the binding strength of this histone, when modified, to chromatin DNA. No differences in solubilization of chromatin components for amidinated and unmodified chromatin were seen when the extraction was performed with 0.15 M NaCl.

Nuclease digestions of chromatin have become one of the more sensitive means of assessment of the nativeness of protein–nucleic acid interactions both within the nucleosome or chromatin subunit (Weintraub et al., 1975; Camerini-Otero et al., 1976) and for interactions of H1 with chromosomal DNA (Varshavsky et al., 1976; Whitlock and Simpson, 1976). DNase I preferentially cleaves within the chromatin core particle, leading to DNA fragments which, when examined as single-strand DNA, are multiples of ten nucleotides in length (Noll, 1974). Partial DNase I digests were performed on unmodified, 50 and 90% amidinated chromatins and DNA samples were analyzed by polyacrylamide gel electrophoresis under denaturing conditions. Typical patterns of ten-nucleotide multiples from 30–200 bases were seen for all samples. No differences were seen in the pattern of DNA fragments generated between control and amidinated chromations (data not shown). Such data strongly suggest integrity of the interactions of the four smaller histones with themselves and with DNA within the core particle of chromatin. Alternations in interaction of H1 with DNA would not be detected by DNase I digestion, since this histone probably binds to the bridge or spacer DNA between particles (Shaw et al., 1976; Varshavsky et al., 1976; Whitlock and Simpson, 1976).

Removal of histone H1 and some nonhistone chromosomal proteins from HeLa cell chromatin by salt extraction leads to a marked increase in the initial rate of digestion by micrococcal nuclease without alternation in the proportion of the DNA acid soluble in a limit digest with micrococcal nuclease (Whitlock and Simpson, 1976). Figure 6 shows the kinetics of micrococcal nuclease digestion of unmodified chromatin, amidinated chromatin, and acetylated chromatin modified to the same degree of lysyl substitution as the amidinated sample. Amidinated chromatin was digested with an initial rate 1.3 times faster than unmodified samples and attained a plateau at 50% acid-soluble nucleotides after 30 min of digestion. Essentially no difference was observed between chromatins amidinated

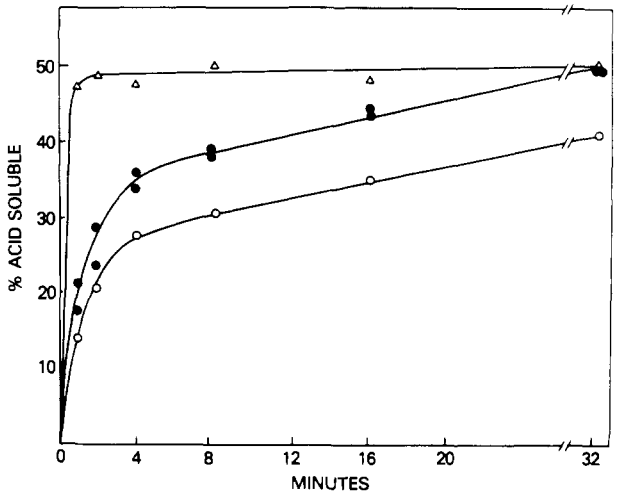

FIGURE 6: Kinetics of digestion of native and modified chromatins. Chromatin at 10 A_{260} units/mL in 1 mM Tris-Cl, pH 8, 2 mM $CaCl_2$ was digested with micrococcal nuclease (200 units/mL) at 37 °C for the times indicated. The increase in A_{260} soluble in 0.6 M perchloric acid-1% Na-DodSO_4 was measured. (△) Acetylated chromatin; (●) amidinated chromatin; (○) control chromatin.

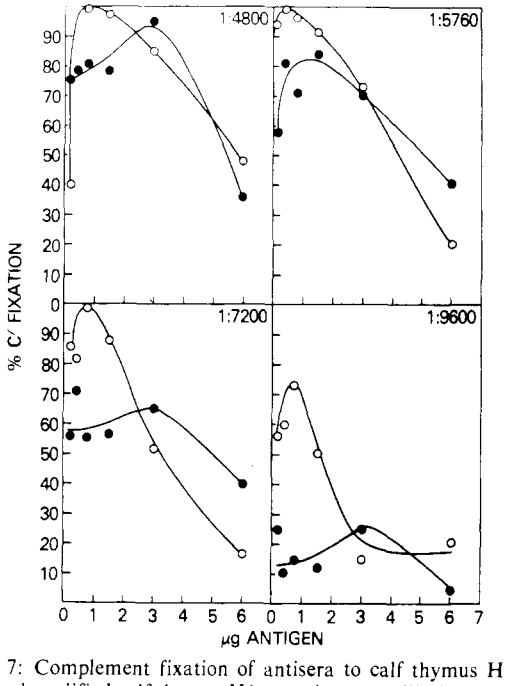

FIGURE 7: Complement fixation of antisera to calf thymus H1 with normal and modified calf thymus H1 at various sera dilutions. (●) 90% amidinated H1; (○) native H1.

to 50 and 90% of total lysines substituted. Acetylated chromatin was digested with an initial rate that is four to six times faster than the control, although it too attained a limit digest level of about 50% acid solubility. The rate changes attending acetylation of chromatin are similar to those observed for chromatin from which H1 has been completely removed. Amidinated chromatin clearly responds to this probe in a fashion more similar to native chromatin than acetylated; the slight increase in initial rate of digestion of amidinated chromatin may be due to a loosening of H1 binding to spacer DNA allowing an increased rate of digestion of chromatin into subunit particles.

Immunochemical Studies of Amidinated H1 and Chromatin. Specific antibodies are sensitive to slight changes in the structure of their corresponding antigens. Thus, maleylation of only two or three lysines per H1 molecule significantly decreases complement fixation with anti-H1 (Burnette et al., 1973). More extensive maleylation (>10 lysines/H1 molecule) resulted in complete loss of complement fixation ability.

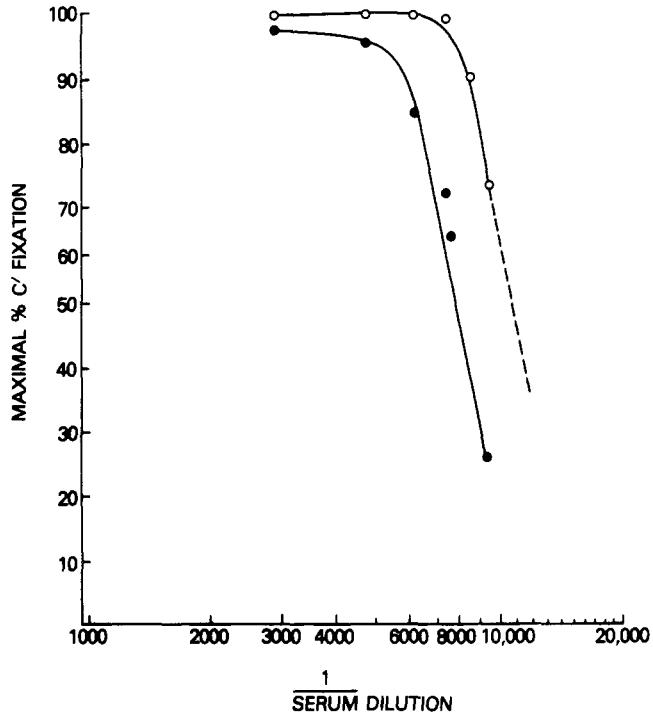

FIGURE 8: Maximal complement fixation of antisera to calf thymus H1 with normal and modified calf thymus H1 at various sera dilutions. Data were obtained from Figure 7. (●) 90% amidinated H1; (○) normal H1.

These data seem to indicate marked conformational lability for H1 after modification with reagents that affect the charge integrity of lysine. It was thus of interest to determine immunochemically the effect of amidination on H1 structure. Accordingly, the immunologic reactivities of amidinated and control H1 were compared. Both H1 antigens were Cl_3AcOH extracted and treated in the same manner. As shown in Figure 7, the complement fixation assay over a wide range of sera dilutions indicate that the solution conformation of amidinated H1 is essentially intact. The immunological relatedness of modified H1 and control H1 (Figure 8) is more similar than the two most closely related subfractions of H1 (Bustin and Stollar, 1972); the index of dissimilarity (given as the ratio of sera dilution resulting in 50% complement fixation for the two samples) for H1-IV to H1-V is 1.5; that for amidinated H1 to H1 is 1.35.

The results of immunoadsorption of amidinated chromatin with anti-H1 sera are shown in Figure 9. Using a serum dilution of 1:10, 200 with 50 μg of amidinated chromatin, approximately 40% or more of the antibody bound by 50 μg of unmodified chromatin was bound by amidinated chromatin. H1-depleted chromatin and 75% acetylated chromatin showed no binding of anti-H1 at this serum dilution. Thus, it appears H1 of highly amidinated chromatin still retains a significant amount of reactivity toward anti-H1. This supports other experimental results, demonstrating that highly amidinated chromatin retains its structural integrity with only slight perturbations due to components (i.e., H1) most susceptible to lysine-specific reagents.

Discussion

Imido esters have been used extensively for structural studies of a variety of proteins (see review by Hunter and Ludwig,

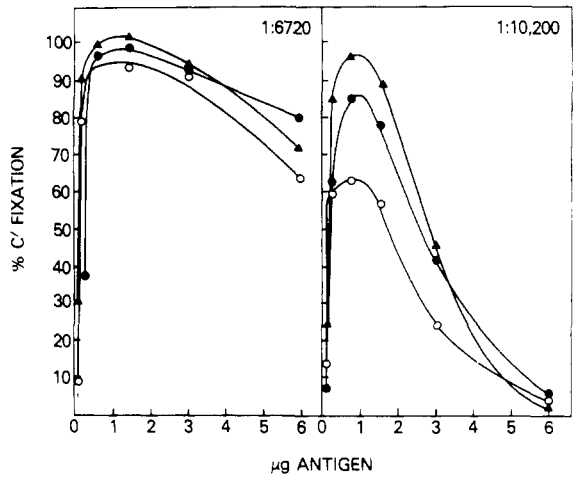

FIGURE 9: Effect of modification of chromatin on its ability to bind anti-H1 sera. (▲) Control reaction between H1 and anti-H1 sera not adsorbed on chromatin; (●) reaction between H1 and anti-H1 sera adsorbed on 50 μg of 90% amidinated chromatin; (○) reaction between H1 and anti-H1 sera adsorbed on 50 μg of unmodified chromatin. Adsorbing anti-H1 sera on 50 μg of 75% acetylated chromatin or H1-depleted chromatin gave the same curve as the control (▲).

1972). A particularly elegant use of these compounds was the labeling of the amino terminus of protamine with a mercapto-containing imido ester which could then be complexed with mercury for subsequent electron microscopic localization (Ottensmeyer et al., 1975). Bifunctional imido esters (Wold, 1967) have been used with great success for cross-linking reactive amino groups in investigation of the subunit structures of proteins (Davies and Stark, 1970). In the study of the organization of nucleoproteins, cross-linked imidinates have been useful in probing ribosomal protein structures (Slobin, 1972; Bickle et al., 1972; Clegg and Hayes, 1974), especially using cleavable cross-linking reagents (Sun et al., 1974; Peretz et al., 1976). In previous studies with chromatin, cross-linking with bisimido esters has aided in delineation of histone–histone interactions in solution (Kornberg and Thomas, 1974; Weintraub et al., 1975) and in chromatin (Thomas and Kornberg, 1975a,b), leading to proposal of a histone octamer as the protein core of the chromatin subunit particle.

Amidination is a gentle and specific modification, leading to retention of the charge of the modified lysyl residue. By variation in the substituents of the imido ester various features may be built into the modifying agent; in the current case acetamidination of lysyl residues leads to products whose size and chemistry closely resemble those of arginyl residues. Physiochemical and functional studies of a variety of proteins modified to varying degrees of lysyl substitution with imido esters have, in general, indicated a high degree of retention of native structure.

It is of major importance to know whether amidination of such a complex structure as chromatin would result in the same conservation of structure as found for simpler systems. Conservation of structure is important in cross-linking studies; cross-linking of amino groups is a two-step reaction and if conformational changes occur following the primary modification, the artifacts generated would greatly affect proximity assignments. In addition, the relative reactivities of histone lysyl residues in chromatin have been assessed with ethyl acetimidate, quantitating the amidinated lysines with respect to sequence location and extent of reactivity, in hopes of assigning accessibility values to different regions of histone molecules (L. O. Tack and R. T. Simpson, manuscript in preparation). It is important to demonstrate that modification with the chemical probe in both of these cases does not lead to structural changes in chromatin which could affect reactivities.

Overall, the results of this study seem to indicate that amidination leads to retention of the integrity of histone–DNA interactions for the four smaller histones with some alteration in interactions of H1 with chromatin DNA. Thus, circular dichroism spectra of native and amidinated chromatins are nearly identical, thermal denaturation profiles are very nearly so, limit levels of digestibility of DNA by micrococcal nuclease are the same, and DNase I cleaved single-stranded DNA fragments are the same for control and experimental samples. Taken together, these data provide rather strong evidence for the integrity of nucleosome core particle structure in amidinated chromatin. On the other hand, several lines of evidence suggest that H1–DNA interactions have been altered in amidinated chromatin. Amidinated H1 is extracted by NaCl concentrations which are without effect on control chromatin H1 content. Rates of micrococcal nuclease digestion of amidinated chromatin are higher than for the control samples, as is seen on dissociation of H1 from chromatin by salt and subsequent digestion (Whitlock and Simpson, 1976). Increased viscosity of amidinated chromatin is also consistent with relaxation of the interaction of H1 with DNA, since H1 is thought to function in the condensation of chromatin. Finally, although the solution conformation of amidinated and native H1 are closely related, as measured immunochemically, the antigenicity of amidinated H1 bound to DNA in the nucleoprotein complex differs more markedly from that of the native material, consistent with an alteration in the spatial relationships between H1 and DNA due to the chemical modification. Because of its high lysine content (about 27% of the molecule) and low content of other basic amino acids, it is not surprising that extensive amidination has its greatest effect on H1–DNA interactions. The apparent change in structure of H1–DNA in chromatin after amidination suggests care in interpretation of cross-linking experiments with bifunctional imido esters involving H1.

In conclusion, amidination appears to be a valuable approach in the study of chromatin structure. Large molar excesses may be used to extensively modify chromatin components with barely detectable changes in the structure of the core particle of the chromosome. It is conceivable that chromatin could be fractionated into its individual components, one or more of these modified with an appropriate imido ester conjugated probe, and then reconstituted to form a modified DNA–protein complex for further structural and functional studies.

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References

Bickle, T. A., Hershey, J. W. B., and Traut, R. R. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69*, 1327–1331.

Bonner, W., and Pollard, H. (1975), *Biochem. Biophys. Res. Commun. 64*, 282–288.

Browne, D. T., and Kent, S. B. (1975a), *Biochem. Biophys. Res. Commun. 67*, 126–132.

Browne, D. T., and Kent, S. B. (1975b), *Biochem. Biophys. Res. Commun. 67*, 133–138.

Burnotte, J., Stollar, B. D., and Fasman, G. D. (1973), *Arch.*

Biochem. Biophys. 155, 428–435.
Bustin, M. (1973), *Nature (London), New Biol. 245,* 207–209.
Bustin, M., and Stollar, B. D. (1973), *J. Biol. Chem. 248,* 3506–3510.
Camerini-Otero, R. D., Sollner-Webb, B., and Felsenfeld, G. (1976), *Cell 8,* 333–347.
Chalkley, R. (1975), *Biochem. Biophys. Res. Commun. 64,* 587–594.
Chalkley, R., and Hunter, C. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1304–1308.
Clegg, C., and Hayes, D. (1974), *Eur. J. Biochem. 42,* 21–28.
Davies, G. E., and Stark, G. R. (1970), *Proc. Natl. Acad. Sci. U.S.A. 66,* 651–656.
DeNooij, E. H., and Westenbrink, H. G. K. (1962), *Biochim. Biophys. Acta 62,* 608–609.
Eigner, J. (1968), *Methods Enzymol. 12B,* 386–429.
Goldblatt, D., and Bustin, M. (1975), *Biochemistry 14,* 1689–1695.
Habeeb, A. F. S. A. (1966), *Anal. Biochem. 14,* 328–336.
Hensen, P., and Walker, I. O. (1970), *Eur. J. Biochem. 14,* 345–350.
Hjelm, R. P., Jr., and Huang, R. C. C. (1974), *Biochemistry 13,* 5275–5283.
Hunter, M. J., and Ludwig, M. L. (1972), *Methods Enzymol. 25,* 585–596.
Hyde, J. E., and Walker, I. O. (1975), *FEBS Lett. 50,* 150–154.
Kornberg, R. D., and Thomas, J. O. (1974), *Science 184,* 865–868.
Layne, E. (1957), *Methods Enzymol. 3,* 447–454.
LeStourgeon, W. M., and Rusch, H. P. (1973), *Arch. Biochem. Biophys. 155,* 144–158.
Martinson, H. G., and McCarthy, B. J. (1975), *Biochemistry 14,* 1073–1078.
Martinson, H. G., Shetlar, M. D., and McCarthy, B. J. (1976), *Biochemistry 15,* 2002–2007.
Noll, M. (1974), *Nucleic Acids Res. 1,* 1573–1578.
Noll, M., Thomas, J. O., and Kornberg, R. D. (1975), *Science 187,* 1203–1206.
Ottensmeyer, F. P., Whiting, R. F., and Korn, A. P. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4953–4955.
Oudet, P., Gross-Bellard, M., and Chambon, P. (1975), *Cell 4,* 281–300.
Peacock, A. C., and Dingman, C. W. (1967), *Biochemistry 6,* 1818–1827.
Peretz, H., Towbin, H., and Elson, D. (1976), *Eur. J. Biochem. 63,* 83–92.
Plapp, B. V., and Kim, J. C. (1974), *Anal. Biochem. 62,* 291–294.
Reynolds, J. H. (1968), *Biochemistry 7,* 3131–3135.
Shaw, B. R., Herman, T. M., Kovacic, R. T., Beaudreau, G. S., and Van Holde, K. E. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 505–509.
Shih, T. Y., and Fasman, G. D. (1970), *J. Mol. Biol. 52,* 125–129.
Simpson, R. T. (1971), *Biochemistry 10,* 4466–4470.
Simpson, R. T. (1972), *Biochemistry 11,* 2003–2008.
Simpson, R. T., and Sober, H. A. (1970), *Biochemistry 9,* 3103–3109.
Slobin, L. I. (1972), *J. Mol. Biol. 64,* 297–303.
Stollar, B. D., and Ward, M. (1970), *J. Biol. Chem. 245,* 1261–1266.
Sun, T. T., Bollen, A., Kahan, L., and Traut, R. R. (1974), *Biochemistry 13,* 2334–2340.
Thomas, J. O., and Kornberg, R. D. (1975a), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2626–2630.
Thomas, J. O., and Kornberg, R. D. (1975b), *FEBS Lett. 58,* 353–358.
Van Lente, F., Jackson, J. F., and Weintraub, H. (1975), *Cell 5,* 45–50.
Varshavsky, A. J., Bakayev, V. V., and Georgiev, G. P. (1976), *Nucleic Acids Res. 3,* 477–492.
Wasserman, E., and Levine, L. (1961), *J. Immunol. 87,* 290–295.
Weintraub, H., Palter, K., and Van Lente, F. (1975), *Cell 6,* 85–110.
Whitlock, J. P., Jr., and Simpson, R. T. (1976), *Biochemistry 15,* 3307–3314.
Wofsy, L., and Singer, S. J. (1963), *Biochemistry 2,* 104–116.
Wold, F. (1967), *Methods Enzymol. 11,* 617–640.
Woodhead, L., and Johns, E. W. (1976), *FEBS Lett. 62,* 115–117.
Zubay, G., and Doty, P. (1959), *J. Mol. Biol. 1,* 1–20.