HISTONE H3 THIOL REACTIVITY AS A PROBE OF NUCLEOSOME STRUCTURE

by

NORMAN TSE NGON LWONG
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Department of Biochemistry

The University of British Columbia
2075 Wesbrook Place
Vancouver, Canada
V6T 1W5

Date Oct. 5, 1978.

ABSTRACT

Nucleosomes were prepared from trout testis nuclei by micrococcal nuclease digestion. The reactivity toward N-[ethyl-^{3}H]maleimide (NEM) of the single sulfhydryl group of histone H3 in the nucleosomes was studied under a variety of conditions.

Under conditions of low ionic strength, there is negligible reaction of nucleosomes with NEM, suggesting that the cysteinyl residue of H3 is buried. Complete denaturation of nucleosomes in 6 M guanidinium chloride leads to reaction of 2 moles of NEM per mole of nucleosomes, in agreement with the expected presence of 2 moles of H3 per particle. Exposure of nucleosomes to 2 M NaCl or 1 M $MgCl_2$ leads to exposure of the thiol group. At higher Mg^{++} concentrations, the thiol group remains exposed, but in NaCl solutions, as the salt concentration is increased beyond 2 M, the thiol group returns to an inaccessible state.

The reactivity of nucleosome thiol groups is relatively unaffected by urea to approximately 5 M. Between 5 and 8 M urea, a rapid increase in thiol reactivity indicates a cooperative unfolding of the nucleosome core. When added together, urea and salt act in a cooperative manner to expose the H3 sulfhydryl group.

Mixtures of oligonucleosomes have also been studied under different conditions. They were found to behave in a

similar fashion to monomers in 6 M guanidine, but their thiols react more slowly than those of monomers in high salt.

Removal of the amino-terminal regions of the core histones by tryptic digestion has no noticeable effect on the accessibility of nucleosome thiol groups. It is concluded that the carboxy-terminal region of H3 containing Cys 110 is masked mainly by histone-histone interactions in the octameric core complex, and is located in a region which is relatively insensitive to the perturbations induced by trypsin or low concentrations of urea.

Nucleosomes reconstituted in the presence of a sulfhydryl reducing agent were indistinguishable from native particles in their reactivity to NEM in low salt buffers, in 2 M NaCl and in 6 M guanidine hydrochloride.

These studies indicate that the degree of exposure of H3 sulfhydryl groups in nucleosomes can be effectively monitored using NEM. The carboxy-terminal region of H3 containing Cys 110 seems to be located in a relatively stable region of the nucleosome core, perhaps at the interface between heterotypic tetramers.

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INTRODUCTION

I. Eukaryotic chromatin

Despite their great variety and complexity, all eukaryotic organisms contain in the nucleus a nucleoprotein complex, the chromatin. This chromosomal material consists of DNA, histones, nonhistones proteins and a small amount of RNA. The structure of the chromatin is maintained by the interactions among DNA and histones. Although the roles of the components in chromatin are not fully established, different functions have been assigned to various components on the basis of previous experiments (1).

The histones, basic proteins of five major classes, are present in approximately equal weight with DNA in chromatin. They are thought to be important in maintaining chromatin structure and to act as a "coarse" control of gene activity. In spite of the little variety in type, histones can be covalently modified by phosphorylation, acetylation, methylation, etc. (2), probably to modulate DNA-histone and histone-histone interactions to bring about changes in chromatin structure and function.

The nonhistone proteins, a heterogeneous population of different protein species, are present in small amounts in interphase chromatin. Although the functions of nonhistone proteins are not understood, there are several lines of evidence to indicate regulatory roles for some of them: these proteins were found to increase in amount in genetically

active tissues (3), induce transcription of active genes (3-4) and alter DNA configuration (5). However, the mode of regulation is yet to be elucidated.

II. Nucleosome structure

A major advance in research on chromatin structure was the discovery of the "nucleosomes" or "ν-bodies" in 1973-4 (6-9). The most convincing evidence emerged from nuclease digestion studies (6, 8). Hewish and Burgoyne (6) found that an endogenous nuclease of rat liver has the capacity to cleave chromatin to multiples of a subunit of about 200 base pairs of DNA in length. This observation was subsequently confirmed by Noll (8). At about the same time, Woodcock (10) and Olins & Olins (9) observed the appearance of a "beads-on-a-string" structure from chromatin in elèctron micrographs. Thus, the presence of a repetitive subunit in chromatin is established both by biochemical and morphological studies.

Since the discovery of the nucleosome, investigators began to ask how the histones and DNA are arranged within the nucleosomes. Experiments were done to measure the DNA content (11-13), to define the relative locations of DNA in the nucleosomes (14), to find the stoichiometry of histones (15-7), and to study the propinquity of histones (16-23) and conformational changes of histones under a variety of condtions (24, 25).

The first measurements of the DNA unit size gave values of 205 base pairs (8) and 180-230 bp (26). These experiments were done by first digesting the chromatin into fragments consisting of multiples of a unit size by commercially available micrococcal nuclease; then the fragments were resolved by gel electrophoresis and their mobilities were compared to DNA standards. The DNA sizes thus obtained varied from 140-220 bp in different organisms and tissues. Since in some experiments the DNA fragments from different sources were analyzed on the same gel, this variation in size was not due to experimental error. It is now generally agreed that monomers of chromatin prepared by mild digestion contain DNA segments of 180-205 bp (27); further digestion of these monomers yields DNA fragments of 140-170 bp bound by whole histones with H1 or H5.

The histone content of the nucleosome has been derived from reconstitution experiments and cross-linking studies (15-7). It has been found that all of the four smaller histones, H2A, H2B, H3 and H4 are required in equal molar ratio to generate the characteristic 125 Å particles in the electron microscope (15). The appearance of a cross-linked octamer of histones observed by Thomas and Kornberg (16-7) further confirmed the existence of two each of the foru small histones. This stoichiometry of histones was obtained from chromatin of different sources (28); however, deviations of 30-50% are frequently obtained, probably due to inaccuracy in measurements.

The role of H1 in association with the nucleosome has been under investigation by various workers. It is assumed that only one H1 molecule is associated with each nucleosome and it is either associated with the DNA spacer region of 30-45 bp or it interacts with the nucleosomal DNA on the outside of the nucleosomes. The association of H1 with DNA was supported by Whitlock & Simpson who demonstrated the exposure of DNA upon the removal of H1 in 0.7 M NaCl (29). Nevertheless, since H1 is the only histone to dissociate from the nucleosomes in low salt concentrations (30) and is most susceptible to proteolysis, many results were greeted with suspicion. Current view favors the suggestion that H1 cross-links between nucleosomes and that H1 is non-essential in maintaining the basic nucleosome structure (27), however, H1 may be involved in the formation of high order structure of chromatin (32).

Extensive digestion of the nucleosome monomer produces the "trimmed" monomer of 140 bp in length. This degradation product with 140 bp of DNA and eight histones (two each of H2A, H2B, H3 and H4) is termed the "core particle" and the remaining DNA which is variable in length in different cell types is termed the "linker" as it connects the nucleosomes. The earliest physical studies suggested the core particle to be roughly spherical and about 110 Å in diameter (33). Neutron scattering data has recently shown that the shape of the core particle to be a flat cylinder with overall dimension of 11 x 11 x 6 nm (34-5). The DNA was found to be either

folded or supercoiled on the outside of the nucleosome (34, 36). More information about nucleosome core shape has now been provided by Finch et al. who were able to isolate the core particles in crystal form (37). X-ray diffraction measurements of the crystals gave similar results to neutron scattering data.

III. Internal arrangement of histones

The arrangement of the histones within the core particle has been under intense investigation, with the aim of elucidating the mechanism of packaging of the DNA and the intermolecular relationships among the histones and DNA.

The locations of the various histones with respect to each other were studied mainly by crosslinking experiments, in which the histones were crosslinked by chemical agents or by UV light (16-23). Analysis of dimers and trimers thus formed defines the relative location of each histone. H1 was found not to be involved in any crosslinks except with other H1 molecules to form a homopolymer. The formation of multiples of crosslinked octamers and the formation of polymeric H1 indicate that the nucleosome is in contact with an adjacent one. Five of the ten possible dimers from the four small histones have also been found, but the occurrence of the others cannot be ruled out, as they may be present in small amounts (31).

Studies of the association properties of histones in solution revealed the tetramer $(H3)_2(H4)_2$, which may itself define the core particle (38-9), e.g. by inducing supercoiling

of the DNA, while the H2A-H2B dimer may aid stabilization of the complete nucleosome (40). The roles of H2A and H2B were supported by reports that the two histones can alter the optical activity and conformation of local regions of DNA (41).

Early attempts to demonstrate the octamer in solution have been unsuccessful because of its instability in low ionic strength; however, hexamers, tetramers, dimers and even heterotypic tetramers comprising each of H2A, H2B, H3 and H4 have all been found (16-7, 42). Recently Chung et al. were able to isolate the core complex in high salts and do analysis on it (43). They showed that the octamer exists in equilibrium with a heterotypic tetramer in 2 M NaCl.

The conservative sequences of H3 and H4 lead to the inference that the tetramer $(H3)_2(H4)_2$ is important in defining the basic fold of DNA in the core while H2A and H2B are for stabilization purpose. H2A and H2B may have other specific functions as well since they are required for the formation of the native nucleosomes. Variation in the linker region is probably due to the variation of H1 sequence in different organisms (1).

IV. The arginine-rich histones

Histones H3 and H4 are similar in terms of arginine content, sequence conservation, salt elution from DNA, etc. They were found to associate in solution to form a tetramer which is most important in packaging the core DNA and inducing supercoiling of DNA (28, 44-6). The $(H3)_2(H4)_2$ tetramer also protects DNA against nuclease digestion in a manner similar to that

found for core particles (47).

Biroc and Reeder (25) have examined the reaction of H4 tyrosines with iodine in Xenopus. When iodinated Xenopus H4 were digested with trypsin and electrophoresed at pH 3.5, four heavily labelled tryptic peptide spots were obtained. By assuming the same H4 sequence in Xenopus as in calf thymus, they were able to assign each of the four tyrosines in the amino acid sequence to a labelled tryptic peptide. Thus the reactivity of individual tyrosine could be analyzed. From analysis of the reactivity of the tyrosine residues under various conditions, they showed that two of the four tyrosines are buried when the histones are attached to native chromatin. However, if the chromatin was put into 2 M NaCl or 5 M urea or both, all of the reactivities went up 5-10 fold, indicating that the supercoiling of the chromatin and histone-histone interactions are both responsible for the protection of the tyrosine residues. One of the tyrosines, tyr 88, increases its reactivity with iodine to maximum at 0.5 M NaCl, the ionic condition for the dissociation of H1, inferring that tyr 88 is involved in interaction between H1 and H4, or is exposed by conformational change upon dissociation of H1.

H3 is the only histone that contains cysteine moieties within its amino acid sequence (48-9). In higher vertebrates, two cysteines are found for each molecule of H3; in lower vertebrates, such as chicken and fishes, only one cysteine is present (50). It is therefore easy to specifically label H3 by using sulfhydryl reagents. Hyde and Walker (24) studied

calf thymus H3 by reacting whole chromatin with 5,5'-ditho-bis-[2-nitrobenzoic acid], a specific thiol reagent. They found that only one of the two cysteine groups is reactive under normal conditions. The rate of reaction as well as the total reactivity increased as the chromatin was put into salt solutions.

Maher and Candido (51) also found that there is negligible reaction between trout testis nucleosomes and p-hydroxy mercurobenzoate under non-denaturing condition. The reaction was greatly enhanced when the nucleosomes were denatured. These results indicate the possibility of studying the thiol reactivity as a probe of H3 and even nucleosome structure.

V. The present investigation

The present investigation concerns the reactivity toward N-ethylmaleimide of the H3 thiol groups in trout testis nucleosomes under various conditions, including different salt concentrations and urea solutions. The reactivity of trypsin-digested and reconstituted nucleosomes are also analyzed. Changes in the thiol reactivity may signify alterations in H3 conformation and consequently, changes in nucleosome structure.

N-ethylmaleimide is a useful sulfhydryl reagent with free cysteine, peptides or proteins. It can be analyzed by spectrophotometric methods or by radioactive labelling. The latter was employed in these studies.

In this thesis, data on the reaction of nucleosome thiol groups with NEM are reported. The results are useful in formulating a detailed picture of histone-histone and histone-DNA interactions in the nucleosome.

MATERIALS AND METHODS

I. Materials and Abbreviations

(a) Materials

Naturally maturing trout testes were obtained from Sun Valley Trout Farm, Mission, B. C. Micrococcal nuclease (E.C.3.1.4.7.), deoxyribonuclease I, trypsin (E.C. 3.4.4.4.), soybean trypsin inhibitor, dithiothreitol were purchased from Sigma. Labelled N-ethyl-[^{3}H]-maleimide was from New England Nuclear and unlabelled N-ethylmaleimide from Aldrich Chemical Co., Wisconsin. Sequencing grade heptane used to dissolve labelled N-ethylmaleimide was obtained from Pierce Chemicals Company. Na-[1-^{14}C]-acetate and aqueous counting scintillant were purchased from Amersham. Minicon B15 concentration cells were from Amicon Corp. Glass fibre filters used for N-ethylmaleimide assays were obtained from Reeve Angel. Acrylamide was from Matheson, Coleman and Bell; N, N'-methylene-bis-acrylamide from Bio-Rad; and TEMED (N,N,N',N'-tetramethyl-ethylenediamine) from Ames Company. All other chemicals and reagents were of the highest purity or reagent grade. Distilled water was used for all solutions.

(b) Abbreviations

EDTA: ethylenediamine-tetraacetic acid

SDS: sodium dodecyl sulfate

TMKS buffer: Tris-HCl (50 mM, pH 7.4), $MgCl_2$ (1 mM), sucrose (0.25 M), and β-mercaptoethanol (15 mM).

Tris-EDTA buffer: Tris-HCl (10mM, pH 7.5), and EDTA (0.7 mM).

PBS buffer: NaCl (0.14 M), KCl (27 mM), Na_2HPO_4 (8 mM), KH_2PO_4 (1.5 mM), $CaCl_2$ (0.9 mM), $MgCl_2$ (0.5 mM), pH 7.2.

NEM: N-ethylmaleimide

Gdn·HCl: guanidinium chloride

TCA-tungstate: 10% trichloroacetic acid, and 0.5% sodium tungstate, pH 2.0.

DTT: dithiothreitol

DNase I: deoxyribonuclease I

II. Preparation of Nucleosomes

(a) Micrococcal nuclease digestion

Nucleosomes were prepared as described by Davie and Candido (53) with some modifications.

Nuclei were isolated from 6-8 g of trout testes by homogenizing in TMKS buffer in a Waring Blendor for 2 minutes. After centrifugation at 3,000 x g for 10 minutes, the pellet was resuspended in TMKS buffer and homogenization and centrifugation repeated. The nuclei in the pellet were suspended in 6 ml of TMKS buffer containing 1 mM $CaCl_2$ (5×10^8 nuclei/ml), and digested with micrococcal nuclease at a final concentration of 300 units/ml for 30 minutes at 37°. The reaction was stopped by adding EDTA to 10 mM, and placing the mixture on ice. The mixture was centrifuged at 12,000 x g for 10 minutes and the supernatant was discarded. Chromatin monomers and multimers were released by vigorous hand-homogenization

of the pellet in a glass-teflon homogenizer in 6 ml of Tris-EDTA buffer. The chromatin subunits (monomers and multimers) were collected in the supernatant after 30 minutes of centrifugation at 12,000 x g.

To separate the monomers from multimers, the supernatant was passed through a Bio-Gel A-5M column (107 cm x 2 cm) (52) equilibrated with Tris-EDTA buffer. The column was run overnight at a flow rate of 8 ml/hr at 4°. The monomer and multimer peak fractions were pooled and stored at -80° until further use. About 40 A_{260} units of monomer and 5 A_{260} units of oligomer were obtained per gram of trout testis.

(b) Preparation of <u>in vitro</u> labelled nucleosomes

Three grams of fresh trout testes were minced and homogenized in PBS buffer in a glass-teflon homogenizer. The homogenate was filtered through cheesecloth and then centrifuged at 3,000 x g for 10 minutes. The cells were suspended in 4.4 ml PBS buffer, 0.6 ml penicillin-streptomycin (100 units/ml), 1.2 ml Waymouth's medium (53), and 0.3 ml sodium 1-[^{14}C]-acetate (50 μCi/ml). The mixture was incubated in a gyratory water bath at 16° for 4 hr, after which time 25 ml PBS buffer was added and the labelled cells collected by centrifugation at 3,000 x g for 10 minutes. When the cells were not used immediately, they were stored at -80°.

Nucleosome monomers were prepared from the labelled cells by digestion with micrococcal nuclease as described above.

III. Reaction of nucleosomes with N-ethylmaleimide

(a) Reaction of monomers in non-denaturing solution

Five microlitres of N-ethyl-[^{3}H]maleimide (80 mCi/mmol) containing 1.25 μCi were added to 0.5 ml of nucleosome monomers (2 A_{260}/ml) at ambient temperature (≈22°) in Tris-EDTA buffer. Fifty microlitre aliquots were taken at time intervals and put into TCA-tungstate at 0° to stop the reaction. The acid precipitable material was collected on glass fibre filters and washed three times with TCA-tungstate, and once each with ethanol and ether. The filter discs were dried and counted in 5 ml of aqueous counting scintillant. Control reactions, in which the concentrations of NEM and nucleosomes were varied, showed that the reagent was not limiting under these conditions.

(b) Reaction of nucleosome monomers in denaturing solutions

One A_{260} unit of nucleosome monomers was concentrated to 0.25 ml in a Minicon B15 cell and added to the appropriate amount of salt, urea or Gdn·HCl. The reaction mixture was brought to 0.5 ml by addition of Tris-EDTA buffer, and the NEM reaction carried out as described above.

(c) Reaction of nucleosome oligomers with NEM

Nucleosome oligomers were collected from the excluded peak after separation of micrococcal nuclease digest products on a Bio-Gel A-5M column. One A_{260} unit of oligonucleosomes was subjected to thiol analysis with NEM under denaturing and non-denaturing conditions as described above.

IV. Trypsin digestion of nucleosomes

Nucleosomes (2 A_{260}/ml) were incubated with 10 µg/ml of trypsin in Tris-EDTA buffer at 20°, for the desired period. To stop the reaction, 0.5 ml of the incubation mixture was added to 10 µl of soybean trypsin inhibitor (1 mg/ml) and the mixture chilled to 0°. After 30 minutes, the NEM reaction was performed as mentioned above.

When the efficiency of trypsin digestion was checked using [^{14}C]-labelled nucleosomes, 1.8 ml of nucleosomes (1.5 A_{260}/ml) was added to 200 microlitres of trypsin (70 µg/ml). At time intervals, 200 microlitre aliquots were taken out and mixed with 5 microlitres of trypsin inhibitor (0.4 mg/ml). Fifty microlitre samples were counted in 5 ml of aqueous counting scintillant after precipitation onto filter discs as described above.

V. Reconstitution of nucleosomes

Monomer nucleosomes (2 ml, 1.6 A_{260}/ml) were dissociated in Tris-EDTA buffer containing 2 M NaCl, 5 M urea and 1 mM DTT (or 10 M urea and 1 mM DTT). The salt and urea were removed by dialysis against 1 litre of Tris-EDTA + 1 mM DTT for 5 hr. The reconstituted nucleosomes were then dialyzed against three 1 litre changes of Tris-EDTA buffer for 3 hr each, to remove the reducing agent. Reactions with NEM were then performed under denaturing and non-denaturing conditions as described above.

VI. DNase I digestion of native and reconstituted nucleosomes

Nucleosomes (20 A_{260}/ml) were preincubated in Tris-EDTA buffer at 37° for 10 minutes. After the incubation period, the solution was made 2.7 mM in $MgCl_2$ + 5.4 mM NaCl to complex the EDTA and to provide the Mg^{++} and Na^+ concentrations for optimal DNase I digestion. DNase I (1 mg/ml) was then added to a final concentration of 0.02 mg/ml and the nucleosomes were digested for 5 minutes at 37°. The reaction was stopped by placing the mixture on ice. The nuclease digested nucleosomes were then analyzed on denaturing polyacrylamide gels.

VII. Gel electrophoresis of nucleosomes

(a) 15% SDS-polyacrylamide slab gel electrophoresis for analysis of nucleosomal proteins

Fifteen per cent polyacrylamide-sodium dodecyl sulfate slab gels were made using a modified Laemmli procedure (52). The following volumes of stock solutions: - 15 ml of I (30.0 g acrylamide, 0.4 g N,N'-methylenebisacrylamide in 100 ml of H_2O), 0.15 ml of II (20 mg ammonium persulfate freshly dissolved in 2 ml H_2O), 0.3 ml of III (10 g SDS in 100 ml of water) and 7.5 ml of IV (1.5 M Tris-Cl, pH 8.8) - were combined with 10 l of TEMED and 7.05 ml of water, and polymerized in 1.5 mm slabs under t-butanol. Nucleosome samples were lyophilized and then heated in 4% SDS, 0.125 M Tris, pH 6.8, 10% β-mercaptoethanol, 20% glycerol and 0.002% bromophenol blue. The gel was run at 15 milliamperes for 7-8 hr in 0.05 M Tris,pH 8.0, 0.384 M glycine and 0.1% SDS. When the gel was to be dissected and solubilized, 0.6% N,N'-diallyltartardiamide was used as crosslinker instead of

bisacrylamide (54). After electrophoresis, the gel was stained in Coomassie Brilliant Blue in methanol:acetic acid: water, 5:1:5, and destained overnight in 5% methanol and 7.5% acetic acid.

(b) Non-denaturing 3% polyacrylamide gel electrophoresis of DNA

Monomeric and oligomeric fractions from A-5M columns were analyzed in 3% polyacrylamide gels as described by Loening (55). The following volumes of stock solutions: - 5 ml of 10x concentrated TEA buffer (tea buffer = 0.04 M Tris-acetic acid, pH 7.8, 2 mM EDTA, 0.02 M sodium acetate), 7.5 ml of 20% acrylamide solution (acrylamide: N,N'-methylene-bisacrylamide, 19:1), 0.5 ml of 10% SDS, 0.4 ml of 10% ammonium persulfate - were combined with 36.6 ml of water and deaerated. Then 40 µl of TEMED was added and the acrylamide was polymerized under t-butanol in 1.5 mm slabs. Nucleosome samples were first precipitated in 10 mM $MgCl_2$, and then protease K mixture (500 µg/ml Protease K, 10 mM EDTA, 2% SDS) was added to digest the proteins. The digestion was carried out at 37° for 1 hr, after which the digestion mixture was made 4% SDS, 30 mM EDTA, 20% glycerol and applied directly to the gel. The gels were prerun at 100 V for 3 hr and running conditions were 50 V for 10 min, followed by 100 V for 4 hr. Gels were stained in ethidium bromide (10 µg/ml) for 10 min and destained briefly in water. Bands were visualized under UV light.

(c) Denaturing 99% formamide, 6% polyacrylamide gel electrophoresis of DNA

These gels were described by Staynov et al. (56). 99% formamide was stirred with Dowex 50W-X8 (3 g/100 ml) for 1 hr, and was then filtered and used the same day. 2.04 g of acrylamide, 0.36 g of N,N'-methylenebisacrylamide and 80 l of TEMED were dissolved in 40 ml of formamide, which was then filtered. The gel was made 20 mM in phosphate and 0.12% in ammonium persulfate by adding 0.8 ml of M sodium phosphate pH 7.0, containing 50 mg of ammonium persulfate. A 15 x 15 x 0.15 cm slab gel was poured. Nucleosome samples were first dissolved in M NaCl, 2.5 M urea; they were then precipitated overnight in 2 volumes of ethanol. The precipitate was taken up in formamide (containing 20 mM sodium phosphate, pH 7.0, 20% sucrose, 0.005% bromophenol blue), heated to 100 , cooled on ice, and electrophoresed in formamide-20 mM phosphate at 160 V for 5 hours. Gels were stained and destained as described above.

RESULTS

Isolation of Nucleosome

Nucleosomes were isolated as described in Materials and Methods. A typical A-5M profile is shown in figure 1. The excluded fraction was pooled to constitute the oligomeric fraction, and the monomer fraction was obtained from the peak fractions as illustrated. The monomer fraction was found to contain only traces of H1 when analyzed on 15% SDS polyacrylamide slab gels (Figure 2). This was taken to mean that most of the DNA "tails" of the particles had been removed (11, 57). Very low amounts of non-histone proteins were observed as reported before (52, 58). The oligomeric fraction is a mixture of oligonucleosomes ranging from dimers (about 50%), trimers (about 30%) to multimers, as observed on a 3% non-denaturing acrylamide slab gel (not shown).

Sulfhydryl reactivity of native nucleosomes

The incorporation of NEM label is a measure of the degree of accessibility of the thiol group in the H3 molecules within the nucleosome core. The modification of H3 was confirmed by analysis of the NEM-treated nucleosomal proteins under denaturing conditions on 15% SDS-polyacrylamide gels. More than 80% of the incorporated label was associated with histone H3 (Figure 3). The counts associated with other histones are probably due to side reactions of NEM with amino and imidazole groups in these proteins (59).

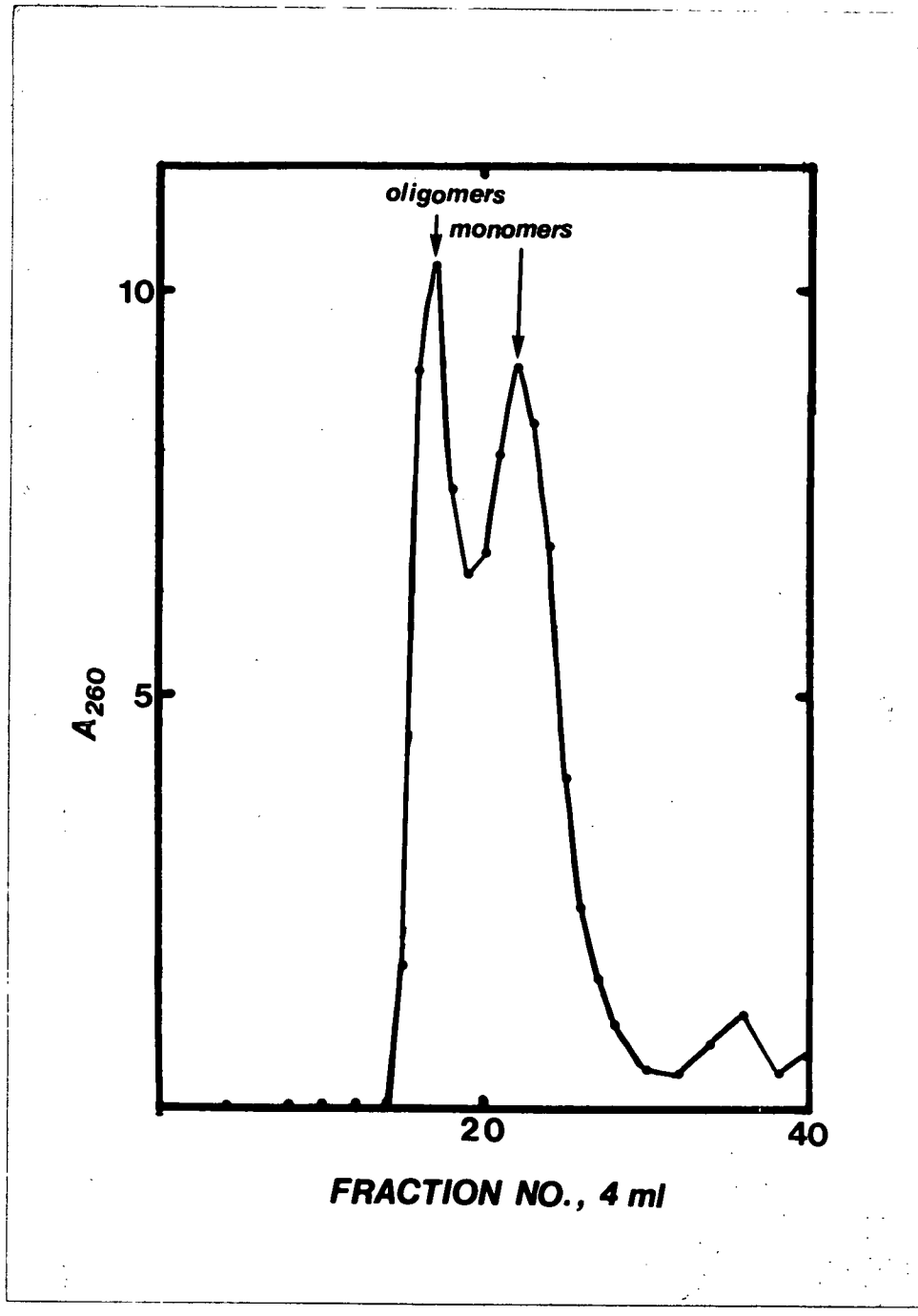

Figure 1. Bio-Gel A-5M column profile of nucleosomes from 30 minutes of micrococcal nuclease digestion. Trout testis nuclei were digested with micrococcal nuclease for 30 minutes as described in "Materials and Methods". The digestion products were fractioned on a Bio-Gel A-5M column. The absorbance at 260 nm of each fraction is plotted vs. the fraction number.

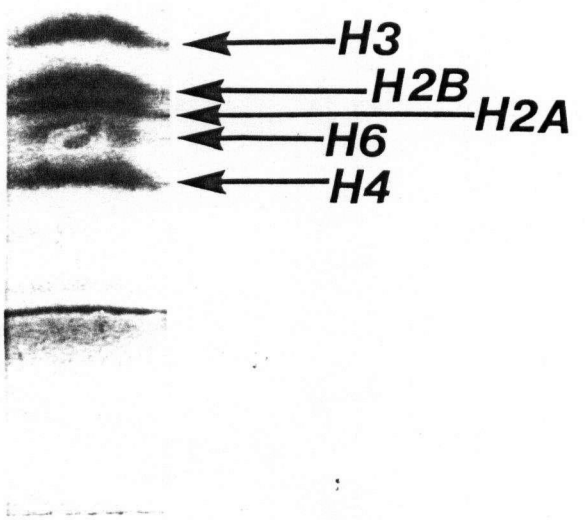

Figure 2. SDS-polyacrylamide gel electrophoresis of the monomer peak fraction from an A-5M column. One A_{260} unit of the monomer peak fraction was lyophilized and then heated in 40 µl of sample buffer. The sample was applied to a 15% SDS-polyacrylamide slab gel and run as described in Materials and Methods. The gel was stained in Coomassie Blue.

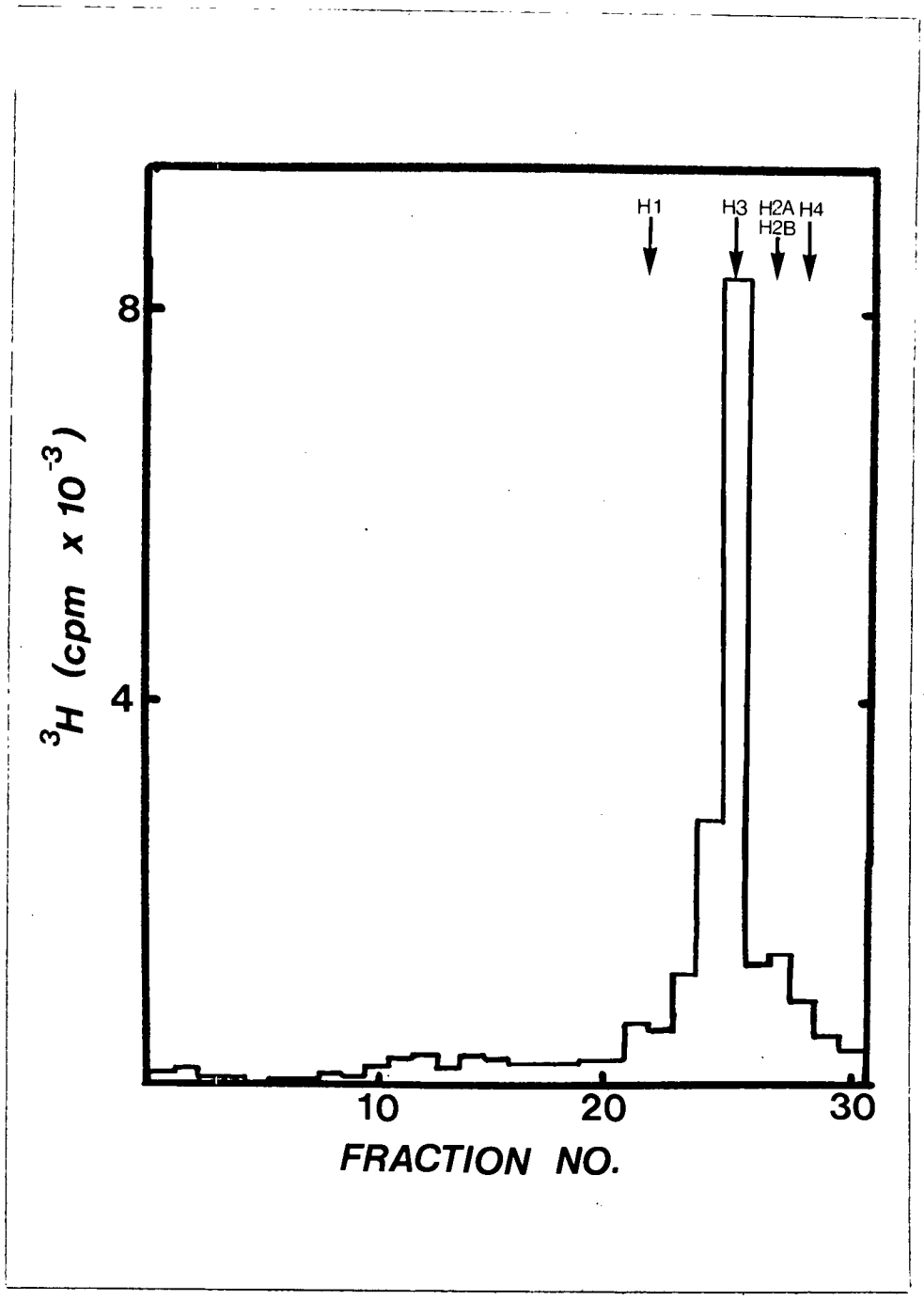

Figure 3. Labelling of H3 with N-ethylmaleimide. Two A_{260} units of nucleosomes were labelled with [^{3}H]N-ethylmaleimide in 10 M urea at ambient temperature for 5 hr. The reaction mixture was dialyzed against Tris-EDTA buffer and lyophilized. The lyophilized material was taken up in 100 µl and 50 µl was applied to a 15% SDS-polyacrylamide slab gel and run as described in Materials and Methods. After electrophoresis, the gel was stained and sliced. The slices were solubilized in 3 ml of 2% periodic acid for 2 days at room temperature. The solubilized gel slices were counted in 10 ml of aqueous counting scintillant.

Hyde and Walker (24) reported the inaccessibility of one of the two cysteines in whole chromatin of calf thymus under non-denaturing conditions. It is thus interesting to observe the behaviour of the sole cysteine residue in trout testis histone H3 under similar conditions. When nucleosomes in Tris-EDTA buffer were exposed to NEM, an extremely low reactivity was observed (Figure 4). The incorporation was very rapid, and essentially complete in 10 minutes. This reaction may be due to traces of denatured nucleosomes or of free H3 or H3 fragments. The possibility of side reactions can be ruled out as these reactions occur much slower. Previous experiments using p-hydroxymercuri-benzoate also showed the H3 sulfhydryl groups of trout testis nucleosomes to be unreactive (51). Olins et al. (60) in their physical studies of the effects of urea on nucleosomes, had also reported the unavailability of the thiol groups in chicken erythrocytes nucleosomes. These results are consistent with the present findings.

Effect of salts on sulfdydryl reactivity

Three different salts were used in the studies of H3 thiol reactivity with NEM: Gdn·HCl, NaCl and $MgCl_2$.

(a) Effect of guanidinium chloride

Guanidinium chloride (Gdn·HCl) is known to dissociate histones from DNA, and to destroy secondary and tertiary structure in histones and proteins in general. When nucleosomes in 6 M Gdn·HCl were allowed to react with NEM, a

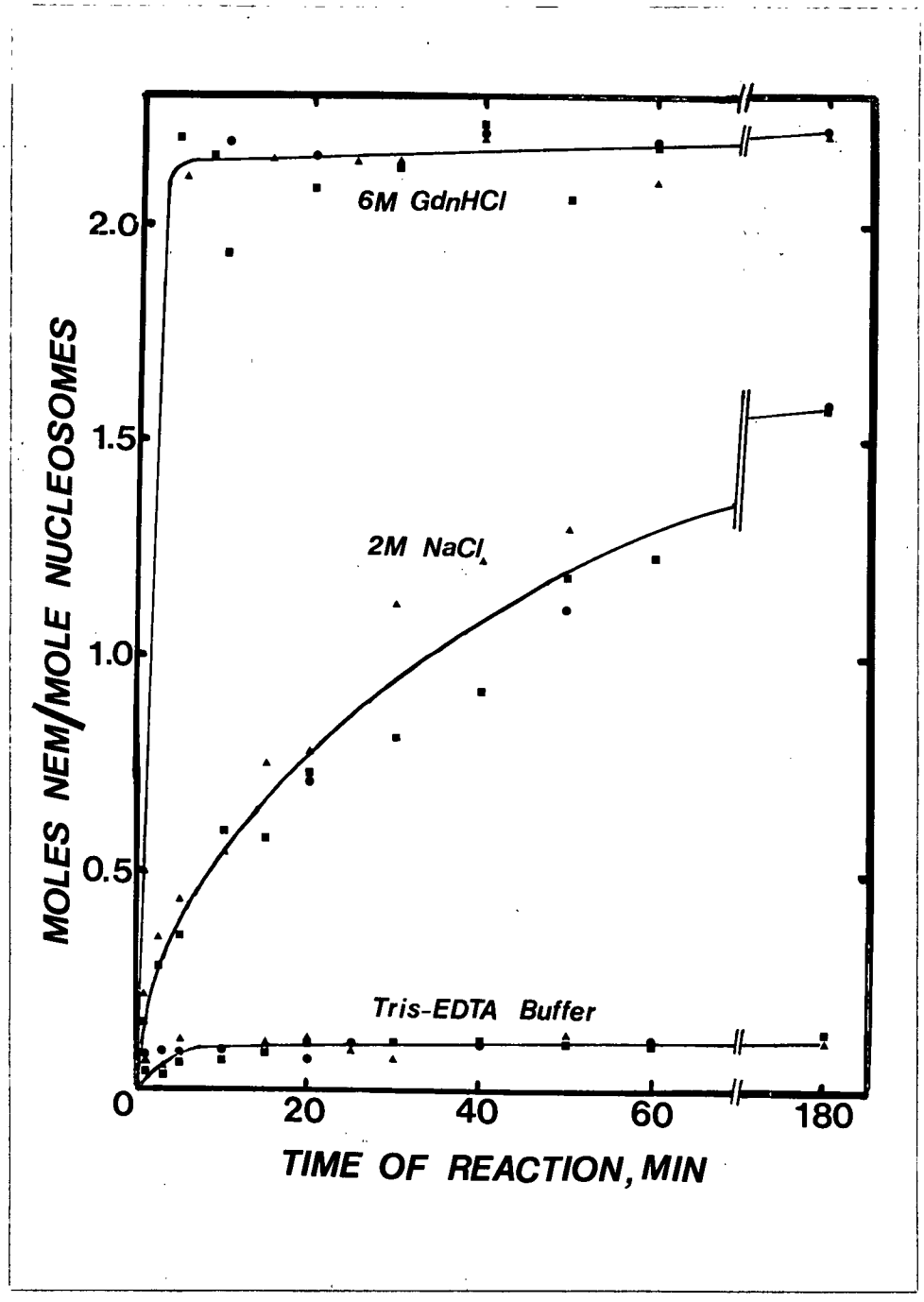

Figure 4. Time course of reaction of nucleosomes in denaturing and non-denaturing conditions. Monomers were reacted with [^{3}H]NEM under the indicated conditions as described in Materials and Methods. The specific reactivity of monomers was estimated by assuming an absorbance of 20 per mg DNA per ml at 260 nm, and a DNA molecular weight of 110,000.

maximum of 2.2 moles NEM per mole nucleosomes was bound within 5 minutes (Figure 4). This maximum is in good agreement with the expected presence of two moles of H3 per nucleosome monomer, and indicates that in 6 M Gdn·HCl the cysteine residues are fully exposed.

The effect of varying molarity of Gdn·HCl on the release of histones from DNA were also studied and the results are shown on figure 5. When nucleosomes were exposed to increasing Gdn·HCl concentrations, negligible reactivity associated with the native particles persisted until the Gdn·HCl concentration exceeded 0.5 M. The reactivity increased rapidly and reached the maximum of two moles NEM per mole nucleosome at 1.2 M. This plateau level did not change even at 6 M Gdn·HCl.

(b) Effect of sodium chloride on thiol reactivity

Nucleosome sulfhydryl reactivity was first analyzed in 2 M NaCl solutions. The nucleosomes exhibited a moderately rapid rate of reaction with NEM, attaining a ratio of 1.0 mole reagent per mole nucleosomes at approximately 30-40 minutes, and reaching a maximum of 1.6 moles/mole at three hours (Figure 4). This large increase in reactivity may be attributed to a direct unmasking of the cysteinyl residue upon removal of the DNA, or to a conformational change in the histone core induced by the removal of DNA. The latter possibility seems more probable when the results of salt concentration studies are taken into account (see below).

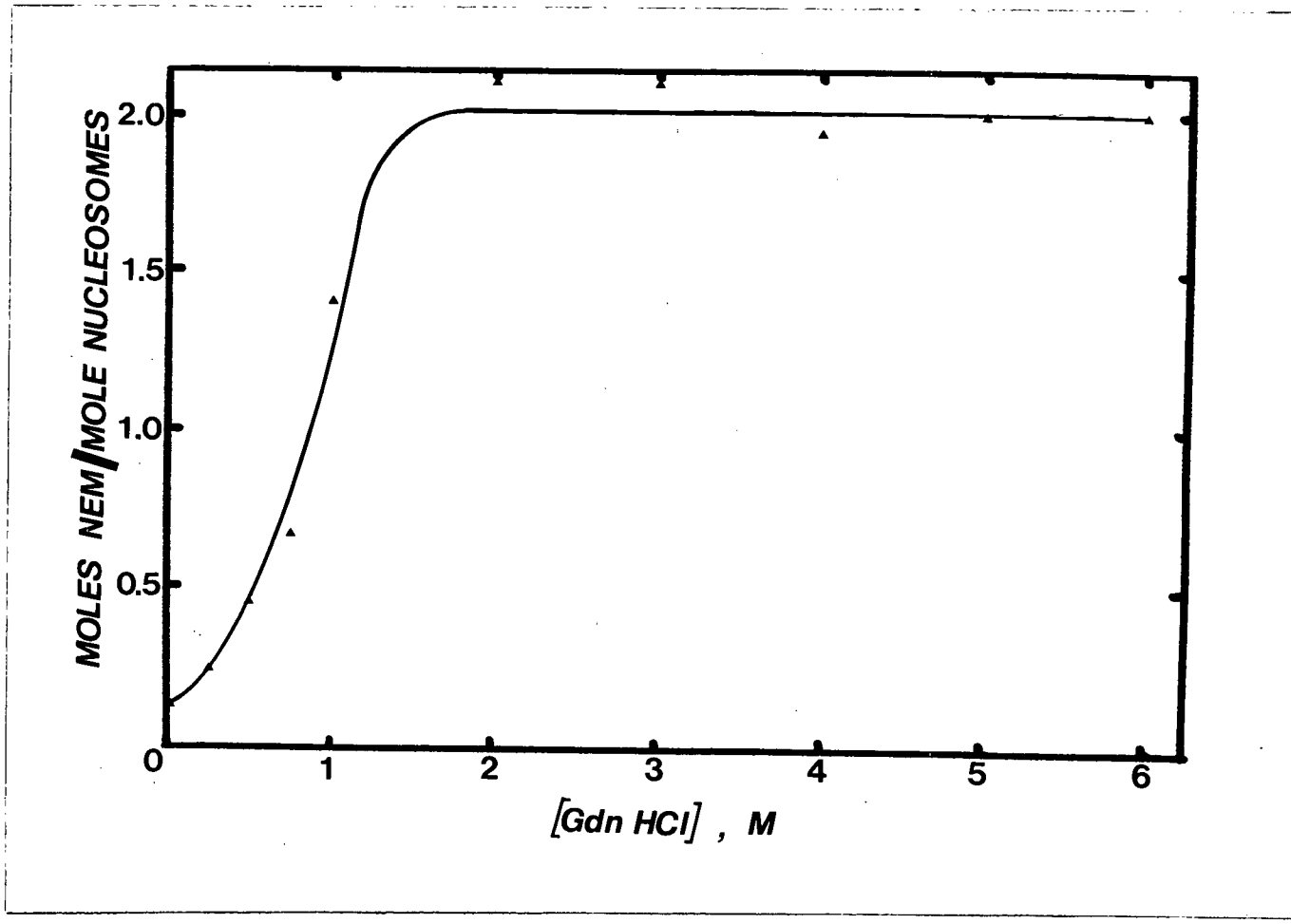

Figure 5. Effect of varying molarity of Gdn·HCl on thiol reactivity of nucleosomes. The specific activity of [^{3}H]NEM incorporated is plotted as a function of Gdn·HCl concentration in Tris-EDTA buffer. The reactions were carried out for 120 minutes.

When nucleosomes were placed in varying NaCl concentrations and the thiol reactivity analyzed, a completely different profile from that of Gdn·HCl was obtained. The reactivity of nucleosomes did not increase until the salt concentration was above 1.0 M (Figure 6), when H3 starts to dissociate from the DNA (30). The reactivity increased from 1.0 M to 1.5 M, the concentration at which histones are completely dissociated. The reactivity then started to drop at 1.75 M and reached a minimum of 0.4 moles reagent per mole nucleosome at 4 M. The maximum reactivity is probably achieved when the histones dissociate from DNA, and before any reassociation occurs. The histones may then reassociate to form heterotypic tetramers and octamers at 2 and 4 M NaCl solutions respectively (61).

(c) Effect of magnesium chloride on thiol reactivity

NEM reactivity of nucleosomes was also examined in $MgCl_2$ solutions of increasing concentration. A similar profile to that of Gdn·HCl was obtained (compare figures 4 and 6). The histones began to dissociate from DNA at an ionic strength of 0.5 as monitored by an increase in thiol reactivity. The reactivity reached a maximum of 2 moles/mole at $\Gamma/2 = 1.2$, which persists as the $MgCl_2$ concentration was increased. The similarity in the behaviour of nucleosomes in Gdn·HCl and $MgCl_2$ solutions is probably due to the fact that like Gdn·HCl, Mg^{++} also has a denaturing effect on the nucleosomal proteins (62).

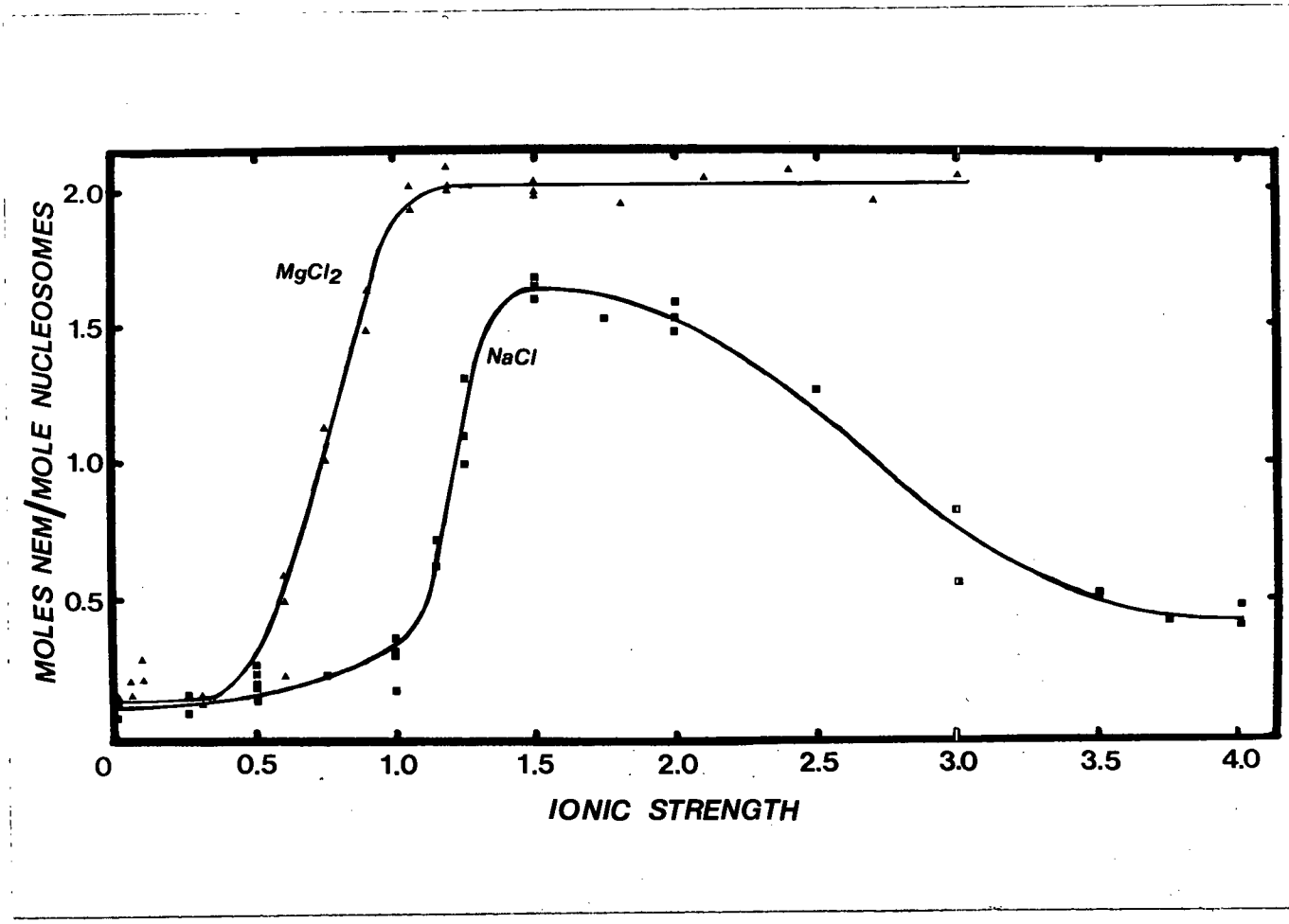

Figure 6. Effect of ionic strength on thiol reactivity of nucleosomes. The nucleosomes were allowed to react with [^{3}H]NEM in $MgCl_2$ (■) and NaCl solutions(●) for 60 minutes. The specific activities are plotted as a function of ionic strength of the solutions.

Effect of urea on sulfhydryl reactivity

Figure 7 shows the effect of increasing urea concentrations on the reactivity of nucleosome thiol groups. Very little change in reactivity was seen until 5-6 M urea was reached, where an abrupt transition began, centered at approximately 8.5 M urea. Since urea has a disruptive effect on hydrophobic interactions, the results suggest that there is a cooperative unfolding of nucleosome structure above 7 M urea, leading to full exposure of H3 thiol groups at 9-10 M urea. Olins et al. have demonstrated the same behaviour with chicken erythrocyte nucleosomes in urea solutions (60). The present data are in good agreement with their results.

Cooperative effect of NaCl and urea on nucleosome conformation

Nucleosomes were exposed to various combinations of salt and urea concentrations, and the reactivity with NEM was measured. The results are summarized in figure 8. A maximum of 2 moles NEM per mole nucleosomes was attained when nucleosomes in 6 M urea were further exposed to 0.5 M NaCl (Figure 8A). Conversely, if nucleosomes in 0.5 M NaCl were exposed to increasing urea concentrations, a steady rise in NEM incorporated was observed until the urea concentration reached 6 M (Figure 8B). Since neither 0.5 M NaCl nor 6 M urea alone allows significant incorporation of the labelled NEM (Figures 6 and 7), they must act cooperatively to alter nucleosome conformation. In 2 M NaCl, only 3-4 M

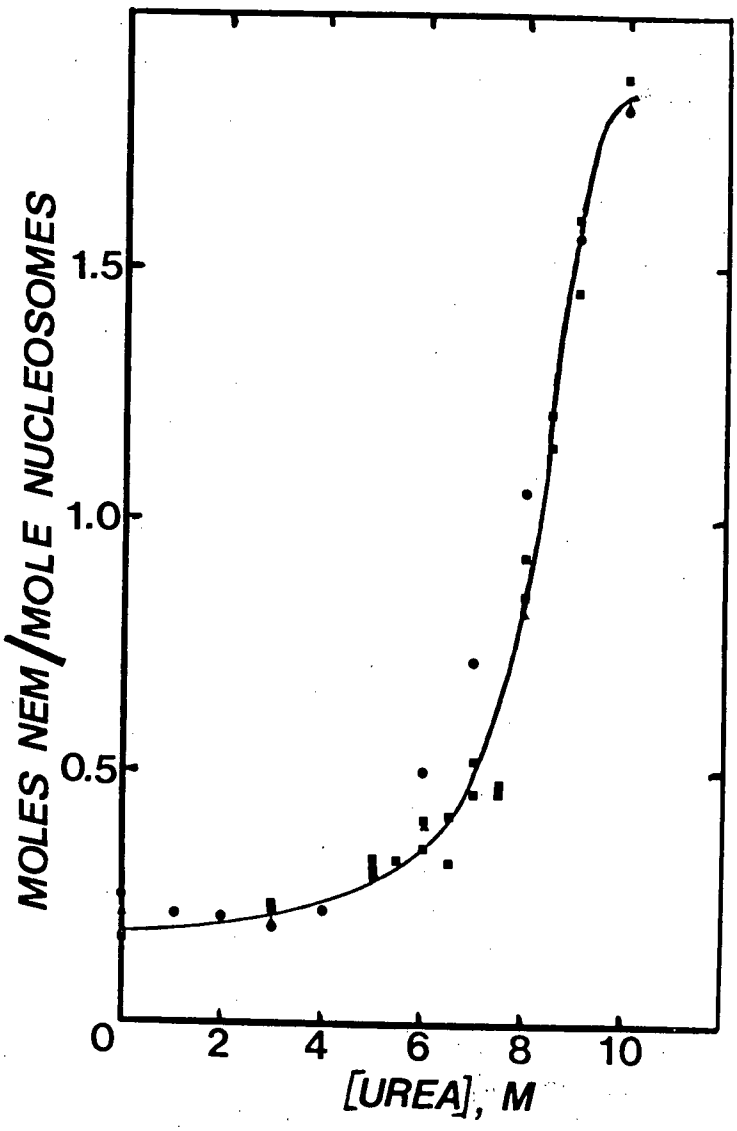

Figure 7. Effect of urea concentration on the reactivity of nucleosomes with NEM. Nucleosomes in solutions of different urea concentrations were reacted with[^{3}H]NEM for 150 minutes. The specific activity is plotted as a function of urea molarity. Different symbols represent different monomer preparations.

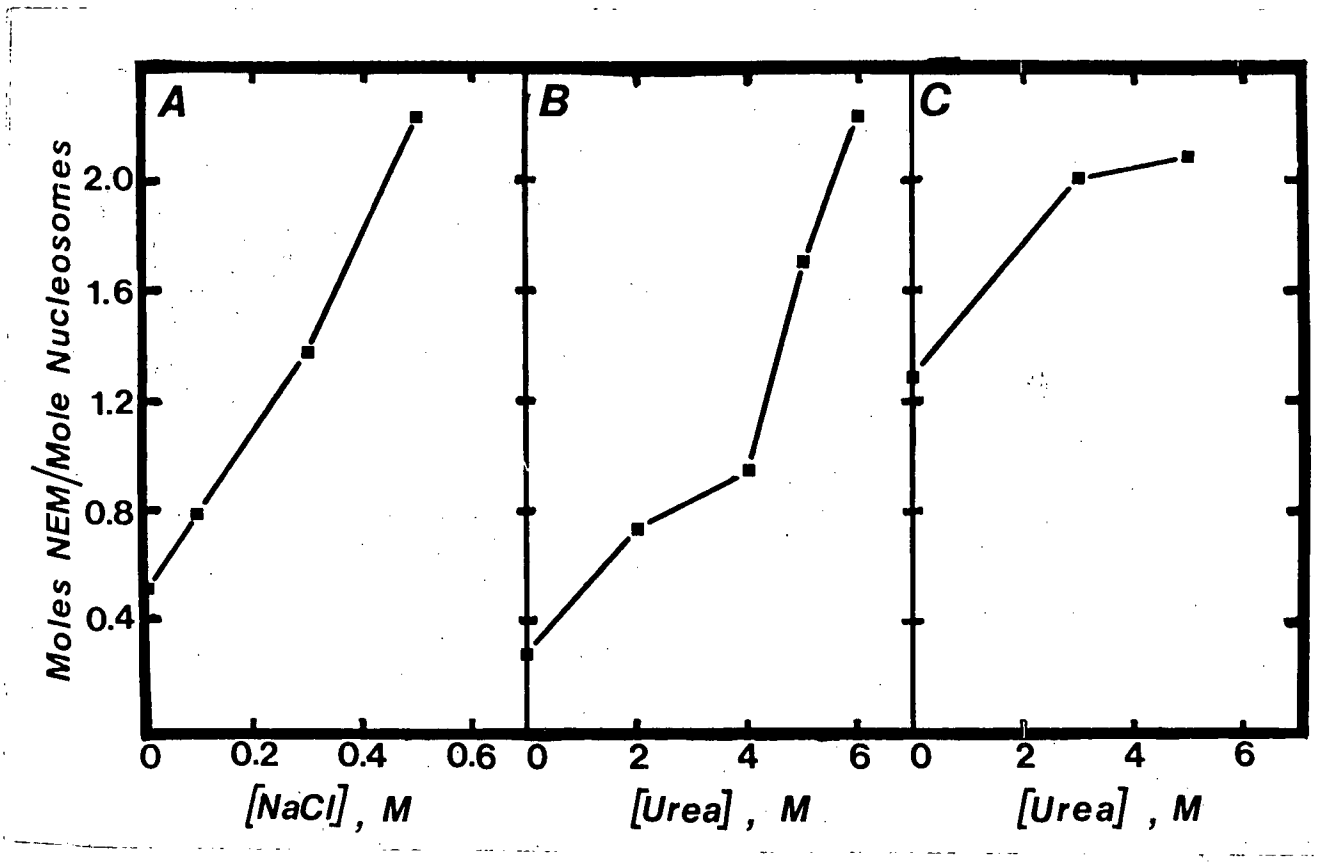

Figure 8. Synergistic effect of salt and urea on exposure of nucleosome thiol groups. Nucleosomes were exposed to various combinations of NaCl and urea concentrations for 60 min, and the reaction with [^{3}H]NEM was carried out for 24 hr at 22°. In panel A, the reaction in 6 M urea is plotted as a function of NaCl concentration. In B and C, the reaction in 0.5 M and 2 M NaCl, respectively, is plotted as a function of urea concentration.

urea was sufficient to allow complete reaction of nucleosomal thiol groups with NEM (Figure 8C).

Sulfhydryl reactivity of nucleosome oligomers

The excluded fraction of an A-5M column, which contains negligible amount of monomers, was allowed to react with NEM under denaturing and non-denaturing conditions. Figure 9 shows the extent of reaction of oligonucleosomes with NEM in 10 mM Tris, pH 7.4 + 0.7 mM EDTA, 2 M NaCl, 6 M urea, 2 M NaCl + 6 M urea and 6 M Gdn·HCl. The behaviour of oligomers under these conditions was very similar, if not identical to, that of monomers, except for the results in 2 M NaCl. Again, the synergistic effect of NaCl and urea was observed. However, in 2 M NaCl, only 0.5 SH groups per mole nucleosome reacted after 60 minutes, using oligonucleosomes, vs. approximately 1.2 SH/mole using monomers (Figure 3). The reason for this behaviour is unclear.

Tryptic digestion of nucleosomes

Sahasrabuddhe and Van Holde (63) found that chromatin particles isolated by nuclease digestion underwent a dramatic change in sedimentation velocity from a value of 11S to approximately 5-7S upon digestion with trypsin. This shift was accompanied by only a small decrease in molecular weight, and was therefore attributed largely to a conformational change, i.e., an unfolding of the particle. Weintraub and Van Lente (64) subsequently showed that tryptic digestion of chromatin leads to the loss of only the N-terminal 20-30

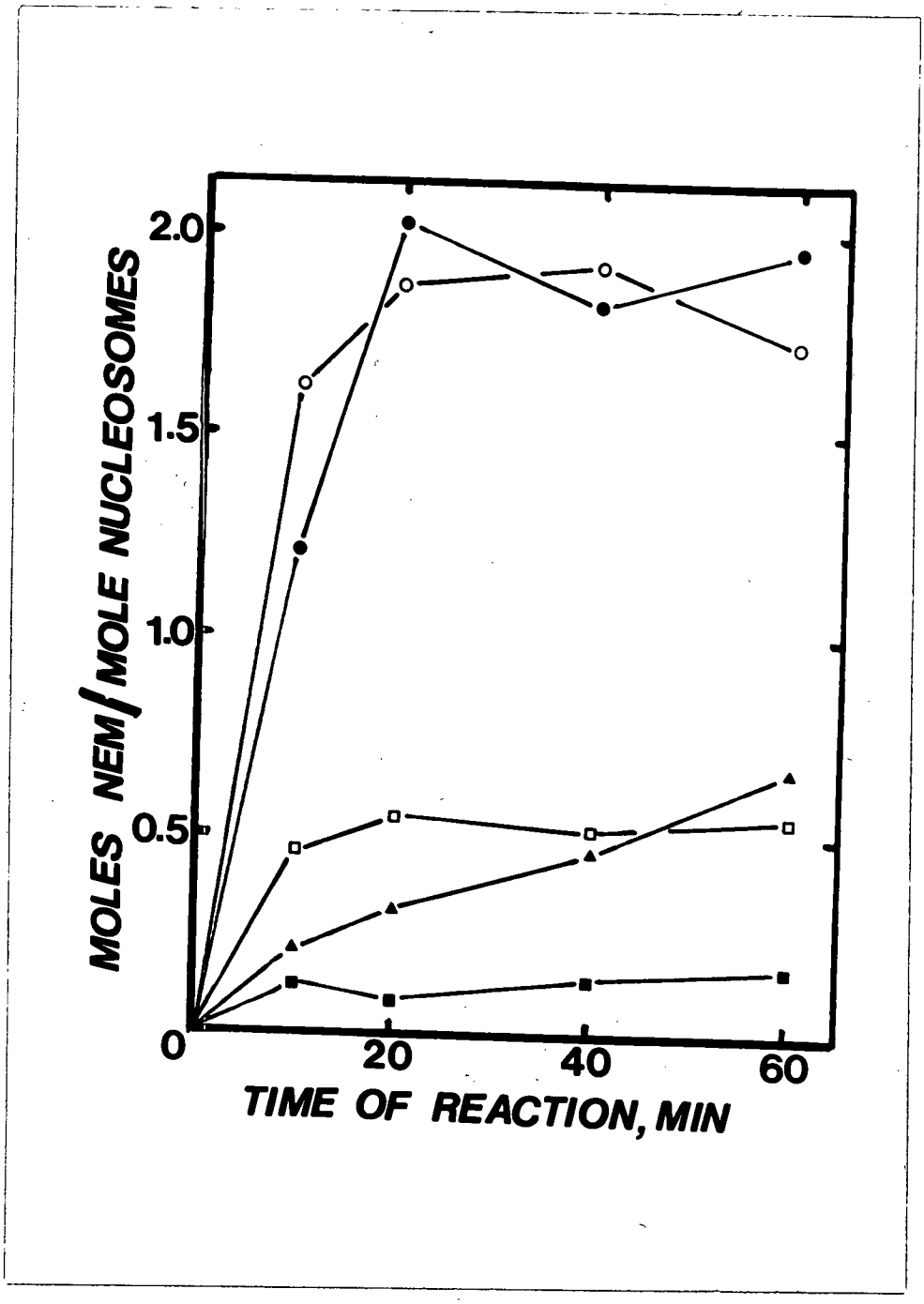

Figure 9. Sulfhydryl reactivity of oligomers in denaturing and non-denaturing solutions. Reactivity of oligomers with NEM was determined in the following solutions: ●, 2 M NaCl and 6 M urea; o, 6 M Gdn·HCl; ▲, 2 M NaCl; □, 6 M urea; ■, Tris-EDTA buffer.

amino acid residues of the core histones. This suggested that the loss of these basic regions could trigger a conformational change in nucleosomes. Since these regions are phosphorylated and extensively acetylated *in vivo* (65), these modifications could provided a mechanism for altering nucleosome conformation during transcription or DNA synthesis.

It was therefore of interest to monitor thiol reactivity in nucleosomes following digestion with trypsin. Table I shows the amount of NEM incorporated after 60 minutes of reaction in Tris-EDTA buffer, 2 M NaCl or in 6 M Gdn·HCl after trypsin digestion for various times. It was observed that trypsin digestion had no noticeable effect on the basal reactivity of monomers to NEM in Tris-EDTA buffer. If Gdn·HCl was added to 6 M after trypsin digestion, essentially stoichiometric reaction of the thiols occurs (1.6-1.75 moles SH/mole nucleosomes), as expected. The reactivity in 2 M NaCl solution also did not change after tryptic digestion (1.0 mole at 60 minutes compared to 1.2 moles/mole before treatment with trypsin) (Figure 3 and Table I). To confirm that the amino-terminal regions were removed under such conditions, *in vitro* [^{14}C]- acetate labelled monomers were subjected to trypsin digestion under similar conditions. It was found that more than 80% of the label was lost at 30-40 minutes of digestion (Figure 10). Since ε-acetyl groups are present only in the amino-terminal regions of the core histones (66-70), trypsin was effective in cleaving these

Table I

Reaction of trypsin-digested mononucleosomes with N-ethylmaleimide

Time of Digestion (min)	Reactivity in		
	Tris-EDTA	2 M NaCl	6 M Gdn·HCl
	(moles N-ethylmaleimide/mole monomer)		
0	0.05	0.82	1.75
2	0.05	1.03	1.51
5	0.05	0.85	1.63
10	0.04	1.03	1.61
30	0.05	1.03	1.61

Nucleosomes digested for 0-30 min with trypsin were allowed to react with NEM for 60 min in the indicated salt solutions at ambient temperature. The acid-precipitable fraction was counted as described in Materials and Methods.

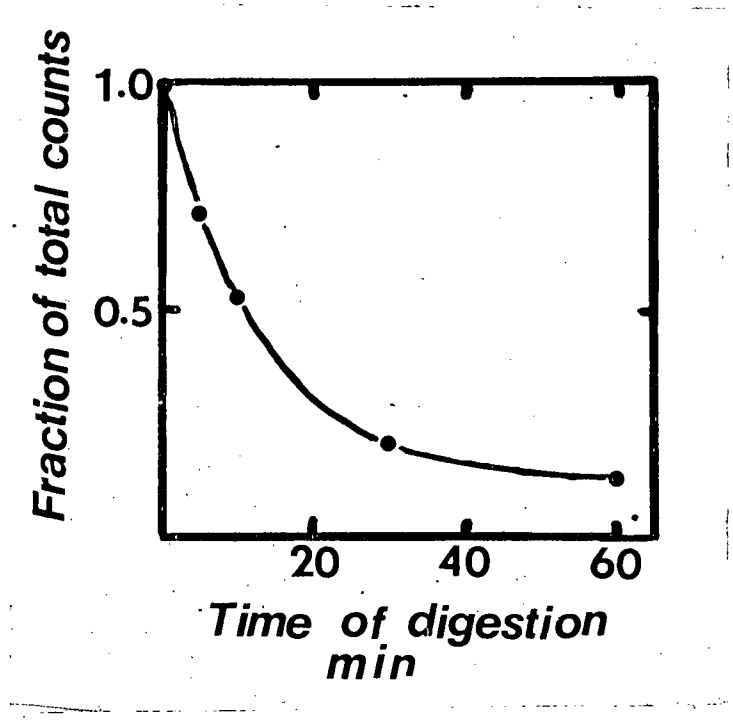

Figure 10. Efficiency of trypsin digestion. [^{14}C]-labelled nucleosomes were digested with trypsin for the appropriate time as described in Materials and Methods. The percentage of label left in the acid-precipitatable fraction is plotted against the time of digestion.

regions from the nucleosomes. The results show that tryptic digestion does not cause any significant conformational changes in the environment of the thiol groups.

Reconstitution of nucleosomes

The reconstitution of nucleosomes from salt-dissociated histones and DNA has been reported by a number of workers (71-74). These reconstituted nucleosomes resemble the native monomers in structure (15) and in nuclease digestion pattern (74). We have reconstituted nucleosomes from 2 M NaCl + 5 M urea (or 10 M urea) solutions by dialysis, and monitored the thiol reactivity using NEM.

Reconstituted nucleosomes showed very little reaction with NEM (Figure 11). Addition of NaCl to 2 M markedly increased the exposure of thiols in the reconstituted particles, giving a time course of reaction much like that of native particles (e.g. compare Figure 4 and 11). Exposure of reconstituted nucleosomes to 6 M Gdn·HCl led to the incorporation of almost 2 moles of NEM per mole of particles, as with native preparations. In order to obtain high yields of reconstituted nucleosomes with normal behaviour towards NEM, it was necessary to carry out the reconstitution in the presence of a reducing agent, e.g. 1 mM DTT. Omission of the reducing agent yielded particles in which a proportion of the H3 thiols was unreactive to NEM even under denaturing conditions, presumably due to formation of H3 intermolecular

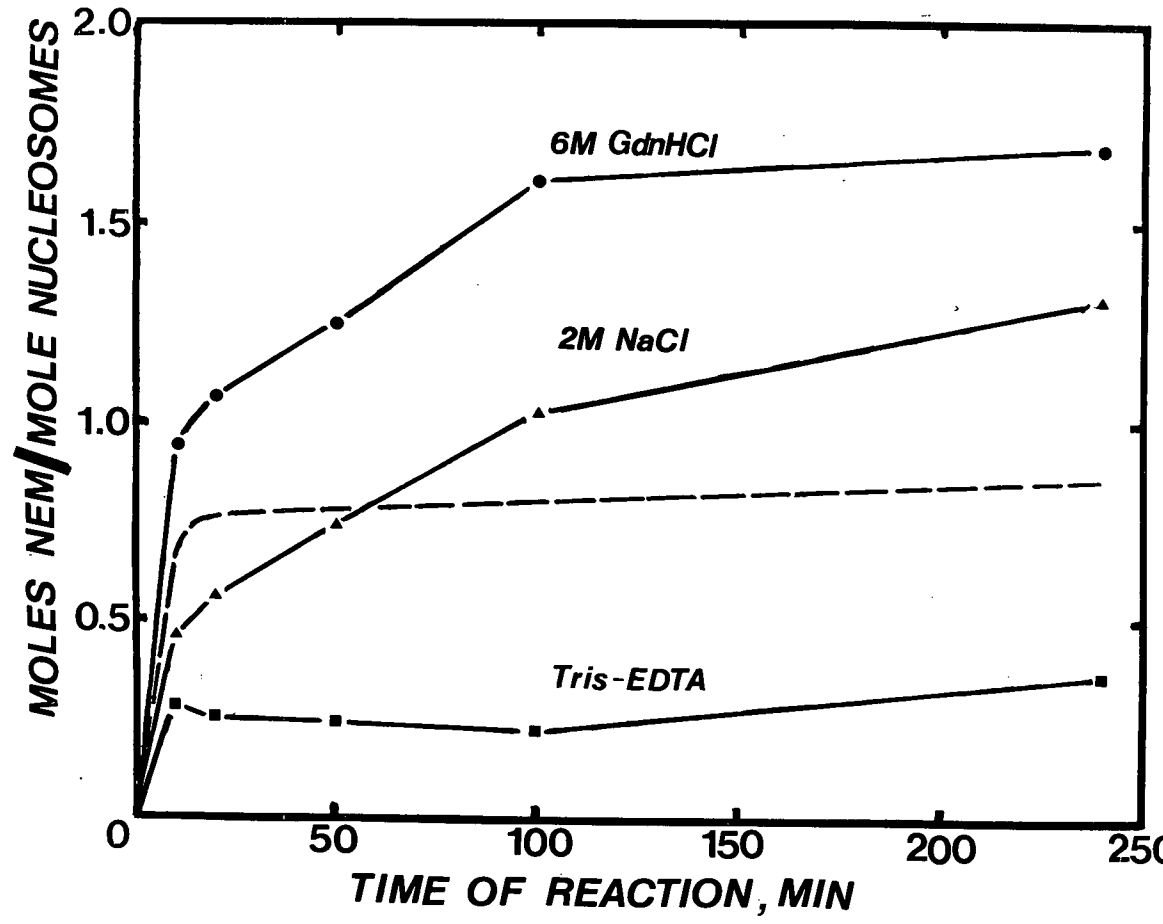

Figure 11. Time course of incorporation of [^{3}H]NEM into reconstituted nucleosomes. The specific activity of NEM incorporated into reconstituted nucleosomes under denaturing and non-denaturing conditions is plotted against the time of reaction. Solid lines, nucleosomes reconstituted in the presence of 1 mM DTT. Dashed line, Nucleosomes reconstituted in the absence of reducing agent.

disulfides (Figure 11, dashed line).

In order to further support the hypothesis that the reconstituted nucleosomes have a similar conformation to the native ones, DNase I digestion was performed and the digest products were analyzed on a 99% formamide, 6% acrylamide gel. As seen from figure 12, the gel pattern of digest products from nuclei, nucleosome monomers (native), and reconstituted nucleosomes are similar. The characteristic "ladder" pattern is clearly visible. This indicates that nucleosomes can be reconstituted after the dissociation of histones from DNA and that the reconstituted nucleosomes resemble the native particles by several criteria.

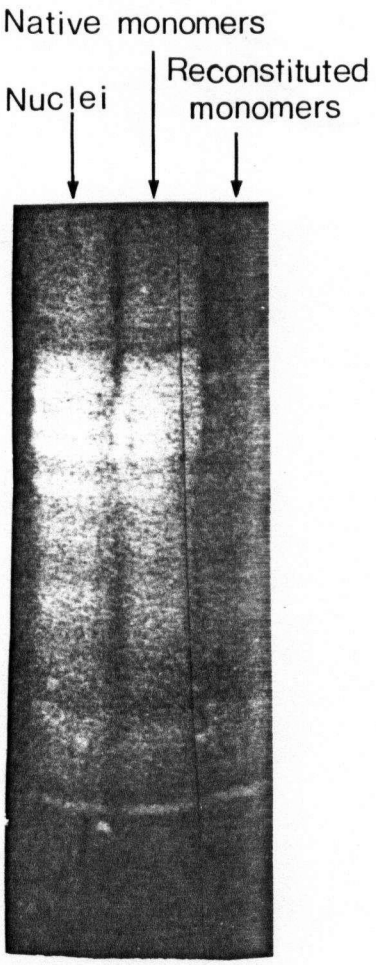

Figure 12. DNase I digestion of nuclei, nucleosome monomers and reconstituted nucleosomes. Nucleosomes were digested with DNase I and analyzed on 99% formamide, 6% polyacrylamide gel as described in Materials and Methods. The gels were stained in ethidium bromide and photographed under UV light.

DISCUSSIONS

Carboxy-terminal region of H3 in native nucleosomes

It is evident from the low reactivity of the H3 thiol group in native nucleosomes that the cysteine residue of H3 in trout testis is buried within the protein core. Although the complete amino acid sequence of trout testis H3 has not been determined, the position of the single cysteine residue is inferred from comparative data from other lower vertebrates to be at position 110 (1). The unreactive cysteine indicates that the carboxyl end of the protein is folded in such a way that the thiol group is masked and unavailable for reaction.

Hyde and Walker (24) reported that one of the two thiol groups in calf thymus H3 was inaccessible to 5,5'-dithiobis-[2-nitrobenzoic acid] in chromatin at low salt concentrations; the other thiol was found to be exposed to solvent. They inferred from primary sequence analysis that the buried thiol group is at position 96. The present data suggest that the protected thiol group in calf thymus H3 very likely corresponds, in its position in the amino acid sequence, to the single sulfhydryl residue of trout testis and chicken erythrocyte H3, which is located at position 110. This is reasonable since the cysteine at position 96 in calf thymus H3 is one of the rare amino acids that are not conserved in the primary sequence of H3. It is likely that this cysteine is non-essential for proper H3 orientation in the nucleosomes.

The unreactive sulfhydryl group in trout testis H3 has previously been demonstrated using p-hydroxymercuri-benzoate (51). However, the previous method of analysis was not as sensitive as the present one; quantitative reaction (i.e. 2 moles reagent per mole nucleosomes) could not be obtained with the former method.

Effect of salts on the structure of H3

Conformational changes of nucleosomes in solutions of different ionic strengths have been studied in various laboratories (61, 75-7). The secondary structure of nucleosomal histones was found to be affected by NaCl and divalent ions at low ionic strengths in hydrodynamic studies (75), electron microscopy (76) and solubility analyses (77). The effect of high salt concentrations on nucleosome structure was studied by Olins (61) who found that the predominant species of the histone core complex at an ionic strength of 2 was a tetramer while at an ionic strength of 4, the octamer predominates.

Three different salts were employed in the present investigation: Gdn·HCl, NaCl and $MgCl_2$. Both Mg^{++} and Gdn·HCl have denaturing effects on proteins and the behaviour of nucleosomal proteins in these salt solutions are similar. A maximal reactivity of two moles reagent per mole nucleosomes was obtained at about 1.2-1.5 ionic strength. At this ionic strength, the histones are dissociated from the DNA and must therefore be denatured by the salts to achieve maximal reactivity. The plateau reaction with these salts was reached

at a lower ionic strength than with NaCl. This is probably due to the fact that both salts are more efficient than Na^+ at displacing histones from DNA. Furthermore, the reaction maximum reached in these salts was higher than that in NaCl solutions. These results may be attributed to an unfolding effect of Mg^{++} and Gdn^+ on histone conformation at high salt concentrations.

Nucleosomes in NaCl solutions exhibit a completely different behaviour in the reaction with NEM. In 2 M NaCl, the thiol groups react with NEM at a moderately rapid rate, attaining a ratio of 1.0 mole reagent/mole nucleosomes at approximately 30-40 min, and reaching a miximum of 1.6 moles/mole at three hours (Figure 4). This is much slower than the reaction in Gdn·HCl which attains the maximum of 2 moles/mole within 5 minutes. Under these conditions, the histones are dissociated from the DNA (30), and several lines of evidence suggest that the native structures of the histones are retained: (i) salt-dissociated histones readily reassociate with DNA to yield normal-appearing nucleosomes once the salt has been removed (11, 57); (ii) Thomas and Kornberg (16) demonstrated the existence of histone octamers in salt dissociated histones, similar to those observed in native chromatin; (iii) Weintraub and Van Lente (64) observed the same tryptic histone cores whether nucleosomes or salt-dissociated histones were digested with trypsin. Two possibilities can explain the behaviour of H3 in 2 M NaCl: either

there is a direct unmasking of the cysteinyl residue upon removal of the DNA,or a conformational change in the histone core occurs as a result of dissociation of the DNA. An example of the latter possibility would be dissociation of a histone octamer to two heterotypic tetramers (42), with the H3 thiols being present at the boundary of interacting tetramers.

Studies on the reactivity of the H3 thiol group in varying salt concentrations favor the latter of the above two alternatives. The reactivity of H3 thiol groups increases from 1.0 M to 1.5 M, the concentration at which the histones are completely dissociated, and falls markedly after reaching a maximum at 2.0 M. At 4.0 M, the reactivity approaches the low level seen in control nucleosomes at low salt concentration. Under these conditions the histones are completely dissociated from the DNA; therefore, the masking of the thiol groups in 4 M NaCl must be due to the formation of histone-histone interactions. These data are consistent with a model in which the thiol groups become exposed due to dissociation of a histone octamer at 1.5-2.0 M NaCl, but become buried again upon reformation of an octameric structure at 3.5-4.0 M NaCl. Olins (61), on the basis of hydrodynamic studies, reached similar conclusions regarding histone interactions over these salt concentrations.

The fact that the maximum reactivity obtained in NaCl solutions is only 1.6 moles SH/mole nucleosomes is probably due to the formation of H3-H3 interactions which promote intermolecular disulfide formation (see below, under "recons-

titution"), thus gradually rendering the thiols unreactive toward NEM. This process would be less favorable with totally denatured H3 in Gdn·HCl solutions (6 M). In support of this interpretation, Hyde and Walker (24) found that both cysteinyl residues of acid-extracted (and therefore presumably denatured) calf thymus H3 reacted rapidly with 5,5'-dithiobis-[2-nitro-benzoic acid], but that upon incubation of the histone in 2 M NaCl, the reaction rate steadily decreased.

That the reaction of nucleosomes with NEM occurs much more slowly in 2 M NaCl than in 6 M Gdn·HCl probably reflects the heterogeneous nature of the histone-histone interactions under these conditions (42). A gradual shift in the octamer-tetramer equilibrium towards the tetramer, and/or the possibility that some of the structures "breathe" may allow the gradual titration of the thiol groups.

Effect of urea on nucleosomes

Olins et al. demonstrated the disruptive effect of urea on chromatin, using hydrodynamic studies (60). They were able to distinguish non-cooperative changes in structure which were attributed to transitions of the outer DNA-rich shell of the particle, and cooperative changes between 5 and 10 M urea, attributed to changes in the protein core. The existence of the latter cooperative effects is thus confirmed by our data on thiol reactivity. In high urea (>8 M), we infer that the histones, although still attached to DNA through basic regions, are completely denatured leading to

exposure of the H3 thiols. A similar scheme was presented by Olins et al.(60).

The inference reached above was further confirmed by the studies in various combinations of salt and urea concentrations. The salt and urea were found to act synergistically in increasing the reactivity of H3 thiols. When nucleosomes were put in 6 M urea and 0.5 M NaCl, the maximum incorporation of 2 moles NEM/mole was attained. Since neither 6 M urea nor 0.5 M NaCl alone allow significant reaction of NEM with H3 sulfhydryl groups, these agents must act cooperatively to alter nucleosome conformation. It is thus concluded that in nucleosomes the H3 sulfhydryl groups at position 110 are protected from NEM by both histone-histone and histone-DNA interactions. In the presence of 0.5 M NaCl and 6 M urea, they may become fully exposed due to a) a rupture of some ionic histone-DNA interactions which de-stabilizes the nucleosome and allows the urea to extensively denature H3, or b) a conformational change in the nucleosome which exposes the sulfhydryl groups without extensive denaturation of the histones.

Thiol reactivity of nucleosome oligomers

Oligonucleosomes differ from nucleosome monomers in the association of H1 and in inclusion of the DNA spacer regions. When such oligomers were subjected the same treatments as nucleosome monomers, similar results were obtained. This indicates that the association with H1 and a longer spacer

region has no significant effect on the buried thiols in H3. The carboxy-terminal region of H3 is therefore probably not involved in interactions among adjacent nucleosomes. There was, however, a slight difference observed in 2 M NaCl, in which the oligomers reacted at a faster rate but attaining lower maximum level. The reason for this discrepancy is unknown. Perhaps other proteins present interact with the dissociated histones. Since quantitative reaction of the thiols of oligonucleosomes occurs in 6 M Gdn·HCl, the lower reactivity in salt is not due to inactivation of the NEM itself.

Trypsin digestion of nucleosomal proteins

Studies of the primary structures of the histones have revealed that the positively charge lysyl and arginyl residues are distributed in clusters near the ends of the protein molecules (1). The amino terminal region has the greatest density of positive charge and was thought to be the primary site of interaction with DNA. This region is more susceptible to proteolysis than other regions of the histones. Earlier investigations had shown a dramatic change in sedimentation velocity of the nucleosomes upon tryptic digestion (63). Subsequently, the conformational change accompanying trypsin digestion was confirmed by nuclease digestion of trypsin-treated nucleosomes (78). Recently, Whitlock and Stein reported that the removal of the NH_2-terminal histone regions with trypsin produces relatively small changes in the folding of core particle

DNA (79). They suggested that the central and COOH-terminal histone regions are important in forming the protein core as well as stabilizing the DNA within the core particle complex.

In our experiments, trypsin digestion of the nucleosomes was found to have no effect on the reactivity of the thiol groups. Since the H3 sulfhydryl groups are situated in the COOH-terminal region, our results suggest that even if there is any large conformational change induced upon removal of the amino-terminal region, these changes must not affect the environment around cysteine 110 of H3 (i.e. the carboxyl end).

Weintraub and Van Lente (64) have shown that the trypsin-resistant region is not affected by 2 M NaCl, but is affected by 6 M urea, which indicates that the trypsin resistance is conferred by intermolecular interactions between histones. Böhm et al also demonstrated, using nuclear magnetic resonance, that the residues 1 to 41 of H3 and 1 to 37 of H4 are not required for the formation of the correct histone-histone interactions in the H3-H4 complex (39). Similar properties were described by Lilley and Tatchell (80) for chicken erythrocyte core particles treated with trypsin.

All evidence accumulated to date supports the suggestion of Whitlock and Stein that the COOH-terminal region is responsible for the organization and stabilization of the DNA in the core particle. This region must be relatively constant in structure, and resistant to proteolysis. The NH_2-terminal

regions, on the other hand, are likely to be non-essential in the organization of the DNA within the core; they may, however, have other functions in maintaining the native chromatin structure since they are relatively free to interact with other proteins such as histones acetylase, methylases and kinases (81) (the amino end is the only site described for core histone modifications).

Reconstitution of nucleosomes

Reconstitution experiments have been reported by many workers (15, 71-78). It has been found that all four histones are required for the reconstitution of a 125 Å particle (15). The reconstituted nucleosomes resemble the native monomers in structure (15) and in nuclease digestion pattern (74). Our results show that the thiol group of reconstituted nucleosomes generally behaves in the same way as that of native monomers under various conditions. These reconstituted nucleosomes must therefore have similar, if not identical, conformational structures as the native nucleosomes.

In order to obtain high yields of the reconstituted nucleosomes with normal behaviour towards NEM, it was necessary to carry out the dialysis in the presence of a reducing agent, e.g. 1 mM DTT. Omission of the reducing agent yielded particles in which a proportion of the H3 thiols was unreactive to NEM even under denaturing conditions. This observation may be due to formation of H3 intermolecular disulfides. Auto-oxidation of the cysteine residues has been reported to occur during homogenization of calf thymus (82).

Thus, although the H3 thiols at position 110 do not form a disulfide bond in the native nucleosomes, they are readily oxidized once H3 is dissociated from DNA. It has recently been shown that dimers of H3 linked through cysteine 110 can be substituted for monomeric H3 in reconstitution experiments, and yield nucleosomes which are indistinguishable from the native particles by a variety of criteria (74). These results establish that the cysteines are close together in the nucleosome; they also confirm crosslinking data indicating that the two H3 are close to each other (16, 22). The fact that they do not form a disulfide *in vivo* suggests that either the reconstituted particles have been distorted slightly to accommodate the H3 dimer, or that the environment around residue 110 in the native nucleosome is not conducive to ionization of the thiol group, perhaps due to a low dielectric constant. If the latter were true, the two cysteines might be in close contact and yet remain reduced.

DNase I digestion products of reconstituted nucleosomes are similar to those of native nucleosomes, indicating that the DNase I sensitive sites are also reconstituted. The assembly of nucleosomes is thus an intrinsic property of the histones and DNA.

Conclusion

The studies reported here indicate that the degree of exposure of H3 thiol groups in nucleosomes can be effectively monitored using a specific thiol reagent such as NEM. This sensitive assay may be used to estimate the amount of histones in isolated nucleosomal fractions such as the "active" fraction (83-4), if full reaction of the thiol groups is assumed under denaturing conditions. Further studies of thiol reactivity of crosslinked histone octamers from core particles may reveal the role of DNA in the masking of the sulfhydryl groups of H3.

The carboxy-terminal region of H3 containing Cys 110 seems to be located in a relatively stable region of the nucleosome core, perhaps at the interface between heterotypic tetramers. This region is unaffected by changes in nucleosome structure induced by tryptic digestion. This region may be essential in the organization and stabilization of the DNA in core particles.

Current efforts to elucidate the three-dimensional structure of nucleosomes by X-ray diffraction (37) should soon allow the definition of the relative positions of histones and DNA in the particles. Knowledge gained from studies of the environment of specific amino acid residues in the histones, such as in the present investigation, should be useful in formulating a detailed picture of histone-histone and histone-DNA interaction in the nucleosomes.

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