STUDIES ON NUCLEOSOMAL HISTONE ACETYLATION
AND NONHISTONE CHROMOSOMAL PROTEINS IN
RELATION TO CHROMATIN STRUCTURE

BY

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to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
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PART A - CHROMATIN SYNTHESIS

Experiments are described that suggest micrococcal nuclease selectively excises from trout testis chromatin a population of mononucleosomes containing increased levels of newly-synthesized histones and, possibly, newly-synthesized DNA. The results suggest that newly-synthesized chromatin has an increased sensitivity to micrococcal nuclease digestion.

PART B - STRUCTURE OF TRANSCRIPTIONALLY ACTIVE CHROMATIN

Different nucleases were used to probe the structure of transcriptionally active chromatin. Micrococcal nuclease and deoxyribonuclease II, enzymes which preferentially digest inter-nucleosomal linker DNA, have both been used successfully to fractionate chromatin into transcriptionally active and inactive regions. Deoxyribonuclease I, an enzyme which digests both intra- and inter-nucleosomal DNA, selectively destroys transcriptionally competent genes. After trout testis nuclei or chromatin was digested with one of the above nucleases, nucleosomes associated with the nuclease sensitive regions (transcriptionally active regions) were selectively eluted from the digested nuclei by the addition of low salt (0.1 M or 0.2 M NaCl); alternatively, nucleosomes were isolated from the digest products by virtue of their solubility in 0.1 M NaCl or 2 mM MgCl₂. The accumulated results suggest that nucleosomes containing highly acetylated histone H4 and normal levels of the acetylated species of histones H3, H2A and
H2B are associated with transcriptionally competent chromatin regions. Furthermore, the internucleosomal linker DNA associated with these regions contains low levels of histone H1, and high levels of HMG-T1, HMG-T2 and HMG-T3, and lower levels of other nonhistone chromosomal proteins. The bulk of chromatin, which is transcriptionally inactive, is associated with nucleosomes containing low levels of acetylated H4 (i.e. unacetylated and monoacetylated H4 are the major species), and the internucleosomal linker DNA is associated mainly with histone H1.

The role of H4 acetylation in transcriptionally competent chromatin may be to reduce interactions between core particles and thus render the extended chromatin region accessible to RNA polymerases. Conversely, unacetylated H4 may be involved in maintaining higher levels of compaction of the chromatin.

PART C - EFFECT OF SODIUM N-BUTYRATE ON HISTONE ACETYLATION

The biochemical mechanisms underlying the increased acetylation of histones found in butyrate - treated tissue culture cells were investigated. The results clearly indicate that the increased acetylation of histone \textit{in vivo} is most probably due to an inhibition of deacetylase enzyme activity caused by butyrate. This inhibition is entirely reversible and appears to be a general phenomenon, since butyrate increases levels of acetylated H3 and H4 in all tissue culture cell types (Xenopus laevis embryonic cells (X58), rat ascites cells (IRC8), mouse fibroblasts (3T3), baby hamster kidney cells (BHK) and Friend
erythroleukemic cells) studied.
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**PART C - EFFECTS OF SODIUM N-BUTYRATE ON HISTONE ACETYLATION**

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The research was supported by grants to Dr. P. Candido from the Medical Research Council.
DEDICATION

to

My Parents

My Brothers, Chris, Phil, Ian and Stephen

and

My Sisters, Elizabeth and Brenda
INTRODUCTION

The fundamental repeating structural unit of chromatin from all eucaryotic cells studied consists of a pair of each of the nucleosomal histones, H2A, H2B, H3 and H4, complexed with 145 base pairs of DNA. These nucleosomal core particles are joined by DNA known as linker DNA to which non-histones such as H1 and H5 can bind (92).

When examined by electron microscopy, chromatin has a "beads on a string" appearance (1), the "beads" being the nucleosomes. The nucleosomes containing the nucleosomal histones, H1 and about 200 base pairs of DNA can be excised from chromatin by mild digestion with micrococcal nuclease. Further digestion with micrococcal nuclease "trims" the nucleosomal DNA with the loss of 40 to 60 base pairs of DNA along with the associated H1 yielding the nucleosomal core particle.

L. The Histones

The histones, a group of basic proteins, are the major structural proteins of chromatin. The nomenclature and some properties of the histones are summarized in Table I. Histone H5 is similar to H1 and is found in the nucleated erythrocytes of birds, amphibians and fish.

Examination of the primary structure of the nucleosomal histones presented in Figures 1 to 4 illustrates the unequal distribution of the
<table>
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<td>I, F1, KAP, lysine-rich</td>
<td>21,000</td>
<td>Acetyl-Ser</td>
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<td>H2A</td>
<td>IIb₁, F2a2, LAK</td>
<td>14,000</td>
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<td>IIb₂, F2b, KAS</td>
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<td>H3</td>
<td>III, F3, ARE</td>
<td>15,300</td>
<td>Ala</td>
</tr>
<tr>
<td>H4</td>
<td>IV, F2a1, GRK</td>
<td>11,300</td>
<td>Acetyl-Ser</td>
</tr>
<tr>
<td>H5</td>
<td>V, F2C, KSA, erythrocyte-specific</td>
<td>20,000</td>
<td>Thr</td>
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basic amino acids. There is a predominance of these amino acids at the amino-terminal portion of the nucleosomal histones, whereas the carboxy-terminal portion contains a large number of hydrophobic amino acids capable of folding into a globular structure. Unlike the nucleosomal histones, both the amino- and carboxy-terminal regions of H1 are highly positively charged (Fig. 5).

One striking feature of the histones is the evolutionary invariability of their amino acid sequences. Histones H3 and H4 are the most conserved, with H4 showing the least variability of all proteins sequenced (2). Though H2A and H2B show variability in the amino-terminal portion, the net positive charge has been retained. The carboxy-terminal portions of the nucleosomal histones have been unusually highly conserved during evolution (3-5). These regions are involved in important histone-histone interactions which form the nucleosomal core particle.

H1 is the least conserved of the histones. Examination of H1's amino acid sequence suggests that its biological function differs from that of the nucleosomal histones. H1 is not involved in nucleosomal formation but may function in the maintenance of the higher order structure of chromatin.
FIG. 1. Amino acid sequence of histone H2A, indicating the presence of acetyl-N-serine at the amino-terminal and of epsilon-N-acetyllysine at lysine-5. Serine residues at positions 1 and 19 are potential sites of phosphorylation (2).
FIG. 2. Amino acid sequence of histone H2B, indicating the presence of multiple epsilon-N-acetyllysine residues at positions 5, 12, 15 and 20. Sites of phosphorylation at serine residues 6, 14, 32 and 36 are also shown (2).
**FIG. 3.** Amino acid sequence of histone H3, indicating sites of acetylation at lysine residues 9 (which is also a site of methylation in some H3 molecules), 14, 18 and 23. Phosphorylation may occur at serine residues in positions 10 and 28. Lysine-27 is subject to methylation (2).
FIG. 4. Amino acid sequence of histone H4, indicating multiple sites of acetylation at lysine residues 5, 8, 12 and 16. The amino-terminal serine residue is stably acetylated and transiently phosphorylated. Lysine-20 is a site of methylation (2).
FIG. 5. The complete sequence of trout testis H1 showing the positions of the repeating pentapeptide subunit (-----) and of the sites of phosphorylation (-----) (241).
II. Histone Modifications

The histones undergo a variety of post-synthetic modifications such as acetylation, phosphorylation, methylation and ADP-ribosylation. The modifications are sequence specific and occur in a specific region of the histone molecule. Modifications of the nucleosomal histones occur in the highly basic amino-terminal parts of the molecule while the modifications to H1 occur in both the amino- and carboxy-terminal portions. The modifications allow for dramatic changes in the chemical nature of the protein's side chains. For example, acetylation of a lysyl side chain will neutralize its positive charge. Thus, the modifications alter the properties of the side chain in the same manner as a radical amino acid substitution.
(a) **Acetylation**

Histones can be enzymatically acetylated in two distinct ways. (6). In the first type, acetylation occurs at the 2-amino group of the aminoterminal serine of histones H1, H2A and H4. This type of acetylation follows soon after synthesis and is essentially irreversible. The second type occurs at the ε-amino groups of the lysyl residues at the amino-terminal portion of the nucleosomal histones. Unlike the first type of acetylation, N⁶-acetylation is a rapid, reversible modification (7, 8) which occurs largely in the S phase of the cell cycle.

N⁶-acetylation occurs at specific lysyl residues in the amino-terminal portion of nucleosomal histones (Fig. 1-4). Comparison of the amino acid sequences around the acetylated lysyl residues suggests that the acetylation sites can be classified into two types. Type A, an ε-acetylated lysine is surrounded by amino acids with small neutral side chains, and type B, the lysine is part of a Lys-Arg, Arg-Lys or Lys-Lys pair.

The sequence about the acetylated lysyl residue may act as recognition sites for the enzymes responsible for the addition and removal of the acetyl groups, namely the histone acetyltransferases and deacetylases (9). These enzymes may also recognize a secondary feature of the histones, i.e. the nucleosomal histone core complex. Histone acetyltransferases and deacetylases have been purified (2). Studies of these enzymes suggest that many different types of histone acetyltransferases and deacetylases may be involved in the process of rapid addition and removal of the acetyl groups.
Neutralization of the positively charged lysyl groups by acetylation would reduce the ionic interactions between the amino-terminal portion of the histone and the negatively charged phosphate backbone of the DNA. Such alterations of the DNA-histone interaction, and hence acetylation, may be required for chromatin assembly, removal of histones from DNA and transcription of chromatin.

The biological role of histone acetylation in chromatin assembly has been studied by Louie and Dixon (10). Kinetic studies indicated that the newly synthesized H4 molecules were subjected to stepwise acetylations followed by deacetylation. Multiple acetylations of H4 would allow the positive charge density of the amino-terminal portion of the molecule to decrease, allowing for the correct alignment of the histone with DNA. Removal of the acetyl groups would then allow the correct ionic bonds to be made between the lysyl group and phosphate backbone of DNA.

Histone acetylation may be required for removal of the histone from DNA. During spermatogenesis in trout testis, the histones are replaced by a highly basic protein, protamine. The possible involvement of histone acetylation during this replacement process has been suggested by Candido and Dixon (11).

The correlation between histone acetylation and transcriptional activity will be discussed in a later section.
(b) Methylation

Of the five major histones only H3 and H4 are methylated. The modification occurs at the $N^\delta$-amino group of lysines with a resulting change in basicity and hydrophobicity but no change in charge at physiological pH. The methyl groups are transferred enzymatically to a lysyl residue in the molecule's amino-terminal portion, and the modification is stable (12).

H4 is methylated at only one site, the lysine at position 20 (13-16), while H3 can be methylated at two major sites, Lys 9 and Lys 27, and sometimes at two minor sites (17), Lys 4 and Lys 36. The modified lysyl residue may contain from one to three methyl groups. In different organs of the rat (14), Ehrlich ascites tumor cells (18), trout testis (13), calf thymus (19), and carp testis (20), H4 is modified mainly as the $N^\delta$-dimethyl lysine, and H3 is modified as the $N^\delta$-monomethyl lysine, $N^\delta$-dimethyl lysine and $N^\delta$-trimethyl lysine with the $N^\delta$-dimethyl lysine predominating. However, H4 from pea seedling is not methylated, and H3 from the same source exists as the $N^\delta$-mono-and $N^\delta$-dimethyl lysine with no $N^\delta$-trimethyl lysine detectable (21,22).

The methyl group is transferred enzymatically with S-adenosylmethionine as the methyl donor. Methylases have been isolated from Hela S-3 cells (23), chicken embryo nuclei (24), and rat brain chromatin (25). The methylases have been found in the nucleus (24, 25) and may be chromatin bound (25).
When the histones are chromatin bound, only H3 and H4 are used as substrates (25), but the specificity is lost when soluble histones are used as substrates. Thus, chromatin bound H3 and H4 may be the true substrates for the methylases.

The temporal sequence of H3 and H4 methylation after synthesis has been examined in Ehrlich ascites tumor cells (18) and trout testis (12). Methylation occurred more slowly than histone synthesis, and the methylation probably occurred after the histone was bound to DNA. H4 methylation probably follows the stepwise acetylations and deacetylations (12).

Methylation may be involved in the final arrangement of histones H3 and H4 on newly replicated DNA (18) and might be involved in histone interactions with other molecules such as histone phosphokinases (12).

(c) Phosphorylation

All five major histones from trout testis (6) and mammalian cells (26,27) are phosphorylated with the possible exception of H2B (26,27). The extent of phosphorylation during the cell cycle varies for each histone (26-31).

For the nucleosomal histones, the sites of phosphorylation all occur in the basic amino-terminal portion of the molecule (Fig. 1-5, Table II), but for H1 the phosphorylation sites occur in both the amino-terminal or carboxy-terminal portions of the molecule.
Temporal studies of histone phosphorylation using synchronized Chinese hamster ovary cells (26) and Hela S-3 cells (27) suggest that H1 and H3 are phosphorylated at different times during the cell cycle while H2A and H4 are phosphorylated at uniform rates during the cell cycle. Kinetic studies of the phosphorylation of H2A and H4 in trout testis indicate these histones are phosphorylated shortly after synthesis (32). Phosphorylation of H4 did not occur appreciably until after a series of acetylations and deacetylations while H2A was phosphorylated shortly after synthesis followed by dephosphorylation.

H3 differs from the other nucleosomal histones in that it is phosphorylated to a greater extent during mitosis than during other parts of the cell cycle (26, 31). During the cell cycle of Chinese hamster cells, H3 was phosphorylated during interphase but the levels of phosphorylated H3 increased dramatically during mitosis. The phosphorylated H3 was dephosphorylated when the cells left anaphase.

H1 has been the most studied histone with regards to its temporal phosphorylation pattern. The phosphorylation of H1 occurs at from one to four sites (Table II). As with H3, H1 is phosphorylated at different times during the cell cycle (26, 31) with maximal phosphorylation occurring during mitosis. During the cell cycle H1 is phosphorylated in different regions of the molecule (31, 33). During interphase, H1 is phosphorylated in the carboxy-terminal portion, but during maximal phosphorylation in mitosis, H1 is phosphorylated at both amino- and carboxy-terminal positions.
**TABLE II**

Phosphorylation Sequences in Histones

<table>
<thead>
<tr>
<th>Histone</th>
<th>Sequence</th>
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<tr>
<td><strong>Group 1:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2A</td>
<td>PO(_4) Ac</td>
<td>trout testis, rat liver in vivo</td>
</tr>
<tr>
<td></td>
<td>Ac-Ser-Gly-Arg-Gly-Lys 1</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>PO(_4) Ac</td>
<td>trout testis</td>
</tr>
<tr>
<td></td>
<td>Ac-Ser-Gly-Arg-Gly-Lys 5</td>
<td></td>
</tr>
<tr>
<td><strong>Group 2:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H2B</td>
<td>Ac PO(_4)</td>
<td>trout testis</td>
</tr>
<tr>
<td></td>
<td>-Ala-Lys-Ser-Ala-Pro 6</td>
<td></td>
</tr>
<tr>
<td>H3</td>
<td>Me PO(_4)</td>
<td>trout testis, calf thymus nuclei in vitro</td>
</tr>
<tr>
<td></td>
<td>-Arg-Lys-Ser-Thr-Gly-10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ac PO(_4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Arg-Lys-Ser-Ala-Pro 28</td>
<td></td>
</tr>
<tr>
<td><strong>Group 3:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1</td>
<td>PO(_4)</td>
<td>rat liver in vivo, rat liver histone kinase I (cyclic AMP) in vitro</td>
</tr>
<tr>
<td></td>
<td>-Lys-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val 38</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PO(_4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Gly-Ala-Ser-Gly-Ser-Phe-Lys 106</td>
<td>rat liver histone kinase (non-cyclic AMP, in vitro)</td>
</tr>
<tr>
<td></td>
<td>PO(_4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-Ala-Ala-Lys-Lys-Ser-Pro-Lys 157</td>
<td>trout testis</td>
</tr>
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Data collected from Dixon *et al.* (6)
Studies of H1 phosphorylation during the cell cycle of *Physarum polycephalum* suggest that the degree of phosphorylation during the cell cycle differs from that of mammalian cells (28, 29). H1 was phosphorylated during G2 and reached a peak at early prophase. The phosphorylated H1 was subsequently dephosphorylated as the cells progressed to metaphase. The results suggested that H1 must be completely phosphorylated before chromosome condensation, and thus may act as a "mitotic trigger". Alternatively, in mammalian cells phosphorylation of H1 during interphase may be a preparative step for chromosome condensation not a "mitotic trigger" (31), or it may be involved in the compaction of heterochromatin (34) while phosphorylation during mitosis may be related to chromosome condensation (27).

Enzymes responsible for histone phosphorylation have been isolated (35-38). The kinases may be either cAMP-dependent (350 or independent (36-38), and in some instances the kinases demonstrate histone specificity (37, 38).

The biological role of histone phosphorylation remains largely a mystery. Probably the role of phosphorylation for histones H4, H2A and H2B differs from that of H3 and H1. Phosphorylation of H4 and H2B may be involved in the correct positioning and binding of the newly synthesized histone to DNA (32) while H3 and H1 may be involved in chromosome condensation during mitosis (26, 27, 31, 33, 39).
(d) N-Phosphorylation

Phosphorylation can occur at the lysyl residue of H1 (N\textsuperscript{\textepsilon}-phosphoryl lysine), and the histidyl of H4 (3-phosphoryl histidine) (40). The modification is acid-labile and alkali-stable and would be destroyed by acid-extraction procedures commonly used for isolating histones.

Histone kinases responsible for the N-phosphorylation have been isolated from regenerating rat liver (40) and Walker-256 carcinosarcoma cells (41). One kinase with pH optimum of 9.5 could phosphorylate the His of H4 while another with a pH optimum of 6.5 could phosphorylate the lysine of H1. Both kinases were unaffected by cAMP (41).

In regenerating rat liver only pre-existing H4 molecules were phosphorylated at the peak of DNA synthesis (42). The modification has a half-life of two hours and has been suggested to be involved in the replication of DNA (40, 42).

(e) Adenosine - Diphospho - Ribosylation (ADP-ribosylation)

Histones are one of the many types of proteins to be ADP-ribosylated. H1 is the major acceptor of ADP-ribose groups (43-46, 102) while the "core" histones are ADP-ribosylated only to a minor extent. In rat (44) and Hela cells (47) H2B, H2A and H3 were found to be modified while ADP-ribosylation of H4 was not detectable.
The linkage of the ADP-ribose group(s) with H1 is alkali-labile (48, 49). For H1 from rat liver the ADP-ribose group is linked to a serine residue (102, 43), but for H1 from trout testis the linkage is to the -COOH of glutamic acid (48). The H1 molecule is ADP-ribosylated in both the amino- and carboxy-terminal portions (48).

The number of ADP ribose units attached to H1 varies with the source and may be from 1 to 15 (49, 50, 46, 48). The number of units attached may depend to some degree on the activity of poly ADP-ribose glycohydrolase (50), an enzyme responsible for the degradation of the poly (ADP-ribose) chain, within a particular tissue (46). H1 has been reported to be cross-linked by a single chain of poly (ADP-ribose) 15 units long in Hela cells and HBL-100 cells (a rat mammary cell line) (46). The activity of the glycohydrolase in the HBL-100 cells was very low. However, the activity of glycohydrolase from rat MTW-9 cells (a rat mammary tumor cell line) was relatively high, and the average number of units attached to H1 was reduced to 1.5. The degradation was not due to the presence of phosphodiesterase, another enzyme which will degrade the ADP-ribose polymer (49).

The enzyme responsible for the synthesis of poly(ADP-ribose), poly (ADP-ribose) polymerase, has been isolated from several sources including rat liver (50), trout testis (50), and Hela cells (51, 47, 52). The enzyme, which has an almost absolute requirement for DNA (50), is tightly associated with chromatin (49) and catalyzes the successive addition of ADP ribose units from NAD to form a polymer. The enzyme is associated with
the linker DNA region between nucleosome core particles (47, 51, 52) and has been suggested to be associated with template "active" chromatin (51).

The biological function of this modification is poorly understood, but it has been suggested to be involved in chromosome condensation (45, 53).

III. The Nucleosome

Since the discovery in 1974 that chromatin has a basic repeating structural unit (called the nucleosome) (1, 54, 55), many research groups have presented evidence to elucidate the fine structure of this repeating unit. Although the fine structure of the nucleosome with regards to conformation of the histones within the nucleosome, histone-histone interactions, and histone-DNA interactions is not fully understood, many known aspects of nucleosome structure have enabled researchers to present models of this repeating chromatin unit.

(a) Histone-Histone Interactions

The histone-histone interactions are important in maintenance of the histone core complex. The histone complement of the complex contains two molecules each of the histones H2A, H2B, H3 and H4 (1). The core histone composition has been examined and confirmed by cross-linking studies of chromatin (46) and of isolated nucleosomal core particles (57). The core histone complex has been isolated free of DNA at high ionic strength (58-61) and has been reported to exist in solution as a tetramer, (H2A)
Eickbush and Moudrianakis (62) favor the octamer form of the histone complex and suggest that the discrepancy may be due to isolating the complex in an unpurified form.

Studies on the interactions of the histones, and cross-linking studies suggest the preferential association of H3 and H4 to form a \((H3)_2(H4)_2\) tetramer (63-66), and the preferential association of H2A and H2B to form a \((H2A)(H2B)\) dimer (67, 68, 69, 62, 70, 63). NMR studies have demonstrated the involvement of the carboxy-terminal portions of the histones in the histone-histone interactions. This region of the molecule adopts a 'globular structure (71) while the amino-terminal portion of the molecule exists as a random coil (71). The amino-terminal portion of the molecule is vulnerable to digestion by trypsin when either associated with the histone core complex or bound to DNA (59, 72). NMR studies suggest the involvement of residues 31-95 for H2A and 37-114 for H2B in the formation of the \((H2A)(H2B)\) dimer (62, 71). Residues 42-120 for H3 and 38-102 for H4 are involved in the tertiary structure of the \((H3)_2(H4)_2\) tetramer (71, 73).

The interactions of H2A or H2B with H3 or H4 have been studied by using chemical crosslinking (67, 68, 70). Through the use of zero length crosslinkers (70), H2B has been found to contain separate binding sites for H2A and H4. The carboxy-terminal half of H2B interacts with the carboxy-terminal portion of H4 while the amino-terminal portion of H2B interacts with...
H2A. The interactions of the histone components of the (H2A) (H2B) dimer or (H3)_2 (H4)_2 tetramer are largely hydrophobic in nature (62) while the interactions between the two (H2A) (H2B) dimers and the (H3)_2 (H4)_2 tetramer to form the histone octamer are possibly maintained by H-bonds.

(b) Histone - DNA Interactions

The nucleosomal DNA is compacted about seven fold (74). The DNA is wrapped around the histone core about 1 3/4 turns (75), with each histone core complexing with 140 base pairs of DNA in a single left-handed, non-interwound DNA supercoil (76).

The nucleosomal histones devoid of their amino-terminal amino acids are capable of compacting the DNA into a nucleoprotein complex (77). Digestion of chromatin with trypsin results in the release of 20 to 30 amino acids from the amino-terminal portion of the nucleosomal histones (72). If the 11 S, nucleosomal core particle, is digested with trypsin, the sedimentation coefficient changes to 9.7 S with an accompanying increased sensitivity to micrococcal nuclease (77, 78). Thus the amino-terminal portions of the nucleosomal histones may not be necessary for the folding of DNA into a nucleoprotein complex but may be involved in the maintenance of the native nucleosomal core particle structure.

H3 and H4 are very important in the organization of the DNA around the histone core complex (79, 80). H3 and H4, when associated with 140
base pairs of DNA, can produce nucleoprotein complexes which are morphologically similar to nucleosomes (79). H4 plays a key role in the correct association of DNA with the histone core (80).

IV. Nucleosomal DNA Size Diversity

When chromatin is mildly digested with micrococcal nuclease, the DNA linker region is most susceptible to attack. The digest products may be separated by gel electrophoresis to yield a repeat of multiples of the lowest unit length, about 200 base pairs (54). After further digestion, the nucleosomal DNA is trimmed to about 140 base pairs, the length of DNA associated with the nucleosomal core particle (81, 82). Extreme digest conditions result in digestion of the intranucleosomal DNA length yielding fragments of about 127, 118, 108, 99, 80, 60 and 52 base pairs in length (80).

Using mild digest conditions, the nucleosomal DNA length has been found to vary with different sources (83-88) (Table III). It has been suggested that the DNA repeat length decreased with increasing transcriptional activity of the cell studied (66). However, studies by Gottesfeld and Melton (85) demonstrate the length of the nucleosomal DNA remains the same for both transcribed and nontranscribed regions of chromatin. In some cases the DNA repeat length of actively dividing cells was shorter than that of the mature cell (83), but this is not a general characteristic of chromatin from actively dividing cells (86).
TABLE III
DNA Content of Nucleosomes

<table>
<thead>
<tr>
<th>Cell type</th>
<th>DNA repeat length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspergillus</td>
<td>154</td>
</tr>
<tr>
<td>Yeast</td>
<td>165, 163</td>
</tr>
<tr>
<td>Rabbit cortical neuron</td>
<td>162</td>
</tr>
<tr>
<td>Neurospora</td>
<td>170</td>
</tr>
<tr>
<td>Physarum</td>
<td>171, 173</td>
</tr>
<tr>
<td>Tetrahymena micronucleus</td>
<td>175</td>
</tr>
<tr>
<td>Cells grown in culture</td>
<td></td>
</tr>
<tr>
<td>CHO</td>
<td>177</td>
</tr>
<tr>
<td>HeLa</td>
<td>183, 188</td>
</tr>
<tr>
<td>hepatoma</td>
<td>188</td>
</tr>
<tr>
<td>teratoma</td>
<td>188</td>
</tr>
<tr>
<td>P815</td>
<td>188</td>
</tr>
<tr>
<td>myoblast</td>
<td>189</td>
</tr>
<tr>
<td>CV1, exponentially growing or confluent</td>
<td>189</td>
</tr>
<tr>
<td>BHK</td>
<td>190</td>
</tr>
<tr>
<td>rat kidney primary culture</td>
<td>191</td>
</tr>
<tr>
<td>myotube</td>
<td>193</td>
</tr>
<tr>
<td>C6, exponentially growing or confluent</td>
<td>198</td>
</tr>
<tr>
<td>Rat bone marrow</td>
<td>192</td>
</tr>
<tr>
<td>Rat fetal liver</td>
<td>193</td>
</tr>
<tr>
<td>Rat liver</td>
<td>198, 196</td>
</tr>
<tr>
<td>Rat kidney</td>
<td>196</td>
</tr>
<tr>
<td>Syrian hamster liver</td>
<td>196</td>
</tr>
<tr>
<td>Syrian hamster kidney</td>
<td>196</td>
</tr>
<tr>
<td>Chick oviduct</td>
<td>196</td>
</tr>
</tbody>
</table>
(TABLE III Continued)

<table>
<thead>
<tr>
<th>Cell type</th>
<th>DNA repeat length (base pairs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrahymena macronucleus</td>
<td>202</td>
</tr>
<tr>
<td>Rabbit cerebellar neuron</td>
<td>200</td>
</tr>
<tr>
<td>Rabbit nonastrocytic glial cells</td>
<td>200</td>
</tr>
<tr>
<td>Stylonychia micronucleus</td>
<td>202</td>
</tr>
<tr>
<td>Chicken erythrocyte</td>
<td>207, 212</td>
</tr>
<tr>
<td>Sea urchin gastrula</td>
<td>218</td>
</tr>
<tr>
<td>Stylonychia macronucleus</td>
<td>220</td>
</tr>
<tr>
<td>Sea urchin sperm</td>
<td>241</td>
</tr>
</tbody>
</table>

For references, see Chambon (92)
What causes the nucleosomal DNA size diversity? Although the role of nucleosomal DNA length variability is unknown, suggestions for the cause of the variability have been made. Variability in the nucleosomal DNA might result from the variability in the primary sequence of H1 (81-84, 89) which binds to the nucleosomal DNA before trimming of the nucleosomal DNA takes place (90). Weintraub (83) has reported that during erythropoiesis in the chick, the nucleosomal DNA length increases from 190 base pairs to 212 base pairs. Accompanying the increase in nucleosomal DNA length is an increase in the levels of the red blood cell specific histone, H5 (a histone similar to H1 but more basic). Thus, the presence of H5 may cause the change in nucleosomal DNA length. Alternatively, Wilhelm et al. (91) doubt that there is a direct correlation between the primary sequence of H1 or H5 and the nucleosomal DNA repeat length. The sequence variability of the nucleosomal histones, H2A and H2B, might influence the size of the nucleosomal DNA (92, 93). There is doubt as to whether or not H2A and H2B are responsible. Spadafora et al. have reconstituted chromatin by complexing the nucleosomal histones from either calf thymus, Chinese hamster ovary cells or sea urchin gastrula cells with SV40 DNA form I by stepwise dialysis from 2 M NaCl (94). In all cases, although the H2A and H2B sequence varied, the DNA repeat length remained the same. Caution should be taken with these types of reconstitution experiments because inaccuracies in the DNA-histone interactions induced by the method of reconstitution used may lead to artifactual results.
The basic structure of the nucleosome core particle is a histone core surrounded by DNA. Noll suggested the DNA was on the exterior of the particle from digestion studies using DNase I (95). This enzyme has the ability to digest both inter- and intranucleosomal DNA at similar rates. The DNA digest products, when denatured, exhibited a 10 base repeat ladder when separated by gel electrophoresis. This repeat length corresponds to the pitch of the DNA helix. Ideally, one would want to know the exact position of cutting sites of the nucleosomal core DNA with this enzyme and, therefore, be able to suggest where histone-DNA interactions interfered with DNase I attack. By labelling the 5' ends of the nucleosomal core particles with $^{32}$P, various workers have shown that sites 30, 60, 70, 80 and 110 exhibit a lower accessibility to digestion (96-100). Using high resolution gel electrophoresis, Lutter reported that the DNase I cutting sites were multiples of about 10.4 bases instead of 10 (101).

Endonucleases other than DNase I (Table IV) were examined for their intranucleosomal core DNA cutting patterns. All of the endonucleases cut at a 10-nucleotide interval (96-99) but the susceptibility of the sites differed. Although the conformation of the DNA within the nucleosome influences its nuclease susceptibility, the actual rate of cleavage at a given site may be a function of the enzyme's catalytic and physicochemical properties. However, all the endonucleases digested sites 30, 60, 80 and 110 base pairs from the 5' end of the nucleosomal core DNA with decreased
### TABLE IV

**Properties of Endonucleases Used**

<table>
<thead>
<tr>
<th>Source</th>
<th>Beef pancreas (DNase I)</th>
<th>Hog spleen (DNase II)</th>
<th>Staphylococcus aureus (Micrococcal nuclease)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mr</strong></td>
<td>31,000</td>
<td>38,000</td>
<td>16,800</td>
</tr>
<tr>
<td><strong>Isoelectric point</strong></td>
<td>4.7 - 5.0</td>
<td>10.2</td>
<td>9.6</td>
</tr>
<tr>
<td><strong>pH optimum</strong></td>
<td>7.0</td>
<td>4.8</td>
<td>9.2</td>
</tr>
<tr>
<td><strong>Activators</strong></td>
<td>Mg$^{+2}$</td>
<td>EDTA</td>
<td>Ca$^{+2}$</td>
</tr>
<tr>
<td><strong>Product</strong></td>
<td>5' - Phosphate terminal</td>
<td>3' - Phosphate terminal</td>
<td>3' - Phosphate terminal</td>
</tr>
</tbody>
</table>
frequencies relative to other sites. Presumably, this variation could be attributed to the orientation of the sugar phosphate bonds to the histone core. From these observations the pitch of the DNA has been estimated to be 10.33 - 10.40 base pairs (100). Also, the results support suggestions that the DNA is wound around the histone core smoothly, without interruption of base-stacking interactions (75).

Small angle neutron scattering measurements (103, 104) and X-ray scattering (105) data of the nucleosome further consolidate the endonuclease results that DNA is on the outside of the histone core. The results indicate the histone core is 32 A in radius surrounded by a DNA rich shell 20 A thick, giving the nucleosome core particle an average radius of 52 A. Crystals of the nucleosome core particle have been studied by electron microscopy (106) and X-ray diffraction (75). The nucleosome core particle has dimensions of 110 x 110 x 57 A, and is somewhat wedge-shaped. The DNA is wound into about 1 3/4 turns of a flat superhelix with a pitch of about 28 A and an average diameter of about 90 A. The pitch of the superhelix is small enough that interactions between two DNA turns might take place if aided by cations and/or histone salt bridges (i.e. amino-terminals of the nucleosomal histones).

VI. Nucleosomal Models

Kornberg's model (1) for the nucleosome, consisting of an histone octamer, (H2A)$_2$ (H2B)$_2$ (H3)$_2$ (H4)$_2$, surrounded by DNA, has been basically unaltered. The model has been further detailed by Weintraub (76) to explain transcription and replication processes in the presence of
nucleosomes (to be discussed in a later section). Based partly on the existence in solution of an heterotypic tetramer histone complex, (H2A)(H2B)(H3)(H4), (61, 59) Weintraub has presented a model for the nucleosomal core particle that consists of two isologously paired heterotypic protein tetramers surrounded by a single non-interwound, left-handed DNA supercoil 140 base pairs long and about 95 base pairs in circumference. The nucleosome is organized about a dyad axis of symmetry, a specification that is supported by X-ray diffraction data (75) and endonuclease digestion studies (100). Although the existence of an heterotypic tetramer histone complex in solution has been questioned (62), no evidence is available to disapprove that the histone octamer when bound to DNA cannot split into symmetrical halves, each containing the heterotypic histone tetramer. This model has the ability to explain how transcription or replication of the DNA might occur without histone displacement.

VII. Higher Order Chromatin Structure: Role of H1

The "beads on a string" form of chromatin consists of the nucleosomal core particles joined by the linker DNA and represents the most extended form of chromatin. Compaction of the linker allows the formation of fibers, 100 Å in diameter (79, 74, 107, 108). Further compaction results in the appearance of a 300 Å diameter fiber (79, 109-111) or "solenoids" (110). Stabilization of the solenoidal structure requires H1 (75, 110, 109, 112) and divalent cations.

Of the five main histones, H1 is the only histone not involved in the formation of the nucleosomal core particle. H1 is associated with about 35-40 base pairs length of DNA in the linker region (90). The main function
assigned to this histone is a crosslinking role in the maintenance of a higher order chromatin structure (113, 109, 112, 75, 114).

DNase II appears to have the ability to recognize higher order chromatin structures (79, 115, 116). Nuclei or chromatin digested with DNase II at appropriate ionic strengths (1 mM CaCl$_2$ or 150 mM NaCl) will produce double-stranded DNA products with a 100 base pair periodicity, indicating nuclease digestion between and within the nucleosome core particles has occurred (115). Ionic conditions of this sort are required for the maintenance of higher order chromatin structures (110, 112, 75, 113, 117, 109). DNase II digestion of chromatin at lower ionic strength results in digestion of only the linker DNA region.

VIII. Transcriptionally Active Chromatin

Although the bulk of chromatin appears to consist of a repeat of nucleosomes, does actively transcribed chromatin have the same nucleosomal structure? Digestion studies of nuclei or chromatin by micrococcal nuclease suggest that actively transcribed chromatin is associated with nucleosomes. Hybridization studies using either total DNA (118), cDNA to cytoplasmic polyadenylated mRNA (119) or cDNA to a specific mRNA indicate that the nucleosomes contain most if not all of the genomic sequences (118), and, in the case of an actively transcribing gene such as the ovalbumin gene in hen oviduct, the nucleosomes protect sequences from digestion (120, 121, 122). Nucleosomes also protect sequences coding for actively transcribing ribosomal RNA genes (123-126). Unlike other actively transcribing genes
studied, the ribosomal RNA gene's protection by nucleosomes decreases with increasing transcriptional activity (123, 126).

Further elaboration of these studies demonstrated that micrococcal nuclease preferentially excises actively transcribing chromatin. Using a cDNA (ovalbumin) probe, sequences coding for the ovalbumin gene were found to be preferentially associated with mononucleosomes generated by micrococcal nuclease digestion of hen oviduct nuclei (122, 121). Dinucleosomes, trinucleosomes, and higher repeats all had diminishing levels of the ovalbumin DNA sequences. However, genes that were not being transcribed (i.e. the globin gene in hen oviduct) did not exhibit the preferential excision by micrococcal nuclease.

The structure of actively transcribing genes has been studied by electronmicroscopy. As the transcriptional activity of the ribosomal RNA gene increases (estimated from the number of nascent RNA chains attached), the frequency of nucleosomes decreases. For very active rRNA genes, the frequency of nucleosomes is either very low or negligible (127-129). Although this would appear to conflict with the biochemical evidence (124), several authors (92, 127) speculate that the compact nucleosomal structure transforms into a more "open" conformation with the histones still present on the DNA offering protection against micrococcal nuclease or DNase II attack (see Nucleosome models). McKnight et al. (212) have presented evidence that the nucleosomal histones are found on transcribed nonribosomal chromatin. The authors demonstrated through the use of immunoelectron microscopy using anti-
histone immunoglobulins that the histones, H2B and H3, remain associated with transcriptionally active chromatin. The chromatin region could therefore be transcribed without loss of the histones as suggested by \textit{in vitro} transcription studies of BK virus chromatin (130) and SV40 chromatin (131), and by examination of the SV40 transcriptional complex (132).

Unlike micrococcal nuclease and DNase II which preferentially digest the linker DNA region, DNase I digests both intra- and internucleosomal DNA with equal frequency. Also unlike the other two nucleases, DNase I will preferentially digest transcriptionally competent genes (120, 133-136). Digestion of nuclei from chick erythrocytes, or hen oviduct with DNase I results in the preferential digestion of the globin (133), or ovalbumin gene (120, 134), respectively. Alternatively, the globin gene from hen oviduct or the ovalbumin gene from chicken erythrocytes were not preferentially digested by DNase I. Although the bulk of chromatin is digested by DNase I, producing a 10 base single-strand DNA repeat when examined by gel electrophoresis, this material from transcriptionally incompetent genes has the ability to hybridize either to total undigested fragmented DNA or to a specific cDNA probe. Thus, the DNA of a transcriptionally competent gene must be digested to acid-soluble oligonucleotides or to small fragments either unable to form stable duplexes, or able to form stable duplexes but at a much reduced rate (120).

Selective digestion by DNase I of transcriptionally active integrated adenovirus genes in transformed hamster cells (135), and of DNA sequences transcribed into poly A mRNA from trout testis (136) has also been reported.
The selective digestion by DNase I does not change with the transcriptional activity of the gene (134). Thus, the nucleosomes associated with a transcriptionally competent gene exist in an altered conformation sensitive to DNase I attack (133).

To further demonstrate the existence of nucleosomes with an altered conformation, mononucleosomes containing sequences of a transcriptionally competent genes were examined for DNase I sensitivity. Mononucleosomes obtained from chicken erythrocyte nuclei had the ability to hybridize to a cDNA (globin) probe but when digested by DNase I, mononucleosomes containing the globin gene were selectively attacked. However, identical experiments using mononucleosomes containing ovalbumin gene sequences from hen oviduct nuclei were not selectively digested by DNase I. This discrepancy might be resolved if mononucleosomes from hen oviduct did not contain HMG proteins (high mobility group proteins: a group of nonhistone chromosomal proteins) while the mononucleosomes from chick erythrocytes did as HMG proteins have been suggested to be involved in maintaining the DNase I sensitive state (137).

Chromatin has been fractionated by many different procedures with the goal of separating transcriptionally active chromatin from the bulk of chromatin (for a review see Ref. 138). Of the methods reported, techniques utilizing selective excision of transcriptionally active chromatin by DNase II (139, 140) or micrococcal nuclease (142-144, 122) have been the most successful. Gottesfeld has reported a method for fractionating chromatin by digestion of chromatin with DNase II followed by the selective precipitation of the
digest products with 2 mM MgCl$_2$. The Mg$^{+2}$ soluble fraction was enriched in DNA sequences complementary to poly A-containing cytoplasmic RNA (141). Mononucleosomes associated with the Mg$^{+2}$-soluble fraction had a higher sedimentation coefficient than that of nucleosomes associated with the bulk of chromatin (14 vs 11 S) (130). The 14 S mononucleosomes had a higher sensitivity to DNase I digestion than did 11 S mononucleosomes.

Levy W. et al. have used micrococcal nuclease to digest trout testis nuclei. The digest products were released by lysis of the nuclei with EDTA followed by the selective precipitation of the products with 0.1 M NaCl (142-144). The 0.1 M NaCl soluble fraction was enriched in transcribed sequences, and this fraction consisted only of mononucleosomes that contained the nucleosomal histones, H6 (a nonhistone chromosomal protein), no H1 and 140 base pairs of DNA (143).

IX. Replication of Chromatin

Several problems in understanding the mechanism of chromatin replication are similar to those encountered for the transcription process. For example, are nucleosomes associated with DNA when it is being replicated? The mode of segregation of the nucleosomes at the replication fork and whether or not newly synthesized histones are assembled without "old" histones to form the histone octamer are also important questions.
Transmission electron microscopic techniques have been used to study chromatin replication in the S phase genome of cellular blastoderm Drosophila melanogaster embryos (145). In this study, nucleosomes were not removed or dissociated prior to replication. Pulse labelling studies using $[^3]H$-thymidine confirmed the presence of nucleosomes on the newly synthesized DNA (146-148). The nucleosomes associated with newly synthesized were excised by micrococcal nuclease at twice the rate of nucleosomes from the bulk of chromatin (146, 148). However, this enhanced sensitivity decreased as the the nucleosomes presumably attained the higher order chromatin structure (146). The nucleosomes associated with newly synthesized differed in structural features from nucleosomes associated with the bulk of chromatin in that they had a smaller DNA repeat length and differed both in micrococcal nuclease and DNase I digest patterns of their core particles (148). Thus, like the nucleosomes associated with actively transcribing chromatin, the nucleosomes associated with newly synthesized chromatin have an altered conformation.

The segregation or distribution of the nucleosomes at the replication fork is conservative. In the presence of the protein synthesis inhibitor, cycloheximide, the DNA synthesized is digested at twice the rate of parental chromatin (146, 149). If the nucleosomes orientated themselves in a dispersive manner at the replication fork, the frequency of micrococcal nuclease attack would increase about five fold due to the increased spacing between the nucleosomes (146).
The nucleosomes associated with newly synthesized DNA consist of only newly synthesized histones which are not mixed with "old" previously synthesized histones. The replicating DNA of SV40 was reported to associate preferentially with newly synthesized histones (150, 151). Also the association of newly synthesized histones with newly synthesized DNA, and the association of "old" histones with the parental DNA from Ehrlich ascites tumor cells has been reported (152).

The preferential association of only newly synthesized histones to form a nucleosome has been demonstrated by Leffak et al. (153). In this study chick myoblast organ cultures were pulsed for one hour with dense amino acids and $[^{3}\text{H}]$-lysine. The nucleosomes were isolated and the histones cross-linked to form a cross-linked octamer core histone complex. Analysis of the cross-linked octamer by density gradient, equilibrium centrifugation demonstrated the conservative assembly of only newly synthesized histones to form the nucleosome.

Through the processes of conservative segregation of the nucleosomes at the replication fork and conservative assembly of the nucleosomes, information about structural chromatin regions, i.e. a transcribing region, might be perpetuated during subsequent cell generations.

The assembly process of histones with DNA to form a nucleosome seems to require an assembly factor \textit{in vivo}. The factor is an acidic, thermostable protein that has been isolated from a supernatant extract of \textit{Xenopus laevis}.
eggs (154, 155), mouse fibroblast nuclei and wheat embryos (155). The factor binds the four nucleosomal histones in equimolar amounts (155). Recently a nicking-closing enzyme has been found to be able to assemble nucleosomes under physiological conditions in the absence of the assembly factor (156). The nicking-closing enzyme will assemble nucleosomes with a greater efficiency than the assembly factor. It is not clear at this time whether or not the assembly factor is a specialized protein capable of aiding the nicking-closing enzyme in nucleosome assembly in only certain systems such as Xenopus oocytes where rapid assembly of nucleosomes from the stored histones is required.

X. Nonhistone Chromosomal Proteins

A large variety of nonhistone chromosomal proteins are also bound to chromatin. These proteins have been classified into two groups based on their electrophoretic mobility on 20% polyacrylamide gels at pH 2.4 (224). They are termed low mobility group (LMG) or high mobility group (HMG) proteins.

The four main HMG proteins, HMG-1, HMG-2, HMG-14 and HMG-17, from calf thymus have been either partially or totally sequenced. The HMG proteins comprise a small fraction of the total chromosomal proteins, being about 3% by weight of DNA (158). HMG-T, a protein isolated from trout testis, has a similar amino-terminal sequence to HMG-1 and HMG-2 (159). Also, H6, formerly thought to be a histone in trout testis, has a sequence
similar to HMG-14 and HMG-17 (160, 168).

Research groups have investigated whether the low levels of the HMG proteins were associated with specialized regions of chromatin (i.e. actively transcribing chromatin). HMG-T from trout testis nuclei and HMG-1 and HMG-2 from calf thymus or mouse brain nuclei were readily released into the supernatant after micrococcal nuclease digestion (161). The results suggest that the proteins are associated with the linker DNA region (161, 162). However, when rabbit thymus nuclei were digested with micrococcal nuclease, only subfractions of HMG-1 and HMG-2 (perhaps modified forms) were rapidly released (163) while HMG-14, 17 and a subpopulation of HMG-1 and HMG-2 were apparently tightly bound to the nucleosome (158, 163). H6 remained bound to the nucleosome after trout testis nuclei were digested with micrococcal nuclease (162), but H6 was released when trout testis nuclei were digested with DNase I (160, 161). DNase I digestion of calf thymus nuclei (161), mouse brain nuclei (161) or duck erythrocyte chromatin (164) released HMG-1 and HMG-2, but the release of these proteins was not observed after DNase I digestion of rabbit thymus or rat liver nuclei (165). The discrepancy may be due to the nuclear isolation and digestion conditions used. The possibility exists that these proteins might be associated with transcriptionally active chromatin by virtue of the fact that HMG proteins that are bound to linker DNA are rapidly released after micrococcal nuclease digestion. Moreover, nucleosomal bound HMG proteins are released after DNase I digestion using conditions known to selectively digest active genes. Interestingly, HMG-14 and HMG-17 have been suggested to be required for maintenance of a DNase I sensitive, transcriptionally active gene (137).
HMG-1 and HMG-2 have been isolated and characterized from several sources including calf thymus, calf kidney, calf liver, chicken thymus, chick red blood cells and duck red blood cells (157). The proteins were found to be very similar despite the source. If the proteins do play a role in maintaining chromatin in a transcriptionally active form, HMG-1 and HMG-2 would presumably be involved as structural or nonspecific gene regulators.

A24 is a unique chromosomal protein containing ubiquitin attached to histone H2A by an isopeptide linkage (166). A24 has been found to be associated with nucleosomes (166). Ubiquitin has been identified in trout testis but the presence of A24 has not yet been reported (167).

XI. Histone Acetylation: Correlation with Transcriptional Activity of Chromatin

The histones are modified post-synthetically by acetylation, phosphorylation and methylation. Acetylation at the ε-lysyl residues of the nucleosomal histones has been suggested to play a role in gene regulation. Studies involving hormonal stimulation such as the aldosterone-stimulated kidney (169), the uterus after estradiol-17β injection (170) and cortisol-stimulated liver (2) have shown increased acetylation of histone H4 and, in some cases, H3. In all cases the increased histone acetylation preceded increased RNA synthesis. Other agents that stimulated RNA
synthesis such as polyamines and mitogenic agents also increased the level of histone acetylation (2).

Highly acetylated forms of the histones have been reported to be associated with transcriptionally active chromatin. Histones H3 and H4 associated with the SV40 minichromosome are highly acetylated while those associated with the minichromosome of a non-transforming mutant have decreased levels of the acetylated histone species (171). Also, the transcriptionally active macronucleus from *Tetrahymena pyriformis* contains highly acetylated histones while the transcriptionally inert micronucleus has low levels of the acetylated histones (172).

XII. The Present Investigation

At the beginning of this project, studies of histone modifications (i.e. acetylation) in relation to nucleosome structure had not yet been reported. The trout testis system was ideal for this investigation and the sites, kinetics and cellular localizations of histone acetylation have been well characterized. The large quantities of tissue available, easy preparation of nuclei and chromatin, and ability of cell suspensions to incorporate radioactive precursors provide more reasons for selecting this system.

Parts A and B of this thesis report on the levels of the acetylated histone species associated with nucleosomes, and the levels of acetylated histone species associated with nucleosome subfractions. The results
indicate that the mononucleosomes associated with the bulk of chromatin contain the full spectrum of acetylated histone species found in whole chromatin. However, nucleosomes associated with nuclease-sensitive (micrococcal nuclease, DNase II or DNase I) regions of chromatin (probably regions of chromatin involved in replication (148) and/or transcription (140, 141, 142, 133, 121, 122)) contained high levels of the acetylated species of histone H4. The other nucleosomal histones H2A, H3 and H2B contained normal levels of their respective acetylated forms. The linker DNA associated with the nuclease-sensitive chromatin regions contained non-histone chromosomal proteins with HMG-T predominating while the linker DNA associated with the bulk of chromatin contained predominantly histone H1.

Part C of this thesis reports on the biochemical mechanisms underlying the increased acetylation of histones found in butyrate-treated tissue culture cells. The results clearly indicate that the increased acetylation of histone in vivo is most probably due to an inhibition of deacetylase enzyme activity caused by butyrate. This inhibition is entirely reversible and appears to be a general phenomenon, since butyrate increases levels of acetylated H3 and H4 in all tissue culture cell types tested.
PARTS A AND B

I. Materials and Abbreviations

(a) Materials

All chemicals obtained commercially were of the highest purity or reagent grade. Special reagents were obtained as follows: micrococcal nuclease (E.C. 3.1.4.7.) from Sigma; deoxyribonuclease I (E.C. 3.1.4.5.) from Sigma; deoxyribonuclease II (E.C. 3.1.4.6.) from Sigma; acrylamide from Bio Rad or Matheson, Coleman and Bell; N, N' -methylene bisacrylamide from Eastman Kodak; N, N' -diallyltartardiamide from Bio Rad; N, N, N', N' -tetramethylethylenediamine from Canal Industrial Corp.; urea from Schwarz/Mann; agarose from Calbiochem; starch from Connaught Laboratories and Electrostarch Company; Minicon B15 concentrator from Amicon; aqueous counting scintillant from Amersham/Searle; NCS tissue solubilizer from Amersham/Searle; glass fibre filters from Reeve Angel and proteinase K from Beckman Instruments, Inc.

Radioactive compounds: - L - $[{}^{14}\text{C}]$ lysine (342 mCi/m mole), D L - [ 4, 5 - $^3\text{H}$] lysine (25 or 40 Ci/m mole), [methyl - $^3\text{H}$] thymidine (47 Ci/m mole), and sodium [ 1 - $^{14}\text{C}$] acetate (60.1 mCi/m mole) - were obtained from Amersham/Searle Corp. N - [ ethyl - 2 - $^3\text{H}$] ethyl maleimide (150-300 mCi/m mole) was obtained from New England Nuclear.
(b) Abbreviations

Tris:  -  Tris (hydroxymethyl) aminomethane
Buffer A:  -  50 mM Tris-HCl, pH 7.4, 25 mM KCl, 0.25 M sucrose and 1 mM MgCl₂
Buffer B:  -  50 mM Tris-HCl, pH 7.4, 25 mM KCl, 0.25 M sucrose, 1 mM MgCl₂ and 15 mM β-mercaptoethanol
Buffer C:  -  phosphate-buffered saline, pH 7.2, containing 0.14 M NaCl, 2.7 mM KCl, 9 mM Na₂HPO₄, 1.5 mM KH₂HPO₄, 0.9 mM CaCl₂ and 0.5 mM MgCl₂
Buffer D:  -  50 mM Tris-HCl, pH 7.4, 25 mM KCl, 0.25 M sucrose and 2 mM MgCl₂
Buffer E:  -  10 mM Tris-HCl, pH 7.4, 3 mM MgCl₂ and 10 mM NaCl
TCA:  -  Trichloroacetic acid
SDS:  -  Sodium dodecyl sulfate
EDTA:  -  (Ethylenedinitrilo) tetraacetic acid (disodium salt)
EGTA:  -  Ethyleneglycol-bis-(β-aminoethyl ether) N, N'-tetraacetic acid
PMSF:  -  Phenylmethylsulfonyl fluoride
NEM:  -  N-Ethyl maleimide
TEMED:  -  N, N, N', N'-Tetramethylethylenediamine
DNA:  -  Deoxyribonucleic acid
RNA:  -  Ribonucleic acid
RNase:  -  Pancreatic ribonuclease
DNase I:  -  Deoxyribonuclease I
DNase II:  -  Deoxyribonuclease II
ACS:  -  Aqueous counting solution
PAGE:  -  Polyacrylamide gel electrophoresis

II. Cell Incubations

Naturally maturing trout testis (Sun Valley Trout Farm, Mission B.C.) or testis obtained from rainbow trout (Salmo gairdnerii) in which
spermatogenesis had been induced by twice-weekly injections of salmon pituitary extract (186) were used. The tissue was scissor-minced in 3 to 4 volumes of either Buffer A, 0.1% glucose or Buffer C. A cell suspension was prepared by gentle hand homogenization (three complete strokes) in a Potter-Elvehjem homogenizer with a Teflon pestle as described by Louie and Dixon (10).

The cells were centrifuged at 1,000 x g for 10 min and resuspended in 2 volumes of Waymouth's medium (187) with 10 mM Tris-HCl buffer (pH 7.2) instead of phosphate buffer. The latter buffer was usually used for incubation times exceeding 4 h. Also added to the suspensions were 100 units/ml of penicillin and streptomycin, and a drop of phenol red. The suspension was preincubated for 10 min at 15 to 16°C before the addition of one or more of the following radioactive labels: sodium [1-14C] acetate to either 5, 15 or 50 µCi/ml final concentration, L-[14C] lysine to 7.5 µCi/ml final concentration, L-[4, 5 (n)-3H] lysine monohydrochloride to 33.3 or 100 µCi/ml final concentration and [methyl-3H] thymidine to 50 µCi/ml final concentration. After various incubation times, the cells were collected by centrifugation at 1,000 x g for 10 min. Cells were either frozen at -80°C or used immediately.

III Preparation of Nuclei and Chromatin

Testis nuclei and chromatin were prepared essentially as described by Honda et al. (188) except that in some instances, the nuclei were treated
with the protease inhibitor, phenylmethylsulfonyl fluoride (0.1 mM) prior to chromatin preparation. The concentration of the nuclei was determined by counting the nuclei on a hemocytometer. Alternatively, a 10.0 μl aliquot of the nuclear suspension was added to a solution containing 4 M urea and 2 M NaCl and the absorbance of the solution at 260 nm was measured. The concentration of the chromatin sample was determined by adding 10 μl of the chromatin preparation to 0.99 ml of 1 N NaOH, and the absorbance of the solution at 260 nm was measured.

IV Enzymatic Digestion of Nuclei or Chromatin and Fractionation of the Digest Products

(a) Preparation of mononucleosomes from micrococcal nuclease digested nuclei

Washed nuclei (5 x 10^8 / ml) were incubated with 300 A_260 units/ml of micrococcal nuclease (Sigma) at 37°C in either Buffer B, 1 mM CaCl_2 or Buffer A (has β-mercaptoethanol omitted), 1 mM CaCl_2 for 8, 15 or 30 min. The digestion was terminated by making the solution 10 mM in EDTA and placing it on ice.

After centrifugation at 12,000 x g for 15 min, the digested nuclei were resuspended vigorously in 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and centrifuged for 30 min at 12,000 x g. The supernatant was made either 7% in sucrose or was applied directly to a Bio-Gel A-5m column (90 x 1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA at 4°C as described by Shaw et al. (189).
(b) Preparation of nucleosome subfractions and micrococcal nuclease digested nuclei (Sanders' procedure)

(i) Digestion of nuclei

Washed nuclei were resuspended to 40 A$_{260}$/ml in Buffer D containing 1 mM CaCl$_2$. The nuclear suspension was incubated with 100 A$_{260}$ units/ml of micrococcal nuclease (Sigma) at 25°C for 4 min. The digestion was terminated by making the solution 1 mM in EGTA, pH 7.0 and placing it on ice. The nuclei were collected by centrifugation at 3,000 x g for 10 min. and the supernatant, S0, placed on ice.

(ii) Stepwise elution of nucleosomes

The nuclei were resuspended in Buffer D containing 0.1 M NaCl and incubated for 20 min. at 0°C as described by Sanders (190). The nuclear suspension was centrifuged at 3,000 x g for 10 min., and the supernatant, SS1, removed. The above steps (resuspension of the nuclei, incubation, and centrifugation) were sequentially repeated using Buffer D containing 0.2 M, 0.4 M and finally, 0.6 M NaCl. The supernatants were saved after each centrifugation yielding SS2, SS4 and SS6 respectively.

Alternatively, digested nuclei were resuspended directly in Buffer D containing 0.4 M NaCl, incubated 20 min. at 0°C, centrifuged and the supernatant, SS4T, removed.
(iii) Fractionation of the salt-eluted products

Supernatants SS1 and SS4T were each applied to a Bio-Gel A-5m column (90 x 1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl at 4°C as described by Shaw et al. (190).

Alternatively, supernatant SS1 was applied to a similar column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.1 M NaCl.

(c) Preparation of nucleosome subfractions from micrococcal nuclease digested nuclei (Levy and Dixon's procedure)

(i) Digestion of nuclei

The conditions used for digesting the nuclei were the same as described in Section b(i). The supernatant is termed SI.

(ii) Preparation and fractionation of nucleosomes

The nuclei were resuspended in 0.2 mM EDTA and incubated for 20 min at 0°C. The nuclear suspension was centrifuged at 12,000 x g for 20 min, and the supernatant, S2, was removed. The resulting pellet was termed P2. Five M NaCl was added slowly to the supernatant S2 to a final concentration of 0.1 M. The supernatant was incubated for 20 min at 0°C and then centrifuged at 17,000 x g for 10 min. The supernatant,
S3, and pellet, P3, were both rescued. The supernatants were dialyzed overnight against 10 mM \( \text{NH}_4\text{CO}_3 \) at 4\(^\circ\)C and lyophilized.

(d) **Fractionation of DNase II digested chromatin (Gottesfeld's procedure)**

(i) **Digestion of chromatin**

Initially, chromatin (10 A\(_{260}\)/ml) suspended in 25 mM sodium acetate (pH 6.6) was digested with DNase II at 100 units/ml for 5 min at 24\(^\circ\)C as described by Gottesfeld et al. (141). In later experiments, chromatin was digested with DNase II at 4 enzyme units per A\(_{260}\) unit for 8 min (or from 4 min to 30 min for digestion course studies) at 24\(^\circ\)C as described by Gottesfeld and Butler (139). The digestion was terminated by raising the pH to 7.5 by adding 50 mM Tris (pH 10) to the solution and placing it on ice. The solution was centrifuged at 12,000 x g for 20 min and the supernatant, S1, and pellet, P1, placed on ice.

(ii) **Fractionation of the chromatin digest products**

To the supernatant, S1, 1 M MgCl\(_2\) was added to 2 mM. The solution was incubated 30 min on ice prior to centrifugation at 12,000 x g for 20 min. The supernatant, S2, and pellet, P2, were placed on ice. In some instances S2 was further fractionated by the addition of 1 M MgCl\(_2\) to 22 mM, and centrifuged at 17,000 x g for 10 min yielding the supernatant, S3, and pellet, P3. Alternatively the Mg\(^{2+}\)-soluble (S2) fraction was divided into two portions, one of which was treated with 10 \( \mu \)g of pancreatic
RNase per ml for 10 min at 37°C, the other of which was treated for 20 min at 24°C. After centrifugation (12,000 x g for 30 min), the pellet (P3) was saved, and the supernatant (S3) was redigested with a further 10 μg of RNase per ml for 20 min (37°C) or 40 min (24°C). The pellet (P4) and supernatant (S4) were recovered after centrifugation at 17,000 x g for 30 min.

(iii) Studies on the efficiency of Mg^{2+}, histone or RNase to precipitate S2 associated nucleosomal material

To the supernatant, S2, either RNase (10 μg/ml), whole histone (10 μg/ml), RNase (10 μg/ml) pretreated with diethyl pyrocarbonate (0.1%) or MgCl$_2$ (22 mM) was added. The solutions were incubated for 2 to 20 min at 37°C. A control solution of S2 was incubated for 60 min at 37°C. After centrifugation at 12,000 x g for 20 min, the supernatant, S3, and pellet, P3, were saved.

(e) Preparation of mononucleosomes from DNase I digested nuclei

Washed nuclei (20 A$_{260}$/ml) were incubated with 2.5 μg/ml of DNase I at 25°C in Buffer E from 2 to 15 min. The digestion was terminated by placing the nuclear suspension on ice.

After centrifugation at 3,000 x g for 10 min, the digested nuclei were resuspended vigorously in 10 mM Tris-HCl, pH7.5, 0.7 mM EDTA, and centrifuged at 12,000 x g for 30 min. The supernatant was applied to a Bio-Gel A-5m column (90 x 1.5 cm)
equilibrated with 10 mM Tris-HCl, 0.7 mM EDTA, pH 7.5, at 4°C as described by Shaw et al. (189).

(f) Preparation of nucleosome subfractions from DNase I digested nuclei (Sanders' procedure)

(i) Digestion of nuclei
Washed nuclei (20 A_{260}/ml) were incubated with 1 μg/ml of DNase I (Sigma) at 15°C in Buffer D for 10 to 60 min (usually 10 min). The digestion was terminated by placing the solution on ice. The nuclei were collected by centrifugation at 3,000 × g for 10 min and the supernatant, SO, placed on ice.

(ii) Stepwise elution of nucleosomes
The stepwise elution of nucleosomes with increasing concentrations of NaCl was the same as described in Section (b)(ii). In addition, digested nuclei were resuspended directly in Buffer D containing 0.2 M NaCl, incubated 20 min at 0°C, centrifuged, and the supernatant, SS2T, removed.

(iii) Fractionation of the salt-eluted products
Supernatants SS1 or SS4T were each applied to a Bio-Gel A-5m column (90 x 1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl at 4°C as described by Shaw et al. (189).

Alternatively, supernatants SS1, SS4 or SS2T were each
applied to a Bio-Gel A-0.5m column (40 x 1.5 cm) equilibrated with either 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl or 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.1 M NaCl, at 4°C.

V DNase I Digestion of Labelled Mononucleosomes

Thirty A$_{260}$ units of mononucleosomes in a 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA buffer, labelled with $^{14}$C- acetate and $^3$H-lysine, were concentrated to a final volume of 1.5 ml (20 A$_{260}$ units/ml) using a Minicon B15 concentrator. To 1.0 ml (20 A$_{260}$) of mononucleosomes, 156 μl of a solution containing 40 mM NaCl, 20 mM MgCl$_2$ (NaCl:5.3 mM, MgCl$_2$:2 mM final concentration) was slowly added with rapid stirring. The mononucleosomes suspension was digested with 20 μg/ml of DNase I for 3 min at 37°C. The digestion was terminated by the addition of EDTA to 15 mM and the addition of urea to 6 M. To 0.5 ml (10 A$_{260}$) of mononucleosomes, 78 μl of 40 mM NaCl, 20 mM MgCl$_2$ (5.3 mM and 2 mM final concentrations for NaCl and MgCl$_2$, respectively) was added with rapid stirring. This sample did not have DNase I added to it, but was incubated for 3 min at 37°C. The suspension was made 15 mM in EDTA and 6 M in urea.

The samples were applied to a Bio-Gel A-0.5m column (90 x 1.0 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 6 M urea, at 4°C.

VI Reaction of Nucleosomes or DNase I Digested Nucleosomes with N-Ethylmaleimide under Denaturing Conditions

Nucleosomes were digested with DNase I as described above. An aliquot of either the digest mixture or undigested nucleosomes was removed and added to the appropriate amount of NaCl (0.5 M) and/or urea (6 M). In
addition, an aliquot from the digested or undigested nucleosome suspensions was added to 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA. All solutions were made to the same final volume. An aliquot was removed and reacted with N-ethyl[³H]maleimide (New England Nuclear, diluted to 80 mCi/mmol) as described by Wong and Candido (191).

VII Gel Exclusion Chromatography of Unknowns PI and PII

Lyophilized samples of either PI or PII were redissolved in 100 mM NH₄HCO₃ and applied to a Sephadex G-25 column (30 x 1.5 cm) equilibrated with 100 mM NH₄HCO₃ at 4°C. Pooled fractions from the Sephadex G-25 column were dialyzed against 50 mM NH₄HCO₃ at 4°C overnight and lyophilized. The lyophilized samples were redissolved in 100 mM NH₄HCO₃ and applied to a Sephadex G-10 column (26 x 1 cm) equilibrated with 100 mM NH₄HCO₃ at 4°C.

VIII TCA Precipitability Tests of Unknowns PI and PII

To a 0.5 ml of a labelled PI or PII sample 25μl of a 2 mg/ml solution of whole histone sample was added as carrier. The solution was made 10% in TCA by adding an equal volume of 20% TCA. The resulting precipitate was collected on a glass fibre filter and sequentially washed with 3 volumes of 20% TCA, 3 volumes of cold ethanol, and 1 volume of ether. The filter was dried and counted for radioactivity after addition of 8.0 ml of ACS (Amersham/Searle). Also, 0.1 ml sample of labelled PI or PII was added to a glass fibre filter. The filter was dried, and radioactivity was
XII Determined after adding 8 ml of ACS to the scintillation vial.

IX High Voltage Paper Electrophoresis

High voltage paper electrophoresis of PI, PII or lysine was carried out on Whatman 3MM paper at pH 6.5 for 2 h at 1,000 volts. The pH 6.5 buffer consisted of pyridine/acetic acid/water, 100:4:900.

X Histone Extraction

Histones were acid extracted in the following ways:

(1) Nuclei were extracted twice with at least 4 volumes of 0.2 N HCl (0°C, 30 min) and the extract was precipitated with 9 volumes of cold acetone. The precipitate was dried under N₂. Histones from nucleosomes were prepared the same way after precipitation of the nucleosomes with MgCl₂ at 10 mM final concentration.

(2) Solutions of nucleosomes were concentrated with an Amicon ultrafiltration unit with either a UM 10 or PM 30 membrane, followed by further concentration in a Minicon B15 cell (Amicon). Alternatively, nucleosomes were precipitated by the addition of MgCl₂ to 10 mM. Nuclei, chromatin, nucleosomal precipitates and concentrated nucleosomal solutions were extracted with 0.2 M H₂SO₄ (30 min on ice), and insoluble material was removed by
centrifugation (3,000 x g for 10 min). Histones were precipitated from the extracts with 4 volumes of 95% ethanol at -20°C; the precipitates were collected by centrifugation and redissolved in distilled water.

(3) Supernatants and pooled column fractions were dialyzed overnight at 4°C against 10 mM NH₄HCO₃, and lyophilized. Lyophilized samples, nuclei and nucleosomal precipitates were extracted with 0.4 N H₂SO₄ (30 min on ice), and insoluble material removed by centrifugation (3,000 x g for 10 min). The acid extracts were dialyzed overnight at 4°C against 0.1 N acetic acid, lyophilized and redissolved in distilled water.

Of the three methods, the third method was the most efficient in extraction. The other methods quite often had losses of the arginine-rich histones, H3 and H4, which, apparently, results from inefficient precipitation of H3 and H4 when acetone or, especially, ethanol is added to the acid extract. When inefficient precipitation occurred, the selective loss of the modified histone species was never observed.

XI. Quantitation of the Histone Sample

The concentration of a histone solution was determined by a turbidity assay (192). The assay tube contained 10 µl of the histone solution, 0.79 ml of distilled water and 0.4 ml of 50% TCA. The contents of the tube were mixed immediately. After incubation for 13 to 15 min, the turbidity
of the solution was monitored at 400 nm (1 A\textsubscript{400} = 107.5\mu g/ml final concentration).

XII. Gel Electrophoresis of Proteins

Four gel systems have been used to separate, identify and/or quantitate chromosomal proteins. The first two systems are used for basic chromosomal proteins.

(a) Starch gel electrophoresis

Histone samples (dissolved in 0.2 N HCl) were applied to the slot of a urea/lactate starch gel (193) and electrophoresis was carried out as described by Louie and Dixon (10). Gels were horizontally trisected, and middle slices were stained 40 min with 0.1% Amido Black containing CoCl\textsubscript{2} and destained in N H\textsubscript{2}SO\textsubscript{4} (193).

(b) Acid/urea gel electrophoresis

Histones were separated on 20cm slab polyacrylamide gels by the system of Panyim and Chalkley (194). The following volumes of stock solutions: 6.25 ml TEMED solution (43.2% acetic acid, 4% TEMED) and 25 ml acrylamide solution (30% acrylamide, 0.4% bisacrylamide) - were added to 19.8 g of urea (6.25 M final). The solution was stirred over low heat, made to 49.5 ml final with distilled water, and filtered. The solution was degassed before the addition of0.625 ml ammonium persulfate solution (10%
ammonium persulfate) and polymerized in a slab gel. Histone samples were dissolved in 20 to 30 μl of sample buffer (0.9 N acetic acid, 15% sucrose). The samples were electrophoresed using an acetic acid running buffer (0.9 N acetic acid) for 30 to 40 h at 170 V at 4°C. The gels were stained with 0.25% Coomassie blue in methanol/acetic acid/water (5:1:5 vol/vol) and destained by diffusion in methanol/acetic acid/water(2:1:5 vol/vol).

(c) SDS polyacrylamide gel electrophoresis

Fifteen percent polyacrylamide-SDS slab gels were made using a modified Laemmli procedure (59). The following volumes of stock solutions were used to prepare the lower 15% polyacrylamide-SDS separating gel: - 15 ml of acrylamide solution (30% acrylamide, 0.4% bisacrylamide), 7.5 ml of Tris buffer (1.5 M Tris-HCl, pH 8.8), 0.3 ml of SDS solution (10% SDS), 0.15 ml of ammonium persulfate solution (10% ammonium persulfate) and 7.05 ml of distilled water - were combined, degassed and polymerized under t-butanol in a slab gel after the addition of 10 μl TEMED. Alternatively, the separating lower gel was prepared using 0.6% N, N'-diallyltartardiamide instead of 0.4% bisacrylamide in the acrylamide solution. This allowed ease of solubilization of the gel after electrophoresis. The following volumes of stock solutions were used to prepare the upper 3% polyacrylamide-SDS stacking gel: - 1.5 ml of acrylamide solution (30% acrylamide,
0.8% bisacrylamide), 3.75 ml of Tris buffer (0.5 M Tris-HCl, pH 6.8), 0.15 ml of SDS solution (10% SDS), 0.15 ml of ammonium persulfate solution (10% ammonium persulfate), and 9.45 ml of distilled water - were combined, degassed and polymerized after the addition of 10 μl of TEMED. The protein sample was added to a sample buffer containing 0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% β-mercaptoethanol and 20% glycerol. After the addition of the running buffer (0.0495 M Tris, 0.384 M glycine, 0.1% SDS), electrophoresis was carried out at 130 V for 6 to 7 h. The gels were stained with 0.25% Coomassie blue in methanol/acetic acid/water (2:1:5 vol/vol).

(d) **Two-dimensional gel electrophoresis**

Histones were separated on 20 cm slab acid/urea polyacrylamide gels by the system of Panyim and Chalkley (194, see above (b)). Gels were run at 170 V for 30 to 40 h at 4°C. Further resolution was achieved by running samples in a second dimension using the sodium dodecyl sulfate (SDS) system of Laemmli (226) as modified by Weintraub et al. (59, see above (c)). Gels from the first dimension were equilibrated for 20 to 30 min in Buffer 0 of O'Farrell (195) (10% glycerol/5% β-mercaptoethanol/2.3% SDS/62.5 mM Tris-HCl, pH 6.8) and then applied horizontally to the top of the SDS slab gel. The slice was sealed with melted 1% agarose in Buffer 0 and electrophoresis was carried out at 130 V for 7 h.
XIII Extraction of Histone from 15% Polyacrylamide – SDS Gels

Stained histone bands were dissected and eluted with 10 volumes of 50 mM \( \text{NH}_4\text{HCO}_3 \), 0.05% SDS, pH 7.8 to 8.0, as described by Weiner et al. (196). The sample was lyophilized and redissolved in 50 mM \( \text{NH}_4\text{HCO}_3 \), 0.05% SDS. The histones were precipitated by the addition of 9 volumes of acetone, and the precipitate was collected by centrifugation at 12,000 X g for 10 min. The pellet was then redissolved in distilled water and reprecipitated with 9 volumes of acetone. The precipitate was centrifuged, dried under \( \text{N}_2 \), and dissolved in 0.2 N HCl. The acetone precipitation removed the SDS and Coomassie blue from the extracted histones. This was found to be necessary for the subsequent resolution of the modified species on starch gels.

XIV Solubilization of Polyacrylamide Gels

Stained, labelled protein bands electrophoresed on 15% polyacrylamide-SDS gels containing bisacrylamide as the cross-linker were dissected, sliced and placed in a scintillation vial. One ml of NCS tissue solubilizer (Amersham/Searle) was added to the vial, and incubated 2 days at room temperature. Alternatively, labelled proteins electrophoresed on 15% polyacrylamide-SDS containing \( \text{N, N'}\text{-diallyltartardiamide} \) as cross-linker were solubilized using 2 ml of 2% periodic acid. The slices were incubated at room temperature for 2 days. The solubilized gel slices were counted in 10 ml of aqueous counting scintillant (Amersham).

XV Analysis of DNA Fragments Produced by Nuclease Digestion

(a) Preparation of DNA fragments
DNA fragments were prepared for electrophoresis in three ways: (i) the DNA was isolated before addition of sample buffer, (ii) the DNA associated protein was digested before addition of sample buffer or (iii) the sample buffer was added directly.

(i) Samples of nucleosomes were precipitated with 10 mM MgCl$_2$, or lyophilized after dialysis against 10 mM NH$_4$HCO$_3$. The samples were redissolved in 1 ml of a solution containing 1 M NaCl, 1% SDS and 20 mM EDTA. An equal volume of chloroform/isoamyl alcohol (24:1) was added, stirred, incubated 10 min at room temperature and centrifuged at 3,000 $\times$ g for 10 min. The aqueous phase was carefully removed and re-extracted as described above. The DNA was precipitated by adding 2 vol of cold ethanol to the aqueous phase at $\sim$20°C.

(ii) A lyophilized sample was redissolved in a small volume (10 µl) of a solution containing 25 mM EDTA and 1% SDS, followed by the addition of proteinase K (Merck) to 8.3 mg/ml. Samples were incubated at 37°C for 3 to 4 h.

(iii) Sample buffers used will be described in the following text.

(b) Non-denaturing polyacrylamide gel electrophoresis of DNA

Three or five percent polyacrylamide – SDS slab gels were made using a modified Loening (197) procedure. To prepare the
3% polyacrylamide-SDS gels, the following volumes of stock solutions were used: - 7.5 ml of acrylamide solution (20% acrylamide, 2.2% bisacrylamide), 5.0 ml of 10 x concentrated TBE buffer (10 x TBE = 0.9 M Tris, 0.9 M borate, 30 mM EDTA, pH 8.3), 0.5 ml of SDS solution (10% SDS), 0.4 ml of ammonium persulfate solution (10% ammonium persulfate), and 36.6 ml of distilled water. These were combined, and the gel polymerized after the addition of 40 µl TEMED. To make 5% polyacrylamide - SDS slab gels, the volume of the acrylamide solution was increased to 12.5 ml, and the volume of distilled water reduced to 31.6 ml. In some instances, the concentration of TBE buffer in the gel was reduced to 50 mM Tris, 50 mM boric acid (pH 8.3) and 0.17 mM EDTA final. The DNA sample was redissolved in sample buffer containing 1 ml of 10 x concentrated TBE buffer, 1% SDS, 20 mM EDTA and 10% sucrose. After the addition of running buffer (10.0 fold or 17.9 fold diluted 10 x concentrated TBE buffer, 0.1% SDS), the 3% polyacrylamide gels were electrophoresed at 100 V for 3 to 4 h, and the 5% gels were electrophoresed at 150 V for 4.5 h. The gels were stained with ethidium bromide (10 µg/ml) for 15 min.

(c) Denaturing polyacrylamide gel electrophoresis of DNA

Ten percent polyacrylamide - SDS slab gels were prepared by the combination of the following stock solutions: - 5 ml of 10 x concentrated TBE buffer (0.9 M Tris, 0.9 M borate (pH 8.3), 30 mM EDTA), 0.5 ml of ammonium persulfate solution (6.4% ammonium persulfate), 0.5 ml of SDS solution (10%), and
26 ml of acrylamide solution (19% acrylamide, 1% bisacrylamide). The solution was added slowly to 21.0 g urea (7 M final concentration), and the urea was dissolved over low heat.

The resulting solution was made to 50 ml with distilled water, filtered and degassed. After the addition of 20 µl TEMED, the solution was poured and polymerized. The sample buffer was prepared by dissolving 1 g of SDS (10% final concentration) and 2 g (20% final concentration) into 1 ml of 10 x concentrated TBE buffer. The buffer solution was made to 10 ml by the addition of 99% deionized formamide. The DNA sample was dissolved in 20 µl of sample buffer, boiled and immediately placed on ice. After the addition of electrophoresis running buffer (10 fold diluted 10 x concentrated TBE buffer, 0.1% SDS), the samples were electrophoresed at 200 V for 4 to 5 h. The gel was stained with ethidium bromide (10 µg/ml for 15 min).
PART C

I. MATERIALS AND ABBREVIATIONS

(a) Materials

All chemicals obtained commercially were of the highest purity or reagent grade. In addition to the special reagents mentioned in Materials and Methods (Part A and Part B I(a)), other special reagents were obtained as follows: sodium penicillin-G and dihydrostreptomycin from GIBCO, culture medium from GIBCO.

Radioactive compounds: - sodium\[^{14}Cl\]acetate (60.1 mCi/mmole) was obtained from Amersham/Searle Corp., and sodium \[^{3}H\]acetate (500 mCi/mmole) and \[^{3}H\]-acetyl coenzyme A (500 mCi/mmole) were obtained from New England Nuclear.

(b) Abbreviations

The following abbreviations were used throughout the text:

TMK: - 50 mM Tris-HCl, pH 7.4, 25 mM KCl and 1 mM MgCl\(_2\)

PBS: - phosphate-buffered saline, pH 7.2 containing 0.14 M NaCl, 2.7 mM KCl, 9 mM Na\(_2\)HPO\(_4\), 1.5 mM KH\(_2\)PO\(_4\), 0.9 mM CaCl\(_2\) and 0.5 mM MgCl\(_2\)

II. Cell Lines and Culture Conditions

The origins and growth characteristics of the various cell lines used in this study are given in the following references: Friend erythroleukemic
cells, clone 745A (198); mouse 3T3 cells (199); hamster BHK-21 cells (200); rat IRC8 ascites cells (201); Xenopus X58 amphibian cells (202). All cell lines (except for X58) were maintained and passaged using standard tissue culture procedures (203) in a medium containing 90% Dulbecco's modified Eagle's medium, 10% fetal calf serum and 100 μg/ml each of sodium penicillin - G and dihydrostreptomycin (DME/FCS). The amphibian X58 cell line was maintained in the above medium diluted by 33% with sterile glass-distilled water. All cultures were periodically monitored and found to be free of mycoplasm contamination.

When required, butyric acid (neutralized with concentrated NaOH) was added to cultures to a final concentration of 5 mM. When cell cultures were labelled with isotopic precursors, cells were incubated in culture medium (DME/FCS) lacking the precursor to maximize de novo incorporation.

Cell counts were made using a hemacytometer, and cell viability was determined by trypan blue dye exclusion (203). It should also be mentioned that after treatment of Friend cells with 5 mM butyrate for 24 h, the rate of recovery (and viability) is slightly less than in comparable cells never exposed to butyrate. Additional experiments, however, also indicate that the great majority of butyrate-treated cells do recover normally after such treatment. Thus, the butyrate effect appears to be reversible when short-term treatments are used.
III Preparation of Histones

Histones were isolated from all cell types by the following modification of the method of Marushige and Bonner (204): frozen (-80°C) cell pellets (10^8–10^9 cells) were homogenized in 2 ml of a standard saline citrate solution (0.075 M NaCl, 0.024 M Na citrate (pH 8.0)) using a glass-Teflon hand homogenizer. The sample was centrifuged at 3,000 x g for 10 min., and the pellet was rehomogenized in TMK as above. After centrifugation as before, the nuclear pellet was homogenized in 2 ml of 10 mM Tris-HCl (pH 7.4) and layered onto 3 ml of 1 M sucrose in 10 mM Tris-HCl (pH 7.4). After centrifugation for 20 min. at 17,000 x g, the gelatinous pellet of chromatin was extracted by the addition of 0.1 to 0.3 ml of 0.4 N H_2SO_4 for 5 – 15 min. on ice. The extract was centrifuged at 12,000 x g for 10 min., and the supernatant was mixed with 4 vol. of 95% ethanol. Histones were allowed to precipitate overnight at -20°C. The precipitated histones were collected by centrifugation at 3,000 x g, redissolved in 0.1 – 0.3 ml of 0.1 M acetic acid, dialyzed against the same solution for 1 h., and lyophilized.

IV Polyacrylamide Gel Electrophoresis

Acid-urea gels were run as described by Panyim and Chalkley (194) using slab gels instead of tubes (see Materials and Methods Part A and B XII (b)). The 1.5 mm slabs were run at 170 V for 20 to 30 h. at 4°C.
V Assay of In Vitro Histone Acetylase and Deacetylase Activity

Naturally maturing trout testis (Sun Valley Trout Farm, Mission, B.C.) were scissor-minced in 3 to 4 volumes of PBS. A cell suspension was prepared by gentle hand homogenization (three complete strokes) in a Potter-Elvehjem homogenizer with Teflon pestle as described by Louie and Dixon (10). The cells were centrifuged at 1,000 x g for 10 min, and resuspended in 2 volumes of PBS containing 0.4 volumes of Waymouth's medium (187) with 10 mM Tris-HCl (pH 7.2) instead of phosphate buffer, 100 units/ml of penicillin and streptomycin and a drop of phenol red. The suspension was subdivided, and to one suspension butyric acid (neutralized with concentrated NaOH) was added to a final concentration of 1 mM. The suspensions were preincubated for 10 min at 16°C before the addition of sodium [1-\(^{14}\)C]acetate to 50 µCi/ml final concentration. Aliquots were removed after various incubation times, placed in an excess of PBS and collected by centrifugation (3,000 x g for 10 min). The histones were acid extracted as described above. An aliquot of the acid extract was added to 1 ml of ACS (Amersham/Searle) and counted for radioactivity.

For assays of histone acetylase activity in crude cell lysates (erythroleukemic cells), equal numbers of control or butyrate-grown (for 24 h in 5 mM butyrate) cells were homogenized in 3 vol of TMK (see above). To these homogenates (100 µl per reaction), 10 µl of \(^{3}\)H]-acetyl-CoA (100 µCi/ml) were added, and the reaction was incubated at room temperature (22°C) for up to 60 min. At various times, reactions were stopped by the addition of 5 µl of concentrated HCl to the 110 µl incubation mixtures. Protein was precipitated from these samples with cold 20% TCA and collected
on glass fibre filters. The filters were washed successively with 20% TCA, 95% ethanol, ether and dried. Radioactivity was determined on an Isocap scintillation counter (Nuclear Chicago). Similar experiments to the above were also performed using purified nuclei from erythroleukemic cells for the determination of acetylase activity. In this case, after the cells were homogenized, the nuclei were pelleted by centrifugation, resuspended in TMK, pelleted a second time and assayed for acetylase activity in TMK, as described above.

For assay of histone deacetylase activity, equal numbers of either control or butyrate-grown erythroleukemic cells (5 mM butyrate for 24 h) were homogenized in TMK as described above. Each lysate was then divided into three aliquots. One of the aliquots was boiled for 1 min to act as a background control. To a second aliquot, sodium butyrate was added to a final concentration of 5 mM. The third aliquot was assayed without further treatment and served for the determination of total deacetylase activity. Each aliquot was incubated with an equal amount of $[^3H]$-acetate-labelled histone (usually 50,000 cpm) prepared by labelling Friend cells in vivo with $[^3H]$-acetate (2 x $10^9$ cells were incubated for 2 h with 150 μCi/ml of $[^3H]$-acetate). These histones were purified as described above. The mixtures (140 μl) were incubated at room temperature (22°C) for 4 h and terminated by the addition of 10 μl of concentrated HCl. This also served to convert any acetate released to the protonated form. The $[^3H]$-acetic acid released was then extracted from the mixtures with 0.5 ml of ethyl acetate, and the ethyl acetate phases were counted in Bray's scintillation fluid (205).
PART A - CHROMATIN SYNTHESIS
I Partial Characterization of Newly-Synthesized Chromatin

The conditions used for labelling DNA and histones from trout testis cell suspensions have been previously described by Louie and Dixon (206) and Candido (207). In the present study, the same conditions were used to examine nucleosome synthesis from labelled histone and DNA. However, the major objective was to study one of the post-synthetic modifications of nucleosomal histones, namely acetylation.

(a) Characterization of micrococcal nuclease digest products

Following the digestion of trout testis nuclei with micrococcal nuclease, the products were fractionated on a Bio-Gel A-5m column as described by Shaw et al. (189). A typical column profile is shown in Fig. 6A. Two main peaks are obtained; the larger peak, eluting at or near the void volume, is termed the multimer fraction and is due to fragments of chromatin which have been only partially digested. As shown in Fig. 6B, this fraction contains the full complement of histones. The second peak, termed the monomer fraction, corresponds to the 11 S particles which have been previously characterized by Honda et al. (208) by sucrose gradient centrifugation and by sedimentation velocity analysis. The monomer fraction contains only trace amounts of histones H10. (Fig. 6B). Fig. 6C shows an SDS gel pattern of histones from monomer particles following more extensive digestion (30 min
FIG. 6. A, Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from an 8-min micrococcal nuclease digest (300 A_{260} units/ml) of trout testis nuclei. B, PAGE SDS separation (15% gel, Coomassie blue stained) of proteins from multimer and monomer peak fractions of the separation illustrated in A. C, PAGE SDS separation of proteins from monomer nucleosomes obtained from a 30-min micrococcal nuclease digest (300 A_{260} units/ml) following fractionation on Bio-Gel A-5m as in A above.
versus 8 min, with 300 AU units/ml) with nuclease. Here HI is totally absent. The loss of HI seems to be associated with digestion of the DNA linker region between nucleosomal core particles (90, 189, 209, 210). Shaw et al. (189) have extensively characterized the nuclease digest products of chicken erythrocyte chromatin using these techniques, and the above results are in agreement with their findings. A small peak of material absorbing at 260 nm is seen emerging later from the column and probably consists of small oligonucleotides.

(b) DNA synthesis

Trout testis cells were incubated with [3H]-thymidine for various times. The isolated nuclei were digested with micrococcal nuclease using mild digestion conditions (300 AU units/ml for 8 min). The digest products from each incubation time were fractionated on a Bio-Gel A-5m column (Fig. 7), and the specific activities of the peak fractions (in counts per min per AU) were calculated for both multimer and monomer fractions (Table V). The incorporation of [3H]-thymidine into monomer and multimer fractions follows a similar time course (Fig. 8), but the specific activity of the monomer peak is consistently higher than that of the multimer peak.
FIG. 7. [³H] Thymidine incorporation into DNA of nucleosomes as a function of time. Trout testis cells were incubated with [methyl - ³H] thymidine (50 µCi/ml); at varying time intervals, aliquots were removed and nuclei were isolated. The nuclei were digested for 8 min with micrococcal nuclease (300 A₂₆₀ units/ml), and the digest products were separated on a Bio-Gel A-5m column (90 X 1.5 cm). Radioactivity was determined by mixing 1.0 ml column fractions with 8 ml of ACS (Amersham/Searle) and counting them on a Unilux liquid scintillation counter. Labelling was for A, 5 min.; B, 30 min.; C, 60 min.
TABLE V

Quantitation of Newly-Synthesized DNA Associated with Nucleosome Fractions. (Data from Fig. 7)

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Peak fraction</th>
<th>Ratio: monomer/multimer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Multimer</td>
<td>Monomer</td>
</tr>
<tr>
<td>cpm/A₂₆₀</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min.</td>
<td>3391</td>
<td>5326</td>
</tr>
<tr>
<td>30 min.</td>
<td>5120</td>
<td>8295</td>
</tr>
<tr>
<td>60 min.</td>
<td>5397</td>
<td>7994</td>
</tr>
</tbody>
</table>
FIG. 8. $[^3H]$ Thymidine incorporation into DNA of multimers and monomers as a function of time. $[^3H]$ Thymidine to $A_{260}$ ratios of labelled peak fractions of monomers and multimers isolated on Bio-Gel A-5m columns in Fig. 7 were plotted versus incubation time. ■ monomers; ● multimers.
The incorporation of label reaches a plateau after 30 minutes incubation time for both monomer and multimer fractions. This would suggest the cessation of DNA synthesis with, possibly, an accompanying loss of cell viability. The result should be examined with caution as with increasing incubation times, cell clumping was evident. Cell clumping would interfere with uniform incorporation of the label.

One possible explanation for the higher specific activity of the monomer peak fraction would be the preference of micrococcal nuclease for A/T rich regions (124). If the linker DNA containing A/T rich regions were selectively digested, the mononucleosomes would contain greater amounts of thymidine residues per DNA fragment length than that of polynucleosomes.

The greater specific activity for the monomer fraction may also reflect a nuclease sensitive state for the newly replicated chromatin as suggested by Seale (148). It is doubtful that the nuclease's ability to selectively excise nucleosomes associated with newly synthesized chromatin is a major contributor to the observed ratios in specific activities. As the time of incubation increases, the nuclease-sensitive nucleosomes containing the newly synthesized, labelled DNA would be transformed into nucleosomes with characteristics similar to those associated with the bulk of chromatin. The labelled DNA associated with nuclease-sensitive nucleosomes should decrease with time. This result
was not observed.

(c) Histone synthesis and histone acetylation

Nuclei isolated from trout testis cells that were labelled with [3H]-lysine and [14C]-acetate for various incubation times were digested with micrococcal nuclease (300 A260/ml for 8 min). The digest products were fractionated on a Bio-Gel A-5m column (Fig. 9). The profiles were similar for all incubation time points. The specific activities of the newly-synthesized histones (3H counts per min per A260) were calculated for the peak fraction of the monomer and oligomer fractions (Table VI). Although the monomer fraction contains reduced amounts of the lysine-rich histone H1, the specific activity of this fraction is greater than that of the multimer fraction at early times of incubation (5 min and 30 min).

This experiment was performed only once so the validity of the measurements is uncertain. However, the trend is in agreement with the idea that newly-synthesized histones are initially associated with nucleosomes associated with nuclease sensitive regions of chromatin, and later they become associated with nucleosomes characteristic of those associated with the bulk of chromatin.

The monomer and multimer fractions are both labelled with the [14C]-acetate label (Fig. 9), indicating that the histones comprising the nucleosomal fractions are modified by acetylation. It should be noted that both newly-synthesized histones and pre-existing histones are susceptible to this modification. This
FIG. 9. [3H] Lysine and [14C] acetate incorporation into histones of nucleosomes as a function of time. Trout testis cells were incubated with L[4,5 (n) - 3H] lysine monohydrochloride (100 µCi/ml) and sodium [1 - 14C] acetate (5 µCi/ml); at varying time intervals, aliquots were removed and nuclei were isolated. The nuclei were digested for 8 min with micrococcal nuclease (300 A260 units/ml), and the digest products were separated on a Bio-Gel A-5m column (90 x 1.5 cm). Radioactivity was determined in the same manner as described for Fig. 7. Labelling was for: A, 5 min; B, 30 min; C, 60 min.
**TABLE VI**

**Quantitation of Newly-Synthesized Histone Associated with Nucleosome Fractions. (Data from Fig. 9)**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Peak fraction</th>
<th>Ratio: monomer / multimer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Multimer</td>
<td>Monomer</td>
</tr>
<tr>
<td>5 min</td>
<td>328</td>
<td>397</td>
</tr>
<tr>
<td>30 min</td>
<td>913</td>
<td>930</td>
</tr>
<tr>
<td>60 min</td>
<td>2179</td>
<td>1993</td>
</tr>
</tbody>
</table>

$^{3}H$ cpm/A$_{260}$
modification will be discussed further in later sections.

(d) Partial characterization of unknowns PI and PII

Two peaks of radioactivity are seen eluting from the columns (Fig. 9) after the monomer fraction. The first peak, PI, eluting in fractions 130 to 160 contains both the $^3$H and $^{14}$C labels. The second peak, PII, eluting in fractions 160 to 190 contains predominantly the $^3$H label. PI and PII did not co-elute with the $A_{260}$ absorbing material, which is presumably oligonucleotides. $[^3H]$-lysine was found to elute between PI and PII (not shown).

The unknowns, PI and PII, could be released into solution after undigested nuclei were resuspended in EDTA (Fig. 10), but the nuclei had to be isolated from freshly labelled cells. Labelled cells frozen at -80°C prior to micrococcal nuclease digestion did not yield PI and PII. Addition of the protease inhibitor, phenylmethylsulfonyl fluoride, to the buffers had no effect on the appearance of PI and PII.

PI was pooled, dialyzed against 50 mM NH$_4$HCO$_3$, lyophilized and applied to a Sephadex G-25 column. The sample eluted at the included volume (Fig. 11A) with both $^{14}$C and $^3$H labels co-eluting. PII, treated identically to PI, also eluted at the included volume (Fig. 11B).
FIG. 10. EDTA solubilized material from nuclei isolated from [\(^{3}\text{H}\)] lysine- and [\(^{14}\text{C}\)] acetate - labelled trout testis cells. Trout testis cells were incubated with L [4,5(n)-\(^{3}\text{H}\)]lysine monohydrochloride (100 \(\mu\)Ci/ml) and sodium [1-\(^{14}\text{C}\)] acetate (50 \(\mu\)Ci/ml) for 4 h., and nuclei isolated. The nuclei were incubated for 15 min at 37\(^{\circ}\)C, centrifuged, and resuspended with 10 mM Tris, pH 7.5, 0.7 mM EDTA. The solubilized material was separated on a Bio-Gel A-5m column (90 X 1.5 cm). Radioactivity was determined in the same manner as described for Fig. 7.
FIG. 11. Sephadex G25 gel exclusion chromatography of unknowns PI and PII. Trout testis cells were incubated with L [4,5(\(n\)) - \(^3\)H] lysine monohydrochloride (100 \(\mu\)Ci/ml) and sodium \([1-{\text{H}}^+\] acetate (15 \(\mu\)Ci/ml) for 5 min, and nuclei isolated. The nuclei were digested with micrococcal nuclease, and the digest products separated on a Bio-Gel A-5m column as described in the legend to Fig. 10. The unknowns PI (fractions 130 to 160) and PII (fractions 160 to 190) were pooled, dialyzed against 50 mM NH\(_4\)HCO\(_3\), and lyophilized. The samples were separately redissolved in 1 ml 100 NH\(_4\)HCO\(_3\), and applied to a Sephadex G25 column (30 x 1.5 cm) equilibrated with 100 mM NH\(_4\)HCO\(_3\). Radioactivity was determined in the same manner as described for Fig. 7 except 0.1 ml column fractions were mixed with 5 ml of ACS (Amersham/Searle). A, PI; B, PII.
The pooled PI and PII fractions from the Sephadex G-25 columns were applied separately to a Sephadex G-10 column after dialysis, lyophilization, and resolubilization. Both PI and PII eluted at the void volume. From the known molecular weight exclusion limits of both columns, PI and PII would be approximately 400 daltons.

When PI and PII were analyzed by high voltage paper electrophoresis, they did not migrate as free lysine. This is in agreement with the fact that neither PI nor PII co-eluted with [³H] -lysine when fractionated on a Bio-Gel A-5m column. Also, the[^14C] label associated with PI was not free[^14C] -acetate, as it was not volatile after acidification.

Both PI and PII were tested for TCA precipitability (Table VII). The majority of material from PI or PII was not retained on the glass fibre filters when treated with 20% TCA. Thus, the label is not free histone.

The identities of PI and PII remain unknown. The results suggest that the materials have a molecular weight of approximately 400 and that lysine, acetate or histone is not associated with either of the unknowns.
TABLE VII
Quantitation of TCA Precipitable Material Associated with Unknowns PI and PII

Unknowns PI and PII were obtained from nuclei isolated from trout testis cells previously incubated with L-[^14C] lysine (7.5 μCi/ml) for 60 min. PI and PII were obtained from a Bio-Gel A-5m column in the same manner as described in Fig. 11. Each 0.5 ml sample with added unlabelled histone carrier (50 μg) was made 10% in TCA. The precipitate was collected on a glass fibre filter, and subsequently washed with 20% TCA, ethanol and ether. A 0.1 ml aliquot of PI or PII was applied directly to a glass fibre filter. The filter discs were dried and counted in 8 ml of ACS (Amersham/Searle).

<table>
<thead>
<tr>
<th>Control</th>
<th>TCA Precipitate</th>
</tr>
</thead>
<tbody>
<tr>
<td>cpm/0.5 ml</td>
<td></td>
</tr>
<tr>
<td>PI</td>
<td>765</td>
</tr>
<tr>
<td>PII</td>
<td>395</td>
</tr>
</tbody>
</table>
PART B - STRUCTURE OF TRANSCRIPTIONALLY ACTIVE CHROMATIN
RESULTS AND DISCUSSION

Both biochemical studies and electron microscopic observations (128, 212, 213) suggest that nucleosomes are associated with transcriptionally active as well as inactive regions of chromatin. Because DNase I selectively digests transcriptionally competent regions of chromatin, the nucleosomes associated with these regions are believed to exist in an altered conformation (120, 133, 135). Possible molecular mechanisms for inducing such conformational changes include histone acetylation and the association of specific nonhistone proteins with transcriptionally competent chromatin regions.

In these studies various methods were used to fractionate chromatin into template active and inactive fractions with the major purpose of examining the levels of the acetylated histone species associated with each nucleosome fraction. By using nuclease probes (DNase I, DNase II and micrococcal nuclease), the structure of chromatin from the two states was compared.

I. Characterization of Nucleosome Subfractions and Chromosomal Proteins Released by Micrococcal Nuclease Digestion of Nuclei

(a) Determination of the levels of acetylated histone species associated with mononucleosomes

In this study the levels of the acetylated histone species associated with mononucleosomes that were excised from chromatin after different extents of micrococcal nuclease digestion were examined. The content of acetylated histone species associated
with mononucleosomes was also compared to those associated with whole chromatin.

1. **Kinetics of $[^{14}\text{C}]$-acetate incorporation into monomer and multimer fractions**

   Fig. 12 shows the column profiles of nuclease digest products obtained from the nuclei of testis cells which were labelled for various times with $[^{14}\text{C}]$-acetate. Both the monomer and multimer peaks are seen to be labelled, and the profiles do not change qualitatively during the course of the incubation from 5 min to 5 h. The specific activities of the peaks (in counts per min per A$_{260}$) for each time point are plotted in Fig. 13. The incorporation of $[^{14}\text{C}]$-acetate into monomer and mutimer follows a similar time course. The specific activity of the monomer peak, however, is consistently higher than that of the multimer peak (Table VIII), the ratios for monomer to mutimer being 1.1 to 1.2.

2. **Effect of extensive nuclease digestion on the relative $[^{14}\text{C}]$-acetate incorporation into monomer and multimer fractions**

   When nuclei labelled with $[^{14}\text{C}]$-acetate are digested more extensively with micrococcal nuclease, the relative specific activities of monomer and multimer peaks remain unchanged (Fig. 14). Here, nuclei have been digested for either 8 or 30 min with
[\textsuperscript{14}C] Acetate incorporation into histones of nucleosomes as a function of time. Trout testis cells were incubated with sodium [1-\textsuperscript{14}C] acetate (5 µCi/ml); at varying time intervals, aliquots were removed and nuclei were isolated. The nuclei were digested for 8 min with micrococcal nuclease (300 AU units/ml), and the digest products were separated on a Bio-Gel A-5m column (90 x 1.5 cm). Radioactivity was determined in the same manner as described for Fig. 7. Labelling was for: A, 5 min.; B, 30 min.; C, 2 h.; D, 4 h.
FIG. 13. $[^{14}C]$ Acetate incorporation into histones of multimers and monomers as a function of time. $[^{14}C]$ Acetate to $A_{260}$ ratios of labelled peak fractions of monomers and multimers isolated on Bio-Gel A-5m columns in Fig. 12 were plotted versus incubation time. ■ monomers; ● multimers.
TABLE VIII

Quantitation of the Levels of Histone Acetylation
Associated with Nucleosomal Fractions
(data from Fig. 13)

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Peak fraction</th>
<th>Ratio: monomer/</th>
<th>cpm/A₂₆₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Multimer</td>
<td>Monomer</td>
<td>multimer</td>
</tr>
<tr>
<td>5 min</td>
<td>309</td>
<td>327</td>
<td>1.1</td>
</tr>
<tr>
<td>30 min</td>
<td>343</td>
<td>386</td>
<td>1.1</td>
</tr>
<tr>
<td>2 h</td>
<td>764</td>
<td>846</td>
<td>1.1</td>
</tr>
<tr>
<td>5 h</td>
<td>982</td>
<td>1149</td>
<td>1.2</td>
</tr>
</tbody>
</table>
FIG. 14. Micrococcal nuclease digestion of nuclei isolated from $^{14}$C acetate - labelled trout testis cells. Trout testis cells were incubated with sodium $[1^{14}$C] acetate (50 μCi/ml) for 90 min., and nuclei were isolated. The nuclei were digested for (A) 8 min with micrococcal nuclease (300 $A_{260}$ units/ml) or (B) 30 min with micrococcal nuclease (300 $A_{260}$ units/ml), and the nucleosomes were separated on a Bio-Gel A-5m column (90 x 1.5cm). Radioactivity was determined in the same manner as described for Fig. 7.
300 $A_{260}$ units/ml of nuclease. In the 30 min sample, the monomer fraction represents 62% of the material (excluding the low molecular weight peak centered at Fraction 140). The ratio of $[^{14}C]$-acetate (in counts per min per $A_{260}$) in monomer versus multimer is still 1.2 as it is in the 8-min digest. Since H1 does not contain $N^e$-acetyl groups and since its $N^\alpha$-acetyl group is not labelled under the conditions used (214), variability in the H1 content of the mononucleosomes will not affect their specific activities, i.e. $[^{14}C]$ acetate counts per min per $A_{260}$. Hence the higher specific activity of monomers must be due to the loss of nucleotides, presumably from the linker DNA between nucleosomal core particles. This would correspond to a 20% decrease in the nucleotide length relative to the nucleosomal DNA, i.e. roughly 40 base pairs. This loss of the internucleosomal DNA seems to be associated with the partial loss of H1 as seen in the 8-min digest of Fig. 6B. The H1 remaining may be bound to the ends of the nucleosomal DNA. Further digestion of these mononucleosomes would lead to "trimming" of the nucleosome DNA and complete loss of H1 (Fig. 6C). Although the complete loss of H1 was observed, the loss of the additional nucleotides during trimming would presumably not be extensive enough to result in a measurable change
in absorbance. Although no attempt was made in these studies to examine the trimming of nucleosome DNA in detail, it is interesting to note that Whitlock and Simpson (210) estimated the length of DNA protected by H1 to be 40 to 50 base pairs.

3. Comparison of the levels of acetylated histone species associated with mononucleosomes and undigested nuclei

In order to compare the extent of labelling of specific histones from monomers and from whole nuclei, histones were extracted, separated by SDS gel electrophoresis, and analysed for radioactivity. As shown in Table IX, the specific activities of H3, H2B + H2A, and H4 are very similar in histones extracted from either whole nuclei or from chromatin monomers after 8 or 30 min of nuclease digestion at 300 μg/ml.

Individual histones from monomers were also examined for their content of modified species by starch gel electrophoresis on urea/aluminum/lactate gels. This technique resolves most of the acetylated and phosphorylated components of purified trout testis histone fractions and their characterization has been described previously (214–216). A photograph of such a gel in which H2A, H2B, H3 and H4 from monomers are
### Quantitation of $[^{14}\text{C}]$ Acetate–Labelled Histone Fractions

Histones labelled with $[^{14}\text{C}]$ acetate were extracted from mononucleosomes or from undigested nuclei, and separated on 15% polyacrylamide SDS gels. The gel slices were scanned at 550 nm after staining with Coomassie blue and counted after solubilization.

<table>
<thead>
<tr>
<th>Histone fraction</th>
<th>Total nuclear histone</th>
<th>Nucleosome monomers</th>
<th>Percent cpm/A$_{550}$ in Each Histone Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>8-min: digest</td>
<td>30-min: digest</td>
</tr>
<tr>
<td>H3</td>
<td>44</td>
<td>52</td>
<td>47</td>
</tr>
<tr>
<td>H2A + H2B$^a$</td>
<td>10</td>
<td>9</td>
<td>13</td>
</tr>
<tr>
<td>H4</td>
<td>46</td>
<td>39</td>
<td>40</td>
</tr>
</tbody>
</table>

$^a$ Histones, H2A and H2B, when extracted from nuclei with 0.2N HCl and precipitated with acetone, co-electrophoresed on 15% polyacrylamide–SDS gels.
compared to the corresponding fractions from whole chromatin, is shown in Fig. 15. In H2A, the unmodified, monoacetyl, and phosphorylated components are visible. H2B is seen to contain the unmodified species, and an additional component which could be either monoacetylated H2B or some contaminating, unmodified H2A. H2B from monomers does contain acetylated species, however, since labelled acetyl groups are present in this fraction (data not shown). The H3 contains unmodified and monoacetylated species, and the H4 contains unmodified, mono-, di-, tri-, and tetraacetylated species. The latter bands are faint and somewhat smeared. The separation of the modified species on these samples is not as distinct as that obtained with histones isolated by larger scale column procedures (214). This is evidently due to the presence of residual SDS in these samples, which were obtained by preparative SDS gel electrophoresis. Furthermore, chromatography on Bio-Gel P10 columns, the method of choice for large histone samples, leads to some fractionation of acetylated species from unmodified ones (214), and the resulting enrichment of modified species makes their visualization on gels much easier.

It is evident from Fig. 15, however, that the starch
FIG. 15. Acetylated components of whole trout testis histones and histones from nucleosomes. Histones isolated by acid-extraction from nucleosomes or nuclei were separated by polyacrylamide - SDS gel electrophoresis and stained with Coomassie blue. The protein bands were dissected, and SDS and Coomassie blue were removed from the histones by acetone extraction before application to a urea/aluminum lactate starch gel. Electrophoresis was for 9 h at 35 mA. The middle slice of the gel was stained with 0.125% Amido black containing CoCl$_2$ for 30 min and destained with 0.5 M H$_2$SO$_4$. a, c, e, and g are histones extracted from nuclei and b, d, f, and h are histones extracted from nucleosomes. "Total" represents whole histones extracted from nucleosomes. Cathode is at the bottom.
gel patterns of histones from whole chromatin and from monomers are very similar.

The above studies, therefore, indicate that chromatin subunits isolated from micrococcal nuclease digestion contain the major acetylated histone species normally found in whole chromatin. In addition, the phosphorylated form of H2A is also present. The levels of these modified species in chromatin monomers are also very close to those found in whole chromatin.

However, these results do not rule out the possibility that major differences may exist in the content of the highly modified species (i.e. species that contain three or four acetyl groups, or which are both acetylated and phosphorylated), since these are present at very low levels, and have not been quantitated in these studies.

(b) Chromatin fractionation (Sanders' procedure)

Recently M. Sanders (190) has reported a procedure for the fractionation of chromatin. The procedure involves stepwise treatments of micrococcal nuclease digested nuclei with increasing salt concentrations (Fig. 16). Using this procedure, the content of the histone acetylated species associated with each nucleosome subfraction, and the non-histone chromosomal proteins
FIG. 16. Experimental procedure for the isolation of nucleosomal subfractions from micrococcal nuclease digested nuclei by successively higher NaCl concentrations.
released were examined.

1. Quantitation of DNA content in salt-extracted chromatin fractions

Following the digestion of trout testis nuclei with micrococcal nuclease, the chromatin was fractionated by sequential nuclear extraction with increasing concentrations of sodium chloride (Fig. 16) as described by Sanders (190), except that the initial NaCl concentration was 0.1 M instead of 0.2 M, and the 0.3 M NaCl extraction step was omitted.

The percentage of total A$_{260}$ absorbing material released in each fraction (Table X) was in close agreement with the yields obtained by Sanders. Furthermore, Sanders (190) reported that the percentage of material released into each fraction was independent of the extent of digestion. Therefore, each fraction represents a distinct nucleosomal population. The DNA released in S0 was almost totally acid soluble. Most of the remaining acid soluble material was released in SS1 and SS2.
TABLE X

Quantitation of DNA Content in Salt-Extracted Chromatin Fractions
Released from Micrococcal Nuclease Digested Nuclei
(Sanders' Procedure)

The $A_{260}$ absorbing material released in the different salt-extracted nucleosome fractions after micrococcal nuclease digestion of trout testis nuclei was quantitated by adding 100 µl of the fraction to 0.9 ml of 0.6 M NaCl in Buffer D and measuring the absorbance at 260 nm. The percentage of acid soluble material in each fraction was measured by adding 100 µl of the fraction to 0.9 ml of 1 N perchloric acid; and measuring the absorbance at 260 nm of the supernatant after centrifugation.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total $A_{260}$ in Each Fraction</th>
<th>Percent $A_{260}$ in Acid Soluble Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0</td>
<td>13.4</td>
<td>83.8</td>
</tr>
<tr>
<td>SS1</td>
<td>7.2</td>
<td>58.5</td>
</tr>
<tr>
<td>SS2</td>
<td>7.8</td>
<td>30.0</td>
</tr>
<tr>
<td>SS4</td>
<td>43.4</td>
<td>2.9</td>
</tr>
<tr>
<td>SS6</td>
<td>7.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>
2. Characterization of the DNA fragments associated with salt-extracted chromatin fractions

The DNA size classes from each salt-extracted fraction were examined on 3% nondenaturing polyacrylamide gels (Fig. 17). In agreement with Sanders' results, the average DNA fragment size increased with each successively higher NaCl concentration. The DNA in SSI and SS2 consisted almost entirely of monomer size fragments. Fragments of less than monomeric size were also observed in the SSI and to a lesser extent in SS2. The SS4 fraction contained very little monomer; it consisted mostly of dimer and lesser amounts of trimer, tetramer, pentamer and hexamer fragments. Fragments greater than octamer in length predominated in the SS6 fraction.

The DNA sizes for SSI and SS2 were further characterized on 5% nondenaturing polyacrylamide gels, and their sizes were calibrated using Fnu DI generated fragments of øX174 DNA as reference markers. The DNA from the SSI shows at least six different fragments (Fig. 18) with a 146 base pair fragment predominating. A larger fragment of 170 base pairs is also evident in
FIG. 17. Polyacrylamide gel electrophoresis of the DNA fragments generated by micrococcal nuclease digestion of trout testis nuclei and released by stepwise increases in NaCl concentrations. DNA (0.2 A₂₆₀) obtained from the stepwise eluted fractions (see "Materials and Methods") was analyzed on a 3% polyacrylamide SDS gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. a, b, c and d are SS1, SS2, SS4 and SS6, respectively. 1a and 1b are the 146 base pair monomer and 170 base pair monomer, respectively; 2, 3 and 4 are dimer, trimer and tetramer, respectively. S is a 124 base pair fragment.
FIG. 18. Polyacrylamide gel electrophoresis of the DNA fragments released by micrococcal nuclease into the SO and SSI fractions (see "Materials and Methods"). (a), digested nuclei were extracted with Buffer D plus 0.1 M NaCl. The SSI fraction was fractionated on a Bio-Gel A-5m column (Fig. 21) and the monomer peak pooled. The DNA (0.05 Å260) was then applied to the 5% polyacrylamide SDS gel. (b) to (f), DNA released into SO from two different preparations of micrococcal nuclease digested nuclei. (b) and (c), DNA (0.2 and 1.2 Å260, respectively) released into SO from one preparation. (d), (e) and (f), DNA (0.2, 0.6 and 1.2 Å260, respectively) released into SO from another preparation. (g) and (h), DNA (0.2 and 0.6 Å260, respectively) released in SSI. Size estimates are based on a calibration using precisely defined Fnu DI generated fragments of φX174 DNA.
the SSI DNA. The 146 base pair and 170 base pair DNA fragments may correspond to the mononucleosomes MN₁ and MN₂ respectively, reported by Bakayev et al. (90).

The DNA fragments of length less than 146 base pairs corresponded to sizes of 124, 102, 92 and 75 base pairs. Similar fragments have been observed by Noll and Kornberg (217), Axel et al. (218), Bakayev et al. (90) and Whitlock (97) and are probably the result of internal nucleosome cleavages.

The DNA from SS2 consisted predominantly of a 170 base pair fragment. Lesser amounts of fragments corresponding to those released in SSI were also observed (data not shown).

The acid solubility measurements for SO (Table X) suggested that some acid precipitable material existed in this fraction. Therefore, the DNA of the SO fraction was examined on 5% non-denaturing polyacrylamide gels (Fig. 18). Low amounts of DNA fragments identical to those released in SSI were observed. This may represent leakage from damaged nuclei of material which is usually extracted in the 0.1 M NaCl fraction.
In agreement with Sanders' observations (190), the results are consistent with the interpretation that the fraction of chromatin which is most sensitive to micrococcal nuclease is eluted at the lowest NaCl concentrations.

3. Quantitation of the acetylated histone species associated with salt-extracted chromatin fractions

To quantitate the content of the modified histone species, Panyim-Chalkley gels (194) were used. This system resolves each histone and its modified forms more effectively than starch-urea gels (193) and does not require the prior separation of each histone by SDS polyacrylamide gel electrophoresis. Fig. 19A shows a photograph of a typical Coomassie blue stained Panyim-Chalkley gel where $[^{14}\text{C}]$-acetate labelled histones have been separated. Accompanying the stained gel is an autoradiogram of the gel (Fig. 19B). The autoradiogram indicates the location of the acetylated histone species. Only histones modified via $\text{N}^\text{c}$-acetylation are labelled; $\text{H}1$, which is modified by $\text{N}^\alpha\text{-acetylation}$, is not labelled.

Acid extracted histones from the different fractions were analyzed on Panyim-Chalkley gels. The histone patterns were examined for differences in the
FIG. 19. Localization of acetylated histone species separated on acid-urea gels. A, Trout testis cells were incubated with sodium [1-\textsuperscript{14}C]acetate (50 \textmu Ci/ml) for 9 h, and nuclei were isolated. Labelled histones were acid-extracted and applied to an acid-urea gel. Electrophoresis was for 20 h at 170 V (\textdegree C). The gel was stained with Coomassie blue and destained. B, After the gel was dried, an autoradiogram of the gel was prepared after a three month exposure time. A\textsubscript{0}, unacetylated H4; A\textsubscript{1}, A\textsubscript{2}, A\textsubscript{3}, and A\textsubscript{4}; mono-, di-, tri-, and tetra-acetylated H4, respectively.
extent of modification (Fig. 20). The most striking differences were found in the content of H1 and in the levels of H4 acetylation.

The H1 content relative to the other histones increased from very low in SSI to maximal in SS4. As the 146 base pair monomer fragment predominates in the SSI fraction, the H1 content would be expected to be low since this nucleosome species is devoid of H1 (189, 217, 219). The H1 present would presumably be associated with the 170 base pair fragment as reported by Bakayev et al. (90) for the mononucleosome MN2.

The content of H1 increased in SS2 with a corresponding increase in the 170 base pair fragment. The SS4 fraction contained the greatest amount of H1 relative to the other four nucleosomal histones. This result is to be expected as H1 is dissociated from chromatin or nuclei between 0.35 and 0.6 M NaCl (220, 221).

The level of H4 acetylation was considerably higher in SSI than in the other fractions, and was found to decrease with sequential extractions. As a check against possible selective losses of histones during extraction, the acid extracted histones from SSI, SS2, SS4 and SS6 were examined on 15% polyacrylamide SDS
FIG. 20. Gel scans of histones analyzed on acid-urea gels. Histones were acid-extracted from nucleosomes that had been released from micrococcal nuclease digested nuclei by stepwise, increasing NaCl concentrations. Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm. MN, micrococcal nuclease. A0, unacetylated H4; $A_1$, $A_2$ and $A_3$, mono-, di- and tri-acetylated H4, respectively.
The measured levels of H4 relative to the other three nucleosomal histones remained constant in all fractions (data not shown). Therefore, the observed level of H4 acetylation in the SS1 fraction was not due to a selective loss of the unacetylated (A₀) and the monoacetylated (A₁) H4 species, but to an increase in the levels of the di- (A₂) and tri-acetylated (A₃) H4 species.

The extent of H4 acetylation for the different fractions was calculated as a ratio of the content of A₂ and A₃ species divided by the sum of the A₁ and A₀ species. The inverse relationship between the levels of H4 acetylation and the increasing concentrations of NaCl used in the sequential extractions is evident (Table XI).

The degree of acetyl group incorporation associated with each fraction in a two hour period in vitro was determined by radioactive labelling of cell suspensions. Nuclei isolated from trout testis cells labelled with [¹³C] -acetate were digested with micrococcal nuclease and subsequently treated with increasing salt concentrations. The histones from fractions SS1, SS2 and SS4 were separated using SDS
**Quantitation of Acetylated Species of Histone H4 in Salt-Extracted Nucleosome Fractions**
*(Sanders' Procedure)*

Histones prepared by acid extraction of the salt-extracted nucleosome fractions were separated on acid-urea gels. The gels were stained with Coomassie blue, and scanned at 550 nm using a Gilford spectrophotometer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ratio of H4</th>
<th>( \frac{A_2 + A_3}{A_0 + A_1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>SS2</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>SS4</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>SS6</td>
<td>0.34</td>
<td></td>
</tr>
</tbody>
</table>

* Determined from the \( A_{550} \) peak heights of the scanned H4 species.
polyacrylamide gel electrophoresis, and their specific activities in terms of $[^{14}\text{C}]$ cpm/A$_{550}$ were determined (Table XII). For H4 the degree of acetate incorporation increases with the increasing NaCl concentrations used in the sequential extractions. Thus, the labelling of H4 acetyl groups does not correspond to the levels of H4 acetylation determined by using scans of stained acid-urea gels (Fig. 20).

Levy et al. (174) have reported a similar result. Micrococcal nuclease digested trout testis chromatin was fractionated to yield a fraction containing high levels of the acetylated H4 species. Although the H4 associated with this fraction was enriched in acetylated species, it was labelled to a lesser extent with $[^{14}\text{C}]$-acetate than the H4 associated with other fractions containing low levels of the acetylated H4 species.

Moore et al. (222) have investigated the turnover kinetics of acetyl groups in eukaryotic cells. Two types of acetylation were reported; one in which 50% of the acetate was removed with a half-life of 3 min and the rest with a much longer half-life of 30 to 40 min. Although the much longer half-life of acetyl groups in trout testis (23 hours (11)) makes it
**TABLE XII**

**Specific Activities of Histones H3 and H4 in Salt-Extracted Nucleosome Fraction**

Histones labelled with $[^{14}\text{C}]$ acetate were extracted from salt-eluted nucleosome fractions and separated on 15% polyacrylamide SDS gels containing 0.6% N, N' - diallyltartardiamide as crosslinker. The gel slices were scanned at 550 nm after staining with Coomassie blue, and counted after solubilization.

<table>
<thead>
<tr>
<th>Salt-Extracted Fraction</th>
<th>Specific Activity of Histone Fraction $[^{14}\text{C}]$ cpm/(A_{550}\times 10^{-3}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SS1</td>
<td>1.90</td>
</tr>
<tr>
<td>SS2</td>
<td>2.07</td>
</tr>
<tr>
<td>SS4</td>
<td>2.44</td>
</tr>
</tbody>
</table>
difficult to relate the kinetics in this system to that of cultured cells, the lower specific activity of the acetylated H4 associated with the SSI fraction suggests that the H4 in this fraction may constitute a subpopulation of molecules which undergoes acetyl group turnover at a relatively slow rate.

4. Quantitation of the acetylated histone species associated with column fractionated salt-extracted nucleosome fractions

To determine if the highly acetylated H4 species are associated with the nucleosomal (146 and 170 base pair) DNA, the SSI fraction was further fractionated on a Bio-Gel A-5m column (Fig. 21). Also, the total monomer population was examined for the extent of H4 acetylation by extracting micrococcal nuclease digested nuclei with Buffer D containing 0.4 M NaCl and fractionating the extract on the column (Fig. 22). Both columns were equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl to prevent aggregation of the SS4T fraction (223).

The first peak eluting at or near the void volume of both columns is termed the multimer fraction and consists of fragments greater than trimer in length.
FIG. 21. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from extraction of micrococcal nuclease digested trout testis nuclei with 0.1 M NaCl in Buffer D.
FIG. 22. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from extraction of micrococcal nuclease digested trout testis nuclei with 0.4 M NaCl in Buffer D.
(Fig. 23, lane a). For the column profile shown in Fig. 21, the second peak contained only monomer size DNA with the 146 base pair DNA fragment predominating (Fig. 18, lane a and Fig. 23, lane e). The two slower eluting peaks probably consisted of particles with DNA fragments of up to 124 base pairs in size and of small acid soluble oligonucleotides, respectively. However, this is not known for certain as the DNA size classes from these peaks were not estimated by polyacrylamide gel electrophoresis. The same profile for the SS1 fractionation was observed whether the column was equilibrated with 0.4 or 0.1 M NaCl.

The second peak of the column profile in Fig. 22 was subdivided into intermediate and monomeric fractions. Fractions 30 to 41 (the intermediate fraction) contained dimer, some trimer and very little monomer (Fig. 23, lane b). The monomer fraction (42 to 51) contained mostly monomer and some dimer (Fig. 23, lane c).

Histones were acid-extracted from SS1 monomeric fraction and from the SS4T multimeric, intermediate and monomeric fractions. The histones were separated on Panyim-Chalkley gels, and the relative degree of H4 acetylation was estimated (Table XIII).
FIG. 23. Polyacrylamide gel electrophoresis of DNA fragments associated with column fractionated, salt-extracted nucleosome fractions.

Salt-extracted fractions SS1 and SS4T were prepared as described in "Materials and Methods" and fractionated on Bio-Gel A-5m columns as presented in Fig. 21 and Fig. 22. The column fractions were pooled as follows: multimer (21-25), intermediate (30-41), monomer (42-51), and oligonucleotide (57-63). DNA (0.2 μg) obtained from the fractions (see "Materials and Methods") was analyzed on a 3% polyacrylamide - SDS gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. a, b, c and d are multimer, intermediate, monomer and oligonucleotide fractions from SS4T fractionated nucleosomes, respectively. e and f are monomer and oligonucleotide fractions from SS1 fractionated nucleosomes, respectively. 1, 2 and 3 are monomer, dimer and trimer, respectively.
Micrococcal nuclease digested trout testis nuclei were extracted with either 0.1 M NaCl in Buffer D (SSI) or 0.4 M NaCl in Buffer D (SS4T). The salt-extracted nucleosome fractions were further fractionated using a Bio-Gel A-5m column equilibrated with 0.4 M NaCl, 10 mM Tris, pH 7.5, and 0.7 mM EDTA. Monomer fractions for SS4T were pooled and acid extracted. The histones were separated on acid-urea gels, stained with Coomassie blue, and scanned at 550 nm using a Gilford spectrophotometer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ratio of H4 $\frac{A_2 + A_3}{A_0 + A_1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSI monomer</td>
<td>0.97</td>
</tr>
<tr>
<td>SS4T multimer</td>
<td>0.45</td>
</tr>
<tr>
<td>SS4T intermediate</td>
<td>0.51</td>
</tr>
<tr>
<td>SS4T monomer</td>
<td>0.58</td>
</tr>
</tbody>
</table>

* Determined from the $A_{550}$ peak heights of the scanned H4 species.
The profiles for the SSI total and SSI monomer fractions were similar for histones H3, H2A, H2B and H4 (data not shown).

The extent of H4 acetylation for the SSI monomer (Table XIII) was slightly higher than that of the SSI total (Table XI). For the SS4T fraction, the levels of H4 acetylation increased as the chromatin fragment size decreased. The SS4T monomer fraction did not contain levels of H4 acetylation as high as those observed for the SSI monomer. This was to be expected, since the monomers containing increased levels of acetylated H4 would be diluted within the total monomer population of this fraction.

The results indicate that low salt elution (0.1 M NaCl) of micrococcal nuclease digested nuclei releases a population of monomers containing increased levels of highly acetylated H4 from nuclease sensitive regions of chromatin. These results are consistent with the observation by Simpson (178) that nucleosomal core particles produced early in the course of micrococcal nuclease digestion of butyrate-treated nuclei (butyrate treatment leads to the enrichment of the acetylated histone species of H3 and H4) had more
highly acetylated H4 than those produced later in the digestion.

5. **Analysis of chromosomal proteins associated with salt-extracted chromatin fractions**

The total protein content of each of the supernatant fractions was analyzed on 15% polyacrylamide SDS gels (Fig. 24). HMG-T, a protein homologous to HMG-1 and HMG-2 in mammals (161, 124), is prominent in the SO fraction. This observation agrees with results of Levy W. et al. (152) that the action of micrococcal nuclease leads to the preferential solubilization of HMG-T. The SO fraction also contains minor amounts of a protein with an apparent molecular weight of 22,000, low levels of nucleosomal histones, and a few non-histone proteins in the 40-90,000 molecular weight range (not shown in Fig. 24). The low level of nucleosomal histones is consistent with the low amount of nucleosomal DNA present (Fig. 18).

SSI contains core histones and low levels of H1 and HMG-T. The SSI monomer (predominantly 146 base pair fragments) obtained from fractionation of SSI on a Bio-Gel A-5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.1 M NaCl contained no HMG-T or H1 when examined on polyacrylamide SDS gels (data
FIG. 24. 15% PAGE SDS separation of proteins released from micrococcal nuclease digested trout testis nuclei by successively higher NaCl concentrations. The salt-extracted fractions were dialyzed against 10 mM NH₄HCO₃ overnight and samples corresponding to 0.6 A₂₆₀ were lyophilized. The samples were redissolved in sample buffer containing SDS and applied to the gel. The gel was stained with Coomassie blue. S₀, fraction released by the initial digestion; S₁ - S₆, fractions successively released by Buffer D containing 0.1 M - 0.6 M NaCl, respectively; MN, micrococcal nuclease.
not shown). Again, these results agree with the suggestion by Levy W. et al. (162) that HMG-T is associated with the linker DNA.

SS2 is similar to SS1 except that the content of HI is higher and the content of HMG-T is lower. Traces of nonhistone proteins in the 40–90,000 molecular weight range were also visible on the original gel for both the SS1 and SS2 fractions.

SS4 and SS6 contained the nucleosomal histones, large amounts of HI (HI being highest in SS4), no HMG-T and an unidentified protein of 29,000 apparent molecular weight.

These results suggest that the internucleosomal linker DNA of nuclease sensitive regions of chromatin may contain HMG-T, and possibly lesser amounts of other nonhistone proteins. Furthermore, the nucleosomes in these regions contain highly acetylated H4.

(c) Chromatin fractionation (Levy and Dixon's procedure)

Levy and Dixon (142) have designed a chromatin fractionation procedure which partially purifies a transcriptionally active nucleosomal subfraction (Fig. 25). The procedure involves mild micrococcal nuclease digestion of trout testis nuclei, and
FIG. 25. Experimental procedure for isolation of a 0.1 M NaCl soluble nucleosomal subfraction released from micrococcal nuclease digested nuclei.
subsequent lysis of the pelleted, digested nuclei with EDTA. The digest products are released into the supernatant which is further fractionated by the addition of 0.1 M NaCl after removal of the insoluble material by centrifugation. The salt-soluble material (S3) was mostly monomeric in size and was enriched over 7 fold in DNA sequences complementary to polyadenylated RNA.

1. **Quantitation of DNA content in the chromatin fractions**

The percentage of total $A_{260}$ absorbing material released in each fraction was quantitated (Table XIV). The yields were similar to those reported by Levy and Dixon (142) except that the percentage of material they obtained in S2 was much lower. The discrepancy may be due to their use of trout testis in a late stage of maturation. Testis at this stage will have some DNA packaged as nucleoprotamine which is resistant to micrococcal nuclease digestion (188).

2. **Quantitation of acetylated histone species associated with the chromatin fractions**

The acid extracted histones from fractions P2, P3 and S3 were analyzed on acid-urea gels, and the scans of the stained gel of the latter two are shown in Fig. 26. The content of H1 was low relative to the core histones for the S3 fraction but was much higher for the P3 and P2 fractions. This is consistent with the fact
**Quantitation of DNA Content in Chromatin Fractions**  
*(Levy and Dixon's Procedure)*

The $A_{260}$ absorbing material released in the different chromatin fractions after micrococcal nuclease digestion of trout testis nuclei was quantitated by adding 100 µl of the fraction to 0.9 ml of 0.6 M NaCl in Buffer D and measuring the absorbance at 260 nm.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total $A_{260}$ in Each Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>9.8</td>
</tr>
<tr>
<td>S2</td>
<td>38.5</td>
</tr>
<tr>
<td>S3</td>
<td>7.1</td>
</tr>
</tbody>
</table>
FIG. 26. Gel scans of histones analyzed on acid-urea gels. Histones were acid-extracted from the 0.1 M NaCl soluble - (S3) and 0.1 M NaCl insoluble - nucleosomes (P3) that had been released from micrococcal nuclease digested nuclei. Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm. $A_0$, unacetylated H4; $A_1$, $A_2$, $A_3$ and $A_4$, mono-, di-, tri- and tetra-acetylated H4, respectively.
that S3 fractions contain predominantly mono-
nucleosomes (142).

The content of the acetylated histone species
associated with each fraction was examined. For all
the fractions the content of the acetylated species of
H2A, H2B and H3 did not differ. However, the level of
the H4 acetylated species was higher in S3 than in the
other fractions (Table XV). For both chromatin
fractionation procedures (Sanders' and Levy's), the 0.1
M NaCl soluble nucleosome fraction is enriched in the
acetylated histone H4 species. The results suggest that
both procedures preferentially solubilize the nucleosomal
subfraction that may be associated with transcriptionally
active chromatin.

(d) Summary

The initial studies (Section I(a)) demonstrated that
mononucleosomes contained normal levels of the major acetylated
histone species compared to those of the total nuclear histone
population. Labelling studies using $[^{14}C]-$acetate also
suggested that the degree of acetylation was similar for the
histones isolated either from mononucleosomes or nuclei. At
that stage it was not realized that the total population of
acetylated histones species was not equally represented by the
label, $[^{14}C]-$acetate: that is, histones (especially H4)
**TABLE XV**

Quantitation of Acetylated Species of Histone H4 in the Chromatin Fractions (Levy and Dixon's Procedure)

Histones prepared by acid extraction of nucleosome fractions were separated on acid-urea gels. The gels were stained with Coomassie blue, and scanned at 550 nm using a Gilford spectrophotometer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ratio of H4 ( \frac{A_2 + A_3}{A_0 + A_1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>0.32</td>
</tr>
<tr>
<td>P3</td>
<td>0.40</td>
</tr>
<tr>
<td>S3</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* Determined from the A\(550\) peak heights of the scanned H4 species.
associated with a nucleosomal subfraction associated with transcriptionally active chromatin were labelled to a lesser extent than nucleosomal subfractiions associated with the bulk of chromatin.

For later experiments the Panyim-Chalkley gel system was used. This method enabled the quantitation of the levels of the acetylated histone species without the use of labelling techniques.

Treatment of micrococcal nuclease digested nuclei with 0.4 M NaCl will solubilize most of the digest products, as does lysis of the nuclei with EDTA. Comparison of the acetylated histone species associated with mononucleosomes with those associated with multimers demonstrated that the monomers were enriched in the acetylated H4 species (A_2 and A_3, Table XIII). As the total histone population would be expected to contain predominantly high levels of the A_0 and A_1 species of H4 similar to SS4 and SS6 (Table XI), the mononucleosomes would contain a greater level of the A_2 and A_3 species of H4 than that of the H4 extracted from undigested nuclei.

The actual degree of enrichment in the acetylated H4 species associated with mononucleosomes would be dependent on the extent of micrococcal nuclease digestion. For example, the mononucleo-
somes obtained from an extensive micrococcal nuclease digestion of nuclei would contain predominantly the $A_0$ and $A_1$ species of H4, while a relatively mild digestion would produce mononucleosomes enriched in the $A_2$ and $A_3$ species of H4. Therefore, it would be doubtful that a difference in the content of the minor acetylated histones species (i.e. H4 $A_2$ and $A_3$) associated with mononucleosomes or nuclei would have been detectable in the earlier experiments (section 1(a)) where quantitation of the minor acetylated histone species was not possible.

Both fractionation procedures used generated a nucleosome fraction soluble in 0.1 M NaCl that contained enriched levels of the acetylated H4 species. The 0.1 M NaCl soluble nucleosomes generated by the Sanders' or Levy's procedures were also similar in that the nucleosome fractions contained low levels of $H_1$, and the nucleosomal DNA fragment was of monomeric size. As the 0.1 M NaCl soluble mononucleosomes obtained by the Levy's procedure represent a transcriptionally active chromatin fraction and as recent reports (121, 122) have demonstrated that active genes are cleaved into mononucleosomes more rapidly than is bulk chromatin by micrococcal nuclease, the 0.1 M NaCl soluble mononucleosome fraction that was obtained by the Sanders' procedure probably corresponds to nucleosomes associated with a transcriptionally active chromatin fraction.
Thus, acetylation of H4 may play a key role in maintaining the structure of transcriptionally active chromatin. Non-histone proteins (i.e. HMG-T) may also be involved in the maintenance of this structure, a structure that seems likely to be an extended state of chromatin.

II Characterization of Nucleosome Subfractions and Chromosomal Proteins Released from DNase II Digested Chromatin

Recently, Gottesfeld and his co-workers have shown that the DNase II fractionation procedure of Marushige and Bonner (225), coupled with Mg\(^{++}\) precipitation (Fig. 27) results in the isolation of a chromatin fraction (S2) enriched in actively transcribing sequences (140, 141). Levy-Wilson et al., applying this procedure to Drosophila cells, observed a 40% increase in the incorporation of labelled acetate into histones of the template-active fraction (173). The patterns of histone acetylation in the different fractions were examined.

(a) Quantitation of DNA content in the chromatin fractions

The percentage of A\(_{260}\) absorbing material associated with each fraction is presented in Table XVI. Although the digest conditions (5 min. with 100 units/ml DNase II) used were identical to those of Gottesfeld et al. (141), the percentage of A\(_{260}\) absorbing material in S2 was lower for trout testis than for rat liver. The results suggest that rat liver chromatin contains a greater percentage of transcriptionally-active chromatin than does trout testis chromatin.
FIG. 27. Experimental procedure for the isolation of a transcriptionally active chromatin fraction released from DNase II digested chromatin.

DNase II DIGESTED CHROMATIN

CENTRIFUGE

P1

S1

1. Slowly add 1 M MgCl₂ to 2 mM final
2. Incubate 20 min. at 0°C
3. Centrifuge

P2

S2 ("ACTIVE" CHROMATIN FRACTION)
The $A_{260}$ absorbing material released in the different fractions after DNase II digestion of trout testis chromatin was quantitated by adding 100 μl of the fraction to 0.9 ml of 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and measuring the absorbance at 260 nm. The distribution of DNA among the various fractions obtained from DNase II digestion of rat liver chromatin is also presented (141).

<table>
<thead>
<tr>
<th>Chromatin Fraction</th>
<th>Total $A_{260}$ in each Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat Liver Chromatin</td>
</tr>
<tr>
<td></td>
<td>%</td>
</tr>
<tr>
<td>P1</td>
<td>84.6 ± 4.8</td>
</tr>
<tr>
<td>S2</td>
<td>11.3 ± 3.9</td>
</tr>
<tr>
<td>P2</td>
<td>4.1 ± 2.5</td>
</tr>
</tbody>
</table>
Kinetics of DNase II digestion

For the following experiments, the concentration of DNase II was lowered to 40 units/ml, and the digestion time was increased to 8 min. A time course of digestion of trout testis chromatin is presented in Fig. 28. Similar to results presented by Gottesfeld et al. (139), the 2 mM Mg\(^{+2}\)-soluble fraction, S2, reaches a steady value early in the digest while the material in S1 increases. Thus, the 2 mM Mg\(^{+2}\)-soluble fraction forms part of the most nuclease sensitive fraction of chromatin DNA.

Trout testis cells contain an enzyme similar to DNase II. To assay for the endogenous DNase II activity, a sample of chromatin was incubated for 30 min at 24°C in the absence of exogenous DNase II. After this incubation time, 3.8% of the chromatin was solubilized, and 64% of this material remained soluble after the addition of 2 mM Mg\(^{+2}\).

Characterization of the DNA fragments associated with each chromatin fraction

The material that remained soluble after the addition of 2 mM Mg\(^{+2}\) (S2) was further fractionated by the addition of 22 mM Mg\(^{+2}\) yielding a 22 mM Mg\(^{+2}\)-soluble fraction, S3, and a 22 mM Mg\(^{+2}\)-insoluble fraction, P3. The addition of 22 mM Mg\(^{+2}\) will precipitate most of the nucleosomal material but not the oligonucleotides present in S2. Fig. 29 presents the percentage of A\(_{260}\) absorbing material associated with each fraction.
FIG. 28. Time course of chromatin fractionation (I).

After various times of incubation with DNase II (4 units/A$_{260}$ unit of DNA), samples were removed, and an appropriate volume of 0.1 M Tris-HCl (pH 11) was added to give a final pH of 7.5. The chromatin was separated into the first supernatant (S1) and subsequent fractionation into 2 mM Mg$^{2+}$-soluble (S2) and insoluble material as described in "Materials and Methods" (Fig. 27). The A$_{260}$ absorbing material was quantitated by adding 100 µl of the fraction to 0.9 ml of 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and measuring the absorbance at 260 nm. •, S1; ■, S2.
Solubilized chromatin fractions were obtained from DNase II digested chromatin as described in the legend to Fig. 28. The 2 mM Mg$^{2+}$-soluble fraction (S$_2$) was further fractionated by the addition of 22 mM Mg$^{2+}$ yielding a 22 mM Mg$^{2+}$-soluble fraction (S3) and a 22 mM Mg$^{2+}$-insoluble fraction (P3). The A$_{260}$ absorbing material was quantitated in the same manner as Fig. 28.

•, S3; ■, P3.
as a function of incubation time. The amount of $A_{260}$ absorbing material precipitated by 22 mM $Mg^{2+}$ (P3) drops to a constant value after 15 min. incubation time while the amount remaining soluble (S3) increases steadily with time. This result is probably a consequence of DNase II digesting the linker DNA which will increase the levels of oligonucleotides that are soluble in $Mg^{2+}$.

The endogenous DNase II generated material that is soluble in S2 can be almost completely precipitated by 22 mM $Mg^{2+}$ suggesting that the level of oligonucleotides is low in S2.

The DNA fragment size of the material associated with P2 and P3 was examined on 3% nondenaturing polyacrylamide gels (Fig. 30). The nucleosomal material excised from chromatin by the endogenous DNase II activity contained DNA fragments greater than pentamer in length for both the P2 and P3 fractions (Fig. 30, lanes a and f). As the incubation time in the presence of exogenous DNase II increases, the P2 fractions maintain a full spectrum of the different nucleosomal DNA size classes with a slight accumulation of monomeric size DNA. The material precipitated by 22 mM $Mg^{2+}$ (P3) shows an accumulation of the monomeric DNA size fragment with increasing incubation time. After a 30 min. incubation, the monomeric DNA fragment is the predominant species. The appearance of the monomer DNA fragment is probably a result of
FIG. 30. Polyacrylamide gel electrophoresis of the DNA fragments solubilized by DNase II digestion of trout testis chromatin.

After various times of incubation with exogeneous DNase II (4 units/A_{260} unit of DNA) or endogeneous DNase II, samples were withdrawn and fractionated as described in "Materials and Methods". DNA (0.5 A_{260}) obtained from the 2 mM Mg^+-insoluble (P2) and 22 mM Mg^+-insoluble (P3) fractions was analyzed in a 3% polyacrylamide SDS gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. b, c, d and e are P2 fractions obtained from chromatin incubated with exogeneous DNase II for 4 min, 8 min, 16 min and 30 min, respectively. g, h, i and j are P3 fractions obtained from chromatin incubated with exogeneous DNase II for 4 min, 8 min, 16 min and 30 min, respectively. a and f are P2 and P3 fractions, respectively, obtained from chromatin incubated 30 min with endogeneous DNase II. 1, 2 and 3 are monomer, dimer and trimer, respectively.
sequential digestion of larger nucleosomal fragments.

The amount of $A_{260}$ absorbing material precipitated by 22 mM Mg$^{2+}$ (P3) was found to diminish to a constant value after 15 min digestion (Fig. 29). The larger values at early incubation times may be due to the initial release of large nucleosomal repeats (i.e. trinucleosomes) (Fig. 30, lane g). As the digestion continues, the linker DNA associated with the polynucleosomes would be excised which would eventually result in the accumulation of mononucleosomes. With the loss of the linker DNA the amount of $A_{260}$ absorbing material precipitated by 22 mM Mg$^{2+}$ would decrease to a constant value when the mononucleosomes should be the major nucleosomal species.

(d) Determination of the levels of acetylated histone species associated with the chromatin fractions

Fig. 31 shows the histone patterns resolved in acid-urea gels obtained from the various stages of the DNase II/Mg$^{2+}$ fractionation procedure. Most of the histone patterns demonstrate low levels of the histone H4 relative to the other nucleosomal histones. This was due to incomplete precipitation of the H4 molecules when ethanol was added to the acid-extracted histone sample. The histone patterns from control chromatin (C), from the undigested pellet (P1) after DNase II treatment, and from
Histone profiles on an acid/urea/polyacrylamide gel.

Histones were prepared from chromatin fractions and separated on a 1.5 mm slab gel by the system of Panyim and Chalkley (194). The gel was stained with Coomassie blue. C, histones from unfractionated chromatin control; P1, histones from chromatin pellet remaining after DNase II digestion; P2, histones from 2 mM Mg$_{+2}$-insoluble fraction of DNase II - released material; S2, histones from 2 mM Mg$_{+2}$-soluble fraction of DNase II - released material; P3, histones from fraction rendered 2 mM Mg$_{+2}$-insoluble by the first RNase digestion of S2; P4, histones from fraction rendered 2 mM Mg$_{+2}$-insoluble by the second RNase digestion of S2. The positions of the histones are indicated in sample C. Histone H3 is resolvable into three components (unmodified, mono- and di-acetylated). H4 is resolvable into four components: A$_0$, unmodified; A$_1$, A$_2$, A$_3$ and A$_4$ are mono-, di-, tri- and tetra-acetylated, respectively. RNase, pancreatic ribonuclease.
the 2 mM Mg$^{+2}$-insoluble fraction (P2) are indistinguishable from each other (Fig. 31). The 2 mM Mg$^{+2}$-soluble fraction (S2) however, is greatly enriched in the diacetylated ($A_2$), triacetylated ($A_3$) and tetraacetylated ($A_4$) species of H4. The solubility of this chromatin fraction in 2 mM Mg$^{+2}$ is believed to be due to its high content of RNA (139). Treatment of this fraction with pancreatic ribonuclease resulted in precipitation of approximately 35% of the material; a sample of histones from this precipitate (P3) is also shown in Fig. 31. Although the H4 region of the P3 sample is not visible on this photograph, the sample is likewise greatly enriched in acetylated H4 species. In this experiment, the yield of H4 in the P3 fraction is especially low due to the inefficient ethanol precipitation of the acid extracted sample; in other experiments the H4 pattern consistently resembled that of the S2 fraction with the addition of the RNase band. A second treatment of the supernatant to P3 with RNase led to precipitation of a further 10 - 15% of the original digest products, and this fraction, P4, resembled the S2 and P3 fractions in its content of acetylated H4 (Fig. 31).

The relative proportions of the acetylated H4 components at each stage of the fractionation are more readily seen in gel scans of the H4 region (Fig. 32). In whole chromatin, the monoacetylated species are most abundant, but the di- and triacetylated species are readily detected; the tetra-acetylated species are not resolved from the adjacent H2B band in these
FIG. 32. Gel scans of the histone H4 regions of an acid/urea gel. The gel is from a different experiment from that illustrated in Fig. 31. A, A₁, A₂ and A₃ are subspecies of histone H4, as described in the legend to Fig. 31. (A) H4 from unfractionated control chromatin; (B) H4 from P1, the chromatin pellet remaining after DNase II digestion; (C) H4 from P2, the 2 mM Mg⁺⁺-insoluble fraction of DNase II released material; (D and E) H4 from P3 and P4, respectively, the fractions rendered 2 mM Mg⁺⁺-insoluble by RNase digestion of the 2 mM Mg⁺⁺-soluble fraction. Gels were stained with Coomassie Blue and scanned in a Gilford spectrophotometer at 550 nm.
scans (Fig. 32A). The chromatin remaining insoluble after DNase II digestion (P1) and the 2 mM Mg^{2+}-insoluble component of the digest (P2) also contain H4 with the above distribution of acetyl bands (Fig. 32 B and C). In the pellet resulting from RNase treatment of the S2 fraction (P3 and P4, Fig. 32, D and E), the A_2 and A_3 species equal or exceed the A_1 in amount. A_3 seems to be the most prominent component in these fractions, however, as will be seen below, an unknown polypeptide migrates very close to A_3 in the first dimension, and the actual amount of A_3 present is approximately equal to the amount of A_2. The A_4 component, visible in the photograph of the original gel (e.g. Fig. 31, P4), was not resolved in the scan.

In order to examine the possibility that the H4 region of the gel might be contaminated with unknown protein species, samples of histones from whole chromatin and from fractions S2 and P4 were analyzed on two-dimensional gels in which components were separated on an acid/urea gel in the first dimension followed by electrophoresis in a SDS polyacrylamide gel in the second dimension. As seen in Fig. 33A all histone fractions are separated from each other in this system. Examination of the S2 fraction confirms that the bulk of the H4 present is in the acetylated forms (Fig. 33B). Furthermore, A_4 is now clearly visible and is present in amounts approximately equal to those of A_3. An unidentified component of slightly lower mobility than H4, designated 12K, migrates between A_2 and A_3 and is barely
FIG. 33. Two-dimensional PAGE separation of histones from chromatin fractions, stained with Coomassie blue. The first (horizontal) dimension consisted of an acid/urea gel (194), and the second (vertical) dimension consisted of the polyacrylamide SDS gel system of Laemmli (226) as modified by Weintraub et al. (59). For the second dimension, a gel strip was excised from a lane of the acid/urea gel containing the histones; the strip was equilibrated in Buffer 0 of O'Farrell (195) for 20 to 30 min, applied to the surface of the SDS slab gel, and sealed with melted 1% agarose in Buffer 0. The remaining region of the first-dimension strip was stained with Coomassie blue and is shown at the top of the corresponding two-dimensional separation. (A) Histones from control unfractionated chromatin; (B) histones from the 2 mM Mg$^{+2}$-soluble DNase II fraction, S2; (C) histones from the material rendered 2 mM Mg$^{+2}$-insoluble by RNase digestion of S2 (P4). 12 K and 29K are unidentified proteins. The positions of the histone fractions are shown in A. Components A$_0$-A$_4$ refer to subspecies of H4 as described in the legend to Fig. 31.
visible in the photograph of this gel (Fig. 33B). This component has not been detected in unfractionated chromatin. An additional unidentified component, designated 29K, coincides exactly with diacetylated H3 in the first dimension, but migrates just ahead of H1 in the SDS dimension (Fig. 33B and C). It also seems to be associated with the template-active chromatin fraction, and on acid/urea gels gives the erroneous impression that the $A_2$ species of H3 is enriched in this fraction (see, for instance, P4 of Fig. 31, arrow). When the experiment was done, the possibility that 12K and 29K components arose from proteolytic degradation, i.e. by a contaminant of the DNase II, could not be ruled out. However, if this is so, they are evidently associated with the 2 mM Mg$^{2+}$-soluble fraction. The absence of other low molecular weight species on the gels, however, argues against this possibility.

A two-dimensional gel of the P4 chromatin fraction is shown in Fig. 33C. The lower amount of protein applied to the gel accentuates the fact that $A_3$ and $A_4$ are predominant H4 species; the $A_0$ and $A_1$ components were clearly visible on the original gels, but do not appear distinct in the photograph. The $A_0$ component was not discernible even on the original gel. The 29K protein is present, but the 12K spot is not visible, possibly due to the lower loading.
The above results clearly show that the DNase II, Mg$^{+2}$-soluble fraction of chromatin is greatly enriched in the multi-acetylated species of H4.

(e) **Behaviour of S2 nucleosomal material in the presence of Mg$^{+2}$, RNase or histone**

Gottesfeld et al. (139) suggested that the solubility of the S2 associated nucleosomal material in 2 mM Mg$^{+2}$ was due to the presence of nascent RNA attached to the nucleosome. Treatment of the S2 fraction with RNase resulted in the precipitation of the 2 mM Mg$^{+2}$-soluble nucleosomes possibly due to the digestion of the RNA. The possibility that RNase, which is positively charged at physiological pH, precipitated the nucleosomes by virtue of its positive charge as opposed to its enzymatic activity was examined.

RNase, histone and Mg$^{+2}$ were studied for their ability to precipitate the S2 nucleosomal material (Table XVII). A control was required because some precipitation occurred normally during the incubation period. In this experiment the percentage of S2 material precipitated by RNase was much lower than previously observed. Both 22 mM Mg$^{+2}$ and whole histone were capable of precipitating the nucleosomal material with Mg$^{+2}$ being the most effective.

The histones associated with the precipitated material were
TABLE XVII

Efficiency of Mg$^{+2}$, Histone or RNase in Precipitation of S2 Associated Nucleosomal Material

A 2 mM Mg$^{+2}$-soluble chromatin fraction (S2) was obtained from DNase II digested chromatin as described in "Materials and Methods". To the S2 fraction either RNase (10 μg/ml), whole histone (10 μg/ml) or Mg$^{+2}$ (22 mM) was added. The solution was incubated for 20 min at 37°C, and centrifuged. A control sample of S2 was incubated for 60 min at 37°C and centrifuged. The $A_{260}$ absorbing material remained soluble was quantitated by adding 100 μl of the supernatant to 0.9 ml of 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and measuring the absorbance at 260 nm.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Percent $A_{260}$ in Precipitated Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>10.3</td>
</tr>
<tr>
<td>RNase (10 μg/ml)</td>
<td>18.9</td>
</tr>
<tr>
<td>Histone (10 μg/ml)</td>
<td>47.8</td>
</tr>
<tr>
<td>Mg$^{+2}$ (22 mM)</td>
<td>68.2</td>
</tr>
</tbody>
</table>
examined on acid-urea gels (not shown). The precipitates that were obtained by adding either RNase, whole histone, or 22 mM Mg\(^{+2}\) to the S2 fraction contained similar high levels of the acetylated histone H4 species, suggesting the same nucleosomal fraction was being precipitated.

The S2 fraction was incubated with RNase for various times, and the percentage of insoluble material was quantitated (Table XVIII). The percentage of material precipitated did not vary appreciably over the time period studied. If the precipitation of the S2 material was caused by enzymatic degradation of the nascent RNA, an increase of insoluble material with increasing incubation time would have been expected.

The results suggest that RNase precipitates the nucleosomal S2 material by virtue of its positive charge rather than by digestion of the nascent RNA.

### Summary

The Mg\(^{+2}\)-soluble DNase II fraction, S2, from rat liver is organized into nucleosomes and is enriched in nascent RNA and non-histone proteins (139). It has also been shown that globin genes are enriched in the Mg\(^{+2}\)-soluble fraction of Friend cells which have been induced to synthesize hemoglobin (228), although there is a disagreement as to whether the same enrich-
TABLE XVIII

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>Percent $A_{260}$ in Precipitated Material</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>17.3</td>
</tr>
<tr>
<td>5</td>
<td>19.7</td>
</tr>
<tr>
<td>10</td>
<td>19.6</td>
</tr>
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</table>
ment occurs in uninduced cells (228, 229). However, in cells that have lost the capacity for hemoglobin induction, no enrichment of globin sequences is seen in this fraction (229). The evidence so far thus suggests that the Mg$^{+2}$-soluble DNase II fraction (S2) is enriched in active gene sequences.

The results suggest that the S2 associated nucleosomal material is associated with a chromatin region that is sensitive to DNase II attack. The nucleosomes in this region are initially released as polynucleosomes, but with continued digestion, the major nucleosome species becomes the mononucleosomes.

The transcriptionally-active nucleosomal fraction contains the four core histones, H2A, H2B, H3 and H4, but only H4 was found to be greatly enriched in muti-acetylated species.

The results suggest that DNase II recognizes and selectively excises nucleosomes enriched in the acetylated H4 species; these nucleosomes are believed to be derived from transcriptionally competent chromatin regions.

III: Characterization of Nucleosome Subfractions and Chromosomal Proteins Released by DNase I Digestion of Nuclei

Weintraub and Groudine (133) have demonstrated that transcriptionally competent genes exhibit an increased susceptibility to
digestion by DNase I. The sensitivity of the genes to DNase I is apparently not dependent on the rate at which it is transcribed (134).

Levy and Dixon (136) have also examined the ability of DNase I to selectively digest transcriptionally-competent genes. DNase I digestion of trout testis nuclei, under conditions in which 10% of the total DNA was digested, resulted in the preferential depletion of the DNA sequences being transcribed into polyadenylated mRNA.

The DNase I sensitivity of transcriptionally-competent genes has led to the proposal that transcriptionally-competent chromatin contains nucleosomes with an altered conformation. The possibility that histone acetylation as well as the presence of other specific proteins might provide a mechanism for the proposed structural changes was investigated.

(a) Characterization of nucleosomes released from DNase I digested nuclei

1. Digest products released from DNase I digested nuclei

The products from a DNase I digestion of nuclei were fractionated on a Bio-Gel A-5m column equilibrated with 10 mM Tris-HCl, pH 7.5, and 0.7 mM EDTA (Fig. 34). The column profile was similar to the fractionation of digest products from nuclei digested mildly with micrococcal nuclease (Fig. 6). The fractionated products were pooled into four nucleosomal size classes: multimer (fractions 18 to 23), intermediate (24 to 27),
FIG. 34. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes isolated from DNase I digested nuclei. Trout testis nuclei (20 A$_{260}$/ml) were digested with DNase I (2.5 µg/ml) for 5 min at 25°C. The digested nuclei were collected by centrifugation, and the digest products released by resuspending the nuclei in 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA.
monomer (28 to 33), and submonomer (34 to 41). It was assumed that the multimer, intermediate, and monomer fractions corresponded to polynucleosomes, dinucleosomes, and mononucleosomes, respectively, as the DNA fragments resolve as smears on nondenaturing polyacrylamide gels.

The single-stranded DNA fragments associated with each fraction were analyzed on denaturing polyacrylamide gels (Fig. 35). Each fraction shows a pattern of bands that are multiples of ten bases and a prominent band at 80 nucleotides. The pattern is due to the nicking of the nucleosomal DNA strands by DNase I at ten bases apart (230). Besides containing the characteristic pattern of bands, the larger nucleosomal size classes contain DNA fragments larger than 140 bases.

Noll (230) has demonstrated that although DNase I attacks both the nucleosomal and linker DNA, the nucleosome still remains intact. Noll has obtained mononucleosomes from DNase I digested rat liver nuclei that sediment at the same rate as intact 11 S chromatin subunits. The mononucleosomes contained single-stranded DNA fragments that were multiples of 10 bases. However, the distribution of single-stranded fragments
FIG. 35. Polyacrylamide gel electrophoresis of single-stranded DNA fragments associated with column fractionated nucleosome fractions. Nucleosomes obtained from DNase I digested nuclei were fractionated as described in the legend to Fig. 34. The column fractions were pooled as follows: multimer (18-23), intermediate (24-27), monomer (28-33) and sub-monomer (34-41). DNA (0.5 A₂₆₀) obtained from the fractions (see "Materials and Methods" Section XV a (i)) was analyzed on a 10% polyacrylamide SDS gel containing 7 M urea. After electrophoresis at 200 V for 4 h , the gel was stained with ethidium bromide and photographed under ultraviolet light. a, b, c and d are multimer, intermediate, monomer and submonomer fractions, respectively.
presented relatively few fragments of 10 and 20 bases. As fragments no smaller than 30 or 40 bases were resolvable on the gels, Noll's observation was not verified.

The possibility of the release of free histones from DNase I digested nuclei was examined. Nuclei obtained from $^{14}$C acetate and $^3$H lysine labelled trout testis cells were digested with DNase I, and the digest products were fractionated on a Bio-Gel A-5m column (Fig. 36). All of the label was associated with the multimer, intermediate and monomer fractions, suggesting that the histones are bound to DNA.

Since the nucleosomal DNA is nicked by DNase I at staggered intervals (98), it should be possible for the nicked DNA strands to remain as an intact double-stranded DNA fragment in the absence of histones. To remove the histones from DNA, sodium lauryl sarcosinate (Sarkosyl) was used (127). The efficiency of Sarkosyl for dissociating the histones was examined by using mononucleosomes containing histones labelled with $^{14}$C acetate isolated from micrococcal nuclease digested nuclei. The labelled mononucleosomes were treated with 1% Sarkosyl and fractionated on a Bio-Gel A-0.5m column
FIG. 36. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes labelled with [\(^{14}\)C] acetate and [\(^3\)H] lysine isolated from DNase I digested nuclei. Trout testis cells were incubated with L [4, 5 (n) - \(^3\)H] lysine monohydrochloride (100 \(\mu\)Ci/ml) and sodium [1-\(^{14}\)C] acetate (50 \(\mu\)Ci/ml) for 7 h, and nuclei were isolated. The nuclei (20 A\(_{260}\)/ml) were digested 15 min with DNase I (2.5 \(\mu\)g/ml) at 25\(^\circ\)C, and the digest products were separated on a Bio-Gel A-5m column. Radioactivity was determined in the same manner as described for Fig. 7.
equilibrated with 10 mM Tris-HCl, (pH 7.5), 0.7 mM EDTA, and 1% Sarkosyl (Fig. 37A). The label elutes after the \( A_{260} \) absorbing material, indicating the intact nucleosomal DNA is free of bound histones. Using the same procedure, unlabelled nucleosomes isolated from a DNase I digested nuclei were fractionated on the Bio-Gel A-0.5m column (Fig. 37B). Only one peak of \( A_{260} \)-absorbing material is seen eluting which suggests the nicked nucleosomal DNA remains intact in the absence of histones.

2. Quantitation of acetylated histone species associated with nucleosomal fractions

The histones associated with the DNase I generated nucleosomal fractions were analyzed for their levels of acetylated species. Trout testis nuclei were digested with DNase I until 6.6, 8.2, or 16.9% of the initial \( A_{260} \)-absorbing material was solubilized and the digest products were fractionated on a Bio-Gel A-5m column. The histones from the multimer fraction and the monomer fraction were acid extracted. The histones remaining with the nuclear pellet after the digest products were released were also acid extracted. The histones from the 6.6% and 8.2% digest fractions were separated on acid-urea gels (Fig. 38). Each fraction contained equivalent proportions of the nucleosomal histones, H2A,
FIG. 37. Bio-Gel A-0.5m (90 x 1.0 cm) fractionation of nucleosomal components. (A) Mononucleosomes (~10 A_{260}) labelled with \([^{14}C]\) acetate were isolated from micrococcal nuclease digested nuclei as described in "Materials and Methods". The mononucleosomes were concentrated by precipitation with 10 mM MgCl₂ and collected by centrifugation. The mononucleosomes were resuspended in 0.5 ml of 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 1% Sarkosyl and applied to the column. Radioactivity was determined by mixing 0.5 ml column fractions with 8 ml ACS (Amersham/Searle). (B) Nucleosomes (~20 A_{260}) were isolated from DNase I digested nuclei as described in the legend to Fig. 34. The nucleosomes were concentrated, collected and resuspended as described above before application to the column.
FIG. 38. Histone profiles on an acid-urea polyacrylamide gel. Histones were acid-extracted from nuclei, polynucleosomes or mononucleosomes that were isolated from DNase I digested nuclei. The histones were separated on an acid-urea polyacrylamide gel by the system of Panyim and Chalkley (194). The gel was stained with Coomassie blue. (a) and (d), histones from nuclear pellet remaining after DNase I digestion until 6.6% or 8.2%, respectively, of the DNA was solubilized, and digest products removed; (b) and (e), histones from multimer fraction of fractionated digest products from nuclei digested with DNase I until 6.6% or 8.2% DNA solubilized, respectively; (c) and (f), histones from monomer fraction of fractionated digest products from nuclei digested with DNase I until 6.6% or 8.2% DNA solubilized, respectively.
H2B, H3 and H4. Examination of the photograph and scans of the gel slices (not shown) suggested the levels of the acetylated species for histones H2A, H2B and H4 were similar for all the fractions and did not vary with the extent of digestion including the 16.9% digestion. All fractions contained predominantly the unacetylated \( (A^0) \) and monoacetylated \( (A_1) \) species of H4. Also the di- \( (A_2) \), tri- \( (A_3) \), and tetraacetylated \( (A_4) \) H4 species were visible but to a much lesser extent than the \( A^0 \) and \( A_1 \) species.

The levels of the acetylated species of histone H3 appeared to diminish in the monomer and multimer fractions relative to the levels of the H3 acetylated species associated with the pellet. The monomer fraction contained the lowest levels of acetylated H3 species. The monomer fraction always had decreased levels of the H3 acetylated species regardless of the extent of digestion. The relatively high levels of the acetylated H3 species associated with the pellet may be deceiving as nonhistone chromosomal proteins are known to run in this position (Fig. 33).

The level of H1 is seen to decrease as the nucleosomal DNA size decreases (Fig. 38). As digestion of
the linker DNA results in the loss of H1, the monomer fraction would be expected to contain the least amount of H1. This expectation is confirmed as the monomer fraction contains the lowest amount of H1.

These studies indicate that mononucleosomes isolated from DNase I digested nuclei contain the four nucleosomal histones, H2A, H2B, H3 and H4. The mononucleosomes contain the same levels of acetylated species of histones H2A, H2B and H4 as those of polynucleosomes but appear to be deficient in the acetylated species of H3.

(b) **Characterization of nucleosome subfractions and chromosomal proteins released from DNase I digested nuclei (Sanders' procedure)**

The Sanders' fractionation procedure involves digestion of the nuclei with micrococcal nuclease followed by successive extractions of the nuclei with buffer solutions containing increasing concentrations of NaCl (Fig. 16). Previous experiments have been described in which micrococcal nuclease was used to digest nuclei followed by subsequent fractionation (see Section I(b)). The experiments were repeated with DNase I as the digesting nuclease. The levels of acetylated histone species and chromosomal proteins associated with the salt released nucleosomal subfractions were examined in the same manner as in the micrococcal nuclease experiments.
1. **Quantitation of the DNA content in salt-extracted chromatin fractions**

Following the digestion of the trout testis nuclei with DNase I, the digested nuclei were sequentially treated with increasing concentrations of NaCl as described by Sanders (190), except that the initial NaCl concentration was 0.1 M instead of 0.2 M, and the 0.3 M NaCl extraction step was omitted.

The percentage of total \( A_{260} \) absorbing material released in each fraction is presented in Table XIX. The values obtained for each salt-extracted fraction did not vary appreciably with the extent of digestion in the range between 1.5% and 30% solubilization of the DNA. The invariability of the amount of DNA associated with each salt-extracted fraction when the nuclei were digested with micrococcal nuclease to different extents has been reported by Sanders (190). Sanders suggested the stability of the internucleosomal interactions was not altered significantly after the chromatin had been digested. This interpretation can be extended to include DNase I.

The percentage of \( A_{260} \) absorbing material associated with each fraction from micrococcal nuclease digested
The $A_{260}$ absorbing material released in the different salt-extracted nucleosome fractions after DNase I digestion of trout testis nuclei was quantitated by adding 100 μl of the fraction to 0.9 ml of 0.6 M NaCl in Buffer D and measuring the absorbance at 260 nm.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total $A_{260}$ in Each Fraction</th>
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<tbody>
<tr>
<td>S0</td>
<td>5.0</td>
</tr>
<tr>
<td>SS1</td>
<td>3.2</td>
</tr>
<tr>
<td>SS2</td>
<td>2.7</td>
</tr>
<tr>
<td>SS4</td>
<td>26.8</td>
</tr>
<tr>
<td>SS6</td>
<td>22.7</td>
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</table>
nuclei or DNase I digested nuclei were quite different (Table X and XIX). These differences may be due to several factors. Firstly, micrococcal nuclease will introduce DNA cuts that are adjacent on both strands while DNase I will introduce staggered cuts (101). Secondly, micrococcal nuclease preferentially digests the linker region while DNase I digests both the linker and nucleosomal DNA at comparable rates (230). Thirdly, the preferential digestion of the linker DNA by micrococcal nuclease will release chromosomal proteins such as HMG-T which are not released by DNase I digestion (162). One or more of the above factors might result in the greater ease of low salt solubilization of nucleosomes from micrococcal nuclease digested nuclei.

The combined percentage of total $A_{260}$ absorbing material associated with SSI (DNase I) and SS2 (DNase I) is 5.9. This value is slightly lower than the percentage of total $A_{260}$ material (7.2) associated with SSI (micrococcal) fraction (Table X). It is possible that the SSI and SS2 fractions released from DNase I digested nuclei represent subsets of the SSI fraction released from micrococcal nuclease digested nuclei.
2. **Characterization of the DNA fragments associated with salt-extracted chromatin fractions**

The size of the DNA fragments associated with each salt-extracted fraction cannot be determined by electrophoresis on nondenaturing polyacrylamide gels as this results in a smear. To obtain an approximate size of the DNA fragments, Bio-Gel A-5m gel exclusion chromatography was used. The elution profiles of A\textsubscript{260} absorbing material associated with the salt-extracted fractions, SS1, SS2 and SS4T (DNase I) are shown in Figures 39, 40 and 41, respectively. The DNA fragment size associated with each column fraction has previously been characterized in experiments in which micrococcal nuclease digested nuclei were further fractionated on Bio-Gel A-5m columns (Section Ib(4)).

The A\textsubscript{260} absorbing material associated with SS1 (DNase I) resolves as three peaks (Fig. 39). The first peak eluting at or near the void volume is termed the multimer fraction, and consists of fragments greater than trimer in length. The second peak (monomer fraction) consists of mononucleosomal size fragments and the third peak consists of oligo-nucleotides. The mononucleosomal fragment is the most abundant of the nucleosome associated DNA fragments.
FIG. 39. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from extraction of DNase I digested trout testis nuclei with 0.1 M NaCl in Buffer D.
FIG. 40. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from extraction of DNase I digested trout testis nuclei with 0.2 M NaCl in Buffer D. The digested nuclei had been previously extracted with 0.1 M NaCl in Buffer D.
FIG. 41. Bio-Gel A-5m (90 X 1.5 cm) separation of nucleosomes obtained from extraction of DNase I digested trout testis nuclei with 0.4 M NaCl in Buffer D.
The SS2 (DNase I) material also resolves into three peaks (Fig. 40). The majority of the material elutes with the monomer fraction (42 to 51). Lower amounts of $A_{260}$ absorbing material elute with the multimer and intermediate (30 to 41) fractions. The intermediate fractions consist of dimer, some trimer and very little monomer (Section Ib(4)).

The SS4T (DNase I) fraction, which contains all of the nucleosomal subfractions normally released in SS1, SS2 and SS4 (DNase I), was fractionated into three peaks (Fig. 41). The majority of the material would be characteristic of the SS4 (DNase I) fraction (Table XIX): that is, about 80% of the material is associated with the SS4 (DNase I). The profile is almost identical to the profile of SS4T (micrococcal) column fractionated material (Fig. 32). The majority of the $A_{260}$-absorbing material elutes with the intermediate fraction.

From the column elution positions, approximate sizes of the nucleosomal DNA fragments were obtained. Both the SS1 and SS2 (DNase I) fractions contain DNA fragments mainly of monomeric size while the SS4T (DNase I) fraction contained mainly fragments of dimer size or
larger. Thus, the results suggest that the fraction of chromatin which is most sensitive to DNase I digestion is eluted at the lowest NaCl concentrations.

The size of the single-stranded DNA fragments associated with the salt-extracted fractions was examined on 15% denaturing polyacrylamide gels (Fig. 42). Fractions SS1, SS2, SS4 and SS6 (DNase I) all presented the 10 base DNA repeat. Visual inspection of the intensity of each band relative to the 80 base band suggested each fraction contained the same quantity of any particular band. However, one observable difference was that the SS4 and SS6 (DNase I) fractions contained a greater abundance of fragments greater than 140 bases. This is consistent with the observation that SS1 and SS2 (DNase I) fractions contain predominately mononucleosomes while the SS4 (DNase I) fraction contains mainly dinucleosomes and other poly-nucleosomes.

The pattern of single-stranded fragments associated with each fraction did not vary appreciably with the extent of digestion in the range between 1.5% and 30% solubilization of the DNA.
FIG. 42. Polyacrylamide gel electrophoresis of single-stranded DNA fragments generated by DNase I digestion of trout testis nuclei and released by stepwise increases in NaCl concentrations. The salt-extracted fractions were dialyzed against 10 mM NH$_4$HCO$_3$ overnight and samples corresponding to 0.5 A$_{260}$ were lyophilized. The samples were redissolved in sample buffer containing SDS and applied to the gel. The gel was stained with ethidium bromide and photographed under ultraviolet light. a–d, fractions successively released by Buffer D containing 0.1 M–0.6 M NaCl (SS1–SS6), respectively.
3. Characterization of the proteins associated with salt-extracted chromatin fractions

The protein associated with each of the supernatant fractions was analyzed on 15% polyacrylamide-SDS gels (Fig. 43). SS1 (DNase I) contains core histones, HMG-T1 (27K, formerly classified as HMG-T (159)), HMG-T2 (25K), and HMG-T3 (22K). (The nomenclature assigned to these proteins is as described by B. S. Bhullar (personal communication)). This fraction also contains nonhistone chromosomal proteins in the 30 - 90,000 molecular weight range. SS2 (DNase I) is similar to SS1 (DNase I) except that the contents of HMG-T2 and HMG-T3 are lower, and low levels of H1 are observed. The spectrum of nonhistone proteins in the 30 - 90,000 molecular weight range is similar to that from the SS1 (DNase I) fraction but there are differences in the content of some proteins.

SS4 and SS6 (DNase I) contained the nucleosomal histones, large amounts of H1 (H1 being highest in SS4), two unidentified proteins of 28,000 and 29,000 apparent molecular weight but no HMG-T proteins. Figure 43 presents a sample (SS4 (II)) which clearly shows the two unidentified proteins associated with the SS4 (DNase I) fraction.
FIG. 43. 15% PAGE SDS separation of proteins released from DNase I digested trout testis nuclei by successively higher NaCl concentrations. Samples were prepared as described in the legend to Fig. 24. The gel was stained with Coomassie blue. a – d, fractions released from DNase I digested nuclei by Buffer D containing 0.1 M – 0.6 M NaCl (SS1 – SS6), respectively; e, fraction released by digestion of nuclei by DNase I (SO (D)); f, fraction released by digestion of nuclei by micrococcal nuclease (SO (M)); and (g) fraction released by Buffer D containing 0.4 M NaCl (SS4 (II)) after previous extractions of DNase I digested nuclei with Buffer D containing 0.1 M NaCl followed by extraction with Buffer D containing 0.2 M NaCl. SO (D), SO (M) and SS4 (II) were all obtained from different experiments than those from which SS1 – SS6 were derived.
The SO (DNase I) fraction contains low levels of the core histones, and lower levels of proteins found associated with the SS1 (DNase I) fraction (Fig. 43). These proteins may be released from damaged nuclei.

The proteins associated with each fraction were examined after the nuclei had been digested to varying extents with DNase I (1.5% to 30% DNA solubilized). Each salt-extracted fraction contained the same characteristic set of proteins regardless of the extent of DNase I digestion. Thus, the extent of nuclear digestion does not appreciably affect the pattern of solubilized proteins released into each salt-extracted fraction.

A comparison of the photographs of the gels presented in Figures 24 and 43 demonstrates similarities and dissimilarities in the salt-extracted fractions released from either micrococcal nuclease or DNase I digested nuclei. The SO (micrococcal) fraction (Fig. 43 and 24) contains HMG-T1, HMG-T2, and HMG-T3 which are not associated with the SO (DNase I) fraction but with the SS1 and SS2 (DNase I) fractions (Fig. 43). Levy W. et al. (162) have
reported similar results. The preferential solubilization of HMG-T by micrococcal nuclease but not DNase I digestion of trout testis nuclei suggested to the authors that HMG-T was located in the internucleosomal region. In the same experiments (162) Levy et al. observed the selective solubilization of H6 from DNase I digested nuclei. The selective solubilization of this protein has not been observed in this laboratory, and the discrepancy may be due to Levy et al. using trout testis that were at a late stage of maturation.

The SS1 (DNase I) and SS2 (DNase I) fractions are qualitatively very similar to the corresponding SS1 (micrococcal) and SS2 (micrococcal) fractions in that they contain the four nucleosomal histones, low levels of H1 and nonhistone chromosomal proteins in the 30–90,000 molecular weight range. The proteins associated with the SS4 (DNase I) and SS6 (DNase I) fractions are also similar to those associated with the corresponding SS4 (micrococcal) and SS6 (micrococcal) fractions with regard to their content of nucleosomal histones, H1 and the unidentified proteins of 28,000 and 29,000 apparent molecular weight. The results suggest that the nucleosomal subfraction and chromosomal proteins solubilized in the salt-extracted fractions from either DNase I digested nuclei or
micrococcal digested nuclei are very similar.

4. Quantitation of the acetylated histone species associated with the salt-extracted chromatin fractions

Acid-extracted histones from the different fractions were analyzed on acid-urea gels (194). The histone patterns were examined for differences in the content of acetylated histone species. However, quantitation of the acetylated species, especially those of H4, for fractions SS1 and SS2 (DNase I) was impossible as nonhistone proteins comigrated with the acetylated histone species (not shown). The levels of acetylated H4 species could be determined for fractions SS4 and SS6 (DNase I). Figure 44 presents gel scans of SS4 (DNase I) and SS6 (DNase I) associated histones respectively. The levels of the acetylated H3 species appear very high but this is due to the comigration of a nonhistone protein designated 29K (Fig. 33) with the acetylated species of H3.

Visual inspection of the scans suggest that the SS6 (DNase I) fraction contains lower levels of the acetylated H4 species than does the SS4 (DNase I) fraction (Fig. 44). The extent of H4 acetylation for the SS4 and SS6 (DNase I) fractions was calculated as
FIG. 44. Gel scans of histones analyzed on acid-urea gels. Histones were acid-extracted from nucleosomes that had been released from DNase I digested nuclei by stepwise, increasing NaCl concentrations (0.1, 0.2, 0.4 and 0.6 M NaCl yielding SS1, SS2, SS4 and SS6, respectively). Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm. Only the gel scans of histones associated with SS4 and SS6 are presented. A₀, unacetylated H4; A₁, A₂, and A₃, mono-, di-, and tri-acetylated H4, respectively.
a ratio of the content of $A_2$ and $A_3$ species divided by the sum of $A_0$ and $A_1$ species (Table XX). The extent of H4 acetylation associated with the SS4 (DNase I) fraction was slightly higher than that of the SS6 (DNase I) fraction. Also, the extent of H4 acetylation for the respective fractions did not vary with the extent of digestion. Interestingly, the content of acetylated H4 species associated with the SS4 and SS6 (DNase I) fractions (Table XX) was similar to that from SS4 and SS6 (micrococcal) fractions (Table XI). These results further suggest that the nucleosomal subfraction solubilized in SS4 or SS6 from DNase I or micrococcal nuclease digested nuclei are very similar.

5. **Bio-Gel A-0.5m column fractionation of salt-extracted nucleosome fractions**

(i) **Column fractionation of the chromosomal proteins associated with the salt-extracted chromatin fractions**

The Bio-Gel A-0.5m column (40 x 1.5 cm) equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA, and 0.4 M NaCl was chosen to separate unbound proteins from the nucleosomal material. SS1 fractions from nuclei that had been digested with either DNase I (4.5% of DNA solubilized) (Fig. 46) or micrococcal nuclease (5.7% of DNA
TABLE XX

Quantitation of Acetylated Species of Histone H4 in Salt-Extracted Nucleosome Fractions Released from DNase I Digested Nuclei

The SS4 and SS6 fractions were obtained as described in "Materials and Methods" from nuclei that had been incubated with DNase I for various times. The histones that were prepared by acid-extraction of the salt-extracted nucleosome fractions were separated on acid-urea gels. The gels were stained with Coomassie blue and scanned at 550 nm using a Gilford spectrophotometer.

<table>
<thead>
<tr>
<th>Percent of Total A_{260} Released into SS0</th>
<th>Fraction</th>
<th>SS4</th>
<th>SS6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio of H4 ( \frac{A_2 + A_3}{A_0 + A_1} ) *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5</td>
<td>0.38</td>
<td>0.26</td>
<td></td>
</tr>
<tr>
<td>2.9</td>
<td>0.32</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>0.36</td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>7.8</td>
<td>0.34</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>AVG.</td>
<td>0.35</td>
<td>AVG. 0.26</td>
<td></td>
</tr>
</tbody>
</table>

* Determined from the A_{550} peak heights of the scanned H4 species.
FIG. 45. Bio-Gel A-0.5m(40 X 1.5 cm) separation of nucleosomes obtained from extraction of micrococcal nuclease digested trout testis nuclei with 0.1 M NaCl in Buffer D. The column eluant was 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA, 0.4 M NaCl.
FIG. 46. Bio-Gel A-0.5m (40 X 1.5 cm) separation of nucleosomes obtained from extraction of DNase I digested trout testis nuclei with 0.1 M NaCl in Buffer D. The column eluant was 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA, 0.4 M NaCl.
solubilized) (Fig. 45) were further fractionated on this column.

The SSI (micrococcal) fraction resolves as four peaks (Fig. 45). Although the size of the DNA fragments was not analyzed on 3% nondenaturing gels, the first (multimer fraction) and second (monomer fraction) peaks probably contain dinucleosomes plus larger oligonucleosomes, and mononucleosomes, respectively. (The Bio-Gel A-0.5m column has an exclusion limit of 500,000 molecular weight; therefore, dinucleosomes would probably be excluded). The third and fourth peaks probably contain oligonucleotides and dinucleotides, respectively. The SSI (DNase I) fraction resolves as three peaks (Fig. 46). The first and second peaks correspond to the multimer and monomer fractions, respectively, while the third peak probably consists of oligonucleotides. The profile for the column fractionated SSI (micrococcal) and SSI (DNase I) fractions remained the same if the Bio-Gel A-0.5m column was equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA, and 0.1 M NaCl (not shown).
Proteins associated with the different fractions were analyzed on 15% polyacrylamide-SDS gels. Figure 47 lane (a) presents the SSI (DNase I) sample before fractionation on the column. This sample contains the core histones, HMG-T1, HMG-T2, and HMG-T3. There is also a multitude of nonhistone proteins in the 30-90,000 molecular weight range. The SSI (DNase I) was fractionated on a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and either 0.1 M NaCl or 0.4 M NaCl. The nucleosomal fraction (Figure 46, fractions 13 to 22) and the oligonucleotide fraction (Fig. 46, fractions 24 to 36) were pooled separately and dialyzed overnight against 10 mM NH₄HCO₃ at 4°C. An equivalent amount of sample (0.6 A₂₆₀ units) from each fraction was examined on a 15% polyacrylamide-SDS gel. The same spectrum of proteins was identified in the corresponding nucleosomal and oligonucleotide fractions regardless of the ionic strength of the column buffer (compare Fig. 47, lane (b) to lane (d), and lane (c) to lane (e)). The nucleosome fractions contained the core histones and some nonhistone proteins in 30-90,000 molecular weight range.
FIG. 47. 15% PAGE SDS separation of chromosomal proteins associated with column fractionated, 0.1 M NaCl extracted nucleosome fractions. DNase I digested trout testis nuclei or micrococcal nuclease digested trout testis nuclei were extracted with 0.1 M NaCl in Buffer D (SSI). The salt-extracted nucleosome fractions were further fractionated using a Bio-Gel A-0.5m column equilibrated with either 0.4 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA or 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA. Nucleosomal (combined multimer and monomer fractions) and oligonucleotide fractions for SSI (DNase I), and nucleosomal, oligonucleotide and dinucleotide fractions for SSI (micrococcal) were pooled separately. The samples were prepared for electrophoresis as described in the legend to Fig. 24. a, SSI (DNase I) unfraccionated; b and c, nucleosomal and oligonucleotide fractions, respectively, from SSI (DNase I) fractionated on a Bio-Gel A-0.5m column equilibrated with 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA; d and e, nucleosomal and oligonucleotide fractions, respectively, from SSI (DNase I) fraction factionated on the column equilibrated with 0.4 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA; f, SSI (micrococcal) unfraccionated; g, h and i, nucleosomal, oligonucleotide and dinucleotide fractions, respectively, from SSI (micrococcal) fraction factionated on the column equilibrated with 0.1 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA; j, l and m, nucleosomal, oligonucleotide and dinucleotide fractions, respectively, from SSI (micrococcal) fraction factionated on the column equilibrated with 0.4 M NaCl, 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA; k, micrococcal nuclease. Lanes k, l and m were from a separate gel than that of the others.
The predominant proteins associated with the oligonucleotide fractions were the HMG proteins (HMG-T1, HMG-T2 and HMG-T3). Low levels of other nonhistone proteins were also contained in this fraction.

Figure 47, lane (f) presents the SSI (micrococcal) sample before column fractionation. This fraction contains the core histones and nonhistone proteins including the HMG proteins. The SSI (micrococcal) fraction was further fractionated on a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and either 0.1 M NaCl or 0.4 M NaCl (Fig. 45). The column fractionated samples were pooled into three fractions: the nucleosome fraction (14 to 22), the oligonucleotide fraction (24 to 29) and the dinucleotide fraction (31 to 38).

The spectrum of proteins associated with each fraction did not alter with the ionic strength of the column buffer (compare Fig. 47, lane (g) to lane (j), lane (h) to lane (l), and lane (i) to lane (m)). The nucleosome fractions contained the core histones and low levels of
nonhistone proteins in the 30 - 90,000 molecular weight range. The oligonucleotide fractions contained nonhistone proteins including HMG-T1, HMG-T2, and HMG-T3, and low levels of the core histones. Micrococcal nuclease was the most abundant protein contained in the dinucleotide fraction (Fig. 47, compare lane (i) or lane (m) to lane (k)).

The SSI (DNase I) nucleosome fraction (Fig. 47, lane (b)) contains a lower amount of core histones but higher amounts of proteins when compared to the SSI (micrococcal) nucleosomal fraction (Fig. 47, lane (g)) per unit of $A_{260}$ absorbing material. This may be due to the fact that the SSI (DNase I) fraction contains a greater amount of nucleosomal material eluting with the multimer fraction relative to the monomer fraction than that of the SSI (micrococcal) fraction (compare Fig. 45 to Fig. 46).

Interestingly, the HMG proteins do not remain bound to either the DNase I or micrococcal nuclease produced nucleosomal material in the presence of 0.1 M NaCl. Although the nucleosome fractions contain nucleosomes with linker DNA
(i.e. dinucleosomes) to which the HMG proteins are supposedly bound (162), the HMG proteins are apparently unable to bind to this region in the presence of 0.1 M NaCl. As HMG proteins are not released from DNase I digested nuclei (Fig. 43), they probably remain bound to the linker DNA at ionic strengths less than 0.1.

(ii) Quantitation of the acetylated histone species associated with nucleosomal subfractions

DNase I digested nuclei were extracted with Buffer D containing 0.1 M NaCl followed by extraction with Buffer D containing 0.4 M NaCl to yield supernatant fractions SSI and SS4T, respectively. The SSI (DNase I) fraction was further fractionated on a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl. The column profile obtained was similar to the profile shown in Fig. 46.

The histone associated with the nucleosomes (fractions 13 to 22) from the column fractionated SSI (DNase I) fraction and the histones associated with the SS4T (DNase I) fraction were acid-extracted and separated on an acid-urea gel in the first dimension followed by electro-
phoresis on a 15% polyacrylamide-SDS gel in the second dimension. As seen in Figure 48A, all histone fractions are separated from each other in this system. Visual inspection of the photographed gel suggests that the levels of the acetylated H4 species, $A_2$ and $A_3$ relative to $A_0$ and $A_1$ are higher for the SSI (DNase I) fraction (Fig. 48A) than for the SS4T (DNase I) fraction (Fig. 48B). Histones H2A and H2B appear to be similar for both fractions, but histone H3 associated with the SSI (DNase I) fraction appears to contain lower levels of acetylated species than the H3 associated with the SS4T (DNase I) fraction. An unidentified component of slightly lower mobility than H4, designated 12K, migrates between $A_2$ and $A_3$, and is barely visible in the photograph of this gel (Fig. 48A and B) for both fractions, SSI and SS4T (DNase I). Another faint, unidentified component, 14K, is observed migrating ahead of H2B for both fractions. The 28K and 29K components associated with the SS4T (DNase I) fraction can be seen migrating slightly faster than H1 (Fig. 48B). Both of these components comigrate with the acetylated species of H3 in the first dimension.
FIG. 48. Two-dimensional PAGE separation of histones from salt-extracted nucleosome fractions.

The first (horizontal) dimension consisted of an acid-urea gel (194) and the second (vertical) dimension consisted of the polyacrylamide SDS gel system of Laemmli (226) as modified by Weintraub et al. (5). The preparation of the first dimension gel for electrophoresis on the second dimension gel was as described in the legend to Fig. 33. (A) Histones from the nucleosomal fraction of a SS1 (DNase I) fraction fractionated on a Bio-Gel A-0.5m column as described in the legend to Fig. 46. (B) Histones from SS4T (DNase I) fraction obtained by extracting DNase I digested nuclei that had been previously extracted with Buffer D containing 0.1 M NaCl, with Buffer D containing 0.4 M NaCl. A0, unacetylated H4; A1, A2, A3 and A4, mono-, di-, tri- and tetra-acetylated H4, respectively.
Based on the percentage of $A_{260}$ absorbing material associated with the SS1 and SS2 (DNase I) fractions (Table XIX), these fractions are possibly the subsets of the SS1 (micrococcal) fraction (see Section III b(1)). Therefore, instead of initially extracting DNase I digested nuclei with Buffer D containing 0.1 M NaCl, Buffer D containing 0.2 M NaCl was used to yield a supernatant designated SS2T. The SS2T (DNase I) was further fractionated on a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 0.4 M NaCl (Fig. 49). The monomer fraction (17 to 21) was pooled, and the histones were acid-extracted. Histones from undigested nuclei (control) were also acid-extracted. Histone samples from the SS2T (DNase I) monomer fraction, control and SS1 (micrococcal)* were analyzed on acid-urea gels, and the histone patterns examined for differences in the extent of modification (Fig. 50).

The levels of the acetylated H4 species appear to be similar for the SS2T (DNase I) and SS1 (micrococcal) fractions (Fig. 50). Also, the content of the acetylated H4 species associated with the control sample is lower than

*The sample was previously prepared for another experiment (section Ib(3)).
FIG. 49. Bio-Gel A-0.5m(40 X 1.5 cm) separation of nucleosomes obtained from extraction of DNase I digested trout testis nuclei with 0.2 M NaCl in Buffer D.
FIG. 50. Gel scans of histones analyzed on acid-urea gels. Histones were acid-extracted from either undigested nuclei (c), nucleosomes that had been released from micrococcal nuclease digested nuclei by Buffer D containing 0.1 M NaCl (SS1 (micrococcal)) or mononucleosomes (SS2T (DNase I)) that were prepared by extracting DNase I digested nuclei with Buffer D containing 0.2 M NaCl and fractionating the solubilized fraction on a Bio-Gel A-0.5m column as described in the legend to Fig. 49. Gels were stained with Coomassie blue and scanned in a Gilford spectrophotometer at 550 nm. MN, micrococcal nuclease. A₀, unacetylated H4; A₁, A₂, A₃ and A₄, mono-, di-, tri- and tetra-acetylated H4, respectively.
that of the SS2T (DNase I) and SS1 (micrococcal) fractions. The extent of H4 acetylation for the different fractions was calculated by dividing the sum of the $A_2$ and $A_3$ species by the sum of the $A_0$ and $A_1$ species (Table XXI). Again, the content of the H4 acetylated species is similar for the SS2T (DNase I) and SS1 (micrococcal) fractions, and the content of the H4 acetylated species is much higher for these fractions than for the control sample. The total H4 population (control) (Table XXI) has low levels of the acetylated H4 species similar to the SS6 (DNase I) fraction (Table XX).

The levels of the acetylated H3 species associated with these fractions appear to differ. The high level of acetylated H3 species associated with the control is deceiving as non-histone proteins, 28K and 29K, migrate in this region. Although fractions SS2T (DNase I) and SS1 (micrococcal) do not contain components 28K and 29K, the level of acetylated H3 species for fraction SS1 (micrococcal) is higher than that of fraction SS2T (DNase I). (Fig. 50).
## TABLE XXI

**Quantitation of Acetylated Species of Histone H4 in Salt-Extracted Nucleosome Fractions released from either Micrococcal Nuclease or DNase I Digested Nuclei**

Histones prepared from either undigested nuclei or salt-extracted nucleosome fractions following micrococcal nuclease or DNase I digestion as described in the legend to Fig. 50 were separated on acid-urea gels. The gels were stained with Coomassie blue and scanned at 550 nm using a Gilford spectrophotometer.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ratio of H4 $\frac{A_2 + A_3}{A_0 + A_1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (undigested nuclei)</td>
<td>0.26</td>
</tr>
<tr>
<td>SS1 (micrococcal)</td>
<td>0.70</td>
</tr>
<tr>
<td>SS2T (DNase I) monomer</td>
<td>0.62</td>
</tr>
</tbody>
</table>

* Determined from the $A_{550}$ peak heights of the scanned H4 species.
To further resolve the proteins, the samples were electrophoresed in a second dimension on a 15% polyacrylamide - SDS gel (Fig. 51). Examination of the SS2T (DNase I) fraction and the SSI (micrococcal) fractions confirms that both fractions contain higher levels of the acetylated H4 species than does the control which has the bulk of the H4 present in the $A_0$ and $A_1$ forms. The level of the acetylated H3 species associated with the SS2T (DNase I) fraction appears to be lower than the level of the acetylated H3 species associated with the SSI (micrococcal) or control samples. Also, the total content of H3 appears to be lower relative to the other nucleosomal histones for the SS2T (DNase I) fraction. Low levels of the acetylated H3 species were also observed for the SSI (DNase I) fraction (Fig. 48).

Several unidentified components associated with the fractions were resolved by this two-dimensional gel system (Fig. 51). The control sample contained the 29K component normally associated with the SS4 (DNase I or micrococcal) fraction. Also, a component that migrated slightly faster than H2B and designated as 14K
FIG. 51. Two-dimensional PAGE separation of histones from salt extracted nucleosome fractions and from undigested nuclei.

The first (horizontal) dimension consisted of an acid-urea gel (194), and the second (vertical) dimension consisted of the polyacrylamide SDS gel system of Laemmli (226) as modified by Weintraub et al. (59). The preparation of the first dimension gel for electrophoresis on the second dimension gel was as described in the legend to Fig. 33. (A) Histones from the 0.1 M NaCl fraction extracted from micrococcal nuclease digested nuclei (SS1 (micrococal)); (B) histones from undigested nuclei; (C) histones from mononucleosomes associated with a fraction prepared by extracting DNase I digested nuclei with Buffer D containing 0.2 M NaCl as described in the legend to Fig. 49. A0, unacetylated H4; A1, A2, A3 and A4, mono-, di-, tri- and tetra-acetylated H4. MN, micrococcal nuclease.
was seen. The 14K component was identified in all three fractions. In addition to the 14K component, several other unidentified components were observed for both the SS2T (DNase I) and SS1 (micrococcal) fractions. These components included a protein migrating ahead of the A₀ species of H4 (11K), and several other proteins positioned between the A₄ species of H4 and H2B. Although the nuclei were treated with the protease inhibitor PMSF, the possibility that these components are proteolytic products (possibly of H3) cannot be ruled out.

6. Determination of the degree of acetyl group incorporation associated with the salt-extracted fractions

The degree of nucleosome acetyl group incorporation associated with each fraction in a two hour period in vitro was determined by radioactive labelling of cell suspensions. Nuclei isolated from trout testis cells labelled with [¹⁴C] acetate were digested with DNase I and subsequently treated with increasing salt concentrations. The histones from fractions SS1, SS2 and SS4 (DNase I) were separated on 15% polyacrylamide-SDS gels, and their specific activities in terms of cpm/A₅₅₀ were determined.
(Table XXII). For H4 the degree of acetate incorporation increased with the increasing NaCl concentration used in the sequential extractions. Thus, the labelling of H4 acetyl groups does not correspond to the levels of H4 acetylation determined by scans of stained acid/urea gels or two dimensional gels. The reasons for this apparent discrepancy are not entirely clear. The simplest explanation would be that histone H4 associated with the SSI fraction constitutes a subpopulation of molecules which undergoes acetyl group turnover at a relatively slow rate (refer p. 107).

The combined results suggest that low salt extraction (0.1 M and 0.2 M NaCl) of DNase I digested nuclei releases a population of nucleosomes that contain increased levels of the highly acetylated species of H4, and, possibly, low levels of the acetylated H3 species from nuclease sensitive regions of chromatin. Also, the nucleosome subfraction associated with SSI and SS2 (DNase I) fractions possibly constitute subpopulations of the nucleosomes associated with SSI (micrococcal). Furthermore, the inter-nucleosomal linker DNA of the DNase I sensitive
Histones labelled with $[^{14}\text{C}]$ acetate were extracted from salt-eluted nucleosome fractions of DNase I digested nuclei. The histones were separated on 15% polyacrylamide SDS gels containing 0.6% N, N'-diallyltartardiamide as cross-linker. The gel slices were scanned at 550 nm after staining with Coomassie blue, and counted after solubilization.

<table>
<thead>
<tr>
<th>Salt-Extracted Fraction</th>
<th>Specific Activity of Histone Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3</td>
</tr>
<tr>
<td></td>
<td>$[^{14}\text{C}]$ cpm/A$_{550} \times 10^{-2}$</td>
</tr>
<tr>
<td>SS1</td>
<td>1.09</td>
</tr>
<tr>
<td>SS2</td>
<td>1.30</td>
</tr>
<tr>
<td>SS4</td>
<td>1.58</td>
</tr>
</tbody>
</table>

**TABLE XXII**

Specific Activities of Histones H3 and H4 in Salt-Extracted Nucleosome Fractions
regions of chromatin may contain nonhistone proteins with the predominant proteins being HMG-T1, HMG-T2 and HMG-T3.

(c) **DNase I digestion of mononucleosome**

Early in the course of this project, the possibility that mononucleosomes containing high levels of the acetylated histone species might be more sensitive to DNase I attack was examined. The $[^{14}C]$-acetate and $[^3H]$-lysine labelled mononucleosomes were digested with DNase I. However, at that time it was not realized that $[^{14}C]$ acetate might not label all nucleosome sub-fractions equally (section IIIb(6)).

Labelled mononucleosomes were digested with DNase I to 50% acid solubility and the digest products fractionated on a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 6 M urea at 4°C (Fig. 52B). The 6 M urea was added to produce a more "loosely" associated nucleosome, and in this way, perhaps, the more extensively digested nucleosomes might be more susceptible to dissociation. Undigested labelled mononucleosomes were fractionated on a Bio-Gel A-0.5m column (Fig. 52A). The undigested mononucleosomes resolved as one peak with maximal $A_{260}$ absorbing material and radioactive counts eluting at fraction 35. The DNase I digested mononucleosomes resolved as two $A_{260}$-absorbing peaks (Fig. 52B).
FIG. 52. Bio-Gel A-0.5m column (90 x 1.0 cm) fractionation of DNase I digested mononucleosomes. (A) Mononucleosomes (10 A₂₆₀), that were isolated from micrococcal nuclease digested nuclei as described in the legend to Fig. 6, labelled with [¹⁴C] acetate and [³H] lysine were applied to a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 6 M urea. (B) Mononucleosomes (20 A₂₆₀), that were prepared as described as above, labelled with [¹⁴C] acetate and [³H] lysine were digested with DNase I until 50% of the DNA was acid soluble. The digests were applied to a Bio-Gel A-0.5m column equilibrated with 10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA and 6 M urea. Radioactivity was determined as described in the legend to Fig. 7.
The specific activity \((^{14}\text{C} \text{cpm}/A_{260})\) of the nucleosome peak fraction (fraction 35) was determined for each profile (Table XXIII). The specific activity of the surviving nucleosomes from the digest is much higher than that of the undigested nucleosomes. Also, the \(^{3}\text{H} \text{cpm}/A_{260}\) ratio remains constant for both fractions while the \(^{14}\text{C} \text{cpm}/A_{260}\) ratio decreases for the digested fraction. These results suggest that the content of histones, as measured by \(^{3}\text{H}\)-lysine label bound to nucleosomal DNA, remains constant but that the nucleosomes containing acetylated histones (\(^{14}\text{C}\)-acetate) are selectively digested. Examination of the column profile of the digested mononucleosomes shown in Figure 52B further strengthens the suggestion. The histones eluting after the nucleosomal peak contain an enrichment of acetylated histones: that is, the \(^{3}\text{H}/^{14}\text{C}\) ratio decreases dramatically after the nucleosomal peak.

This experiment has been repeated with two other preparations of labelled mononucleosomes, and each yielded identical results. However, one particular preparation of labelled mononucleosomes did not demonstrate the increase in the \(^{3}\text{H}/^{14}\text{C}\) ratio of the nucleosomal peak after DNase I digestion. The reason for this result is unclear.

The histones eluting after the nucleosomal peak in Fig. 52B were possibly bound to DNA fragments rather than free in solution. N-ethyl \(^{3}\text{H}\) maleimide (NEM) was used to measure
### TABLE XXIII

**Specific Activity of Histones Associated with Mononucleosomes Before and After DNase I Digestion**

Mononucleosomes and DNase I digested mononucleosomes labelled with both $[^{14}\text{C}]$ acetate and $[^{3}\text{H}]$ lysine were fractionated on Bio-Gel A-0.5m columns, and the radioactivity of the peak fractions (fraction 35) determined as described in the legend to Fig. 52.

<table>
<thead>
<tr>
<th>Fraction 35</th>
<th>Specific Activity of Fraction</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$[^{3}\text{H}]$ cpm/A$_{260}$</td>
<td>$[^{14}\text{C}]$ cpm/A$_{260}$</td>
</tr>
<tr>
<td>Undigested mononucleosomes</td>
<td>32,688</td>
<td>7,912</td>
</tr>
<tr>
<td>DNase I digested mononucleosomes</td>
<td>33,959</td>
<td>5,106</td>
</tr>
</tbody>
</table>
the degree of accessibility of the thiol group in the H3 molecule (191). Mononucleosomes were digested by DNase I to 50% acid solubility followed by reaction with NEM in the presence of either a low ionic strength buffer (10 mM Tris-HCl, pH 7.5, 0.7 mM EDTA), 6 M urea, 0.5 M NaCl or a combination of 6 M urea and 0.5 M NaCl. Undigested nucleosomes (control) were treated in a similar manner (Table XXIV). The reactivity of the H3 thiol groups of the control and digested nucleosomes in either Tris/EDTA, 6 M urea or 0.5 M NaCl was extremely low. Maximal reactivity of the H3 thiol groups for both the control and nucleosomes was obtained in 6 M urea and 0.5 M NaCl. Thus, the H3 sulfhydryl groups are fully exposed in the presence of 0.5 M NaCl and 6 M urea, but the thiol groups are protected by histone-histone interactions and/or histone-DNA interactions in the presence of Tris/EDTA, 6 M urea, or 0.5 M NaCl. As the thiol groups of the digested nucleosomes did not exhibit a greater reactivity to NEM in the presence of 6 M urea than that of the control, the histones trailing after the nucleosomal peak (Fig. 52B) are probably bound to DNA: that is, DNase I digestion of nucleosomes followed by exposure of the digested nucleosomes to 6 M urea does not lead to the release of free histones.

Simpson (178) has reported that nucleosome core particles containing high levels of the acetylated H3 and H4 species are digested by DNase I at a rate equivalent to that of core
TABLE XXIV

N-Ethyl [\(^{3}\)H] Maleimide Reactivity of the Cysteinyl Residue of Histone H3 Associated with Either Mononucleosomes or DNase I Digested Mononucleosomes in Various Denaturing Solutions

Mononucleosomes were prepared as described in the legend to Fig. 6, and digested with DNase I as described in "Materials and Methods". Reaction of the nucleosomes (digested or undigested) with N-ethyl [\(^{3}\)H] maleimide was as described in "Materials and Methods".

<table>
<thead>
<tr>
<th>Solution</th>
<th>Undigested Mononucleosomes</th>
<th>DNase I Digested Mononucleosomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(^{3})H cpm</td>
<td></td>
</tr>
<tr>
<td>Tris/EDTA</td>
<td>173</td>
<td>186</td>
</tr>
<tr>
<td>6 M urea</td>
<td>356</td>
<td>132</td>
</tr>
<tr>
<td>0.5 M NaCl</td>
<td>222</td>
<td>221</td>
</tr>
<tr>
<td>6 M urea and 0.5 M NaCl</td>
<td>3,104</td>
<td>2,854</td>
</tr>
</tbody>
</table>
particles containing much lower levels of the acetylated H3 and H4 species. Thus, the contents of acetylated histones did not alter the rate of digestion of the core particle. However, the DNA site, 60 nucleotides from the 5' end of the nucleosome core particle, was more susceptible to DNase I digestion for those particles containing the increased levels of the acetylated H3 and H4 species. The evidence suggests that acetylation of the amino-terminal position of the histones results in loss of protection from DNase I attack at this DNA site. The presence of a perturbing agent such as urea might possibly enable the more extensively nicked nucleosomes containing enriched levels of the acetylated histone species to readily denature. Preliminary results provide some support for this possibility. Ideally, the experiment should be repeated using acid-urea gels to examine the content of the acetylated histone species instead of using radioactive labels.

(d) **Summary**

Although DNase I digests both inter- and intranucleosomal DNA at approximately equivalent rates, nucleosomes can still be obtained from the DNase I digested nuclei. When the single-stranded DNA fragments associated with the mononucleosomal or polynucleosomal fraction were examined on denaturing polyacrylamide gels, both nucleosome fractions were found to contain fragments that were multiples of ten bases apart. In addition, the polynucleosomal fractions contained fragments greater than
The content of acetylated histone species was similar for both mono- and polynucleosomes, although the content of the acetylated H3 species appeared low for the mononucleosome fraction. Both fractions contained low levels of the acetylated H4 species (Fig. 38). This result is expected as the nucleosomes that are released into solution by lysing micrococcal nuclease digested nuclei with EDTA are representative of the nucleosomes associated with the bulk of chromatin (Fig. 44): that is, the majority of nucleosomes associated with chromatin contain low levels of the highly acetylated H4 species (A$_2$ and A$_3$) (Table XIX).

Fractionation of the nucleosome population into subfractions was made possible by extracting DNase I digested nuclei with sequentially increasing salt concentrations. Results from these experiments suggested that the inter-nucleosomal linker DNA of DNase I sensitive regions of chromatin may contain HMG-T1, HMG-T2, HMG-T3 and low levels of other nonhistone proteins. Also, the nucleosomes in these regions contain highly acetylated H4 and possibly low levels of the acetylated H3 species. In addition to the selective excision of the nucleosomes containing highly acetylated H4, preliminary evidence suggests that nucleosomes containing highly acetylated
histones are more susceptible to internal DNase I attack than nucleosomes containing low levels of the acetylated histone species.

Weintraub and Groudine (133) have demonstrated that transcriptionally competent genes exhibit an increased susceptibility to DNase I. The results may be interpreted as indicating an alteration in nucleosome structure or packaging at transcriptionally competent loci. Histone acetylation (178 - 182) and HMG proteins (137) have been implicated in producing a DNase I sensitive chromatin structure. The results suggest that histone acetylation, principally of H4, and nonhistone proteins, such as HMG-T1, HMG-T2, and HMG-T3, might be involved in maintaining the DNase I sensitive conformation of chromatin.
PART C - EFFECT OF SODIUM N-BUTYRATE ON HISTONE ACETYLATION
Sodium butyrate in millimolar concentrations has been reported to have a wide variety of morphological and physiological effects on mammalian cells grown in tissue culture. Furthermore, it appears that most of the observed changes are readily reversible once this short chain fatty acid has been removed from the cell culture medium. (For a recent review of this subject, see Prasad and Sinha (231)).

Of all the reported effects of butyrate on cultured cells, perhaps the most interesting have been those linking low concentrations of this fatty acid to the induction of Friend erythroleukemic cells, causing these cells to differentiate into nondividing, hemoglobin-synthesizing orthochromatophilic normoblast-like cells (232). During this butyrate induction, the histones, particularly H3 and H4, become highly acetylated (175) in parallel with a decrease in the rate of DNA replication (233). The reasons why butyrate causes induction of cellular differentiation are unknown. Since, however, a considerable body of experimental evidence has suggested that acetylation of histones may have an important role in either the control of chromatin transcriptional activity (234) or chromatin assembly (32), it was of considerable interest to elucidate the biochemical mechanisms underlying these effects of butyrate.

The following set of experiments was done in collaboration with Dr. Reeves who maintained the cell lines (except the hamster BHK-21 cell line which was maintained by Dr. Richardson), treated the cell lines with butyrate
(except the hamster BHK-21 cell line) and labelled the tissue cultures and with Dr. E. P. M. Candido who isolated the histones from all the cell lines except the hamster BHK-21 cell line. Both Dr. R. Reeves and Dr. E. P. M. Candido performed the assays of histone acetylase and deacetylase activity associated with erythroleukemic cells.

I. Effects of Butyrate on Erythroleukemic Cell Histones

Figure 53 shows the profiles of histones extracted from Friend erythroleukemic cells (subline FrC18 obtained from clone 745A) and separated on acid-urea gels by electrophoresis. It is evident from this photograph that in erythroleukemic cells that have been exposed to 5 mM butyrate for 24 h (gel slot B), the histones, particularly H3 and H4, have become very highly acetylated compared with the histones from control cells not exposed to butyrate (gel slot A). The mono-, di- and triacetylated species of H4 are particularly noticeable in this photograph, but it can also be seen that H3 has increased its population of acetylated species relative to the control cells. This point is more clearly evident in the optical scans shown in Figure 54. In contrast to the increased acetylation of H3 and H4, H2A and H2B do not seem to exhibit increased levels of acetylation in butyrate-treated Friend cells. These findings are in agreement with those reported by Ingram's laboratory (175, 233).

In addition, two further points can be made from Figures 53 and 54. The first is that in erythroleukemic cells exposed for 24 h or more to 2% dimethylsulfoxide (DMSO), a known inducer of erythroid
FIG. 53. Histone patterns of Friend erythroleukemic cells.

(A) Electrophoretic pattern of histones from control, uninduced cells;
(B) Histone pattern from cells induced with butyrate (5 mM for 24 h);
(C) Histone pattern from cells induced with DMSO (2% for 96 h).
Chromatin and histone preparation were as described in "Materials and Methods" (Part C Section III). Electrophoresis was carried out in 15% acid-urea gels (194). The gel was stained with Coomassie blue.
FIG. 54. Optical scans of histones on acid-urea gels.

(A) Histones from uninduced Friend erythroleukemic cells; (B) histones from Friend cells induced with butyrate (5 mM for 24 h); (C) histones from trout testis cells for comparison. The positions of H4 and H3 are indicated. Peak 1 is unacetylated H4, and peaks 2 to 4 represent mono-, di- and triacetylated H4, respectively. The gels were stained with Coomassie blue and scanned at 550 nm in a Gilford spectrophotometer.
differentiation for these cells (198), no increase in the amount of histone acetylation is observed (Fig. 53, gel C) relative to untreated control cells. The second point to be made is evident from the gel scan of trout testis histones shown in Figure 54C. When the scan is compared with that of histones isolated from butyrate-treated Friend cells shown above it in Figure 54B, it is rather striking that the amount of acetylation found in trout testis H3 and H4 histones is analogous to that found in the same histones from butyrate-treated cells. The possible significance of this observation is treated further in the Discussion.

II. Effects of Butyrate on Other Cell Lines

To investigate the effect of butyrate treatment on histone acetylation in other culture cell types, a number of permanent vertebrate cell lines with radically different morphologies and growth characteristics were studied. The cell lines that were tested for response to butyrate treatment were: 3T3 cells, a strongly contact-inhibited cell line of embryonic mouse fibroblast origin (199); BHK-21 cells, a fibroblast-like cell line that is partially contact-inhibited, derived from the culture of kidney cells from baby Syrian hamsters (200); IRC8 cells, a lymphoid leukemia cell line obtained from Fisher rats (line 334) that can grow either in suspension culture or as ascites tumors when injected into isologous hosts (201); and X58 cells, partially contact-inhibited epitheloid cells obtained from wild-type embryos of the amphibian *Xenopus laevis* (202). Figures
55 and 56 show the results of experiments in which histones isolated from untreated control cultures of these cell lines are compared with histones isolated from parallel cultures treated for about 24 h with 5 mM sodium butyrate. From the photograph of the stained histones separated by electrophoresis on acid-urea gels shown in Figure 55, it is apparent that all of the tissue culture cell lines tested, regardless of their morphology or growth characteristics, responded to butyrate treatment by accumulating increased levels of acetylated histones H3 and H4. It should be noted, however, that the amount of butyrate-induced histone acetylation varied from cell line to cell line. Thus, the embryonic amphibian cell line from Xenopus (X58) showed the lowest response to butyrate with respect to histone acetylation, whereas the rat cell line IRC8 not only extensively acetylated histones H3 and H4, but also appeared to have acetylated histones H2A and H2B (Figure 55, gels 3 and 4; Figure 56, second panel). A quantitative comparison with untreated control cell histones reveals that after 24 h at 5 mM butyrate treatment, a maximum of 20 - 25% of histone H4 is left unacetylated in the treated IRC8 cells, the remaining 75 - 80% of the H4 being in the mono-, di-, tri- and perhaps tetraacetylated forms. A similar amount of histone H4 is converted to acetylated forms in butyrate-treated Friend erythroleukemic cells (Figure 53, slot B; Figure 54B; (175)).

These results confirm and extend those obtained with human cancer cells (Hela cells; 175) and with chick embryo fibroblast cells (233) and indicate that the induction of histone acetylation by butyrate
Cells (see "Materials and Methods" (Part C)) were exposed to 5 mM butyrate in culture for 24 h. Chromatin was prepared and histones were extracted as described in "Materials and Methods". All samples were analyzed on a single acid-urea 15% acrylamide slab gel (194). (C) Control cells grown in the absence of butyrate; (BA) cells treated with 5 mM butyrate for 24 h; (X58) Xenopus laevis embryonic cells; (IRC8) rat ascites cells; (3T3) mouse fibroblasts; (BHK) baby hamster kidney cells.
FIG. 56. Optical scans of histones from various cell types analyzed on acid-urea acrylamide gels.

Abbreviations are as in the legend to Figure 55. For each pair, the top scan is of histones from control cells. Arrows indicate acetylated components which are increased in amount in cells exposed to 5 mM butyrate for 24 h. (lower scan). The gels were stained with Coomassie blue and scanned at 550 nm in a Gilford spectrophotometer.
treatment of tissue culture cells is a general phenomenon. It is not dependent upon the degree of contact inhibition shown by the cells, the rate of growth or the growth characteristics of the cells, nor is it a function of the vertebrate class from which the cells are derived.

Given the ubiquity and conservation of the core structure of the nucleosome and the generality of acetylation of the core histones H2A, H2B, H3 and H4 (32, 227, 234), it seems probable that the mode of action of butyrate on histone acetylation is similar in all vertebrate cell types affected.

III. Histone Acetylase Activity in Trout Testis Cell Suspensions and in Erythroleukemic Cell Lysates

To examine the effect of butyrate on histone acetylase activity, the rates of incorporation of radioactive acetate into histones in either trout testis cell suspensions, whole erythroleukemic cell homogenates or isolated erythroleukemic cell nuclei were measured. Trout testis cells were labelled with $[^{14}C] \text{acetate}$ in the presence or absence of 1 mM butyrate. After various labelling times, the histones from the control and butyrate-treated cells were isolated and counted for radioactivity. Examination of the results shown in Figure 57 suggests both the control and butyrate-treated trout testis cells incorporated the $[^{14}C] \text{acetate}$ label into histone at equivalent rates.

In other experiments, either control erythroleukemic cells or cells treated for 24 h previously with 5 mM butyrate were homo-
FIG. 57. Histone acetylase activity in suspensions of trout testis cells.

Cell suspensions from either control cells ( ● - ●) or butyrate-treated cells ( ○ - ○) were assayed using $[^{14}C]$ acetate as substrate as described in "Materials and Methods" (See Part C Section V).
genized in an appropriate buffer solution, and $[^3H]$-acetyl-CoA was added to the disrupted cells. After various labelling periods, the histones were isolated from these mixtures and counted for radioactivity. The results of one such experiment are shown in Figure 58. From this graph, it is apparent that both control cells and butyrate-treated cells acetyluate histones at the same rates when they are disrupted and supplied with radioactive acetyl-CoA. Similar results were obtained for the incorporation of acetate into isolated nuclei from these two cell populations (data not shown).

Thus, butyrate does not activate histone acetylases. The same conclusion was reached by Hagopian et al. (233) from studies of in vivo rates of acetate incorporation.

IV. Histone Deacetylase Activity in Cell Lysates

Given the results of the above experiments, the possibility that butyrate in millimolar concentrations might inhibit the activity of histone deacetylases in crude extracts of erythroleukemic cells was examined. The experiments involved the addition of $[^3H]$-acetate labelled histones (obtained from in vivo labelled erythroleukemic cells) to crude lysates of either control cells or butyrate-induced cells, and monitoring the release of $[^3H]$-acetic acid after a period of incubation at 22°C. The results of such an experiment are shown in Figure 59. Crude cell lysates from either control cells or cells previously grown in 5 mM butyrate is present in the lysates. This
FIG. 58. Histone acetylase activity in cell-free extracts of Friend erythroleukemic cells.

Cell lysates from either control cells (● - ●) or butyrate-induced cells (○---○) were assayed using [3H]-acetyl-CoA as substrate (see "Materials and Methods" Part C Section V).
Cell lysates from either control or butyrate-induced cells were incubated with 50,000 cpm of $[^3\text{H}]$ acetate, in vivo labelled histone. Reaction volumes were 140 μl, and incubation was for 4 h at 22°C. $[^3\text{H}]$ acetic acid release was measured by ethylacetate extraction, as described in "Materials and Methods". (a and d) Boiled lysates of control and induced cells, respectively; (b and e) total deacetylase activity in lysates of control and induced cells, respectively; (c and f) deacetylase activity in the presence of 5 mM butyrate for control and induced cell lysates, respectively.

FIG. 59. Histone deacetylase activity in cell-free extracts of Friend erythroleukemic cells.
clearly indicates that growth of erythroleukemic cells in butyrate does not result in the loss of deacetylating enzymes. Rather, the butyrate must somehow inhibit the existing enzymes; this is confirmed by the finding that 5 mM butyrate added to lysates of either control or butyrate-grown cells greatly inhibits histone deacetylation. This is also shown in Figure 59. The extent of inhibition of histone deacetylation by butyrate is virtually identical in the two lysates: — that is, 82% inhibition for control cells and 83% for butyrate-grown cells.

DISCUSSION

The experiments that have been described demonstrate that millimolar concentrations of butyrate inhibit histone deacetylase activities in a reversible manner, and that it is probably this inhibition which leads to hyperacetylation of histones in vivo during butyrate treatment of vertebrate cells in culture. The reversible nature of this inhibition agrees with the findings of other investigators that the effects of butyrate on histone acetylation are reversible in vivo (185, 233).

Several recent reports agree with the conclusion that butyrate exerts its effects by inhibition of the histone deacetylase(s) (176, 177, 185). In addition, Cousens et al. (185) have recently demonstrated that butyrate is a noncompetitive inhibitor of histone deacetylase, and the authors suggest that butyrate may be acting as a tight binding detergent in inhibiting the deacetylase(s).
The only other reported situation in which butyrate apparently acts to inhibit enzyme activities directly is in the case of liver cells, where low concentrations of this fatty acid inhibit the glucose-phosphorylating enzymes, glucokinase and hexokinase, when added to cell homogenates (231). In this case, it is not known whether the effect on the enzymes is reversible, although given the general reversibility of most butyrate effects on cultured cells (231), this seems to be probable. Thus, the findings reported here for the reversible inhibition of deacetylase activity by butyrate may be indicative of a much larger class of reversible enzyme inhibitions brought about in cultured vertebrate cells by low concentrations of this fatty acid.

Butyrate causes an accumulation of acetylated histones in the chromatin of all vertebrate cell types examined, but the extent of this effect varies with different cell types. Large increases in acetylated H3 and H4 occur in Friend erythroleukemic cells, 3T3 cells and IRC8 cells; moderate increases are observed in BHK cells, and the lowest response was observed in Xenopus X58 cells. The rat lymphoid cell line IRC8 gave the most dramatic overall response, in which acetylated species probably corresponding to H2A and H2B also accumulated. Whether these individual responses to butyrate are related to the types of histone metabolism normally occurring in these cell types is unknown. It is interesting to note that the histones of control IRC8 cells show a prominent band in the
position of triacetylated H4, as seen in Figure 56. It is tempting to speculate that this may be related to the extensive hyperacetylation in these cells in response to butyrate. If histone acetylation within a nucleosome core particle were cooperative: —that is, if modification of H4 to the triacetyl level promoted acetylation of H2A and H2B — this might account for the observed results.

The differential effect of butyrate on deacetylation indicates that there is no simple feedback control on histone acetylation: — that is, an increase in the level of acetylated histones does not lead to a decrease in the activity of acetylating enzymes. It is probable that the two processes are controlled independently and are coupled to other key nuclear events.

Hagopian et al. (233) have found that inhibition of DNA synthesis and the accumulation of acetylated H4 follow similar time courses in butyrate-treated Hela cells, and they have suggested that the two processes may be linked. Whereas inhibition of DNA synthesis may be a necessary condition for the accumulation of acetylated histones, however, it clearly is not sufficient. since the inhibition of DNA synthesis by other means has very little effect on histone acetylation (227). The converse, however, may be true:— that is, histone acetylation may lead to decreased DNA synthesis, as suggested by Hagopian et al. (233). This possibility is more difficult to test since levels of histone acetylation are not readily manipulatable other than by butyrate. It is interesting to note that Zlantanova and Swetly (240) have reported that the histones from erythroleukemic mouse
spleen cells (Friend cells) treated with n-butyrate are synthesized at a time when DNA synthesis is blocked, indicating that the synthesis of histones has been uncoupled from that of DNA in the treated cells. Also in this context, it is interesting to note that trout testis histones normally contain high levels of acetylated histones as seen in Figure 54. The predominant cell types in this tissue have generation times of 3 - 7 days (10) and therefore may spend a shorter proportion of their cycle in S phase. Thus the higher levels of histone acetylation may correspond with the long periods during which DNA synthesis is quiescent in these cells.

Histone acetylation is a complex process, and at least two different phases can be distinguished: acetylation of de novo synthesized histones and acetylation of "old" or preformed histones. Louie and Dixon (10) showed that newly-synthesized H4 is rapidly acetylated in cells from developing trout testis, and Ruiz-Carillo et al. (234) and Jackson et al. (227) have shown that newly-synthesized histones are rapidly acetylated and deacetylated in duck erythroblasts and HTC cells, respectively. These results argue for a role of histone acetylation and deacetylation in the assembly of new chromatin. On the other hand, trout spermatid cells, which are completely inactive in DNA synthesis, acetylate histones at significant rates at a time when the protamine replacement process is occurring (11); mature avian erythrocytes, also inactive in DNA synthesis, similarly carry out extensive histone acetylation (235), and a "late" phase of H4 acetylation, involving preformed molecules occurs in duck erythroblasts (234). Thus, histone acetylation may also be needed for post-S phase events in the
cell cycle which require changes in nucleosome conformation, nucleosome sliding or histone removal. The availability of newly synthesized and preformed histones has been shown to be different, with the newly synthesized histones being more available to the histone acetyltransferase(s) (177). In addition, the accessibility of the preformed histones to the histone acetyltransferase(s) differs markedly, probably, depending on the nucleosome environment in which it is located (185). At this time, it is not known if butyrate affects deacetylation of both de novo synthesized and preformed histones or only one of these classes. In view of the magnitude of the butyrate response and of the in vitro deacetylase results, however, it seems probable that both processes are affected.

In summary, the results reported here indicate that butyrate treatment of a wide variety of vertebrate tissue culture cells leads to a direct inhibition of histone deacetylase enzyme activity and that this effect is readily reversible after removal of the fatty acid. This reversible effect of butyrate on the deacetylase enzymes occurs both in vivo and in vitro, and should therefore provide a useful tool for the further investigation of the roles that histone acetylation may have in nuclear metabolism and chromatin structure.
CONCLUDING REMARKS

The nuclease sensitive regions of chromatin may be either newly replicated regions (148) or transcriptionally active regions (120, 133, 202). Seale (148) has reported that newly synthesized DNA was digested at a greater rate than the bulk of the DNA in Hela cells. Alternatively, transcriptionally active regions are excised preferentially by nucleases since recent reports (139, 121, 122) demonstrate that active genes are cleaved into mononucleosomes more rapidly than is bulk chromatin by micrococcal nuclease or DNase II.

In the present study, experiments have been described that suggest micrococcal nuclease selectively excises a population of mononucleosomes containing increased levels of newly synthesized histones. The possibility that micrococcal nuclease selectively excises nucleosomes containing newly synthesized DNA from chromatin was difficult to determine, since the preferential micrococcal nuclease digestion of A/T rich regions may have produced mononucleosomes containing greater amounts of thymidine residues per DNA fragment length than that of polynucleosomes. Ideally, the experiment should be repeated by labelling trout testis cells for a brief period with $^{3}$H thymidine. As nucleosomes associated with nuclease sensitive regions of chromatin can be selectively solubilized by extracting micrococcal nuclease digested nuclei with low concentrations of NaCl (0.1 M or 0.2 M), the specific activity ($^{3}$H cpm/A$_{260}$) of the low salt extracted fraction may be compared to the high salt (0.4 and 0.6 M NaCl) extracted fraction which represents nucleosomes associated with the bulk of chromatin (190). Thus, the nuclease sensitivity of newly replicated chromatin regions
may be examined.

Both micrococcal nuclease and deoxyribonuclease II have been used successively to fractionate chromatin into transcriptionally active and inactive regions. (142, 122, 139). Both enzymes preferentially cleave active regions by digestion between nucleosomal core particles.

In the present study, the nuclei were digested with micrococcal nuclease followed by the following manipulations:

1. sequential extraction of the nuclei with increasing concentrations of NaCl (0.1, 0.2, 0.4 and 0.6 M NaCl), or
2. lysis of the nuclei with EDTA followed by the addition of NaCl (final concentration, 0.1 M) to the solubilized digest products.

The results indicate that the 0.1 M NaCl-soluble nucleosome subfraction contains increased levels of highly acetylated H4 and that the nucleosomes are associated with nuclease sensitive regions of chromatin. Furthermore, the results suggest that the internucleosomal linker DNA of the nuclease sensitive regions of chromatin may contain increased levels of HMG-T1, HMG-T2 and HMG-T3 and possibly lesser amounts of other nonhistone proteins.

Levy W. and Dixon (142) have reported that the 0.1 M NaCl soluble nucleosome subfraction obtained from micrococcal nuclease digested trout testis nuclei was enriched 10-fold for DNA sequences complementary to polyadenylated RNA. Recently, Levy et al. (174) have reported also that nucleosomes associated with nuclease sensitive regions of chromatin contain high levels of the acetylated H4 species. Thus, the results from the
present study are in agreement with those from Levy et al. (174) and support the general idea that nucleosomes associated with transcriptionally competent chromatin contain highly acetylated H4.

By digesting chromatin with deoxyribonuclease II followed by fractionation of the products with 2 mM Mg\(^{+2}\), it has been shown that highly acetylated H4 is associated with a transcriptionally active chromatin fraction. The results suggest that the 0.1 M NaCl-soluble nucleosome subfraction obtained from micrococcal nuclease digested nuclei is probably the same fraction as obtained from DNase II digested chromatin. It is interesting to note that the percentage of the total DNA remaining soluble after deoxyribonuclease II digestion and 2 mM Mg\(^{+2}\) fractionation of chromatin (approximately 7%) corresponds to the percentage of total DNA associated with the 0.1 M NaCl-soluble fraction obtained from micrococcal nuclease digested nuclei.

Deoxyribonuclease I, an enzyme which digests both intra- and internucleosomal DNA, selectively destroys active genes. Weintraub and Groudine (133) have suggested that the nucleosomes associated with transcriptionally competent chromatin regions are in an altered conformation. Results from the present study suggest that both histone acetylation and nonhistone chromosomal proteins may be involved in the maintenance of the altered structure.

DNase I digested nuclei were extracted with sequentially increasing concentrations of NaCl (0.1, 0.2, 0.4 and 0.6 M). The results indicate that low salt elution (0.1 or 0.2 M NaCl) of DNase I digested nuclei releases
a population of nucleosomes containing increased levels of highly acetylated H4 and, perhaps, decreased levels of acetylated H3 species. In addition, the internucleosomal linker DNA of nuclease sensitive regions of chromatin may contain HMG-T1, HMG-T2 and HMG-T3 and many other nonhistone chromosomal proteins. The results suggest that the low salt eluted nucleosome subfraction is associated with chromatin that is in a transcriptionally competent, deoxyribonuclease I sensitive state.

Therefore, the accumulated results suggest that nucleosomes containing highly acetylated H4 are associated with transcriptionally competent chromatin regions. Furthermore, the internucleosomal linker DNA associated with these regions seems to contain little, if any, histone H1 and high levels of HMG-T1, HMG-T2, HMG-T3 and other nonhistone chromosomal proteins. The bulk of chromatin, which is transcriptionally inactive, is associated with nucleosomes containing low levels of acetylated H4 (i.e. unacetylated and monoacetylated H4 are the major species), and the internucleosomal linker DNA is associated mainly with histone H1. The transcriptionally inactive nucleosomal fraction is also distinguished by the presence of two nonhistone chromosomal proteins designated as 28K and 29K.

Unlike the 0.1 NaCl solubilized nucleosome subfraction eluted from micrococcal nuclease or DNase I digested nuclei, the 2 mM Mg$^{2+}$ -soluble fraction obtained from DNase II digested chromatin contained histone H1 and the unidentified protein designated 29K which are both normally associated with transcriptionally inactive chromatin. One
explanation for the above result may be inefficiency of the extraction procedure used. The extraction procedure involved ethanol precipitation of an acid extract which, in some instances, selectively precipitated the lysine-rich histones.

Since the discovery of Riggs et al. (175) that the addition of low levels of the short chain fatty acid, sodium n-butyrate, to Hela or Friend erythroleukaemia cells results in hyperacetylation of histones H3 and H4, sodium n-butyrate treatment of cells has been a useful tool for further investigation of the roles that histone acetylation may have in chromatin structure. Concurrently with other laboratories (176, 177, 185), it was found that sodium n-butyrate is an inhibitor of histone deacetylase(s) and that it is due to the inhibition of this enzyme(s) that the acetylated H3 and H4 species accumulate. By using butyrate to elevate the levels of acetylated histones, Simpson (178), Mathis et al. (179) and Vidali et al. (182) have reported that chromatin containing hyperacetylated histones is digested by DNase I at a greater rate than chromatin containing normal levels of the acetylated histones. Also, although the rate of micrococcal nuclease digestion of nuclei from sodium n-butyrate treated Hela cells was identical to the rate of digestion of nuclei from untreated cells (178, 179), the nucleosomes containing the highest levels of acetylated histones were excised from the treated chromatin preferentially (178).

Shewmaker et al. (181) have reported that treatment of chromatin with acetyl adenylate results in chemical hyperacetylation of the histones. Calf thymus chromatin treated in such a manner had an increased sensitivity to
DNase I relative to that of untreated chromatin. The preferential DNase I digestion of chromatin regions containing naturally-occurring hyperacetylated histones has also been demonstrated (180-183). Thus, the consensus of the above reports is that histone acetylation might convert chromatin into a transcriptionally active, deoxyribonuclease I sensitive state.

Recent experiments by Reeves and Cserjesi (184) have demonstrated that sodium n-butyrate treatment of Friend erythroleukemic cells leads to the synthesis of new RNA transcripts and accompanying synthesis of new nuclear and cytoplasmic proteins. These results provide strong evidence that histone acetylation is probably a part of the required biochemical mechanisms for the expression of a gene.

In agreement with the above recent reports, the present results indicate that highly acetylated H4 is involved in maintaining a region of chromatin in a transcriptionally competent form. The role of H4 acetylation in transcriptionally competent chromatin may be to reduce interactions between core particles as suggested by Whitlock and Stein (77), Simpson (178) and Pospelov et al. (236) and thus render the extended chromatin region accessible to RNA polymerases. Conversely, unacetylated H4 may be involved in maintaining higher levels of compaction of the chromatin.

It would be interesting to further examine the levels of chromatin compaction for regions associated with either high or low levels of the acetylated H4 species. Altenburger et al. (115) have reported that
deoxyribonuclease II may recognize different conformation states of chromatin. DNase II digestion of chromatin leads to a cleavage pattern with a 200-nucleotide pair periodicity. However, if chromatin was digested in the presence of 150 mM NaCl or 1 mM CaCl$_2$, a 100-nucleotide pair repeat pattern appeared. The latter ionic conditions are known to contract chromatin. Thus, if chromatin containing highly acetylated H4 is in an extended form, the low salt extracted fraction (0.1 M NaCl) eluted from DNase II digested nuclei containing highly acetylated H4 should present the 200-nucleotide pair repeat pattern, while the high salt extracted fraction (0.4 M NaCl) containing low levels of the acetylated H4 species should present the 100-nucleotide pair repeat pattern.

In addition to the present results which suggest that the nucleases DNase I, DNase II and micrococcal nuclease recognize the extended form of chromatin containing highly acetylated H4, Gross and Simpkins (237) have reported that there is an altered accessibility of histone H4 to enzymatically - or chemically - catalyzed iodination of chromatin. If iodination of chromatin is catalyzed through the use of the large molecular weight enzyme (80,000 M.W.)$_2$ lactoperoxidase, the mono- and diacetylated forms of H4 are predominantly labelled. However, if the iodination reaction is catalyzed by chloramine T, the mono-, di-, and unacetylated H4 species are labelled to approximately the same extent. Thus, the acetylated species of H4 are possibly associated with chromatin that exists in an extended form.

Through the aid of histone acetylation, the transcriptionally
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competent chromatin that is in an extended state would be accessible to RNA polymerases, regulators and nuclease. DNase II and micrococcal nuclease would digest internucleosomal DNA, and this would release nucleosomes containing the transcriptionally competent DNA sequences. However, DNase I would digest both inter- and intranucleosomal DNA, and this would result in the selective destruction of transcriptionally competent gene sequences: that is, DNase I would digest the DNA to such an extent that the digested DNA products would be unable to hybridize to a specific cDNA probe.

Acetyl groups are incorporated into both newly synthesized histones (10, 238) and pre-existing, "old" histones. Louie and Dixon (10) have suggested that the role of H4 acetylation in assembly may be to reduce the positive charge density in the N-terminal portion of the molecule and allow proper histone-DNA binding. Woodland (238) has reported that although H4 is acetylated before assembly, H3 is not acetylated before assembly. Thus, H3 and H4 do not behave in a similar manner with regard to their pattern of acetylation before assembly.

The nuclease sensitive fractions of chromatin which have been shown in these studies to contain elevated levels of acetylated H4 could also include newly replicated chromatin. This is a possibility because newly replicated chromatin as well as transcriptionally competent chromatin has been found to be nuclease sensitive (148). Thus, histone acetylation may also be involved in chromatin replication: that is, the extended form of chromatin maintained by histone acetylation would be accessible to
DNA polymerases.

It is puzzling that the highly acetylated H4 associated with extended chromatin undergoes acetylation at a slow rate: that is, the extended chromatin form should be highly accessible to histone acetyltransferases and deacetylases. Recently, Reeves and Candido (23) have presented interesting evidence of a deacetylase inhibitor associated with DNase I sensitive chromatin. The association of such an inhibitor with transcriptionally competent chromatin would explain the slow rate of acetyl group turnover, and the inhibition of the deacetylases, perhaps one specific for H4, would lead to a localized region of chromatin containing hyperacetylated H4 molecules.

Although histone acetylation is implicated in inducing and maintaining a transcriptionally functional state in chromatin, it is doubtful that this reaction alone is involved in these processes. Non-histone proteins have also been suggested as regulators of genetic activity. HMG-1, HMG-2, HMG-14 and HMG-17 in mammals and HMG-T in trout are suggested to be associated with transcriptionally active regions of chromatin (137, 161, 162). HMG-T1, HMG-T2, HMG-T3 and other nonhistone proteins (H6, ubiquitin (167)) in conjunction with histone may play an important role in maintaining a region of trout testis chromatin in an extended form, a structure probably necessary for the functioning of an actively transcribing gene.
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