Release of Histone Lysine Methyltransferases from Rat Brain Chromatin by Nuclease Digestion

Dale V. Onisk

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Release of Histone Lysine Methyltransferases From Rat Brain Chromatin by Nuclease Digestion

Dale V. Onisk, M.S.
The University of North Dakota, 1983

Faculty Advisor: Professor John A. Duerre

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The enzymes were extracted by limited digestion (12-16%) of chromosomal DNA from rapidly proliferating rat brain chromatin with micrococcal nuclease. The enzymes were further purified by gel filtration, ammonium sulfate fractionation and DEAE-cellulose chromatography. The histone methyltransferases were resolved into two distinct fractions by Sepharose 6B-100 and DEAE-cellulose chromatography. One enzyme fractionated by DEAE-cellulose chromatography was specific for histone H3, while the other enzyme was specific for histone H4.

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1.9:1.0:0.08.

The histone H4 lysine methyltransferase which was detectible in the crude nuclease digest, was extremely labile loosing all activity upon further purification. The enzyme specific for histone H4 after DEAE-cellulose chromatography methylated only arginyl residues in histone H4 and would not methylate lysyl residues in histone H4.

The pH optimum for histone H3 lysine methyltransferase with soluble rat brain histone H3 as substrate was 8.5 with little variation from pH 8.2 to 8.7. The pH optimum for histone H4 arginine methyltransferase with soluble histone H4 as the methyl acceptor was 7.5 with little variation from pH 7.3 to 7.8.

After DEAE-cellulose chromatography both enzymes were extremely unstable. Complete removal of DNA by DNAase I digestion resulted in the complete loss of enzyme activity. However, when the enzymes remained associated with DNA fragments they were quite stable. Indicating that the enzymes require DNA for stability and/or activity. The requirement for DNA may only be important to prevent hydrophobic interactions involving the enzyme with itself and/or other non-histone chromosomal proteins.
RELEASE OF HISTONE LYSINE METHYLTRANSFERASES FROM RAT BRAIN CHROMATIN BY NUCLEASE DIGESTION

by

Dale V. Onisk

Bachelor of Science, South Dakota State University, 1981

A Thesis
Submitted to the Graduate Faculty
of the
University of North Dakota
in partial fulfillment of the requirements
for the degree of
Master of Science

Grand Forks, North Dakota

August
1983
This thesis submitted by Dale V. Onisk in partial fulfillment of the requirements for the Degree of Masters of Science from the University of North Dakota is hereby approved by the Faculty Advisory Committee under whom the work has been done.

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This thesis meets the standards for appearance and conforms to the style and format requirements of the Graduate School of the University of North Dakota, and is hereby approved.

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Signature Dale V. Olson

Date 7/31/83
**TABLE OF CONTENTS**

**LIST OF TABLES**................................................................. v
**LIST OF ILLUSTRATIONS**..................................................... vi
**ACKNOWLEDGEMENTS**........................................................... vii
**ABSTRACT**........................................................................... ix
**INTRODUCTION**.................................................................... 1
**HISTORICAL REVIEW**............................................................ 3
**MATERIALS AND METHODS**................................................... 12
- Chemicals.............................................................................. 12
- Preparation of Rat Brain Histones H1, H2A, H2B, H3 and H4.................................................. 13
- Preparation of Chromatin....................................................... 16
- Preparation of DNA Containing Methyltransferases......................................................... 19
- Purification of the Histone Lysine Methyltransferases...................................................... 20
- Enzyme Assay.......................................................................... 22
- Acrylamide Gel Electrophoresis.................................................. 23
- Molecular Weight Determination........................................................................... 26
- Basic Amino Acid Analysis.............................................................................. 26
- Determination of Protein Concentration...................................................................... 29
- Determination of DNA Concentration................................................................... 30

**RESULTS**.............................................................................. 37
- Properties of the Histone Methyltransferases.................................................................. 50
- Effects of Polyamines and Cations............................................................................ 63
- Effects of Puromycin on Histone Methylation 
  In Vivo.................................................................................... 75

**DISCUSSION**.......................................................................... 78

**SUMMARY**............................................................................ 83

**APPENDICES**........................................................................ 85

**APPENDIX I.** Solutions.......................................................... 86

**APPENDIX II.** Key to Abbreviations........................................ 88

**LITERATURE CITED**............................................................. 91
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Cellular Distribution of the Histone Lysine Methyltransferases</td>
<td>38</td>
</tr>
<tr>
<td>2.</td>
<td>Activity of Rat Brain Histone Methyltransferases Against Soluble Rat Brain Histones as Substrates</td>
<td>57</td>
</tr>
<tr>
<td>3.</td>
<td>Distribution of $[^3H]$-Methyl Groups in Basic Amino Acid Residues from Soluble and Chromosomal Bound Histones Incubated with Partially Purified Enzyme</td>
<td>58</td>
</tr>
<tr>
<td>4.</td>
<td>Distribution of $[^3H]$-Methyl Groups in Basic Amino Acid Residues from Soluble and Chromosomal Bound Histones Incubated with Crude Enzyme</td>
<td>60</td>
</tr>
<tr>
<td>5.</td>
<td>Effects of Polyamines, NaCl and MgCl$_2$, on the Extent of In Vitro Incorporation of $[^3H]$-Methyl Groups into Mono- and Dimethyllysine in Chromosomal Bound Histone H4</td>
<td>71</td>
</tr>
<tr>
<td>6.</td>
<td>Effect of Polyamines, NaCl and MgCl$_2$, on the Rate of In Vitro Methylation of Soluble Histones</td>
<td>72</td>
</tr>
<tr>
<td>7.</td>
<td>Effect of Polyamines, NaCl and MgCl$_2$, on the Rate of In Vitro Methylation of Chromosomal Bound Histones</td>
<td>73</td>
</tr>
<tr>
<td>8.</td>
<td>Effects of Polyamines, NaCl and MgCl$_2$, on the Extent of In Vitro Incorporation of $[^3H]$-Methyl Groups into Mono-, Di- and Trimethyllysine in Chromosomal Bound Histone H3</td>
<td>74</td>
</tr>
<tr>
<td>9.</td>
<td>Key to Abbreviations</td>
<td>89</td>
</tr>
</tbody>
</table>
# LIST OF ILLUSTRATIONS

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Diagram Showing how Histones May Align Within the Nucleosome</td>
<td>6</td>
</tr>
<tr>
<td>2.</td>
<td>Elution Profile of Total Rat Brain Histones on Bio-Gel P-10 and Elution Profile of Rat Brain Histones H2A-H4 on Bio-Gel P-10</td>
<td>18</td>
</tr>
<tr>
<td>3.</td>
<td>Elution Profile of Standard Molecular Weight Markers on Bio-Gel A-1.5 M</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Elution Profile of Standard Amino Acid Samples on Beckman PA-35</td>
<td>32</td>
</tr>
<tr>
<td>5.</td>
<td>Reference Curve for the Determination of Protein Concentration</td>
<td>34</td>
</tr>
<tr>
<td>6.</td>
<td>Reference Curve for the Determination of DNA Concentration</td>
<td>36</td>
</tr>
<tr>
<td>7.</td>
<td>Micrococcal Nuclease Digestion of Chromatin from 4-6-Day Old Rat Brains</td>
<td>40</td>
</tr>
<tr>
<td>8.</td>
<td>Fractionation of Nucleosomes by Gel Filtration on Sepharose 4B-200</td>
<td>42</td>
</tr>
<tr>
<td>9.</td>
<td>Fractionation of the Histone Lysine Methyltransferase(s) by Gel Filtration on Sepharose 6B-100</td>
<td>44</td>
</tr>
<tr>
<td>10.</td>
<td>Fractionation of the Histone Lysine Methyltransferase(s) by Gel Filtration on Sepharose 6B-100</td>
<td>47</td>
</tr>
<tr>
<td>11.</td>
<td>Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis of Proteins Under the Methylase Peak from Sepharose 6B-100 Chromatography</td>
<td>49</td>
</tr>
<tr>
<td>12.</td>
<td>Fractionation of the Histone Lysine Methyltransferase(s) (Nuclease Extracted) on DEAE-Cellulose</td>
<td>52</td>
</tr>
<tr>
<td>13.</td>
<td>Fractionation of the Histone Lysine Methyltransferase(s) (NaCl Extracted) on DEAE-Cellulose</td>
<td>54</td>
</tr>
<tr>
<td>14.</td>
<td>Polyacrylamide Gel Electrophoresis of Histone H3 and H4 Methyltransferases Following DEAE-Cellulose Chromatography</td>
<td>56</td>
</tr>
<tr>
<td>15.</td>
<td>Methylation of Free and Bound Histones by Chromosomal Bound Histone Methyltransferases</td>
<td>62</td>
</tr>
</tbody>
</table>
16. Effect of Calf Thymus DNA on Histone Lysine Methyltransferase Activity................................. 65

17. Activity of Histone Methyltransferase(s) as a Function of pH............................................... 67

18. Time-Course of Incorporation of $[^3H]$-Methyl Groups into Chromosomal Bound Histones H3 and H4 in the Absence and Presence of 1.5 mM Spermidine or 0.1 M NaCl........................................ 70

19. Effects of Puromycin on Histone Synthesis in Isolated Rat Livers......................................... 77
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ABSTRACT

The histone lysine methyltransferases catalyze the transfer of methyl groups from S-adenosyl-L-methionine to specific ε-N-lysyl residues in the N-terminal regions of histones H3 and H4. These enzymes are located exclusively within the nucleus and are firmly bound to chromatin. The chromosomal bound enzymes do not methylate free or loosely bound histones. However, histones H3 and H4 associated within the nucleosomes are methylated.

The enzymes were extracted by limited digestion (12-16%) of chromosomal DNA from rapidly proliferating rat brain chromatin with micrococcal nuclease. The enzymes were further purified by gel filtration, ammonium sulfate fractionation and DEAE-cellulose chromatography. The histone methyltransferases were resolved into two distinct fractions by Sepharose 6B-100 and DEAE-cellulose chromatography. One enzyme fractionated by DEAE-cellulose chromatography was specific for histone H3, while the other enzyme was specific for histone H4.

Histone H3 lysine methyltransferase was shown to methylate only the lysyl residues of chromosomal bound or soluble histone H3. The molar ratio of mono- to di- to trimethyllysine in the soluble system was 1.0:2.1:1.0, while the ratio with chromosomal bound histone H3 was 1.9:1.0:0.08.

The histone H4 lysine methyltransferase which was detectible in the crude nuclease digest, was extremely labile loosing all activity upon further purification. The enzyme specific for histone H4 after DEAE-cellulose chromatography methylated only arginyl residues in histone H4 and would not methylate lysyl residues in histone H4.
The pH optimum for histone H3 lysine methyltransferase with soluble rat brain histone H3 as substrate was 8.5 with little variation from pH 8.2 to 8.7. The pH optimum for histone H4 arginine methyltransferase with soluble histone H4 as the methyl acceptor was 7.5 with little variation from pH 7.3 to 7.8.

After DEAE-cellulose chromatography both enzymes were extremely unstable. Complete removal of DNA by DNAase I digestion resulted in the complete loss of enzyme activity. However, when the enzymes remained associated with DNA fragments they were quite stable. Indicating that the enzymes require DNA for stability and/or activity.

The requirement for DNA may only be important to prevent hydrophobic interactions involving the enzyme with itself and/or other non-histone chromosomal proteins.
INTRODUCTION

Our knowledge of eucaryotic chromatin has increased markedly over the past few years. The model of chromatin as "beads on a string" first proposed by Olins and Olins (1) has been greatly expanded. The nucleosome (bead) consists of a flat particle in which the DNA is wrapped around a histone core. Brief micrococcal nuclease digestion of rat liver chromatin yields particles (200 base pairs) containing one molecule of histone H1 and two molecules each of the other four histones, H2A, H2B, H3 and H4 (2). Exhaustive digestion leads to a stable limit particle (146 base pairs) containing all the histones except H1. Since histones H2A and H2B are quite resistant to trypsin digestion they appear to be completely buried in the nucleosome. In contrast the N-terminal regions (20-30 amino acid residues) of histone H3 and H4 are susceptible to trypsin digestion (3). The N-terminal regions of these histones contain the methylated lysyl residues.

Histone H4 from mammalian systems contains a single methylated lysyl residue at position 20, while histone H3 contains methylated lysyl residues at positions 9 and 27 (4). Both sites on histone H3 contain ε-N-mono, ε-N-di and ε-N-trimethyllysine, while ε-N-trimethyllysine is absent from histone H4. Histone H4 from mammalian species contain both methylated and unmethylated residues at position 20, while this site is unmethylated in peas (5,6). In the rat, methylation of histones H3 and H4 does not vary from organ to organ (7). In histone H3 the molar ratio of mono- to di- to trimethyllysine is 0.55:1.0:0.35. In histone H4 the ratio of mono- to dimethyllysine is about 0.1:0.9.
The histone lysine methyltransferases catalyze the transfer of methyl groups from S-adenosyl-L-methionine to specific ε-N-lysyl residues in the flexible N-terminal regions of histones H3 and H4. These enzymes are located exclusively within the nuclei and are firmly bound to chromatin (8).

It is the purpose of this investigation to isolate and characterize the histone lysine methyltransferase(s) from rapidly proliferating rat brain chromatin. The isolation and purification of the histone methyltransferase(s) will help further the understanding of chromatin structure and the biochemical significance of such post-transcriptional modifications.
HISTORICAL REVIEW

It is now well established that eucaryotic chromatin is made up of two parts the nucleosomal particle and the linker region (Beads on a string). The nucleosomal particle consists of 145 base pairs wound around a histone octomer, made up of equimolar amounts of histones H2A, H2B, H3 and H4. The length of the linker region depends on the species and varies between 15 and 100 base pairs (9). One molecule of histone H1 and some 20 to 200 non-histone chromosomal proteins are associated with the linker region.

Various models have been proposed for the alignment of the core histones along the major and minor grooves of the DNA (10-14). One common feature of these models is that the N-terminal residues of the core histones are flexible and can bind electrostatically to DNA. Weintraub et al. (3) have shown that histones H2A and H2B are quite resistant to trypsin digestion. In contrast the N-terminal regions (20-30 amino acids) of histones H3 and H4 are susceptible to trypsin. The N-terminal regions of these histones contain the methylated lysyl residues. Recent studies suggest that the histones are laterally arranged along the minor groove, while the non-histones are located in the major groove (15). Trypsin digestion suggests that histone H1 binds to residues 35-120 and may be involved in a nucleosome "seal" (16,17). The same region (residues 74-106) may be cross linked to the globular region of histone H2A (18). Histone H1 also may be cross linked to histone H3 (19). If the histones are laterally aligned along the minor groove (14,15) then the histones H3 and H2A should lie close to the "seal" while histones H2B and H4 should lie on the other side of
the coil (Fig. 1).

Histones are perhaps the most conserved molecules in nature. However, it has become clear that there is a significant amount of variability (20). Histone H1 is by far the most variable of the five types. The number of subfractions varies from tissue to tissue in a given species. The subfractions appear to be almost species specific in its sequences (21). Histone H1 contains a number of highly conserved regions. One such region of about 75 base pairs has a sequence typical of globular proteins (20).

Very little is known about the variability of the structures of histone H2A. In rat chloroleukemia cells there appear to be three molecular species of histone H2A (22). One species accounts for about 60% of histone H2A; it has a serine residue at position 16 and a lysine residue at position 99. The other two species contain an arginine residue at position 99 and differ from one another at position 16. One type contains a threonine residue whereas the other has a serine residue at position 16. Tryptic and thermolytic peptide maps of calf thymus histone H2A resolved it into two distinct types (23). The types vary with the serine-threonine substitution at position 16 and a methionine-leucine substitution at position 51.

In histone H2B two-thirds of the carboxyl terminal region is conserved, while the amino acid terminal region is not (20). Calf thymus H2B has been resolved into three variants (23). The three variants differ in their amino acid sequence at positions 75 and 76. Histone H2B.1 contains the amino acids glycine-glutamic acid, H2B.2 contains serine-glutamic acid and histone H2B.3 contains glycine-glutamic respectively.
Figure 1: Diagram showing how histones may align within the nucleosome.
Histone H3 from calf thymus also contains three variants. H3.1 and H3.2 are identical except the latter contains a serine residue at position 96 in contrast to a cysteine residue in the other (24). This also has been shown to occur in carp and chicken histone H3 (25, 26). Histone H3.3 contains the above substitution of serine for cysteine at position 96 and it also contains the residues isoleucine-glycine at positions 89-90 instead of valine-methionine as in H3.1 and H3.2 (23). Glycine is considered to be a "helix-breaker" at position 90. Pea histone H3 also contains a "helix-breaker" (serine) at position 90 (27). The reason for this alteration is not understood. Pea histone H3 contains three other structural differences as compared to calf thymus (24).

Except for histone H2B.2 which has been found only in the mouse, all the above variants of histones H2A, H2B and H3 have been found in all tissues of mammals examined, although in different relative amounts (28). Due to the limited amount of knowledge the reasons for such changes only can be speculative.

Histone H4 is the most highly conserved of all the histone molecules. Calf thymus histone H4 varies from pea by two conservative substitutions, isoleucine for valine at residue 60 and arginine for lysine at residue 77 (5,29,30).

Although histones H3 and H4 are highly conserved, in their amino acid sequences, they have been found to be extensively modified. Five types of postsynthetic modifications are known to occur to the histones: acetylation, phosphorylation, methylation, poly(ADP)ribosylation and the formation of protein A24. It has been found that modifications in the form of phosphorylated serine, histidine, lysine and threonine
residues, acetylated serine and lysine residues, methylated lysine and arginine residues and the presence of ADP-ribosyl groups all exert specific effects on the histones and their surrounding environment. The presence of protein A24 seems to be common. It has been estimated one in ten nucleosomes is altered in this way (20).

The methylation of histones appears to be a very specific event. The first identification of an ε-N-methyllysine in histones was observed by Murray in 1964 (31). In the following years an ε-N-dimethyllysine derivative was found in histones (32), as was an ε-N-trimethyllysine derivative (33). It has been established that of the five major histones, the methylation of amino acids occurs mainly in the arginine-rich histones H3 and H4. Histone H3 from calf thymus and fish contains methylated lysine residues at position 9 and 27 (4,26, 29,34). A minor site at lysine residue 4 in histone H3 is methylated in developing trout testis (34). Histone H4 from the above sources contains only one methylated lysine residue at position 20 (29,35). Both sites of histone H3 contain ε-N-mono-, ε-N-di, and ε-N-trimethyllysine, while the trimethyl derivative is not found in histone H4. The methylation sites and the amino acid sequences of histone H3 and H4 are highly conserved (4,5,24,26,34-38). It has been shown that methylation of a particular lysyl residue need not occur. In the pea, histone H4 is unmethylated and trimethyllysine is absent from histone H3 (5,6).

The methylation of histones is relatively a late event in cellular division occurring after DNA synthesis (39-41). Studies on the time course of histone methylation during hepatic regeneration in the rat indicated that the rate of histone methylation reaches a peak at
about 30 hours, whereas the rate of DNA synthesis reaches a peak at about 24 hours (40). Similar results were obtained using thymidine-synchronized HeLa S-3 cells (42). Methylation occurred mainly from the late S-phase through the G2-phase. It was determined that the peak of methylation occurred about 3 hours after DNA synthesis.

Investigators on the kinetics of histone methylation in Ehrlich ascites tumor cells revealed that methylation of histones commenced just after their biosynthesis (43). The methylation of histone H4 proceeded slower than histone H3. Thomas et al. (43) found that monomethylation of histone H3 begins in the S-phase, whereas dimethylation of histone H4 appears to occur mainly in the G2-phase. All the methylation sites in histones H3 and H4 are fully methylated after one generation. The ratio of mono- to di- to trimethyllysine in histone H3 is about 1:3:1, while histone H4 contained mostly dimethyllysine. The methylation rates of histones H3 and H4 in tumor cells do not differ significantly (8,44). Bryvoet et al. (45) demonstrated that methylation of histones H3 and H4 in Chinese hamster ovary cells occurs preferentially in the S-phase. They found that histones H3 and H4 in the newly assembled nucleosomes are undermethylated.

In the rat, methylation of histones H3 and H4 does not vary from organ to organ (7). In histone H3 the molar ratio of mono- to di- to trimethyllysine is 0.55:1.0:0.35. In histone H4 the ratio of mono- to dimethyllysine is 0.1:0.9. In rat brain nuclei, methylation has been found to proceed step-wise from mono- to di- to trimethyllysine in histone H3 and from mono- to dimethyllysine in histone H4 (44).

Rat brain histones H3 and H4 do not turnover in the absence of cell division, nor does the \( \epsilon \)-N-methyl groups turnover independently
of the polypeptide chain (46,47). Similar results were obtained with
Novikoff hepatomas cells (47), Ehrlich ascites tumor cells (48),
Chinese hamster ovary cells (49) and developing trout testis (50).
However, Hempel et al. (51) reported methyl groups on lysyl residues
in histones H3 and H4 turnover independently of the polypeptide
chains from nonproliferating adult cat kidney cells. Paik and Kim
(52,53) have reported that a histone demethylase is present in rat
kidney and liver cells.

The enzyme catalyzing the transfer of methyl groups from S-
adenosyl-L-methionine to the ε-N-lysine residues of histones H3 and H4
was first found in calf thymus nuclei in 1970 (54). Paik and Kim
called the enzyme "protein methylase III". The Enzyme Commission has
since given it the name S-adenosyl-methionine: protein lysine methyl-
transferase (EC 2.1.1.25). The enzyme has been found in a number of
tissues including frog liver, rat brain, liver, testis and kidney,
Tetrahymena, calf lymphocytes and thymus, and Krebs 2 ascites cells
(8,41,54-57).

Early methods of extraction of the enzyme included the use of
high salt concentrations, large volumes of water and solubilization
from acetone powders. The enzymes prepared by any of the above methods
are extremely unstable. In 1977 Wallwork et al. (8) isolated the
histone lysine methyltransferase from young rat brain chromatin by
repeated water extractions. They determined that the enzyme was bound
to chromatin. With the use of soluble histones H3 and H4 as sub-
strates, the pH optima was 8.2 to 8.7 and 7.2 to 8.0, respectively.
The Km values of the enzyme for S-adenosyl-L-methionine are 11.5 ± 1.1
μM and 12.5 ± 1.3 μM with histones H3 and H4 as methyl acceptors,
of the polypeptide chain (46, 47). Similar results were obtained with Novikoff hepatomas cells (47), Ehrlich ascites tumor cells (48), Chinese hamster ovary cells (49) and developing trout testis (50). However, Hempel et al. (51) reported methyl groups on lysyl residues in histones H3 and H4 turnover independently of the polypeptide chains from nonproliferating adult cat kidney cells. Paik and Kim (52, 53) have reported that a histone demethylase is present in rat kidney and liver cells.

The enzyme catalyzing the transfer of methyl groups from S-adenosyl-L-methionine to the e-N-lysine residues of histones H3 and H4 was first found in calf thymus nuclei in 1970 (54). Paik and Kim called the enzyme "protein methylase III". The Enzyme Commission has since given it the name S-adenosyl-methionine: protein lysine methyltransferase (EC 2.1.1.25). The enzyme has been found in a number of tissues including frog liver, rat brain, liver, testis and kidney, Tetrahymena, calf lymphocytes and thymus, and Krebs 2 ascites cells (8, 41, 54-57).

Early methods of extraction of the enzyme included the use of high salt concentrations, large volumes of water and solubilization from acetone powders. The enzymes prepared by any of the above methods are extremely unstable. In 1977 Duerre et al. (8) isolated the histone lysine methyltransferase from young rat brain chromatin by repeated water extractions. They determined that the enzyme was bound to chromatin. With the use of soluble histones H3 and H4 as substrates, the pH optima was 8.2 to 8.7 and 7.2 to 8.0, respectively. The Km values of the enzyme for S-adenosyl-L-methionine are 11.5 ± 1.1 μM and 12.5 ± 1.3 μM with histones H3 and H4 as methyl acceptors,
respectively (44). S-Adenosyl-L-homocysteine, one of the products of the reaction, is a competitive inhibitor with respect to S-adenosyl-L-

methionine. The $K_i$ values for S-adenosyl-L-homocysteine are $5.5 \pm 0.4$ 

$\mu$M and $5.9 \pm 0.5$ $\mu$M with histones H3 and H4 as methyl acceptors, respectively.
MATERIALS AND METHODS

Chemicals

S-Adenosyl-L-[\textsuperscript{3}H-methyl]methionine (7.0 Ci/mmol) was obtained from ICN Chemical and Radioisotopes Division, Irvin, California. It was diluted to 1.0 Ci/mmol with unlabeled S-adenosyl-L-methionine prepared by the method of Schlenk and De Palma (58). The unlabeled S-adenosyl-L-methionine was a gift from Dr. J. Duerre, University of North Dakota, Grand Forks, North Dakota. Ammonium sulfate (ultrapure) was obtained from Schwarz/Mann, Inc., Spring Valley, New York. Triton X-100, 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyl-oxazoly)-benzene (POPOP) were obtained from Research Products International Corporation, Mount Prospect, Illinois. Deoxyribonuclease was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Sodium dodecyl sulfate, Bio-Gel P-10, Bio-Gel A-0.5 M, Bio-Gel A-1.5 M and Cellex D (diethylaminoethyl (DEAE)-cellulose) were obtained from Bio-Rad Laboratories, Richmond, California. Bacto-brom phenol blue was obtained from DIFCO Laboratories, Inc., Detroit, Michigan. Acrylamide, N-tris(hydroxymethyl)methyl glycine (Tricene), Napthol Blue Black, N,N\textsuperscript{'} methylene-bis-acrylamide, N,N,N\textsuperscript{'}N\textsuperscript{''}-tetramethylenediamine (TEMED), 2-mercaptoethanol, blue-dextran, phenylmethyl sulfonyl fluoride, dithiothreitol, sodium ethyleneglycol-bis(\textsuperscript{8}-aminoethyl ether)N,N\textsuperscript{'}-tetraacetic acid (EGTA), deoxyribonucleic acid (calf thymus), bovine serum albumin, catalase (bovine liver), ribonuclease-A (bovine pancreas), pepsin (hog stomach mucosa), micrococcal nuclease, deoxyribonuclease I, Type 1 (bovine pancreas), deoxyribonuclease II, Type 4 (porcine spleen), and Sepharose 6B-100 were
obtained from Sigma Chemical Company, St. Louis, Missouri.

All other general chemicals were purchased from Curtin Matheson Scientific, Inc., Minneapolis, Minnesota or J.T. Baker Chemical Company, Phillipsburg, New Jersey.

Preparation of Rat Brain Histones H1, H2A, H2B, H3 and H4

Rat brain histones were prepared as outlined by Duerre and Chakrabarty (7). Nuclei from 4-6 day-old rat brains were lysed in 10 mM potassium phosphate buffer (pH 7.5)-1.0 mM dithiothreitol (1.0 mg DNA/ml). Chromatin was recovered by centrifugation a 10,000 X g for 10 minutes and washed twice with the same buffer. The chromatin was washed with excess Folsch (chloroform: methanol (2:1, v/v) and filtered on a course sintered glass filter with suction. The chromatin was washed twice with methanol, twice with acetone and twice with acetic acid, pH 3.0. The histones were extracted with 0.4 M HCl for 10 minutes with intermittent stirring, followed by centrifugation at 27,000 X g for 10 min. The supernatant fluid containing the histones was decanted and the pellet extracted with 0.4 M HCl. The histones were pooled and precipitated by the addition of 7 volumes of acetone. After standing overnight the supernatant fluid was decanted and the pellet washed once with acetone. The histones were recovered by centrifugation at 10,000 X g for 10 min, air dried and dissolved in a minimal volume of 10 mM HCl.

The histone fractions were isolated using the procedure of Johns as modified by Oliver _et al._ (59). Total histones (500 mg) were diluted to 1.0 mM HCl with distilled water. Concentrated perchloric acid (HClO4) was added dropwise with shaking to make the final solution 0.5 M. The solution was allowed to stand for 15 min, followed by
centrifugation at 12,000 X g for 5 min. The supernatant fluid containing histone H1 was decanted. The precipitate containing histones H2A, H2B, H3 and H4 was retained. To the supernatant fluid, 3.0 M \( \text{H}_2\text{SO}_4 \) was added until a concentration of 0.2 M was reached. Histone H1 was precipitated by the addition of 6 volumes of acetone and storage overnight at -14°C. The precipitate was collected by centrifugation at 12,000 X g for 10 min and washed once with acetone. The histones were air dried and dissolved in a minimal volume of 10 mM HCl.

Histone H2B was fractionated from the initial precipitate as follows: To remove any additional H1 the precipitate was dissolved in 1.0 mM HCl. Concentrated HCIO\(_4\) was added to a final concentration of 0.5 M and allowed to stand for 15 min. The precipitate was collected by centrifugation at 12,000 X g for 5 min and the supernatant fluid was discarded. This procedure was repeated 3 times. The precipitate was washed with 50 ml of 0.5 M HCIO\(_4\) and centrifuged at 12,000 X g for 10 min. The supernatant fluid was discarded and the precipitate extracted with 60 ml of ethanol-HCl, (a solution containing 10 ml concentrated HCl, 90 ml of water and 400 ml of anhydrous ethanol). After homogenizing with a Teflon-glass homogenizer, the solution was allowed to stand for 10 min. The extract was centrifuged at 12,000 X g for 10 min and the supernatant fluid containing histones H2A, H3 and H4 was saved. The precipitated H2B was dissolved in 1.0 mM HCl and treated with 0.5 M HCIO\(_4\) as described above. The precipitate was extracted an additional 5 times with decreasing amounts of ethanol-HCl. The histone H2B precipitate was washed with acetone, centrifuged at 12,000 X g for 10 min and dried in vacuo.
Only the supernatant fluids, containing histones H2A, H3 and H4 from the first three H2B extracts were pooled. Prior to any further extractions, remaining traces of histone H2B were removed as follows: 3.0 M H$_2$SO$_4$ was added to the pooled histones until a final concentration of 0.2 M was reached. The histones were precipitated with 4 volumes of acetone, allowed to stand overnight at 4°C, collected by centrifugation at 12,000 X g for 5 min, washed once with acetone and air dried. The histones were extracted once in a small volume of ethanol-HCl as described above. The precipitate was discarded.

Histones H2A, H3 and H4 were precipitated from the supernatant fluid by the addition of H$_2$SO$_4$-acetone as described above, except the precipitate was dried in vacuo. Prior to the next step drying was essential, otherwise the presence of acetone gives an artificially high 230 nm reading.

Histone H3 was extracted from the precipitate as follows: The precipitate was dissolved in 10-20 ml of ethanol-HCl. The absorbance at 230 nm of the solution was recorded and adjusted by the addition of ethanol-HCl to a reading of 6 (1.8 mg/ml). The solution was dialysed against 7 volumes of anhydrous ethanol for 5 h with vigorous stirring. The contents of the dialysis bag were removed and centrifuged at 12,000 X g for 5 min. The precipitate (histone H3) was washed once with the supernatant fluid and centrifuged at 12,000 X g for 5 min. Histone H3 was washed with acetone and centrifuged at 12,000 X g for 5 min. Histone H3 was washed an additional 3 times with acetone and air dried. The supernatant fluid containing histones H2A and H4 was dialysed overnight against 7 volumes of 95% ethanol. Any precipitate formed was discarded. Histones H2A and H4 were precipitated.
with the addition of \( \text{H}_2\text{SO}_4 \)-acetone as described above, washed once with acetone and air dried. The histones were dissolved in 10 mM HCl and the protein concentration determined.

Histones H2A and H4 were fractionated on Bio-Gel P-10 columns as follows:

Bio-Gel P-10 (equal amounts of 100-200 and 300-400 mesh) was swollen overnight in distilled water. A column (1.6 cm x 3.5 m) was packed and equilibrated with 500 ml of 10 mM HCl. To remove any extraneous proteins, 2.0 ml of 6.0 M urea was applied to the column and the proteins eluted with 10 mM HCl. A sample of histones H2A and H4 (10-15 mg) in 1.0 ml of 10 mM HCl was applied to the column and the proteins eluted with the same solvent at a flow rate of 5.2 ml/h. Fractions (6.5 ml) were collected with the aid of a fraction collector. The elution profile of proteins was determined by measuring the absorbance at 230 nm (Fig. 2). Fractions under the peaks were pooled and the protein recovered by freeze-drying.

The other histone fractions (H1, H2B and H3) were also purified employing Bio-Gel P-10 chromatography. After freeze-drying, the histones were dissolved in a minimal amount of 10 mM HCl. The purity of each histone fraction was determined using sodium dodecyl sulfate acrylamide gel electrophoresis.

**Preparation of Chromatin**

Long-Evans rats were killed by decapitation at 4-6 days of age. Their brains were removed and placed in 0.32 M sucrose-1.0 mM MgCl\(_2\) at 4°C. The tissue was weighed and divided into 20 g aliquots. Each aliquot of tissue was placed in 200 ml of 0.32 M sucrose-1.0 mM MgCl\(_2\) and homogenized employing a Teflon-glass type homogenizer for 10
Figure 2: A) Elution profile of total rat brain histones on Bio-Gel P-10 column (1.6 cm x 3.5 m). Total rat brain histones (15-18 mg) were applied to the column, eluted with 10 mM HCl at a flow rate of 5.2 ml/h. Fractions (6.5 ml) were collected and followed by reading the effluent at 230 nm. B) Elution profile of rat brain histones H2A-H4 on Bio-Gel P-10 column (1.6 cm x 3.5 m). Rat brain histones H2A-H4 (10-15 mg) were applied to the column and eluted as described above.
strokes. The homogenate was filtered through 4 layers of cheese cloth as rapidly as possible to avoid loss of nuclei. Nuclei were sedimented by centrifugation at 800 X g for 10 min in a horizontal type centrifuge. The supernatant fluid was removed with the aid of an aspirator and discarded. The crude nuclei were placed in 0.32 M sucrose-1.0 mM MgCl₂ (1 ml sucrose-MgCl₂/original g tissue) and homogenized 3-4 strokes. To the suspended nuclei 2.5 M sucrose-1.0 mM MgCl₂ was added to give a final concentration of 1.63 M sucrose (1 volume 0.32 M sucrose/1.5 volumes 2.5 M sucrose). After mixing, the nuclei were purified by centrifugation at 80,000 X g for 60 min. The supernatant fluid was decanted and the sides of the tubes were wiped clean. The nuclei from several tubes were pooled and lysed with 10 mM potassium phosphate buffer, (pH 7.5)-1.0 mM dithiothreitol (1 ml buffer/original g tissue). The chromatin was recovered by centrifugation at 27,000 X g for 15 min. The supernatant fluid was decanted and the precipitate washed with one half volume of the same buffer. The chromatin was sedimented as above and washed with one fourth volume of the same buffer. The chromatin was sedimented as above and stored at -90°C until used.

Preparation of the DNA Containing Methyltransferases

Rat brain chromatin containing about 100 mg DNA was suspended in 30 ml of 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride-1.0 mM MgCl₂ by homogenization (4-5 strokes) using a loose fitting Teflon-glass type homogenizer. The DNA concentration was estimated spectrophotometrically. Samples (0.2 ml) of chromatin were solublized in 19.8 ml of 5 M urea-2 M NaCl by homogenization (4-5 strokes). The samples were read against a reagent
blank at 260 nm (A value of 24 O.D. units/mg DNA was obtained by measuring DNA concentration by using diphenylamine reagent and comparing it to spectrophotometric values.).

Limited digestion of the chromatin with micrococcal nuclease was carried out as follows: The reaction mixture contained chromatin (3.0 mg DNA/ml), 50 mM Tricine-HCl buffer, (pH 7.5), 1.0 mM dithiothreitol, 1.0 mM MgCl₂ and 0.5 mM phenylmethyl sulfonyl fluoride. The reaction mixture was equilibrated to 37°C in a water bath prior to the addition of CaCl₂ (2.0 mM). Micrococcal nuclease was added with stirring (1.2/µg nuclease/mg DNA) and the reaction mixture was incubated for 30 min at 37°C with intermittent stirring.

The reaction was terminated with the addition of sodium ethylene-glycol-bis(β-aminoethyl ether)N,N'-tetraacetic acid, pH 7.14, to a concentration of 10 mM followed by cooling to 4°C in an ice-water bath. The nucleosomes and protein aggregates were sedimented by centrifugation at 80,000 X g for 60 min. The supernatant fluid containing the DNA-methylase complex was decanted, and stored at 4°C.

Purification of the Histone Lysine Methyltransferases

The DNA-methylase fragments were concentrated with an Amicon Diaflo Apparatus using an YM-10 filter, to approximately 20 mg protein/ml. The crude enzymes were fractionated by gel filtration employing Sepharose 6B-100 chromatography as follows: Sepharose 6B-100 having an exclusion limit of 4 x 10⁶ daltons was suspended in distilled water. A siliconized column (1.7 x 170 cm) was packed with resin and equilibrated with 500 ml of 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride. The crude enzyme (60-90 mg protein) was applied to the column and eluted with
50 mM Tricene (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylethyl sulfonyl fluoride. Fractions (6.8 ml) were collected with the aid of a fraction collector. The elution profile was followed spectrophotometrically at 260 nm and 280 nm. Aliquots (0.1 ml) of the effluent were assayed for methylase activity using soluble rat brain histones as substrate.

Fractions containing methylase activity were pooled. Ammonium sulfate was added to the pooled fractions to effect 20% saturation (0.143 g ammonium sulfate/ml). The fraction was stirred for 20 min in a ice-water bath. The fraction was transferred to tubes and centrifuged at 28,000 X g for 15 min. The supernatant fluid was decanted and ammonium sulfate added to effect 45% saturation (0.107 g ammonium sulfate/ml). The solution was stirred for 20 min in an ice-water bath and centrifuged as above.

The precipitates from each of the above centrifugation steps was diluted in a minimal volume of 50 mM Tricene (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylethyl sulfonyl fluoride. Aliquots (0.5 ml) of each fraction were dialyzed overnight against the same buffer with three changes. The fractions were assayed for methylase activity.

The ammonium sulfate fraction 20-45% was dialyzed against 20 volumes of 50 mM Tricene (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylethyl sulfonyl fluoride overnight with three changes. The proteins (16 mg) were applied to a diethylaminoethyl-cellulose (DEAE-cellulose) (1.0 x 20 cm), which had been previously equilibrated with 100 ml of the same buffer. The proteins were eluted with a linear NaCl gradient formed by mixing 100 ml of 50 mM Tricene (pH 7.5)-1.0 mM dithiothreitol-0.6 M NaCl with 100 ml of the same buffer.
minus the NaCl. Fractions were collected with the aid of a fraction collector. The effluent was followed spectrophotometrically by reading fractions at 260 nm and 280 nm. Aliquots (1.0 ml) of the effluent were dialyzed against 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride overnight with one buffer change. This step is essential to remove the NaCl which inhibits the activity of both enzymes (60). The fractions containing methylase activity for histone H3 and histone H4 were pooled separately and ammonium sulfate was added to effect 50% saturation (0.357 g ammonium sulfate/ml). After stirring for 20 min the precipitate was removed by centrifugation at 28,000 X g for 15 min. The supernatant fluid was decanted and the precipitate was dissolved in 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride. The dissolved precipitates were dialyzed against the same buffer overnight with three buffer changes.

**Enzyme Assay**

The activity of the histone lysine methyltransferases was determined utilizing soluble rat brain histones as methyl acceptors as previously described by Wallwork et al. (61). The assay mixtures contained 200 μg soluble rat brain total histones, 0.2 M Tris(hydroxymethyl)aminomethane buffer (pH 8.0), 20 μM S-adenosyl-L-[3H-methyl]methionine (1.0 Ci/mm mol) and enzyme in a final volume of 0.2 ml [when individual histones were assayed as substrates, 0.2 M Tris(hydroxymethyl)aminomethane buffer (pH 8.7) was used with 30 μg rat brain histone H3 and 0.2 M Tricine buffer (pH 7.5) was used with 65 μg rat brain histone H4]. The reaction mixtures were placed in a water bath at 37°C for 5 min and the reaction initiated by the addition of S-adenosyl-L-
[\textsuperscript{3}H-methyl] methionine. At zero time, 0.025 ml of the reaction mixture was removed and placed on chromatography paper (2 cm\textsuperscript{2}, Whatman No. 3MM). The reaction was terminated by immersing the chromatography paper in 200 ml of 1.1 M trichloroacetic acid (TCA) at 70°C. Samples were removed at 3 min intervals. After the final sample paper had been immersed in the hot TCA, the papers were incubated for 20 min at 70°C. The hot TCA was decanted, the papers were immersed in 100 ml of fresh 1.1 M TCA at 70°C and incubated for 5 min at 70°C. The papers were washed three times with 1.1 M TCA, rinsed three times with acetone, dried and placed in 10 ml of Bray's counting fluid (Appendix). Radioactivity was determined using a Packard liquid scintillation spectrometer. Radioactivity associated with endogenous controls was subtracted from the other values before enzyme activity was determined. Activity was expressed as pmol \textsuperscript{3}H-methyl incorporated/mg protein/min at 37°C.

Acrylamide Gel Electrophoresis

Sodium dodecyl sulfate electrophoresis was performed using the method of Laemmli (62). The gels contain 12.9% acrylamide, 0.11% N,N\textquotesingle-methylene-bis-acrylamide, 0.385 M Tris(hydroxymethyl)aminomethane buffer (pH 8.8) and 0.1% sodium dodecyl sulfate (Na Dod S\textsubscript{4}). The gels were polymerized by the addition of 0.034% by volume of N,N,N\textquotesingle,N\textquotesingle-tetramethylenediamine (TEMED) and 0.034% by volume of ammonium persulfate and allowed to stand for 90 min. The sample buffer contained 0.025 M Tris(hydroxymethyl)-aminomethane (pH 6.8), 2.0% Na Dod S\textsubscript{4}, 10% glycerol, 5% 2-mercaptoethanol and 0.001% bromophenol blue. The electrode buffer contained 0.025 M Tris(hydroxymethyl)aminomethane(pH 8.4), 0.192 M glycine and 0.1% sodium dodecyl sulfate. Slab gel electrophoresis was carried out at 10°C with a current of 25 mA until the
tracking dye, bromophenol blue, reached the bottom of the gel. The gels were fixed with 40% ethanol in 5% acetic acid for 60 min prior to staining. The proteins were stained for 90 min at room temperature with 0.1% Coomassie brilliant blue in methanol/acetic acid/water (5:1:5, v/v/v). The gels were destained in methanol/acetic acid/water (1:1.5:17.5, v/v/v).

Gel electrophoresis of histones (acid-urea) was performed using the method of Panyim and Chalkey (63). Three separate solutions (A, B and C) were prepared and when used gave a 12% polyacrylamide gel in 5.0 M urea at a final pH of 3.2. Solution A contained 48% acrylamide (w/v) and 0.4% N,N'-methylene-bis-acrylamide (w/v) in distilled water. Solution B contained 43.2% glacial acetic acid (v/v) and 4% TEMED in distilled water. Solution C contained 0.2% ammonium persulfate (w/v) in 10 M urea, made fresh daily. Solution A and B were stored at 0°C prior to use and were warmed to room temperature before mixing. The ratio of A to B to C was 2:1:5 respectively. The gels were allowed to polymerize for 60-90 min. Electrophoresis was performed at room temperature in a standard vertical gel system, using tubes 0.6 cm in diameter and 12 cm in length. The electrode buffer was 0.9 N acetic acid. All gels were subjected to pre-electrophoresis, for about two hours, to remove charged particles. The histones (20-50 µg) were dissolved in 0.9 N acetic acid. To the dissolved histones an equal volume of the tracking dye was added (0.1% safranin in 7% acetic acid plus 50% sucrose w/v) and the solution applied to the gel.

The system was run at 2 mA/7.5 cm gel, with a voltage range between 80-120 V (depending on the urea concentration). The system was stopped after the tracking dye had traveled the length of the
gels. The gels were removed, fixed and stained as described above.

Standard disc gel electrophoresis of proteins was performed using the method of Davis (64). Three separate solutions (A, B and C) were prepared and when used gave a 7.5% polyacrylamide gel at a final pH of 9.5. Solution A contained 48% 1 M HCl (v/v), 0.46% TEMED (v/v) and 36.3% Tris (hydroxymethyl)aminomethane (w/v) in distilled water. Solution B contained 30% acrylamide (w/v), 0.8% N,N'-methylene-bis-acrylamide (w/v), 0.015% potassium ferricyanide (w/v) in distilled water. Solution C contained 0.14% ammonium persulfate (w/v) in distilled water, made fresh daily. Solution A and B were stored at 4°C prior to use and were warmed to room temperature before mixing.

Solutions A and B and distilled water were mixed to a ratio of 1:2:1 respectively. An equal quantity of the above was mix with solution C and a partial vacuum was pulled on the mixture. The gel mixture was placed into columns 0.6 cm in diameter and 12 cm in length and allowed to polymerize for 60-90 min. Electrophoresis was performed at room temperature in a standard vertical gel system. The electrode buffer contained 50 mM Tris(hydroxymethyl)aminomethane buffer and 0.38 M glycine.

Tracking dye (0.1% safranin in 7% acetic acid and 50% sucrose w/v) was added to an equal volume of the protein solution (10 to 100 μg protein in 8 M urea) and applied to the gels. The system was run at 5 mA/7.5 cm gel. The system was stopped after the tracking dye had traveled the length of the gel. The gels were removed from the glass columns and placed in the fixative-stain solution (1% Naphthol Blue Black in 7% acetic acid). After 1 h the excess dye was removed and the gels destained with 7.5% acetic acid.
Molecular Weight Determination

Bio-Gel A-1.5 M (200-400 mesh) which is capable of separating 10,000-1,500,000 molecular weight proteins was diluted with distilled water. The resin was packed into a siliconized column (1.0 x 170 cm) and equilibrated with 500 ml of 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyle fluoride. Standard proteins (3.0 mg) of known molecular weights were applied to the column and eluted with the same buffer, at a flow rate of 1.6 ml/h. The elution profile was determined by reading fractions of the effluent spectrophotometrically at 280 nm (Fig. 3).

The enzymes from the 20-45% ammonium sulfate fractionation (23 mg protein) were applied to the Bio-Gel A-1.5 M column. The proteins were eluted with the above buffer at a flow rate of 5.7 ml/h. Fractions were collected and the elution profile followed spectrophotometrically by reading the effluent at 260 nm and 280 nm. The effluent fractions were assayed for enzyme activity.

Basic Amino Acid Analysis

Quantitative analysis of the basic amino acids of the histones was performed with the aid of a Technicon automatic amino acid analyzer. The samples were prepared for hydrolysis as follows: Histone H3 and H4 samples were made 6 N with respect to HCl in a 10 ml indented round bottom flask. The samples were shell frozen by rotating the flasks in an alcohol dry-ice bath. The flasks were connected to a vacuum pump and flushed with nitrogen while under vacuum. The samples were thawed and the process repeated three times. The flasks were sealed under vacuum and placed in a oil bath at 110 ± 1°C for 22 hours.

HCl was removed from the histone hydrolysate as follows: Samples
Figure 3: Elution profile of standard molecular weight markers on Bio-Gel A-1.5 M (1.0 x 170 cm). Protein markers (3.0 mg) were applied to the column and eluted with 50 mM Tricine (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride, at a flow rate of 1.6 ml/h.
Effluent, ml

Absorbance (280nm)

Methylase

Blue Dextran
(MW 2,000,000)

Catalase
(MW 250,000)

Bovine Albumin
(MW 65,000)

Deoxyribonuclease
(MW 31,000)

Methyltransferase (units/ml ——)
process repeated three times by the addition and removal of water. The dried hydrolysates were dissolved in 0.3 ml of 0.2 M sodium citrate buffer, pH 2.2. Samples were applied to a column (0.8 x 40 cm) of Beckman PA-35 resin which had previously been equilibrated with 0.35 M sodium citrate buffer, pH 5.82. Amino acids were eluted from the column by 0.35 M sodium citrate buffer, pH 5.82, at 26°C under 250 psi. The effluent from the column was divided with a stream splitter with half of the effluent passing through the auto analyzer, while the other half was collected with the aid of a fraction collector. Ten-minute fractions were collected until lysine started to be eluted, after which five minute fractions were collected and all the effluent diverted to the fraction collector. Five minute fractions were collected through histidine, followed by twenty minute fractions through the elution of arginine.

Scintillation spectrometry was used to determine the distribution of the $^3$H-methyl groups in the basic amino acid residues. This was accomplished by counting 1 ml aliquots of the fractions collected in Bray's counting fluid (Appendix) in a Packard Tri-carb Model scintillation spectrometer.

The elution profile of a standard amino acid sample containing 1.0 µmol arginine, 0.5 µmol each of lysine, monomethyllysine and histidine and 0.2 µmol each of dimethyllysine and trimethyllysine are shown in Fig. 4.

**Determination of Protein**

The method of Lowry et al. (65) was used to determine the protein concentrations. Samples containing 10 to 180 µg of protein were analyzed using a Technicon Auto Analyzer. A standard curve was
prepared using bovine serum albumin and was found to be linear between concentrations of 10 to 180 μg of bovine serum albumin per ml (Fig. 5).

**Determination of DNA**

The method of Burton (66) was used for the determination of DNA. Ten volumes of 1.0 N perchloric acid was added to the chromatin. The chromatin was hydrolysed at 70°C for 20 min, the hydrolysate was clarified by centrifugation at 755 X g for 20 min. The supernatant fluid (containing the DNA) was decanted and DNA concentration determined with diphenylamine. Samples containing 10 to 100 mg of DNA were made up to 1.0 ml with a final concentration of 0.5 N perchloric acid. Two ml of diaphenylamine reagent (Appendix) were added and the solution mixed. The solutions were incubated at 30°C for 16 h. Following incubation, absorbance was measured at 600 nm in a Beckman 25 spectrophotometer. A standard curve was prepared from calf thymus DNA (Fig. 6).
Figure 4: Elution profile of a standard amino acid sample containing 1.0 μmol arginine, 0.5 μmol each of lysine, histidine and monomethyllysine, and 0.2 μmol each of dimethyllysine and trimethyllysine from a column (0.8 x 40 cm) of Beckman PA-35 resin. The amino acids were eluted with 0.35 M sodium citrate buffer, pH 5.82 at 26°C. The flow rate was 26.0 ml/h. The column effluent was divided, one half passing through a Technicon automatic amino acid analyzer and the remaining half was collected with the aid of a fraction collector (10 min fractions).
Figure 5: Standard reference curve for the determination of protein concentration, using bovine serum albumin.
Absorbance (660nm)

Bovine Serum Albumin (μg)

0 40 80 120 160

0 0.1 0.2 0.3

Diagram showing a linear relationship between absorbance (660nm) and bovine serum albumin (μg).
Figure 6: Standard reference curve for determination of DNA concentration, using calf thymus DNA.
Absorbance (600 nm) vs. Calf Thymus DNA (μg)

0.6
0.5
0.4
0.3
0.2
0.1
0

0 20 40 60 80 100

Calf Thymus DNA (μg)
RESULTS

The histone lysine methyltransferases are bound to chromatin (Table 1). The enzyme(s) can be solubilized by limited digestion of chromatin with micrococcal nuclease (Fig. 7). Maximum solublization of the enzyme(s) occurred after 12-16% digestion. If digestion was continued a decrease in soluble protein occurred with a concurrent loss in methylase activity. These proteins apparently aggregate with the nucleosome particles or with one another. If the aggregates were treated with 0.4 M NaCl, methylase activity can be recovered in the soluble fraction. However, the enzyme(s) dissociated with salt was extremely unstable and removal of the salt by dialysis results in complete loss of activity.

Exhaustive digestion of rat brain chromatin solubilized 45% of the DNA, (Fig. 7) with little or no methylase recoverable in the soluble fraction. Nor was there any significant methylase recovered upon sodium chloride treatment of the particulate fraction. Gel filtration of this fraction on Sepharose 4B-200 revealed a limit digest particle (nucleosome) (Fig. 8). The particle consisted of 30% DNA, equal amounts of histones H2A, H2B, H3 and H4 and traces of non-histones chromosomal proteins, but no histone lysine methyltransferase. The composition of the nucleosomes from rat brain is similar to that reported for liver by Thoma et al. (2).

The soluble fraction from a 12-16% nuclease digest of chromatin was subjected to chromatography on Sepharose 6B-100 (Fig. 9A). The molecular weight of the enzyme(s) was determined to be approximately 2
## TABLE 1

CELLULAR DISTRIBUTION OF THE HISTONE LYSINE METHYLTRANSFERASES

<table>
<thead>
<tr>
<th>Fraction</th>
<th>pmol $[^3]$H-methyl]/mg/min$^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>Cytoplasm$^1$</td>
<td>0</td>
</tr>
<tr>
<td>Mitochondria$^1$</td>
<td>0</td>
</tr>
<tr>
<td>Microsomes$^1$</td>
<td>0</td>
</tr>
<tr>
<td>Nucleoplasm$^1$</td>
<td>0</td>
</tr>
<tr>
<td>Chromatin$^2$</td>
<td>10.5</td>
</tr>
</tbody>
</table>

1 The reaction mixture contained 0.2 M Tris-HCl buffer (pH 8.0), 20 µM S-adenosyl-$[^3]$H-methyl]-methionine, 0.2 mg rat brain total histone and 1-2 mg protein.

2 Assayed against chromosomal bound histones.

3 Endogenous activity has been subtracted out.
Figure 7: Micrococcal nuclease digestion of chromatin from 4-6-day old rat brains. The reaction mixture contained chromatin (3.0 mg DNA/ml), 1.0 mM MgCl₂, 2.0 mM CaCl₂, 0.5 mM phenylmethyl sulfonyl fluoride, 50 mM Tricine-HCl buffer (pH 7.5), 1.0 mM dithiothreitol and varying concentrations of micrococcal nuclease. The reaction mixture was incubated for 30 min at 37°C after which time sodium ethylene-glycol-bis(β-amoioethyl ether)N,N'-tetraacetic acid (pH 7.1) was added to stop the reaction. The nucleosomes and protein aggregates were sedimented by centrifugation at 120,000 X g for 60 min. The supernatant fluid containing the histone lysine methyltransferase-DNA complex was assayed for enzyme activity utilizing soluble rat brain histones as substrates as indicated in 'Materials and Methods'.
Figure 8: Fractionation of nucleosomes by gel filtration. A fraction of the nucleosomal pellet from a 45% nuclease digestion of rat brain chromatin was dissolved in 0.6 M NaCl-1.0 mM sodium ethylene-glycol-bis (β-aminoethyl ether)N,N'-tetraacetic acid-5.0 mM sodium phosphate buffer (pH 7.5)-0.5 mM dithiothreitol-0.1 mM phenylmethylsulfonyl fluoride. After centrifugation at 34,800 X g, the supernatant fluid was loaded upon a Sepharose 4B-200 column (1.2 x 100 cm). The nucleosomes and proteins were eluted with the above buffer at a flow rate of 7.8 ml/h. Fractions were collected with the aid of a fraction collector. Aliquots (1.0 ml) were dialyzed overnight against 50 mM Tricine-HCl buffer (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethylsulfonyl fluoride. Aliquots (0.1 ml) of the dialyzed fractions were assayed for methylase activity using soluble histones as substrates. The assays were carried out as described under "Materials and Methods".
Absorbance

260 nm (---)
280 nm (--)

Nucleosomes

Effluent, ml

Methylase, Units/ml (x--x)
Figure 9: Fractionation of the histone lysine methyltransferase(s) by gel filtration. The crude enzyme fraction (50 mg protein) obtained from 4-6-day old rat brain chromatin with either 0.4 M NaCl (B) or 16% micrococcal nuclease digestion (A) was applied to a Sepharose 6B-100 column (2 x 170 cm) and eluted with 50 mM Tricine-HCl buffer (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride at a flow rate of 11.0 ml/h. Aliquots (0.1 ml) of the effluent were assayed for methylase activity using soluble rat brain total histones as substrate.
A. Nuclease Digest

Absorbance

Methyltransferase, Units/ml

B. NaCl Extract

Absorbance

Methyltransferase, Units/ml

Effluent, ml

260 nm

280 nm
x $10^6$ daltons (Fig. 9A). Similar results were obtained when the enzyme(s) were extract from rat brain chromatin by 0.4 M NaCl (Fig. 9B).

The fractions under the peaks were assayed against soluble histones H3 and H4. The histone H3 lysine methyltransferase eluted earlier than histone H4 methyltransferase (Fig. 10). The fractions under peak 2 contained activity against histone H3, while fractions under peak 3 contained activity against both histones H3 and H4. This indicated that there are two distinct enzymes, one specific for histone H3 and the other specific for histone H4. Both enzyme fractions were found to contain 10-15% DNA.

Electrophoresis on sodium dodecyl sulfate polyacrylamide gels revealed that the fractions contained 20-30 non-histone chromosomal proteins (Fig. 11). The molecular weights of the proteins ranged from about 10,000 to 205,000 daltons, with the majority of the proteins in the range of 30,000 to 100,000 daltons. The exact molecular weight of the DNA fragments has not been determined. However, they are less than 10,000 daltons since they pass through a Amicon YM-10 membrane (exclusion limit 10,000).

From these data we concluded that the histone lysine methyltransferases are associated with protein DNA-complexes.

If the DNA associated with the complex was removed by complete digestion with deoxyribonuclease I all methylase activity was lost. As the digestion proceeded a concurrent increase in protein aggregation was observed. No trace of methylase activity was found following 0.4 M NaCl treatment of the aggregates. No loss of enzyme activity or aggregation of proteins was observed when the fragments were treated with ribonuclease-A. This indicated that the histone lysine methyl-
Figure 10: Gel filtration of the histone lysine methyltransferases, employing Sepharose 6B-100 column chromatography (1.7 x 160 cm). The concentrated (Amicon YM-10) fraction (76 mg enzyme protein) from the micrococcal nuclease digestion was applied to the Sepharose 6B-100 column which had been previously equilibrated with 50 mM Tricine-HCl buffer (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride. The proteins were eluted with the same buffer at a flow rate of 10.2 ml/h.

Fractions were collected with the aid of a fraction collector. Aliquots (0.1 ml) were assayed for the histone lysine methyltransferases using soluble histones H3 and H4 as methyl acceptors. The assays were carried out as described under "Materials and Methods".
Absorbance

260 nm

Effluent, ml

Methyltransferase, units/ml

Histone - H3 Methylase

Histone - H4 Methylase

Absorbance

2.0

1.6

1.2

0.8

0.4

0

100 180 260 340 420

Effluent, ml

Methyltransferase, units/ml

260 nm

280 nm
Figure 11: Sodium dodecyl sulfate polyacrylamide gel electrophoresis of proteins under the methylase peak from Sepharose 6B-100 chromatography. After fractionation of the nuclease solubilized methylase-DNA fraction on Sepharose 6B-100 resin, the proteins under the methylase peak were concentrated with ammonium sulfate (20-45%) and subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described in "Materials and Methods". Lane 1 contains 84 µg enzyme protein and lane 2 contains 112 µg enzyme protein.
• a

\( \text{Myosin \ (205,000)} \)

\( \beta\)-Galactosidase \ (116,000) \)

\( \text{Phosphorylase } \beta \ (97,400) \)

\( \text{Bovine Albumin \ (66,000)} \)

\( \text{Egg Albumin \ (45,000)} \)

\( \text{Carbonic Anhydrase \ (29,000)} \)
transferase(s) requires DNA for activation and/or stability.

If the protein DNA complexes were bound to DEAE-cellulose the non-histone proteins could be selectively eluted with sodium chloride (Fig. 12). Methyltransferase activity was found under two separate peaks. Similar results were obtained when salt extracted methyltransferases were treated as above (Fig. 13).

As determined from polyacrylamide gel electrophoresis the histone H3 lysine methyltransferase fraction (lane 3) consists of two major components and two minor components (Fig. 14), whereas the histone H4 methyltransferase fraction (lane 2) consists of one major component and a number of minor components. We have not established which of the components are the methyltransferase enzymes.

Properties of the Histone Methyltransferases

The substrate specificity of the two enzymes was determined against several possible methyl acceptor proteins. The histone H3 lysine methyltransferase was specific for soluble histone H3, with traces of activity with histone H1 (Table 2). The histone H4 methyltransferase was specific for soluble histone H4, with traces of activity with histones H1 and H2A. No significant activity of either enzyme was obtained with protamine sulfate, gelatin, cytochrome c, poly(L-arginine), poly(L-lysine) or γ-globulin.

When the histone H3 lysine methyltransferase from DEAE-cellulose was incubated with soluble histone H3 only the lysyl residues were methylated. The molar ratio of mono- to di- to trimethyllysine was 1.0:2.1:1.0 (Table 3). This ratio is quite similar to that which was obtained in vitro by Duerre and Chakrabarty (7). When histone H3 lysine methyltransferase was incubated with Folsch treated chromatin
Figure 12: Fractionation of the histone lysine methyltransferase(s) (nuclease extracted) using DEAE-cellulose chromatography. The ammonium sulfate fraction (16 mg) was applied to a column (1 x 20 cm) which had previously been equilibrated with 50 mM Tricine-HCl buffer (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethyl sulfonyl fluoride. The proteins were eluted with a linear NaCl gradient in the above buffer. Fractions were collected with the aid of a fraction collector. Aliquots (1.0 ml) were dialyzed to remove the NaCl and the enzyme activity assayed as outlined in "Materials and Methods", employing soluble rat brain histones H3 and H4 as methyl acceptors.
Figure 13: Fractionation of the histone lysine methyltransferase(s) (0.4 M NaCl extracted) on DEAE-cellulose. The ammonium sulfate fraction (16 mg protein) was applied to a column (1 x 20 cm) and eluted with a linear NaCl gradient. Fractions under the various peaks were dialyzed to remove NaCl and enzyme activity assayed employing soluble rat brain histones H3 and H4.
Figure 14: Polyacrylamide gel electrophoresis of histone H3 and H4 lysine methyltransferases after fractionation on DEAE-cellulose. Electrophoresis was carried out as outlined in "Materials and Methods". Lane 1 contains 20 µg of each indicated standards. Lane 2 contains 92 µg of the histone H4 methyltransferase fraction. Lane 3 contains 56 µg of the histone H3 lysine methyltransferase fraction.
<table>
<thead>
<tr>
<th>Substrates</th>
<th>Concentration (mg/ml)</th>
<th>Activity (pmol[^H-methyl]/mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Histone H3 methyltransferase</td>
</tr>
<tr>
<td>H1</td>
<td>0.20</td>
<td>4</td>
</tr>
<tr>
<td>H2A</td>
<td>0.20</td>
<td>0</td>
</tr>
<tr>
<td>H2B</td>
<td>0.20</td>
<td>0</td>
</tr>
<tr>
<td>H3</td>
<td>0.03</td>
<td>105</td>
</tr>
<tr>
<td>H4</td>
<td>0.07</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>0.20</td>
<td>192</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 7.5 μg of histone H3 lysine methyltransferase or 28.6 μg of histone H4 methyltransferase from a 0-50% ammonium sulfate fractionation of the methyltransferase fractions from the DEAE-cellulose column, 20 μM S-adenosyl-L-[^3H-methyl]methionine, 0.2 M Tris-HCl buffer (pH 8.0) and substrates listed in the table in a final volume of 0.2 ml. The assay was performed as outlined in "Materials and Methods".
The histone H3 lysine methyltransferase (120 units) was incubated for 60 min at 37° C with Folsch treated 4-6-day old rat brain chromatin (12 mg DNA/60 mg chromatin) in the presence of 20 μM S-adenosyl-L-[3H-methyl]methionine and 50 mM Tris-HCl buffer (pH 8.0). The reaction was terminated by the addition of trichloroacetic acid to 1.1 M and chilling to 4° C. The chromatin was subjected to centrifugation at 27,750 X g for 15 min. The precipitate (chromatin) was washed 3 times with 1.1 M trichloroacetic acid and 3 times with distilled water. The histones were extracted and the basic amino acids analyzed as outlined under "Materials and Methods".

The histone H4 methyltransferase (133 units) was incubated with Folsch treated chromatin as outlined above.

The histone H3 lysine methyltransferase (15 units) was incubated for 60 min at 37° C with soluble histone H3 (410 μg) in the presence of 20 μM S-adenosyl-L-[3H-methyl]methionine and 50 mM Tris-HCl buffer (pH 8.5). The reaction was terminated by the addition of trichloroacetic acid to 1.1 M and chilling to 4° C. The mixture was subjected to centrifugation at 27,750 X g for 15 min. The precipitate (histones) was washed 3 times with 1.1 M trichloroacetic acid. The histones were placed in 6 N HCl and the basic amino acids analyzed as outlined under "Materials and Methods".

The histone H4 methyltransferase (16 units) was incubated with soluble histone H4 (415 μg) as outlined above, except 50 mM Tricene-HCl buffer (pH 7.5) was used instead of the Tris-HCl buffer.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>μmol [3H-methyl]/mol histone</th>
<th>Chromatin</th>
<th>Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3¹</td>
<td>H4²</td>
<td>H3³</td>
</tr>
<tr>
<td>ε-N-Monomethyllysine</td>
<td>227</td>
<td>4</td>
<td>750</td>
</tr>
<tr>
<td>ε-N-Dimethyllysine</td>
<td>120</td>
<td>0</td>
<td>1,564</td>
</tr>
<tr>
<td>ε-N-Trimethyllysine</td>
<td>90</td>
<td>0</td>
<td>727</td>
</tr>
<tr>
<td>1-Methyl-histidine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N⁰,N⁰-Dimethyl-arginine</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>N⁰,N¹⁰-Dimethyl-arginine</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

¹The histone H3 lysine methyltransferase (120 units) was incubated for 60 min at 37° C with Folsch treated 4-6-day old rat brain chromatin (12 mg DNA/60 mg chromatin) in the presence of 20 μM S-adenosyl-L-[3H-methyl]methionine and 50 mM Tris-HCl buffer (pH 8.0). The reaction was terminated by the addition of trichloroacetic acid to 1.1 M and chilling to 4° C. The chromatin was subjected to centrifugation at 27,750 X g for 15 min. The precipitate (chromatin) was washed 3 times with 1.1 M trichloroacetic acid and 3 times with distilled water. The histones were extracted and the basic amino acids analyzed as outlined under "Materials and Methods".

²The histone H4 methyltransferase (133 units) was incubated with Folsch treated chromatin as outlined above.

³The histone H3 lysine methyltransferase (15 units) was incubated for 60 min at 37° C with soluble histone H3 (410 μg) in the presence of 20 μM S-adenosyl-L-[3H-methyl]methionine and 50 mM Tris-HCl buffer (pH 8.5). The reaction was terminated by the addition of trichloroacetic acid to 1.1 M and chilling to 4° C. The mixture was subjected to centrifugation at 27,750 X g for 15 min. The precipitate (histones) was washed 3 times with 1.1 M trichloroacetic acid. The histones were placed in 6 N HCl and the basic amino acids analyzed as outlined under "Materials and Methods".

⁴The histone H4 methyltransferase (16 units) was incubated with soluble histone H4 (415 μg) as outlined above, except 50 mM Tricene-HCl buffer (pH 7.5) was used instead of the Tris-HCl buffer.
similar results were obtained, however there was a shift towards the under methylated forms of lysine. The molar ratio of mono- to di- to trimethyllysine was 1.9:1.0:0.75. When Duerre et al. (44) incubated native chromatin with S-adenosyl methionine they also found a similar shift in the molar ratio of mono- to di- to trimethyllysine, 0.93:1.0:0.17.

When the histone H4 methyltransferase from DEAE-cellulose was incubated with soluble histone H4 only $N^G, N'^G$-dimethyl-arginine was obtained (Table 3). It would appear that we have isolated a arginine methyltransferase. When this enzyme was incubated with Folsch treated chromatin no significant incorporation of methyl groups was found in any of the basic amino acid residues.

When the crude enzyme fraction was incubated with soluble histone H4 the arginyl residues were methylated and lysyl methylation also was observed (Table 4). When the crude enzyme fraction was incubated with Folsch treated chromatin only lysyl residues were methylated. This would suggest that the crude enzyme fraction contains two enzymes, one specific for histone H4 lysyl residues and the other specific for the methylation of arginyl residues in soluble histone H4.

The possibility that soluble histones are methylated prior to their incorporation into the nucleosome was investigated. When crude or partially purified histone H3 lysine methyltransferase or histone H4 arginine methyltransferase was incubated with nucleosomes prepared from a limit digest, no methylation of basic amino acid residues was observed. However, when soluble histones were incubated with native chromatin rich in histone methyltransferases no methylation of the soluble histones was observed (Fig. 15, top). It was found that a
TABLE 4

DISTRIBUTION OF [\(^{3}\)H-METHYL] GROUPS IN BASIC AMINO ACID RESIDUES FROM SOLUBLE AND CHROMOSOMAL BOUND HISTONES INCUBATED WITH CRUDE ENZYME

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>μmol [(^{3})H-methyl] groups/mol histone</th>
<th>Chromatin(^1)</th>
<th>Soluble(^2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H3</td>
<td>H4</td>
</tr>
<tr>
<td>(\varepsilon)-N-Monomethyllysine</td>
<td></td>
<td>320</td>
<td>35</td>
</tr>
<tr>
<td>(\varepsilon)-N-Dimethyllysine</td>
<td></td>
<td>160</td>
<td>60</td>
</tr>
<tr>
<td>(\varepsilon)-N-Trimethyllysine</td>
<td></td>
<td>45</td>
<td>0</td>
</tr>
<tr>
<td>1-Methyl-histidine</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(N^G,\text{^{\text{(N))}}})-Dimethyl-arginine</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(N^G,\text{^{\text{(N))}}})-Dimethyl-arginine</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\)The crude histone methyltransferase (11 units) was incubated for 90 min at 37° C with Folsch treated 12-day old rat brain chromatin (7.5 mg DNA) in the presence of 20 μM S-adenosyl-L-[\(^{3}\)H-methyl]-methionine, 0.1 M Tris-HCl buffer (pH 8.0). The reaction was terminated by the addition of S-adenosyl-L-homocysteine to a final concentration of 10 mM and chilling to 0° C. The chromatin was washed three times by repeated centrifugation (27,750 X g) in 20 mM potassium phosphate buffer (pH 7.6). The histones were extracted and the basic amino acids analyzed as outlined under "Materials and Methods".

\(^2\)Soluble histones H3 and H4 (1.5 mg) were incubated for 90 min at 37° C in the presence of 20 μM S-adenosyl-L-[\(^{3}\)H-methyl]methionine, 0.2 M Tris-HCl buffer (pH 8.0) and 90 and 30 units of crude histone methyltransferase, respectively. The reaction was terminated and the histone-enzyme complex dissociated by adjusting the pH to 9.7 and adding urea to a final concentration of 8.0 M. The histones were purified on DEAE-cellulose as outlined by Duerre and Gaitonde (67). They were hydrolyzed and the basic amino acids analyzed as outlined under "Materials and Methods".
Figure 15: Methylation of free and bound histones by chromosomal bound histone methyltransferases. Newborn Long-Evans rat pups were given 5.0 μCi [\(^{3}\text{H}\text{]lysine/g body weight by intraperitoneal injection. On day 11 an additional 5.0 μCi [\(^{3}\text{H}\text{]lysine/g body weight was administered by the same route. The animals were killed by decapitation on day 14 and the histones extracted as outlined in "Materials and Methods".}

Native rat brain chromatin (12 mg DNA) from 6 to 8-day old rat pups was incubated with 4.2 mg of the [\(^{3}\text{H-lysyl}\)] histones, 20 μM S-adenosyl-L-[\(^{14}\text{C-methyl}\)]methionine and 50 mM Tris-HCl buffer (pH 7.6) at 37°C for 60 min. The reaction was terminated by chilling to 0°C in an alcohol-solid CO\(_2\) bath. The reaction mixture was subjected to centrifugation at 27,750 X g for 15 min. The supernatant fluid (free histones) was decanted, fraction 1. The chromatin was washed with acetic acid (pH 3.0), followed by centrifugation as above. The supernatant fluid (loosely bound histones) was decanted, fraction 2. The firmly bound histones (nucleosome associated) were extracted from the precipitate with 0.4 M HCl, fraction 3. The histones from fractions 1, 2 and 3 were precipitated by the addition of 7 volumes of acetone and stored overnight at 4°C, followed by one wash with acetone. The histones were dissolved in 0.01 M HCl and subjected to Bio-Gel P-10 chromatography as outlined in "Materials and Methods".
Free Histones

Bound Histones (pH 3.0 Extracted)

Bound Histones (HCl Extracted)
considerable amount of the added histones particularly histones H3 and H4 bound to the chromatin. These histones were not associated with the nucleosomes and could be readily resolved with dilute acetic acid (pH 3.0). Furthermore, these histones were not methylated to any significant degree (Fig. 15, middle). In contrast nucleosomal associated histones H3 and H4 were readily methylated (Fig. 15, bottom).

The histone H3 lysine methyltransferase and the histone H4 arginine methyltransferase are quite labile after DEAE-cellulose chromatography. With histone H3 lysine methyltransferase between 50 and 60% of the activity was lost in 24 h and 80-90% was lost after 72 h in 50 mM Tricine-HCl (pH 7.5)-1.0 mM dithiothreitol-0.1 mM phenylmethylsulfonyl fluoride. Histone H4 methyltransferase was somewhat more stable, retaining between 40-50% of its' original activity up to 72 h. Attempts were made to stabilize the enzymes with sheared calf thymus DNA (Fig. 16). This resulted in non-specific binding of the enzymes to the DNA and subsequent loss of methyltransferase activity.

The pH optimum for histone H3 lysine methyltransferase with soluble rat brain histone H3 as substrate was 8.5 with little variation from pH 8.2 to 8.7 (Fig. 17, bottom). The pH optimum for histone H4 methyltransferase with soluble histone H4 as the methyl acceptor was 7.5 with little variation from pH 7.3 to 7.8 (Fig. 17, top). These results are similar to those obtained by Wallwork et al. (8) with their water extracted histone lysine methyltransferase.

Effects of Polyamines and Cations

We investigated the effects polyamines and cations on the rate and extent of the incorporation of methyl groups into the lysyl residues of histones H3 and H4. The time-course of in vitro methylation
Figure 16: Effect of calf thymus DNA on histone lysine methyltransferase activity. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.0), 20 μM S-adenosyl-L-[3H-methyl]methionine, 30 μg histone H3 or H4 methyltransferase and either 29 μg histone H3 or 66 μg histone H4.
Methyltransferase Activity (units/mg/min)

A. Histone-H4 Methylase

B. Histone-H3 Methylase

Calf Thymus DNA, μg

Methyltransferase Activity (units/mg/min)
Figure 17: Activity of histone methyltransferases as a function of pH. Histone H3 and H4 methyltransferase (DEAE-cellulose fraction) were assayed with their respective substrates at varying pH values. The assays are similar to those outlined in "Methods and Materials" except the pH was varied. Potassium phosphate was used below pH 7.5, Tris-HCl between pH 8.0 and 10.3 and cyclohexylamino-propane-sulfinic acid-HCl above 10.3.
of chromosomal bound histones H3 and H4 in the presence of 1.0 mM MgCl₂ was determined (Fig. 18). When spermidine was added to a concentration of 1.5 mM it altered not only the rate, but inhibited the extent of methylation of histone H4, while the ratio of mono- to dimethyllysine shifted towards a more highly methylated form (Table 5). This ratio closely approximated that which Duerre and Chakrabarty (7) observed in vivo. The rate and extent of methylation of histone H4 was not affected by putrescine, but the effects of spermidine could be mimicked by increased concentrations of MgCl₂ (Table 5, 6 and 7). The addition of CaCl₂ gave similar results.

All the methylation sites on the arginine-rich histones appeared to be saturated after incubating nuclei for 1 h with S-adenosylmethionine in the presence of 1.0 mM MgCl₂ at pH 7.0 (Fig. 18). These results are consistent with those reported by Wallwork et al. (61). However, the total number of [³H] methyl groups incorporated into the lysyl residues of histone H4 increased in the presence of 0.1 M NaCl. The ratio of mono- to dimethyllysine remained essentially unaffected by the presence of the NaCl (Table 5). Interestingly, concentrations of NaCl between 0.05-0.15 M had no significant affect on either the rate or extent of methylation of histone H3 (Table 6, 7 and 8). Nor did the NaCl affect the ratio of mono- to di- to trimethyllysine in histone H3 (Table 8).

It was proposed by Duerre et al. (60) that NaCl could possibly alter the conformation of the histone H4 methyltransferase or interfere with its binding to DNA. When similar experiments were carried out using the DNA-enzyme complex and soluble histone H4 as substrate, NaCl between 0.05 to 0.15 M had little or no affect on the reaction
Figure 18: Time-course of incorporation of $[^3H]$methyl groups into chromosomal bound histone H3 and H4 in the absence and presence of 1.5 mM spermidine or 0.1 M NaCl. The reaction mixtures contained 0.32 M sucrose, 1.0 mM MgCl$_2$, nuclei (8-10 mg DNA), 2.0 mM Tris-HCl buffer (pH 8.4), 15 μM S-adenosyl-L-$[^3H]$methylmethionine and the compounds listed. The final pH of the reaction mixtures was 7.0. The reaction was initiated by the addition of S-adenosylmethionine. The reaction was run at 37° C for varying time periods and terminated by adding adenosylhomocysteine to a final concentration of 2.0 mM and chilling to 4° C in an alcohol-solid CO$_2$ bath. The histones were fractionated as outlined in the "Materials and Methods" section.
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>monomethyllysine</td>
<td>dimethyllysine</td>
</tr>
<tr>
<td>None</td>
<td>196</td>
<td>219</td>
</tr>
<tr>
<td>0.1 M NaCl</td>
<td>685</td>
<td>686</td>
</tr>
<tr>
<td>1.5 mM spermidine</td>
<td>64</td>
<td>139</td>
</tr>
<tr>
<td>1.5 mM putrescine</td>
<td>250</td>
<td>219</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>37</td>
<td>53</td>
</tr>
</tbody>
</table>

The reaction mixtures contained 0.32 M sucrose, 1.0 mM MgCl₂, nuclei (8-10 mg DNA), 2.0 mM Tris-HCl buffer (pH 8.4), 15 μM S-adenosyl-L-[[^3]H-methyl]methionine and the compounds listed. The final pH of the reaction mixture was 7.0. The reaction was initiated by the addition of S-adenosylmethionine and was incubated at 37° C for 60 min. The reaction was terminated by the addition of adenosylhomocysteine to a final concentration of 2.0 mM and chilling to 4° C in an alcohol-solid CO₂ bath. Histones were fractionated as outlined in "Materials and Methods."
### TABLE 6

**EFFECT OF POLYAMINES, NaCl AND MgCl₂ ON THE RATE OF IN VITRO METHYLATION OF SOLUBLE HISTONES**

<table>
<thead>
<tr>
<th>Addition</th>
<th>Soluble Histones&lt;sup&gt;1&lt;/sup&gt; pmol[&lt;sup&gt;3&lt;/sup&gt;H-methyl]</th>
<th>mg/min</th>
<th>H3</th>
<th>H4</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>28</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 M NaCl</td>
<td>28</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.10 M NaCl</td>
<td>28</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>26</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20 M NaCl</td>
<td>18</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25 M NaCl</td>
<td>13</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.35 M NaCl</td>
<td>7</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.0 mM MgCl₂</td>
<td>29</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0 mM MgCl₂</td>
<td>28</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25.0 mM MgCl₂</td>
<td>28</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50.0 mM MgCl₂</td>
<td>11</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 mM Spermidine</td>
<td>25</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 mM Spermidine</td>
<td>27</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 mM Spermidine</td>
<td>27</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 mM Putrescine</td>
<td>26</td>
<td>13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.0 mM Putrescine</td>
<td>23</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>1</sup>The reaction mixtures contained 0.2 M Tris-HCl buffer (pH 8.0), 20 μM S-adenosyl-L-[<sup>3</sup>H-methyl]methionine, 62 μg enzyme protein and either 15 μg of histone H3 or 66 μg of histone H4 plus the additions indicated in the table, in a final volume of 0.2 ml. Values are the mean of two separate assays.
TABLE 7

EFFECT OF POLYAMINES, NaCl AND MgCl2 ON THE RATE OF IN VITRO METHYLATION OF CHROMOSOMAL BOUND HISTONES

<table>
<thead>
<tr>
<th>Addition</th>
<th>Nuclei pmol $[^3]$H-methyl]/mg/min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H3</td>
</tr>
<tr>
<td>None</td>
<td>4.6</td>
</tr>
<tr>
<td>0.05 M NaCl</td>
<td>5.1</td>
</tr>
<tr>
<td>0.10 M NaCl</td>
<td>6.5</td>
</tr>
<tr>
<td>0.15 M NaCl</td>
<td>4.6</td>
</tr>
<tr>
<td>0.20 M NaCl</td>
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<td>5.0 mM MgCl2</td>
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<td>4.3</td>
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<tr>
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<td>5.6</td>
</tr>
<tr>
<td>6.0 mM Putrescine</td>
<td>4.6</td>
</tr>
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1The reaction mixtures contained 0.32 M sucrose, nuclei (8-10 mg DNA), 2.0 mM Tris-HCl buffer (pH 8.4), 15 µM S-adenosyl-L-[^3]$H-methyl]-methionine and the compounds listed. The final pH of the reaction was 7.0. The reaction was initiated by the addition of S-adenosylmethionine. The reaction was run for 5 min at 37° C and terminated by the addition of adenosylhomocysteine to a final concentration of 2.0 mM and chilling to 4° C in an alcohol-solid CO2 bath. Histones were fractionated as outlined in "Materials and Methods" and specific activity determined.
### TABLE 8

Effects of Polyamines, NaCl and MgCl₂ on the Extent of In Vitro Incorporation of [³H]Methyl Groups into Mono-, Di- and Trimethyllysine in Chromosomal Bound Histone H3

<table>
<thead>
<tr>
<th>Addition</th>
<th>µmol [³H-methyl]methyllysine/mol histone H3</th>
<th>Ratio mono-:di-:tri-methyllysine</th>
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<tr>
<td></td>
<td>monomethyllysine</td>
<td>dimethyllysine</td>
</tr>
<tr>
<td>None</td>
<td>654</td>
<td>517</td>
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<tr>
<td>0.1 M NaCl</td>
<td>684</td>
<td>517</td>
</tr>
<tr>
<td>1.5 mM spermidine</td>
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<td>621</td>
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<td>730</td>
<td>546</td>
</tr>
<tr>
<td>10 mM MgCl₂</td>
<td>676</td>
<td>458</td>
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The reaction mixtures contained 0.32 M sucrose, 1.0 mM MgCl₂, nuclei (8–10 mg DNA), 2.0 mM Tris-HCl buffer (pH 8.4), 15 µM S-adenosyl-L-[³H-methyl]methionine and the compounds listed. The final pH of the reaction mixture was 7.0. The reaction was initiated by the addition of S-adenosylmethionine and was incubated at 37°C for 60 min. The reaction was terminated by the addition of adenosylhomocysteine to a final concentration of 2.0 mM and chilling to 4°C in an alcohol-solid CO₂ bath. Histones were fractionated as outlined in "Materials and Methods".
When the concentration of NaCl was increased above 0.2 M, methylation of free and chromosomal bound histone H4 was inhibited (Table 6 and 7). Similar results were observed with free and chromosomal bound histone H3. Apparently NaCl alters the conformation of chromatin. As a result methylation sites on histone H4 were made accessible to the enzyme (Table 5). To determine if these sites were specific or nonspecific the experiments were repeated using nuclei from adult rats. Duerre et al. (7) have shown that histones from adult rat brains are fully methylated in vivo. The results of these experiments revealed that only traces of $[^3H]$ methyl groups are incorporated into histones H3 and H4 in the presence or absence of NaCl. Therefore the sites exposed on chromosomal bound histone H4 from rapidly proliferating tissue in the presence of NaCl appear to be specific.

**Effects of Puromycin on Histone Methylation In Vivo**

When adult rat livers were perfused with puromycin (125 µg/ml) for one hour the incorporation of amino acids into chromosomal proteins was essentially nil (95-98% inhibition). Under these conditions the methylation of histones was inhibited by 75% (Fig. 19). The incorporation of methyl residues in the absence of protein synthesis suggested that methylation is a late event or methyl groups turnover independent of the polypeptide chain. After a two hour incubation in the presence of puromycin the incorporation of methyl residues decreases by 90%. These data indicated that the methylation of histones is a late event, occurring some time after their synthesis. Similar results are obtained by Allfrey et al. (68) using isolated calf thymus nuclei.
Figure 19: Effects of puromycin on histone synthesis in isolated rat livers. Normal adult rat livers were perfused with puromycin (125 µg/ml) for varying lengths of time, after which all essential amino acids were added at twice normal plasma levels. L-Methionine (6.4 µmol) contained 12.8 µCi/µmol [³H-methyl]methionine and 1.6 µCi/µmol L-[¹⁴C-carboxyl]methionine. After 60 min livers were frozen in liquid nitrogen. The histones were fractionated as outlined under "Materials and Methods" and specific radioactivity determined.
nmol [\(^3\)H-methyl] methionine/mg histone (○○)

nmol L-[\(^{14}\)C-carboxyl] methionine/mg histone (××)

Time Preperfused with Puromycin (min)
DISCUSSION

The enzymes involved in the methylation of histones H3 and H4 are located exclusively within the nucleus and are firmly bound to chromatin (Table 1). Other enzymes have been shown to be associated with chromatin such as acetyltransferase (69) and certain nuclear-protein kinases (70).

The histone lysine methyltransferase(s) can be solubilized from chromatin by partial digestion with micrococcal nuclease (Fig. 7), NaCl (61), distilled water (8) or mechanical shearing (71). We have found that all these preparations contain DNA fragments. If the DNA fragments were digested with DNAase I all methylase activity was lost. Wallwork et al. (8) reported the presence of histone methyltransferase(s) in the nucleoplasm. When this fraction was treated with DNAase I similar results were obtained. The enzymes apparently required DNA for stabilization and/or activation. Attempts to recover the enzymes by the addition of DNA or prevention of hydrophobic interaction by the addition of NaCl have failed. However, the enzymes, particularly histone H3 lysine methyltransferase, were quite stable while they remain associated with the small DNA fragments.

When nucleosomes were incubated with partially purified histone methyltransferase(s) in the presence of S-adenosyl-L-[\(^3\)H-methyl] methionine no methyl groups were incorporated into the lysyl, arginyl or histidyl residues of the histones. In contrast the same enzyme preparation readily methylated soluble histones. These observations have led Sarnow et al. (71) to conclude that histones are methylated in the soluble form prior to nucleosome assembly.
However, when soluble histones were incubated with native chromatin rich in histone methyltransferase(s), the soluble histones were not methylated (Fig. 15), while the firmly bound histones methylated readily. Apparently the histones were methylated after binding to DNA during nucleosome assembly. When the DNA-methyltransferase complexes were incubated with chromatin from rapidly proliferating cells in the presence of S-adenosyl-L-methionine, both histones H3 and H4 were methylated, whereas when chromatin from adult rats is incubated as above no methylation of histones H3 and H4 is observed (41). Similar results are obtained by Tidwell et al. (40) when brain nuclei from adult animals are incubated with radiolabeled S-adenosyl-L-methionine.

When one day old rats are given radiolabeled lysine and methyl-labeled methionine, significant quantities of the radioactivity are incorporated into brain histones (41). In contrast, when adult rats are treated similarly only trace quantities of radioactivity is incorporated into rat brain histones. Duerre et al. (44) estimated that in rat brain tissue from 12-14-day old animals, 0.024% of the total methylation sites on histone H3 and 0.013% of the methylation sites on histone H4 are undermethylated at the time the nuclei are isolated. This indicates that histones from non-proliferating tissue are fully methylated and that only the histones in newly formed nucleosomes are able to accept methyl groups. All of the observations are consistent with the conclusion that the histones are methylated during the assembly or shortly after assembly of the nucleosome.

It has been well established that nascent DNA is very susceptible to nuclease digestion. Micrococcal nuclease has been shown to preferentially degrade nascent DNA in chromatin of swimming blastulae of
sea urchins (72), Chinese hamster cells (73, 74), HeLa cells (75) and chick erythrocytes (76). Brövvoet has found that the histones associated with the newly formed nucleosomes are undermethylated and this region is quite susceptible to nuclease digestion (personal communication).

Duerre et al. (60) found that NaCl increased the extent of methylation of histone H4 when nuclei are incubated with S-adenosyl-L-methionine. NaCl alters the conformation of chromatin in such a way that there is an increase in the number of sites available for methylation in histone H4. Whereas MgCl₂ or spermidine acts in an opposite manner. This indicates that the methylation of the N-terminal region of histone H4 may be involved in some higher order of chromatin structure, perhaps formation of the superhelix (solenoid). Histone H3 is unaffected by altering the types or concentrations of the cations or polyamines. Therefore the N-terminal region of histone H3 may be located in a region which is unaffected by the conformation of the nucleosome in the solenoid. The N-terminal region of histone H3 may be involved in some higher order of chromatin structure.

If methylation is involved in the formation of the superhelix (solenoid), then the relaxation of this structure during transcription may involve demethylation. Paik and Kim (77) have reported the presence of histone demethylase in rat kidney and liver. Failure to detect turnover of methyl group in non-proliferating cells would in no way negate this possibility. The majority of the operons which are actively transcribing should be turned on at the time of cell differentiation and should continue to transcribe throughout the lifetime of the cell. Consequently, little or no turnover of methyl groups
would be detectable in most non-proliferating cells.

The turnover of c-N-methyl groups on lysine residues of histones H3 and H4 have been studied by a number of different laboratories (46,48-51). After the simultaneous administration of 4,5-[\(^3\)H]lysine and L-[\(^{14}\)C-methyl]methionine to 12 day old rat pups the turnover of the methyl groups [\(^{14}\)C] in brain histones did not differ significantly from that of the lysyl residues [\(^3\)H]. Nor did histone H3 or H4 turnover in the absence of cell division (46). Attempts to detect the turnover of methyl groups on histones in vitro with Ehrlich-ascites tumor cells (48), Chinese hamster ovary cells (49) and developing trout testis (50) have also been unsuccessful. Contrariwise, Hempel et al. (51) reported that methyl groups on the lysyl residues turned over independently of the the polypeptide chains in histones H3 and H4 from non-proliferating adult cat kidney cells. They reported that from 2-3% of histones H3 and H4 turnover daily, while a much higher percentage (16-36%) of the methyl groups turnover. Considering that methylation of histones is a relatively late event occurring sometime after histone biosynthesis (39-41) and from the results obtained from the puromycin studies, we conclude that the methyl residues on histones H3 and H4 do not turnover in the absence of protein synthesis.

The histone H4 methyltransferase isolated by DEAE-cellulose chromatography catalyzes the methylation of arginyl not lysyl residues in soluble histone H4. This enzyme was considerably more stable than the histone H3 lysine methyltransferase and was not inhibited by the addition of calf thymus DNA. Gupta et al. (78) have reported the presence of an arginyl methyltransferase in wheat germ and Lee et al. (79) reported the presence of this enzyme in calf brain.
We have reported that the crude enzyme extract from young rat brain chromatin would catalyze the methylation of arginyl residues in soluble histone H4 (Table 4). Similar results are obtained by Wallwork et al. (61). They indicated that when the histone H4 lysine methyltransferase is dissociated from chromatin it lost specificity. However the enzyme that we have isolated from DEAE-cellulose will not catalyze the methylation of chromosomal bound histones (Table 3), whereas the crude preparation will methylate chromosomal bound histones. Apparently the histone H4 lysine methyltransferase was extremely unstable and we have isolated the arginyl methyltransferase.

The majority of the arginyl methyltransferase has been reported to be present within the cytoplasm (80-83). Considering that a large quantity of methylated arginyl residues have been found in other fractions, particularly the microsomal fraction (84), it may well be that this enzyme is a contaminant. This observation is consistent with in vivo results from work done by a large number of laboratories (7,34,85). These laboratories have not been able to detect the presence of methylated arginyl residues within histones from a varied number of sources. However, this does not negate the possibility that this enzyme may play a role in denaturation of histones during their turnover or some other role.
SUMMARY

The histone lysine methyltransferases catalyze the transfer of methyl groups from S-adenosyl-L-methionine to specific ε-N-lysyl residues in the N-terminal regions of histones H3 and H4. These enzymes are located exclusively within the nucleus and are firmly bound to chromatin. The chromosomal bound enzymes do not methylate free or loosely bound histones. However, histones H3 and H4 associated within the nucleosomes are methylated.

The enzymes were extracted by limited digestion (12-16%) of chromosomal DNA from rapidly proliferating rat brain chromatin with micrococcal nuclease. The enzymes were further purified by gel filtration, ammonium sulfate fractionation and DEAE-cellulose chromatography. The histone methyltransferases were resolved into two distinct fractions by Sepharose 6B-100 and DEAE-cellulose chromatography. One enzyme fractionated by DEAE-cellulose chromatography was specific for histone H3, while the other enzyme was specific for histone H4.

Histone H3 lysine methyltransferase was shown to methylate only the lysyl residues of chromosomal bound or soluble histone H3. The molar ratio of mono- to di- to trimethyllysine in the soluble system was 1.0:2.1:1.0, while the ratio with chromosomal bound histone H3 was 1.9:1.0:0.08.

The histone H4 lysine methyltransferase which was detectible in the crude nuclease digest, was extremely labile losing all activity upon further purification. The enzyme specific for histone H4 after DEAE-cellulose chromatography methylated only arginyl residues in histone H4 and would not methylate lysyl residues in histone H4.
The pH optimum for histone H3 lysine methyltransferase with soluble rat brain histone H3 as substrate was 8.5 with little variation from pH 8.2 to 8.7. The pH optimum for histone H4 arginine methyltransferase with soluble histone H4 as the methyl acceptor was 7.5 with little variation from pH 7.3 to 7.8.

After DEAE-cellulose chromatography both enzymes were extremely unstable. Complete removal of DNA by DNAase I digestion resulted in the complete loss of enzyme activity. However, when the enzymes remained associated with DNA fragments they were quite stable, indicating that the enzymes require DNA for stability and/or activity. The requirement for DNA may only be important to prevent hydrophobic interactions involving the enzyme with itself and/or other non-histone chromosomal proteins.
APPENDIX I
SOLUTIONS
Bray's Scintillation Counting Solution

To make 1 liter:

4 g 2-5-diphenyloxazole (PPO)
100 mg 1,4-bis-2-(5phenyloxazolyl)-benzene (POPOP)
333 ml Triton X-100
666 ml Toluene (scintillation grade)

Diphenylamine Reagent

1.5 g diphenylamine
100 ml concentrated acetic acid
1.5 ml concentrated sulfuric acid

The reagent was stored in the dark until used. Aqueous acetaldehyde (16 mg/ml) was added to the reagent just prior to use (0.1 ml aqueous acetaldehyde/20 ml reagent).
APPENDIX II

KEY TO ABBREVIATIONS
## KEY TO ABBREVIATIONS

<table>
<thead>
<tr>
<th>Term</th>
<th>Abbreviation or Symbol</th>
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</tr>
<tr>
<td>nano ((10^{-9}))</td>
<td>n</td>
</tr>
<tr>
<td>micro ((10^{-6}))</td>
<td>u</td>
</tr>
<tr>
<td>milli ((10^{-3}))</td>
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<td>inhibitor constant</td>
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<td>and others</td>
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<tr>
<td>within a living organism</td>
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<tr>
<td>within an artificial environment</td>
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<td>optical density</td>
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<td>deoxyribonucleic acid</td>
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<td>diethylaminoethyl-cellulose</td>
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<td>N,N,N',N'-tetramethylenediamine</td>
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<td>2,5-diphenyloxazole</td>
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<td>1,4-bis-2-(5-phenyloxazoly)-benzene</td>
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<td>sodium ethyleneglycol-bis(β-aminoethyl ether) N,N'-tetraacetic acid</td>
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<td>chloroform: methanol (2:1, v/v)</td>
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REFERENCES


