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Carboxyl Methylation of Non-Histone Chromosomal Proteins

Douglas P. Quick

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IN VITRO STUDIES ON THE
METHYLATION OF DNA
IN RAT BRAIN AND LIVER CHROMATIN

by
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Bachelor of Science, University of Iowa, 1975

A Thesis
Submitted to the Graduate Faculty
of the
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in partial fulfillment of the requirements
for the degree of
Masters of Science

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1978

This thesis submitted by Douglas P. Quick in partial fulfillment of the requirements for the Degree of Master 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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Title In Vitro Studies on the Methylation of DNA in Rat Brain
and Liver Chromatin

Department Microbiology

Degree Master of Science

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Date 4-26-78

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ABSTRACT

The properties of DNA methyltransferase in various age rats were investigated to better characterize the enzyme. When purified chromatin from 5-day-old rat brain or liver nuclei was incubated with S-adenosyl-L-methyl- ^3H -methionine, 5-methyl- ^3H -methylcytosine was the only product of methylation. The optimum pH of the DNA methyltransferase was approximately 8.0 with little or no change in activity over a pH range of 6.9 to 8.3. The K_m value of the chromosomal bound DNA methyltransferase for S-adenosyl-L-methionine (AdoMet) was $3.5 \pm 0.15 \mu\text{M}$ and $4.2 \pm 0.13 \mu\text{M}$, in the liver and brain, respectively. The V_{max} values for the enzyme from liver and brain of 5-day-old rats were 0.17 ± 0.04 and $0.23 \pm 0.09 \text{ pmol of } ^3\text{H-methyl groups incorporated/mg DNA/min}$, respectively. S-Adenosyl-L-homocysteine was found to inhibit competitively the DNA methyltransferase in the brain with a K_i value of $3.5 \pm 1.6 \mu\text{M}$. The enzyme was firmly bound to chromatin. After ten extractions with water or 20 mM potassium phosphate buffer, pH 7.0, only trace amounts of enzyme could be eluted from the chromatin. Measurable amounts of the enzyme could be extracted from chromatin with 0.4 M NaCl. However, measurable amounts of the DNA methyltransferase were still associated with the NaCl extracted chromatin.

The rate of the reaction catalyzed by the chromosomal-bound DNA methyltransferase was dependent on the age of the rat and the type of

cell from which the chromatin was isolated. In the liver, the rate declined from 0.28 pmol of methyl- ^3H incorporated/mg DNA/min at birth to 0.13 pmol of methyl- ^3H incorporated/mg DNA/min at 30 days. Enzyme activity remained at this level throughout life. In the brain, the rate decreased rapidly from 0.48 pmol of methyl- ^3H incorporated/mg DNA/min at birth to 0.05 pmol of methyl- ^3H incorporated/mg DNA/min at 30 days. Little or no activity could be detected after 30 days.

Chromosomal DNA methyltransferase activity was found to be dependent on the availability of methyl acceptors and enzyme concentration. The number of methylation sites available for in vitro methylation in liver decreased from 3.5 pmol of 5- $\overline{\text{methyl-}}^3\text{H}\overline{\text{methyl-}}$ cytosine/mg DNA at birth to 1.5 pmol of 5- $\overline{\text{methyl-}}^3\text{H}\overline{\text{methyl-}}$ cytosine/mg DNA at 14 days, remaining constant thereafter. The number of methyl acceptors in the brain decreased from 8.5 pmol of 5- $\overline{\text{methyl-}}^3\text{H}\overline{\text{methyl-}}$ cytosine/mg DNA at birth to 2.5 pmol of 5- $\overline{\text{methyl-}}^3\text{H}\overline{\text{methyl-}}$ cytosine/mg DNA at 40 days, remaining constant thereafter.

The level of extractable enzyme from the cerebellum increased from 0.13 pmol of methyl- ^3H incorporated/mg protein/min at birth to 0.29 pmol of methyl- ^3H incorporated/mg protein/min at 10 days. The level of the enzyme decreased thereafter to 0.07 pmol of methyl- ^3H incorporated/mg protein/min in 30-day-old rats, remaining at this level throughout life. The amount of extractable enzyme from the cerebrum decreased from 0.13 pmol of methyl- ^3H incorporated/mg protein/min at birth to 0.07 pmol of methyl- ^3H incorporated/mg DNA/min in 30-day-old rats, remaining at this level of activity thereafter.

The extractable enzyme from the liver increased from 0.45 pmol of methyl-³H incorporated/mg protein/min at birth to 0.60 pmol of methyl-³H incorporated/mg protein/min at 16 days. In 30-day-old rat liver the enzyme decreased to 0.10 pmol of methyl-³H incorporated/mg protein/min, remaining at this level until 100 days. At this time the extractable enzyme increased to 0.25 pmol of methyl-³H incorporated/mg protein/min at 300 days, remaining at approximately this level, thereafter.

The level of DNA methyltransferase and the number of in vitro methylation sites available on DNA correlated well with the rate of DNA synthesis in these organs. The synthesis of DNA methyltransferase appears to be coupled to DNA synthesis.

INTRODUCTION

The presence of 5-methylcytosine in nucleic acid was first discovered in 1925, in bacteria (1). Methylation of DNA is now well defined in prokaryotic cells, where it functions in modification-restriction system, allowing for the select destruction of foreign DNA. If the DNA methyltransferase is not modified in a specific nucleotide sequence by DNA methyltransferase, an endonuclease will cleave the DNA, leading to its destruction.

A function for DNA methylation is not known in eukaryotic cells. The product of methylation, 5-methylcytosine, varies in concentration in different eukaryotic cells from 0.65 to 2.6% of the total DNA bases. Cytosine bases in specific nucleotide sequences in DNA contain 5-methylcytosine, suggesting that methylation of DNA does not occur randomly throughout the DNA. In vivo studies have been limited largely to tissue culture cells, where cytosine methylation occurs rapidly up to 2 min after DNA synthesis, remaining at a detectable level for 2 h after DNA replication. The variation in 5-methylcytosine content in DNA isolated from eukaryotic cells, along with the changing rates of methylation in developing sea urchin embryos, led several investigators to propose various theories involving cytosine methylation in cell differentiation (2-4).

Methylation of DNA in eukaryotic cells is not well understood. Questions still remain concerning where the DNA methyltransferase is

located in the cell, when methylation occurs in relation to cell age, and with respect to cell differentiation. The purpose of this investigation is to answer some of these questions, and to characterize the basic properties of the rat DNA methyltransferase in the brain and liver.

HISTORICAL REVIEW

The presence of 5-methylcytosine in nucleic acids was first reported by Johnson and Coghill in tuberculinic acid, in 1925 (1). Wyatt, in 1951, showed that 5-methylcytosine was present in deoxy-ribonucleic acid (DNA) of higher animals and plants (5). Seven years later, this unusual base was found to be present in the DNA of bacteria and bacteriophages (6). However, the presence of this base could not be explained by de novo DNA synthesis (7). In 1963, Borek (8) discovered an enzyme that catalyzed the post-transcriptional methylation of transfer ribonucleic acid. Later in the same year, Gold and colleagues (9) at the Albert Einstein College of Medicine discovered a DNA methylase in Escherichia coli W. Crude extracts of E. coli W catalyzed the incorporation of radioactive methyl groups into DNA, in the presence of radioactive methionine and ATP (9), as outlined in reaction 1 and 2.

Of all the known methyl donors, including 5, 6-dimethylbenzimidazolylcobamide (B_{12}) and N^5 -methyltetrahydrofolic acid, only S-adenosyl-L-methionine (AdoMet) functions as a methyl donor for DNA (reaction 2). DNA methylase has no other requirements for enzymatic activity, as suggested by reaction 2 (10-12).

Deoxyribonucleic acid from E. coli, Micrococcus lysodeikticus, Clostridium pasteurianum, Mycobacterium phlei, Salmonella typhimurium, and Staphylococcus aureus, are not methylated when incubated with homologous enzyme (13). All methylation sites appear to be saturated in vivo prior to extraction of the DNA. When mutants of E. coli that require methionine are grown in media containing low levels of methionine, the resultant DNA is methyl deficient (14). This DNA, and DNA from other species (15), is capable of accepting methyl groups from E. coli DNA methylase. DNA melting experiments have demonstrated that the secondary structure of DNA is required for enzymatic activity (14). When DNA is heated at different temperatures, cooled rapidly, and incubated with an E. coli DNA methyltransferase at 30° C, methylation decreased with increasing temperatures. If the DNA was heated and allowed to cool slowly, methylation increased. Slow cooling of melted DNA allows for reannealing of the single strands to a secondary configuration similar to native DNA.

Incubation of E. coli B, E. coli K 12, λ phage, M. phlei, rat brain, and wheat germ DNA with $\sqrt{\text{methyl-}^{14}\text{C}}/\text{Ado-met}$, and E. coli W DNA methylase resulted in a unique methylation pattern with each source of DNA (15,16). Since the ratio of A + T/G + C was the same

for E. coli B, E. coli K 12, and λ phage, it is concluded that the A + T/G + C ratio is not related to the methylation pattern. Broches, et al. (17) have isolated a sequence recognized by a DNA methylase in bacteriophage PI. From direct radioautography of partially digested $\overline{\text{methyl-}}^{14}\text{C}\overline{\text{oligonucleotides}}$, the following sequences were identified as having ^{14}C -methyl groups: $\text{pG-}\overset{*}{\text{A}}$, $\text{pA-}\overset{*}{\text{T}}$, $\text{pA-G-}\overset{*}{\text{A}}$, $\text{G-}\overset{*}{\text{A-T-C}}$,¹ and $\overset{*}{\text{A-T-C-T}}$. If $\overset{*}{\text{A-T-C}}$ and $\text{A-G-}\overset{*}{\text{A}}$ are assumed to be from the same sequence, the longest defined sequence would be $\text{pA-G-}\overset{*}{\text{A-T-C-T}}$.

Survival of heterologous DNA in a bacterial host depends upon the restriction and modification enzymes present. Methylation of certain nucleotides is thought to prevent restriction endonucleases from cleaving DNA. At a given sequence, an endonuclease makes a double stranded cleavage. If a specific nucleotide sequence is modified by methylation, the endonuclease can not cleave at that site (18-20). Interest in restriction-modification systems began with Luria's work in 1953 (21). Presently, two types of restriction endonucleases have been defined and classified (22). The DNA sequence recognized by Hemophilus restriction endonuclease has been determined by Kelly and Smith (23), as follows. The 5' phosphoryl group of the termini were isotopically labelled using a polynucleotide kinase. The DNA was then hydrolyzed with a specific nuclease and the following sequence determined by chromatographic techniques (the arrows indicate where the 5'-phosphoryl 3'-hydroxyl termini are located, where pu and py stand for purines and pyrimidines, respectively).

^{1*} denote radioactive label

Notice the palindromic nature of this sequence. A palindrome in DNA is an inverted duplication, with a two-fold rotational symmetry.

Arber (24) first demonstrated that methylation prevented cleavage of DNA. Partially purified DNA methylase from E. coli B enhanced the ability of unmethylated double stranded λ fd DNA to infect spheroplasts of E. coli B. The complete methylation of λ fd DNA contained 4 methyl groups, indicating 2 modification-restriction sites. Mutants of λ fd which have either none or 1 restriction-modification site, incorporated none or 2 methyl groups into their DNA, respectively (25).

The only function of DNA methylation in bacteria that has been found is its role in protecting native DNA from endonuclease cleavage. Ribonucleic acid and DNA polymerase activity is not affected by methylation (14,25,26). Partially purified DNA methylase from E. coli W was used to methylate DNA from various strains of Bacillus subtilis and bacteriophage 105 (26). Of the three strains of B. subtilis used, 2 had a 50% decrease in transforming activity, with no change in the other. A 50% decrease in the ability of phage λ 105 DNA to rescue defective phage strains was observed after methylation. However, there was no physiochemical change due to methylation, as measured by hyperchromicity on heating, formaldehyde denaturation, viscosity, or sedimentation analysis. From the above research, no new role for methylation could be suggested.

DNA methyltransferase activity in eukaryotic cells was not demonstrated until 1968, when Sheid, et al. (27) detected activity in cells from the liver, kidney, and spleen of rats. Their research and subsequent work in eukaryotic cells has shown that 5-methylcytosine

is the only product of methylation, except for N⁶-methyladenine, which has been found in Tetrahymena pyriformis. 5-Methylcytosine has been found in DNA throughout the animal kingdom (27-29), in tissue culture cell lines (30-34), and in plants (35,36). From 0.65 to 2.6 bases of 5-methylcytosine per 100 bases of DNA were found in organs of 26 different species in the animal kingdom, including sperm cells (28). Mitochondrial DNA, isolated from hamster cells, also contains this base (29).

The function of DNA methylase has not been established in eukaryotic cells. In mouse fibroblast cells methylation of DNA was found to be coupled to DNA synthesis (30). In synchronized cells, cytosine methylation occurred within one hour of DNA synthesis in the S phase of the cell cycle. As previously found in prokaryotic cells, methylation of eukaryotic DNA was irreversible. Kappler used a sensitive method of relating DNA methylation to DNA synthesis (37). Tissue culture cells were grown in the presence of [¹⁴C]deoxycytidine. Methylation of cytosine was followed by the incorporation of [¹⁴C]deoxycytidine into 5-methylcytosine, as detected by paper chromatography. Methylation was found to occur within a few min of DNA synthesis. Methylation of DNA during hormonal stimulation in organ cultures of mouse mammary cell supported Kappler's results (38). It was found that 96% of mammary epithelial cells in the G₁ phase of the cell cycle were induced to initiate DNA synthesis by incubation with insulin. However, insulin by itself does not cause mammary cells to differentiate into secretory cells. When insulin, hydrocortisone, and prolactin were used together, the cells

differentiated. This study demonstrated that methylation of cytosine in DNA occurs only in relation to DNA synthesis in the S phase of the cell cycle and was not related to mammary cell differentiation.

Other investigations using polyoma infected mouse kidney culture (39) and Novikoff Hepatoma cells (33) demonstrated that DNA methylation was dependent on DNA synthesis. In cultured plant cells methylation of cytosine occurred within 2 min of the incorporation of deoxycytidylate into DNA (36). In mouse fibroblast cells (L 929), newly synthesized DNA was methylated 40 times faster than DNA 2 min old (40). Methylation of DNA was still detectable after 2 h.

[$\overline{\text{Methyl-}}^3\text{H}$]methionine was injected into newborn (approximately 8g), 40g, 90g and 160g rats (41). After 4 h, the rats were killed and the amount of ^3H -methyl incorporated into the thymus, spleen, and liver DNA was measured. In all three tissues, methylation of DNA was higher in the 40g rats than the newborn, 90g and 140g rats. Both Morris (42) and Cox (43) demonstrated that the specific enzyme activity of the DNA methylase increased 3 to 4 fold upon hepatectomy.

Di- and tripyrimidine sequences containing 5-methylcytosine were measured in mouse DNA, in order to establish the methylation sequence in eukaryotes (32). Newborn mice or mouse embryo cells were cultured in the presence of [$\overline{\text{methyl-}}^3\text{H}$]methionine. By the use of high-voltage electrophoresis, paper chromatography, and hydrolysis with snake venom phosphodiesterase, di- and tripyrimidine base sequences were analyzed. Of the nine possible dipyrimidine combinations, T p M (M = 5-methylcytosine) contained 40% of the radioactivity. With 27 possible tripyrimidine sequences, CpTpM, TpMpC,

MpTpT, and TpTpM were the only sequences that contained 5-methylcytosine, where MpTpT contained 38% of the radioactivity.

Drahovsky and Morris (44) have suggested that rat liver DNA cytosine methyltransferase methylates several cytosine residues without dissociating from the DNA, and is able to "walk" along the DNA at a rate of 1.5 to 3.5 base pairs per sec. He found that a 50% inhibition in the rate of methylation occurred when 0.05M NaCl was added to the incubation mixture. In the presence of 0.2 M NaCl, little, if any enzymatic activity was observed. However, when the salt was added, after the DNA and enzyme had been allowed to incubate together for 2 min, activity was not affected. From experiments where various concentrations of enzyme, DNA, AdoMet, and NaCl were added to the incubation mixture, in different orders, and at different temperatures, a 0.2M salt-resistant-enzyme-DNA complex was formed at 37°C. When different molecular weight DNA was used in the assay, the linearity of the reaction was directly proportional to the length of DNA. Since 0.2 M NaCl was added to the DNA-enzyme complex, inhibiting the formation of any new DNA-enzyme complexes, the enzyme can not dissociate from the DNA. Knowing the molecular weight of the DNA used, and the number of methyl groups donated per given length, one may estimate the number of methyl groups attached per sec. Assuming that the enzyme methylates in one direction, the rate of movement of the enzyme along the strand was from 1.5-3.5 bases per sec. Turnbull and Adams (34) could not verify the mechanism suggested by Morris for DNA methylation. They found no 0.2 M NaCl resistant DNA-enzyme complex, and suggested that DNA methylase attacks randomly to a

specific nucleotide sequence, methylates cytosine, and dissociates from the DNA before it methylates another site.

Cells isolated from the brain and liver of various age rats were used to investigate the characteristic of the DNA methyltransferase. After birth rapid developmental changes were observed in both the brain and liver. Information concerning the synthesis of DNA is required when DNA is a potential substrate for the DNA methyltransferase. Post and Hoffman (45) have shown that cell replication in the rat liver exponentially declines after birth, measured by the percentage of cells in mitosis. Past 168 days of age, less than 0.2% of the cells in the liver are going through mitosis. Although mitosis virtually stops after 168 days in the liver, $\text{[}^3\text{H]}\text{-thymidine}$ incorporation into DNA increases in the livers isolated from 124-day-old rats to 1000-day-old rats (46). Repair of damaged DNA could explain this increase in $\text{[}^3\text{H]}\text{-thymidine}$ incorporation in liver DNA, when mitosis is not occurring. Double stranded DNA was found to decrease from birth to 400 days, with single stranded DNA increasing over this same time period (47). Because DNA is being made when repair of DNA occurs, the increase in $\text{[}^3\text{H]}\text{-thymidine}$ incorporation is explainable.

By the use of autoradiographic and histological studies, it is known that replication and differentiation of cells in the rat brain continues postnatally (48,49). When rats were given $\text{[}^3\text{H]}\text{-thymidine}$ by intravenous injection, 6 h or 2 days after birth, the granular layers of the olfactory, hippocampal dentate gyrus, and cerebellar cortex nuclei were isotopically labelled. The major area of cell replication

was in the ventricular or subpial germinal zones. The internal and external granular cells of the cerebellum were observed to undergo rapid cell replication from birth to 15 days of age, with mitosis continuing in the external granular cells until 20 days, and in the internal granular cells, well past 20 days. The cortex of the brain in a 1-day-old rat showed only a 1-2% labelling of cells after $[^3\text{H}]$ thymidine injection. The cerebrum showed low amounts of detectable mitosis in its cells after birth. The hippocampal dentate gyrus cells and the granular layers of the olfactory, showed a rapid rate of cell replication up to approximately 6 days after birth, with little cell replication after 6 days.

MATERIALS

Chemicals

S-Adenosyl-L- $\bar{\text{methyl}}\text{-}^3\text{H}\bar{\text{methionine}}$ ($\bar{\text{methyl}}\text{-}^3\text{H}\bar{\text{AdoMet}}$) was purchased from ICN Chemical and Radioisotopes Division, Irvin, California, or from Schwartz/Mann, a division of Becton, Dickinson and Company, Orangeburg, New York, and was diluted with unlabelled S-adenosylmethionine (AdoMet) to a specific activity of 1.0 Ci/mmol. Ribonuclease was obtained from Worthington and was heated to 70°C for 5 min to inactivate any DNase present (50). All other chemicals except AdoMet, 5'-methylthioadenosine, and S-adenosylhomocysteine (AdoHom), were purchased from Mallinckrodt Chemical Works, St. Louis, Missouri.

Preparation of S-Adenosyl-L-homocysteine

S-Adenosyl-L-homocysteine was prepared enzymatically as described by Duerre (51). In a final volume of 100 ml, 1 g of adenosine, 1.5 g of DL-homocysteine (free base), and 0.1 M phosphate buffer (pH 6.5) was added. After nitrogen gas was bubbled through the above solution for 5 min, 5 ml of crude rat liver homogenate, prepared as described by De La Haba and Cantoni (52), was added and incubated at 37°C. After 180 min, 0.025 ml of thiodiglycol was added to maintain the S-adenosyl-L-homocysteine in a reduced state. The solution was heated in a boiling water bath for 5 min to denature protein and degrade S-adenosyl-L-methionine present. The S-adenosyl-L-homocysteine

was separated from the crude extract by the use of column chromatography. A 15 cm² cross-sectional column with Dowex 50 (H⁺ form) resin, 7 to 10 cm high, was sufficient to purify 100 ml of the reaction mixture. Undesirable nucleotides, nucleosides, bases, and amino acids were eluted from the column with 3 to 4 l of 0.5 M H₂SO₄. Adenine was eluted with the passage of 200 ml of 1.5 M H₂SO₄ and S-adenosyl-L-homocysteine was eluted with the passage of approximately 3 l of 3 M H₂SO₄. S-Adenosyl-L-homocysteine was precipitated from this solution by the addition of 20 ml of 20% phosphotungstic acid solution for each 100 μ mol of compound expected. After the phosphotungstate-S-adenosyl-L-homocysteine complex settled, the supernatant fluid was decanted. The precipitin was collected by centrifuging at 5000 X G for 5 min. The precipitate was washed with 5 ml of cold distilled water and then dissolved in 5 volumes of acetone-water mixture (1:1). The phosphotungstic acid was separated from the AdoHom by the addition of 4 volumes of a 1:1 solution of isoamyl alcohol and ether, in a separatory funnel. AdoHom remained in the aqueous phase. This process was repeated 3 times with 3 volumes of the isoamyl alcohol : ether solution. The aqueous phase was extracted in a separatory funnel 4 times with 3 volumes of ether. Nitrogen gas was bubbled through the aqueous phase to remove all traces of ether. This solution was neutralized with freshly prepared BaCO₃ (Appendix) to a pH of 4.5. The BaSO₄ was removed by the filtration of the precipitin and was washed with a small amount of distilled H₂O. The H₂O wash was combined with the filtrate, and the AdoHom crystallized by standing at 4°C overnight.

The purity of the compound was tested by paper chromatography using butanol-water-acetic acid (65:34:1), and found to be 95% pure.

Preparation of S-Adenosyl-L-Methionine

AdoMet was prepared by the method of Schlenk and De Palma (53). Yeast cells grown in the presence of methionine were extracted with 4 volumes of 1.5 M perchloric acid for 1 h at room temperature with constant stirring. Cellular debris was removed by centrifugation at 10,000 X G for 10 min and discarded. AdoMet was precipitated from the supernatant fluid by the addition of $\frac{1}{2}$ volume of cold, freshly prepared saturated solution of ammonium reineckate. After storage at 4°C for 8 h, the precipitate was collected by filtration, washed with a small amount of cold water and dissolved in 10-20 volumes of a 1:1 mixture of acetone and 0.5 M H₂SO₄. AdoMet was applied to a column of Dowex 50 (H⁺ form) resin and eluted as described for the preparation of AdoHom. The compound was precipitated with phosphotungstic acid and separated from phosphotungstate as described by the purification of AdoHom. The compound was checked for purity by paper chromatography, and found to contain trace amounts of ultra-violet absorbing impurity.

METHODS

Determination of Protein

The method of Lowry et al. (54) was used to determine the protein concentrations. Samples containing 10 to 60 μg of proteins were brought to a volume of 0.6 ml with distilled water. Three ml of Reagent D (Appendix) was added. The solution was mixed, and allowed to stand at room temperature. After 10 min, 0.3 ml of Folin-Ciocalteu Phenol reagent (0.5 M with respect to H_2SO_4) was added, mixed immediately, and allowed to stand for 30 min at room temperature. The samples were read at 690 nm against a reagent blank in a Coleman Junior spectrophotometer. A standard curve was prepared using Bovine serum albumin, and was found to be linear between concentration of 10 to 60 μg of bovine serum albumin per ml, as shown in Figure 1.

Determination of DNA

The method of Burton (55) was used for the determination of DNA content. A sample containing 10 to 100 μg of DNA was made up to 1.0 ml with a final concentration of 0.5 M perchloric acid. Two ml of diphenylamine reagent (Appendix) was added and the solution was mixed and incubated at 30°C . After 16 h, samples were read against a reagent blank at 600 nm in a Coleman Junior spectrophotometer. A standard curve was prepared from calf thymus DNA, in 0.5 M perchloric acid and was found to be linear between 10 and 100 μg of DNA, as shown on Figure 2.

Figure 1. Standard reference curve for the determination of protein concentration, using bovine serum albumin.

Figure 2. Standard reference curve for
determination of DNA concentration, using calf thymus DNA.

Preparation of Purified Nuclei

Long Evans rats were killed at the desired age, the brains and livers removed, and placed in 0.32 M sucrose containing, 1.0 mM phosphate buffer (pH 7.6) and 1.0 mM MgCl_2 (0.32 M buffered sucrose). The brains and livers were blotted dry on filter paper, weighed, and homogenized in 15 and 10 volumes of 0.32 M buffered sucrose, respectively. The homogenates were filtered through 4 layers of cheese cloth and the crude nuclei pelleted by centrifugation at 800 X G. The nuclei were suspended by homogenization (2 or 3 strokes) in 0.32 M buffered sucrose, and buffered 2.5 M sucrose was added to obtain a 1.77 M solution. Nuclei were recovered by centrifugation at 78,500 X G for 60 min in a Spinco ultracentrifuge.

Preparation of Mitochondria, Microsomes, Cytoplasm and Nucleoplasm

The 800 X G supernatant fluid from above was centrifuged for 15 min at 30,000 X G to pellet the mitochondria, and then at 78,000 X G for 60 min to recover the microsomes. The remaining supernatant fluid was considered the cytoplasm.

Sucrose gradient purified nuclei were lysed with 10 mM Tris-HCl, pH 7.8, in 1.0 mM MgCl_2 . The chromatin was recovered by centrifugation, and the supernatant fluid saved. The chromatin was washed with the above solution, and centrifuged. The supernatant fluids were combined and called nucleoplasm.

Assay of Chromatin-Bound DNA Methyltransferase

The chromatin was sheared in a Virtus homogenizer operated at maximum speed for 15 sec. The standard assay mixture contained 20 mM

Tris-HCl, pH 8.4 (final pH of 8.0), 10 mM MgCl_2 , 80 μg of RNase, and sheared a chromatin (0.5 to 1.0 mg of DNA), in a total volume of 0.5 ml. The assay mixture was equilibrated to a temperature of 37°C , and the reaction initiated with the addition of $[\text{methyl-}^3\text{H}]\text{-AdoMet}$. Incubation was stopped by freezing the reaction mixtures in a dry ice-ethanol bath. The reaction mixtures could be stored at -40°C for 2 weeks, without any loss in activity. The reaction mixtures were thawed, the chromatin collected by centrifugation, and washed in distilled H_2O .

Preparation of Soluble DNA Methyltransferase

The DNA methyltransferase was extracted from the chromatin with 0.4 M NaCl containing 10 mM Tris-HCl (pH 7.8) and 1.0 mM dithiothreitol. After centrifugation, the supernatant fluid was saved and the chromatin was reextracted with buffered saline. The supernatant fluid was collected and pooled with the first extract. The extracts were dialyzed overnight against 100 volumes of 10 mM Tris-HCl, pH 7.8 in 1.0 mM dithiothreitol, with two changes.

Assay of Soluble DNA Methyltransferase

The reaction mixture contained 50 mM Tris-HCl (pH 7.8) 400 μg of *E. Coli* DNA (Appendix), 0.5 mg of RNase, 10 μM $[\text{methyl-}^3\text{H}]\text{-AdoMet}$ (1 Ci/mmol), and 0.15-0.7 mg of crude DNA methyltransferase in a total volume of 0.4 ml. The reaction was initiated by the addition of $[\text{methyl-}^3\text{H}]\text{-AdoMet}$, and stopped by the addition of 0.6 ml of 15% sodium dodecyl sulfate (SDS) in 0.14 M NaCl, and 0.01 M sodium citrate.

The DNA was purified by a modification of the procedure of Kalousek (56), Zamenhof et al. (57), and Lehman (58). After the reaction was stopped with SDS the DNA was precipitated with 2 volumes 95% ethanol, and collected by centrifugation at 20,000 X G for 5 min. The crude DNA was solubilized in 1.0 ml of 1.4 M NaCl by intermittent stirring for 1 h. After centrifugation at 25,000 X G for 10 min, the precipitate was discarded. The DNA was precipitated by the addition of 1.5 volumes of 95% ethanol. The DNA was collected by centrifugation, dissolved in 0.5 ml of 0.5 M NaOH and heated at 60°C for 10 min. The DNA was precipitated by the addition of 15 µl of concentrated HCl and 0.5 ml of 1.0 M perchloric acid. The DNA was collected by centrifugation, and washed twice with 2.5 ml of 0.5 M perchloric acid at a temperature of 0°C. The DNA was hydrolyzed with 2.0 ml of 1.0 M perchloric acid at 70°C for 25 min. One ml of the hydrolysate was placed in 10 ml of a scintillation counting solution (Appendix) for determination of radioactivity, and the remainder was used for the determination of the DNA content. Zero time controls contained up to 10 cpm/ml. One enzyme unit is defined as that amount catalyzing the incorporation of 1.0 pmol of H³-methyl/min at 37°C. Specific activity is expressed as units/mg protein.

DNA Base Analysis

Chromatin, obtained from purified rat liver or brain nuclei, was incubated for 180 min as described above. The DNA was purified as previously described, and 0.1 ml of 70% perchloric acid was added

to 1 mg of DNA. This solution was heated in a boiling water for 30 min with intermittent stirring. The solution was allowed to cool, neutralized (a pH from 5 to 9 was acceptable) with 5M KOH, and centrifuged. The supernatant fluid devoid of black material and salt, was analyzed by paper chromatography using Whatman 3 mm paper. The developing solvent used was isopropanol, concentrated HCl, and water (67:17:18). Ultraviolet light was used to identify the position of the standards and unknown bases.

RESULTS

Cellular Distribution of the DNA Methyltransferase

Different cell fractions were assayed for the presence of DNA methyltransferase activity. This enzyme was found only in the nucleus (Table 1), associated with the chromatin. Nass (29) has shown that mitochondria contain DNA methyltransferase activity in vivo. Nucleoplasm was essentially devoid of enzyme activity, and when added to chromatin it was found to be inhibitory. The degree of inhibition varied from experiment to experiment. This inhibition may be due to the presence of S-adenosyl-L-homocysteine, a known inhibitor of methyltransferase (59-61).

Properties of Chromosomal-Bound DNA Methyltransferase

The pH optimum of DNA methylase was found to be from 7.0 to 8.5 (Figure 3). Outside this pH range, activity dropped off markedly.

The incorporation of ^3H -methyl groups into DNA increased with time in the liver and brain (Figure 4). The rate of DNA methylation in the liver was significantly higher than in the brain. The rate of methylation dropped off rapidly after 5 min in both organs. Saturation of the DNA occurred at approximately 45 and 100 min in the liver and brain, respectively. The further addition of $\text{[methyl-}^3\text{H]AdoMet}$ at this point had no effect on methylation; therefore, all available methylation sites were saturated. The number of available methylation sites in liver DNA were about half the

TABLE 1
CELLULAR DISTRIBUTION OF DNA METHYLTRANSFERASE IN
5-DAY-OLD RAT BRAIN CELLS

Cell Fraction	pmol of ^3H -Methyl/mg DNA/min
Cytoplasm ¹	None
Microsomal ¹	Trace
Nuclei ²	0.15 ± 0.02
Nucleoplasm ¹	Trace
Chromatin ²	0.15 ± 0.02
Chromatin ² + Nucleoplasm	0.13 ± 0.04

¹The reaction mixtures contained 400 μg of *E. coli* DNA, 0.5 mg RNase, 50 mM Tris-HCl buffer, pH 7.8, 10.0 μM S-adenosyl-L-/methyl- ^3H /methionine (1.0 Ci/mmol) and 1.2 mg cytoplasmic protein, or 0.82 mg nucleoplasmic protein or 0.43 mg of microsomal protein in a total volume of 0.4 ml. The reaction was initiated by the addition of AdoMet and terminated after incubating 10 min at 37° by the addition of 15% sodium dodecyl sulfate containing 0.14 M NaCl and 0.01 M sodium citrate. The DNA was purified and radioactivity determined as described under "Materials and Methods".

²The reaction mixtures contained sheared chromatin (0.5 mg DNA) or nuclei, 80 μg RNase, 20 mM Tris-HCl buffer, pH 8.4, 20.0 μM S-adenosyl-L-/methyl- ^3H /methionine (1.0 Ci/mmol) and 1.0 mM MgCl_2 . The final pH of reaction mixture was 8.0. The reaction was terminated after 5 min and the DNA purified as described under "Materials and Methods".

Figure 3. Activity of chromatin-bound DNA methyl-transferase with pH.

Reaction mixture contained sheared chromatin, 80 μ g RNase, 20 mM Tris-HCl, at the designated pH, 10 μ M [α -methyl- 3 H]AdoMet (1.0 Ci/mmol), and 1.0 mM MgCl_2 . The reaction was started by the addition of [α -methyl- 3 H]AdoMet, and stopped after 10 min by freezing in a dry ice-methanol bath. Purification of DNA and determination of radioactivity was carried out as described in "Materials and Methods".

Figure 4. Activity of chromatin-bound DNA methyl-transferase with time.

Reaction mixture contained sheared chromatin from 5-day-old rat brain or liver, 80 μ g RNase, 20 mM Tris-HCl, pH 8.4 (final pH 8.0), 20 μ M $\overline{\text{methyl-}}^3\text{H}/\text{AdoMet}$ (1.0 Ci/mmol) and 1.0 mM MgCl_2 . The reaction was started by the addition of $\overline{\text{methyl-}}^3\text{H}/\text{AdoMet}$, and stopped at various time intervals by freezing in a dry ice-ethanol bath. The DNA was purified and radioactivity determined as described in "Materials and Methods".

number in brain DNA.

After incubation of brain chromatin with $\overline{\text{methyl}}\text{-}^3\text{H}/\text{AdoMet}$ for 180 min, the DNA was purified as previously described, and hydrolyzed. The purine and pyrimidine bases were separated by ascending paper chromatography (Figure 5). The only compound that contained detectable radioactivity was 5-methylcytosine, indicating that cytosine was the only base methylated. Uracil was not detected in the hydrolysate, hence it can be concluded that the sample was devoid of RNA. Although not presented, similar results were obtained using liver chromatin.

The K_m values of the chromosomal-bound enzyme from 5-day-old rats for AdoMet was $4.2 \pm 0.13 \mu\text{M}$ and $3.5 \pm 0.15 \mu\text{M}$, in the brain and liver, respectively (Figures 6 and 7). The V_{max} values were 0.23 ± 0.09 and $0.17 \pm 0.04 \text{ pmol of } ^3\text{H-methyl groups incorporated/mg DNA/min}$, in the brain and liver, respectively. The standard deviations observed resulted from low incorporation of $^3\text{H-methyl}$ groups into DNA during 5 min incubation periods. The incubation time could not be increased, due to the rapid decline in the rate of methylation, after 5 min (Figure 4). The low incorporation of methyl groups into DNA allowed for only an estimate of the inhibition constant (K_i) for AdoHom to be measured (Figure 6). This inhibition appears to be a competitive with a K_i value of $3.5 \pm 1.6 \mu\text{M}$.

The DNA methyltransferase is tightly bound to chromatin. Repeated washes of chromatin with H_2O or a dilute potassium phosphate buffer, pH 7.6, solubilizes only trace amounts of the enzyme. However, 0.45 mg of protein/mg DNA is solubilized from chromatin when it is

Figure 5. Distribution of ^3H -methyl groups in methylated DNA.

Chromatin isolated from the brains of 1-day-old rats, was incubated for 180 min and the DNA purified as previously described. The DNA was hydrolyzed with 0.1 ml of 70% perchloric acid/mg DNA, in a boiling water bath for 30 min. The solution was allowed to cool, the pH adjusted to between 5 and 9 with KOH, and centrifuged. The supernatant fluid was analyzed by ascending paper chromatography, using a developing solvent of isopropanol, concentrated HCl, and H_2O (67:19:18). Ultraviolet light was used to identify the position of the standard, and unknown bases. The amount of radioactivity present was determined by counting each cm of the paper from the base line to the solvent front.

Figure 6. Plots of reciprocal reaction velocity (v) against reciprocal micromolar concentration of $\text{[methyl-}^3\text{H]AdoMet}$ in the absence ($\bullet\text{---}\bullet$) and presence ($\times\text{---}\times$) of 10.0 micromolar S-adenosyl-L-homocysteine.

Reaction mixtures contained sheared chromatin from 5-day-old rat brains, 80 μg RNase, 20 mM Tris-HCl, pH 8.4 (final pH of 8.0), various concentration of $\text{[methyl-}^3\text{H]AdoMet}$ (1 Ci/mmol), and 1 mM MgCl_2 . The reaction was started by the addition of $\text{[methyl-}^3\text{H]AdoMet}$, and stopped after 5 min by freezing in a dry ice-ethanol bath. The DNA was purified and the radioactivity determined as described in "Materials and Methods".

Figure 7. Plots of reciprocal reaction velocity (v) against reciprocal micromolar concentrations of $\overline{\text{methyl-}^3\text{H}}\overline{\text{AdoMet}}$.

Reaction mixtures contained sheared chromatin from 5-day-old rat liver, 80 μg RNase, 20 mM Tris-HCl, pH 8.4 (final pH of 8.0), various concentrations of $\overline{\text{methyl-}^3\text{H}}\overline{\text{AdoMet}}$ (1 Ci/mmol), and 1 mM MgCl_2 . The reaction was started by the addition of the $\overline{\text{methyl-}^3\text{H}}\overline{\text{AdoMet}}$, and stopped after 5 min by freezing in a dry ice-ethanol bath. The DNA was purified and the radioactivity determined as described by "Materials and Methods".

repeatedly washed with H₂O (62).

A fraction of the DNA methyltransferase can be solubilized with NaCl. When 4-day-old rat chromatin was extracted with a 0.4 M NaCl solution, 0.27, 0.12 and 0.022 units of enzyme were solubilized/mg DNA from the liver, cerebrum, and cerebellum, respectively. (A unit is defined as that amount of enzyme catalyzing the incorporation of 1.0 pmol of ³H-methyl/min). Repeated extractions with NaCl solubilized only trace amounts of enzyme. Increasing the concentrations of NaCl was found to solubilize additional chromosomal proteins. However, the DNA was also solubilized.

Properties of Soluble DNA Methyltransferase

Activity of soluble DNA methyltransferase from the liver and brain was measured using E. coli DNA as a methyl acceptor. This enzyme has a pH optimum of 7.0 to 8.5, identical to the bound enzyme. A linear relationship existed between enzyme activity and protein concentration. Activity was linear in the cerebrum and cerebellum from 0.20 to 0.70 mg of protein (Figure 8). Activity of the enzyme from the liver was linear from 0.15 to 0.53 mg of protein.

Incorporation of ³H-methyl groups into E. coli DNA increased with time when the enzyme was prepared from the cerebrum, cerebellum, or liver (Figure 9). Enzyme activity was higher in the liver than in the cerebrum or cerebellum, in 1-day-old rats.

DNA Methyltransferase Activity with Respect to Age

Rats were killed at various ages, and the chromatin assayed for enzymatic activity. The rate of DNA methylation at birth was

Figure 8. Activity of soluble DNA methyltransferase with respect to the concentration of chromosomal proteins derived from cerebrum, cerebellum, or liver of 1-day-old rats.

The reaction mixture contains 400 μ g of *E. coli* DNA, 50 mM Tris-HCl (pH 7.8), 0.5 mg of RNase, 10 μ M α -methyl- 3 H-AdoMet (1 Ci/mmol), and the specified protein concentrations of cerebrum or liver, in a total volume of 0.4 ml. The reaction was started by the addition of α -methyl- 3 H-AdoMet and after 10 min of incubation, the reaction was stopped by the addition of 0.6 ml of 15% sodium dodecyl sulfate, in 0.14 M NaCl, and 0.01 M sodium citrate. The DNA was purified and radioactivity determined as described in "Materials and Methods". The dashed lines represent the protein concentrations containing enzymic activity too low to accurately measure.

Figure 9. Activity of soluble DNA methyltransferase from chromosomal proteins derived from the cerebrum or liver of 1-day-old rats, with respect to time.

The reaction mixture contains 400 μ g of *E. coli* DNA, 50 mM Tris-HCl (pH 7.8), 0.5 mg of RNase, 10 μ M [$\bar{\text{methyl}}\text{-}^3\text{H}\bar{\text{AdoMet}}$] (1.0 Ci/mmol), and 0.20 mg of cerebrum chromosomal proteins or 0.14 mg of liver chromosomal proteins, in a total volume of 0.4 ml. The reaction was started by the addition of [$\bar{\text{methyl}}\text{-}^3\text{H}\bar{\text{AdoMet}}$], and after various amounts of time, was stopped by the addition of 0.6 ml of 15% sodium dodecyl sulfate, in 0.14 M NaCl, and 0.01 M sodium citrate. The DNA was purified and radioactivity determined as described in "Materials and Methods".

markedly higher in the brain than in the liver (Figure 10). A rapid decrease in the rate of methylation was observed in the brain during the first 5 days after birth. The rate of methylation continued to decrease until about 30 days, then remained at a low level throughout the rest of the rat's life. In the liver the rate of methylation decreased gradually over the first 30 days, remaining relatively constant, thereafter, at approximately twice the level in the brain.

From the data presented in Figure 10, it can not be determined whether the decrease in the rate of methylation was caused by a decrease in enzyme concentration, and/or methylation sites available. The number of sites available for methylation was measured by incubating chromatin for 180 min, with $\overline{\text{methyl-}^3\text{H}}\text{AdoMet}$. The extent of methylation decreased rapidly after birth until approximately 8 days, in both the brain and liver (Figure 11). After 2 weeks, no detectable change in the extent of methylation was observed in either organ. The extent of methylation in the brain was almost twice as high as that found in the liver. The rate and extent of methylation in the brain parallel each other closely in different age animals. In contrast, the extent of methylation in liver DNA decreases much more rapidly than the rate of methylation.

Enzyme levels in the brain and liver of different age rats were measured by extracting chromatin with 0.4 M NaCl. One difficulty encountered in these experiments was that not all of the enzyme could be extracted from chromatin with NaCl. Residual DNA methyltransferase activity was measured by incubating the NaCl

Figure 10. Rate of chromosomal-bound DNA methyl-transferase activity with respect to age.

Reaction mixture contains sheared chromatin (0.5 mg DNA), 80 μ g of RNase, 20 mM Tris-HCl, pH 8.4 (final pH 8.0), 20 μ M γ -methyl- 3 H-AdoMet (1.0 Ci/mmol), and 1.0 mM MgCl_2 . The reaction was started by the addition of γ -methyl- 3 H-AdoMet, and stopped after 5 min of incubation by freezing in a dry ice-ethanol bath. The DNA was purified and radioactivity determined as described in "Materials and Methods".

Figure 11. Extent of DNA methylation with age.

Reaction mixture contains sheared chromatin (0.5 mg DNA), 80 μ g of RNase, 20 mM Tris-HCl, pH 8.4 (final pH 8.0), 20 μ M γ -methyl- 3 H-AdoMet (1.0 Ci/mmole), and 1.0 mM of MgCl_2 . The reaction was started by the addition of γ -methyl- 3 H-AdoMet (1 Ci/mmole), and stopped after 180 min of incubation by freezing in a dry ice-ethanol bath. The DNA was purified and radioactivity determined as described in the "Materials and Methods".

extracted chromatin with $\bar{\text{methyl}}\text{-}^3\text{H}/\text{AdoMet}$. A 30% decrease in enzyme activity was observed in chromatin prepared from the cerebrum or cerebellum. Salt extracts from liver chromatin always contained more enzyme activity than those prepared from either the cerebrum or cerebellum chromatin; however no detectable decrease in chromosomal activity was observed after the liver chromatin was extracted with NaCl.

The amount of soluble enzyme from the cerebellum increased from 0.13 units/mg protein at birth to 0.29 units/mg protein in 10-day-old rats (Figure 12). From 10 to 30 days after birth, the amount of extractable enzyme decreased to 0.06 units/mg protein. No detectable change in enzyme level occurred between 30 and 700-day-old rats. In contrast, the level of enzyme in the cerebrum decreased from 0.13 units/mg protein at birth to 0.06 units/mg protein in 30-day-old rats. After 30 days, no detectable change in the level was observed.

The amount of extractable enzyme in the liver increased from 0.45 units/mg protein at birth to 0.6 units/mg protein in 17-day-old rats (Figure 13). The enzyme level then decreased to about 0.1 units/mg protein at 100 days. After 100 days, the enzyme level increased up to approximately 300 days, remaining at that level, thereafter.

Figure 12. Activity of soluble DNA methyltransferase from the cerebrum or cerebellum of various age rats.

Reaction mixtures contained 400 μ g of *E. coli* DNA, 50 mM Tris-HCl (pH 7.8), 0.5 mg RNase, 10 μ M $\overline{\text{methyl-}}^3\text{H}\overline{\text{AdoMet}}$ (1.0 Ci/mmol), and 0.20-0.50 mg protein from the cerebrum or cerebellum, in a total volume of 0.4 ml. The reaction was started by the addition of $\overline{\text{methyl-}}^3\text{H}\overline{\text{AdoMet}}$, and stopped in 10 min by the addition of 0.6 ml of 15% sodium dodecyl sulfate, in 0.14 NaCl, and 0.01 M sodium citrate. The DNA was purified and the radioactivity determined as described in "Materials and Methods".

Figure 13. Activity of soluble DNA methyltransferase from the liver of various age rats.

Reaction mixtures contained 400 μ g of E. coli DNA, 50 mM Tris-HCl (pH 7.8), 0.5 mg RNase, 10 μ M [methyl-³H]AdoMet (1.0 Ci/mmol), and 0.20-0.60 mg of protein from the liver, in a total volume of 0.4 ml. The reaction was started by the addition of [methyl-³H]AdoMet, and stopped in 10 min by the addition of 0.6 ml of 15% sodium dodecyl sulfate, in 0.14 M NaCl, and 0.01 M sodium citrate. The DNA was purified and the radioactivity determined as described in "Materials and Methods".

DISCUSSION

One of the major difficulties in studies on the in vitro methylation of DNA is obtaining a suitable methyl acceptor. Tissue culture experiments indicate that the majority of the sites are methylated within two min after the synthesis of DNA, with methylation still detectable two h after DNA replication (31,36-40). It has also been shown that these methyl groups do not turn over (31). Consequently, DNA becomes saturated with methyl groups within a short period of time after DNA synthesis, allowing for only a limited number of methyl sites available for in vitro studies. Chromatin isolated from rat brain or liver nuclei accept a limited number of methyl groups from $\overline{\text{methyl-}^3\text{H}}\overline{\text{AdoMet}}$ (Figure 4). Brain DNA appears to contain more unmethylated sites than liver DNA (Figure 11).

When rat brain or liver chromatin was incubated with isotopically labelled AdoMet, the only detectable product was 5-methylcytosine. These results are similar to those reported for all higher eukaryotic cells (27-29).

The DNA methyltransferase is tightly bound to the chromatin derived from liver or brain cells. Repeated extraction with water or dilute potassium phosphate buffer failed to solubilize significant amounts of enzyme. Such procedures do release from 35-45% of the chromosomal proteins, including the histone methyltransferase (62).

Extraction of the chromatin with 0.4 M NaCl released measurable amounts of the DNA methyltransferase. However, measurable amounts of this enzyme were still associated with the NaCl-extracted chromatin. Increasing the concentration of NaCl resulted in the release of more enzyme, with the solubilization of the DNA, also.

The K_m value of the liver chromatin-bound DNA methyltransferase for AdoMet is $3.5 \pm 0.15 \mu\text{M}$. A similar value was obtained with the brain enzyme. A three-fold lower K_m value of $1.33 \mu\text{M}$ for AdoMet with the liver DNA methyltransferase previously has been reported by Cox *et al.* (43). Our reaction conditions appear to be very similar to that of Cox *et al.*, yet a V_{max} of $2.7 \text{ pmol } ^3\text{H-methyl incorporated/mg DNA/min}$ was reported by Cox *et al.* This value is more than 10 times higher than the value we obtained ($0.17 \text{ pmol of } ^3\text{H-methyl incorporated/mg DNA/min}$). A possible explanation for the differences in results could be the method used to isolate the DNA after chromatin had been incubated with $[\text{methyl-}^3\text{H}]\text{AdoMet}$. Research in our laboratory and by Kolousek and Morris (56), has shown that different methods used to isolate the DNA can cause variation in results. Histones (57,58), non-histone chromosomal protein (58), and transfer RNA (13), have been shown to accept methyl groups from AdoMet. Contamination of purified DNA with $[\text{methyl-}^3\text{H}]$ histone, $[\text{methyl-}^3\text{H}]$ non-histone chromosomal proteins, and $[\text{methyl-}^3\text{H}]$ transfer RNA, would give erroneously high values. The method used in our laboratory to isolate DNA from chromatin has been tested for possible contaminants. Our purified DNA was found to be devoid of RNA (Figure 5). After HeLa cells were grown in the presence of $[\text{methyl-}^3\text{H}]$ -

thymidine, and L- $[\text{I}^{14}\text{C}]$ arginine, the DNA was purified as previously described under "Materials and Methods", and found to be free of $[\text{I}^{14}\text{C}]$ protein.

When the rate of the DNA methyltransferase was measured in rats of varying ages a decrease in activity was observed over the first 20 days of life (Figure 10). The apparent decrease in methylation of brain and liver DNA with age could be due to a decrease in the level of the DNA methyltransferase and/or the availability of sites on the DNA to accept methyl groups. To delineate between either of these possibilities, the number of available sites and the level of the enzyme, were measured.

The DNA methyltransferase was considerably more active in extracts prepared from liver and brain chromatin of young rats. There also was a significant difference in the ability of DNA prepared from brain and liver chromatin of various age rats to accept methyl groups. DNA prepared from the brain of young rats was a much better methyl acceptor than that from old rats. Similar results were found in the liver, but the difference between young and old rats was not as significant as in the brain. Therefore, the decrease in methylation in chromatin derived from the brain and liver with age, was due to a decrease in methyl acceptors and a decrease in the level of enzyme on the chromatin.

The number of sites available for DNA methylation correlates well with the rate of DNA synthesis in these organs. Cellular proliferation in the cerebellum continues postnatally until approximately 16 days (48,49). In contrast, cerebrum cell

proliferation is nearly complete at birth, with only a few cells in the hippocampal dentate gyrus and in the granular layers of the olfactory still undergoing replication up to 6 days after birth. In the liver, cell proliferation declines exponentially after birth (45).

Massie et al. (47) found that rat liver duplex DNA has an increased tendency to become single stranded with increasing age. DNA synthesis, as measured by $[^3\text{H}]$ -thymidine incorporation, was found to increase beyond 125 days (46). Interestingly, the DNA methyltransferase was found to increase beyond 125 days. Apparently the synthesis of DNA methyltransferase is closely coupled to DNA synthesis. It is postulated that the newly incorporated cytosine bases are rapidly methylated by the newly formed DNA methyltransferase. The synthesis of this enzyme may be controlled at the transcriptional level by a regulator gene that also controls DNA synthesis. The genome for the DNA methyltransferase may be located in an operon that also contains genomes of the enzymes and factors required for DNA replication.

The concentration of S-adenosyl-L-methionine, one of the substrates for the DNA methyltransferase, was measured in the cells of the brain and liver. The liver contains approximately 70 nmol of AdoMet/g wet tissue throughout life (63). The level of AdoMet decreased in the cerebrum at birth from 45 nmol/g wet tissue to 20 nmol/g wet tissue at 50 days, remaining at this level thereafter. AdoMet increased in the cerebellum at birth, from 50 nmol/g wet tissue to 60 nmol/g wet tissue at 10 days. This compound decreased to 25 nmol/g wet tissue at 30 days, and was found at this level in adult rats.

It can be concluded, from the K_m value of this enzyme for AdoMet, and the μM concentration of AdoMet in the cells, that the DNA methyltransferase is always saturated with AdoMet.

S-Adenosyl-L-homocysteine, a product of the methylation reaction, inhibits many methyltransferases (59-61), and has been proposed to be a bioregulatory molecule (59,60). The level of this compound in the liver was 5 to 10 nmol/g wet tissue throughout the life of the rat (63). AdoHom also remained constant in the cerebrum and cerebellum, at about 1 nmol/g wet tissue. The ratio of AdoMet/AdoHom was never below 8 to 1, throughout the age of the rat in both the liver and brain. A ratio of AdoMet/AdoHom of 4 to 1 did not affect the rate of DNA methylation. It is doubtful that under normal conditions AdoHom would have any affect on DNA methylation.

Several theories concerned with the mechanism of cell differentiation have been proposed, based on observations concerning methylation during differentiation. Scarano, et al. (2) postulated that eukaryotic DNA is organized into units of ontogenic information called synchrons. A synchron can be located on different chromosomes, and is composed of 3 types of genes; the development programming gene (DPG), promoter gene, and structural gene. The DPG controls the synchron, coding for a DNA modifying enzyme, possibly a DNA methyltransferase. The RNA polymerase must bind to the promoter region, preceding each structural gene before that structural gene can be transcribed. Structural genes code for the enzymes required by that particular cell, or can induce further differentiation. After the promoter gene is modified by the DPG enzyme, the RNA polymerase

binds, the transcribes the structural genes. By induction of the next DPG gene in another synchron, ontogeny is continued in that cell.

Another theory of cell differentiation, involving methylation, proposes that an operator or promoter site exists in 2 alternative states (3). With a shift in a base pair at the appropriate site, the RNA polymerase can bind and transcribe the gene. The shift in states is due to an A-T to G-C base pair change, or vice versa, mediated by enzymes. Removal of an amino group at the 6-position of adenine produces inosine. After cell replication, inosine pairs with cytosine, and with another round of DNA synthesis, the original A-T pair is changed to G-C. A base pair change in the opposite direction is started with the methylation of cytosine to 5-methyl-cytosine. After removal of the amino group from the 6 position, thymine is produced. The G-C to A-T shift can also be completed by removal of the amino group from the 6 position of cytosine, producing uridine. After 2 rounds of replication, the shift is completed. A change in base pairs at the promoter, or operator site of a gene, is theorized to turn on the synthesis of an enzyme. The first enzymes made in ontologic development of a cell could be another methylase that induces a certain set of enzymes by modifying a specific nucleotide sequence. The strongest evidence supporting the idea that a change in DNA composition occurs in differentiation comes from the use of a thymidine analog, bromodeoxyuridine (64). It has been shown that this analog inhibits the formation of hemoglobin in erythroblasts. The same concentration of bromodeoxyuridine does not

inhibit transcription or translation of hemoglobin, in cells that have already started to produce hemoglobin. No effect of bromodeoxyuridine is seen when excess thymidine is added at the same time. It is proposed that the effect of this analog comes about by the substitution of a bromine atom for the methyl group on 5 position of thymine, preventing the normal modifications of controlling sequences during development (64).

A third theory involving DNA methylation in the control of eukaryotic cell differentiation is based on a modification-restriction system similar to that found in bacteria (4). Silencing of genetic information during differentiation has been observed in heterochromatization, chromosomal elimination in insects, and X-chromosome inactivation in placental mammals. The turning off of genetic material is postulated to be carried out by modification-restriction enzyme(s) that either protect against, or stimulate, the inactivation or elimination of genetic material. Support of this theory comes from studies involving inheritance of chloroplast DNA in Chlamydomonas. In this genome, the chloroplast genome from the mt^+ , female parent, is transmitted to all progeny in sexual crosses, where the mt^- , male genome is not transmitted. Radioactive labelling of each parental DNA before mating, allows for the monitoring of the male and female DNA to be followed in the zygote. The total DNA from the zygote was isolated at varying time points after mating, and examined in a CsCl gradient. It was found that the mt^+ female DNA undergoes a density shift from 1.695 g/cm^3 to 1.690 g/cm^3 within 6 h after zygote formation. In this same period of time, the mt^- genome for the

chloroplast disappears. It is posulated that the density shift observed in the mt^+ DNA, occurs with methylation, protecting the DNA from endonuclease attack. It is estimated that 5% methylation of the mt^+ DNA could account in a density shift change of 0.005 g/cm^3 .

Summary

The properties of DNA methyltransferase were investigated in the liver and brain from various age rats. The enzyme appears to be firmly bound to chromatin. When purified chromatin from 5-day-old rat brain or liver cells was incubated with S-adenosyl-L-[methyl- ^3H]-methionine, 5-[methyl- ^3H]methylcytosine was the only product of DNA methylation. The enzyme from brain or liver has a broad pH optimum from 7.0-8.5. The K_m value of the chromosomal-bound DNA methyltransferase for S-adenosyl-L-methionine was $3.5 \pm 0.15 \mu\text{M}$ and $4.2 \pm 0.13 \mu\text{M}$ in the liver and brain, respectively. The rate and extent of the reaction catalyzed by the chromosomal-bound enzyme was dependent on the age of the rat and the type of cell from which the chromatin was isolated. The $V_{\max}$ values for the chromosomal-bound enzyme with 5-day-old rat liver and brain DNA as methyl acceptor were 0.17 ± 0.04 and $0.23 \pm 0.09 \text{ pmol } ^3\text{H-methyl incorporated/mg DNA/min}$, respectively. S-Adenosyl-L-homocysteine, one of the products of the reaction, was a competitive inhibitor with respect to S-adenosyl-L-methionine. The K_i value for S-adenosyl-L-homocystine was $3.5 \pm 1.6 \mu\text{M}$ with brain DNA as methyl acceptor. The enzyme could be eluted from chromatin with 0.4 M NaCl; however, measurable amounts of the enzyme remained bound to chromatin. The properties of the soluble DNA methyltransferase were similar to those of the bound enzyme. The specific activity of DNA methyltransferase and the number of unmethylated sites on DNA

correlated well with the rate of DNA synthesis in these organs. The synthesis of DNA methyltransferase appears to be coupled to DNA synthesis.

APPENDIX

BaCO₃ Synthesis

1. Dissolve 37.5 gm of BaOH in 500 ml of H₂O. Heat the solution until it almost boils.
2. Add ry ice to BaOH solution until the pH if 6.5.

Determination of Protein (LOWRY)Reagent A

2% Na₂CO₃ in 0.1N NaOH

Reagent B

1.0% CaSO₄·5H₂O in water

Reagent C

2.0% Na or K tartrate in water

Reagent D

Reagent A - 50 ml

Reagent B - 0.5 ml

Reagent C - 0.5 ml

Diphenylamine Reagent

1.5 gm diphenylamine

100 ml concentrated acetic acid

1.5 ml concentrated sulfuric acid

Preparation of *E. coli* DNA

Add 9 ml of 0.01 M sodium citrate in 9 g of frozen *E. coli* cell paste, and stir until cells are suspended in the solution. Add 90 ml of 15% sodium dodecyl sulfate in 0.14M NaCl:0.01 M sodium citrate. Homogenize the solution until the *E. coli* cells are lysed.

Slowly add 2 volumes of absolute ethanol, stirring constantly. Collect the resultant crude DNA by centrifugation. Using a glass homogenizer, blend the DNA in 1.5 volumes of 1.4 M NaCl and centrifugation at 25,000 X G for 10 min. Precipitate the purified DNA from the supernatant fluid by slowly adding 2 volumes of absolute ethanol, while stirring the solution with a glass stirring rod. The DNA was dried under a vacuum before it was weighed.

Scintillation Counting Solution

To make 1 liter:

4 g PPO

100 mg POPOP

333 ml TRITON X-100

666 ml Toluene

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