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S-adenosylmethionine: DNA-cytosine 5-methyltransferase from a Novikoff rat hepatoma cell line

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ABSTRACT

Partial purification of DNA methylase from Novikoff rat hepatoma cells is described. Contamination with other proteins persists although the enzyme preparation has a high specific activity and is purified 980-fold over homogenate activity. Evidence suggests, but does not prove, that there may be more than one species of DNA methylase in these cells. The enzyme has two broad pH optima at pH 7.0 and 7.5 and most readily methylates heterologous denatured DNAs although complex reaction kinetics indicate that native DNAs may eventually be methylated to an equal or greater level. The preparation of undermethylated DNA from Novikoff cells is also described. Undermethylated homologous DNA is an 85-fold greater acceptor of methyl groups than fully methylated Novikoff cell DNA. In contrast to other DNA substrates, the enzyme preparation methylates native undermethylated homologous DNA at a 3.5-fold greater rate than denatured undermethylated homologous DNA.

INTRODUCTION

Transmethylation of base residues in polymeric DNA has been recognized for almost fifteen years[5,6]. Except for the DNA modification restriction phenomenon in prokaryotes[7] the biological functions served by the process are not known. *In vivo* approaches to the study of eukaryotic DNA methylation have been limited since the methyl donor in the reaction, S-adenosylmethionine, is involved in many vital transmethylation reactions and since no specific inhibitors of the DNA transmethylation reaction are yet known. If a eukaryotic DNA methylase could be purified to homogeneity, the *in vitro* study of its properties would be of interest *per se* and could lead to the development of inhibitors specifically directed against the DNA methylation reaction. Earlier workers studied the *in vitro* reaction using crude chromatin[8] or isolated nuclear systems[9,10]. Sheid *et al.*[11] and Morris and coworkers[12-14] reported the partial purification of DNA methylase from mammalian and plant sources. Drahovsky and Morris have noted some *in vitro* DNA methylation properties of an ammonium sulfate fraction from regenerating rat liver[15-17].

All studies to date, however, used quite heterogeneous enzyme preparations that make interpretation of *in vitro* results somewhat difficult. Therefore, we have attempted to purify to homogeneity DNA methylase from a Novikoff rat hepatoma cell line. This enzyme source was chosen since prior work from this laboratory established the DNA methylation pattern in this organism[18,19] and since preliminary studies (see below) indicate that homologous Novikoff cell DNA non-methylated in at least one strand might be obtainable for enzyme mechanism studies. This report is an extension of studies detailed elsewhere[20].

MATERIALS AND METHODS

A cloned line of Novikoff rat hepatoma cells (N1-S1) was grown in suspension culture as previously described[21]. Possible microbial, fungal or *Mycoplasma* sp. contamination was routinely monitored. Cells grown for enzyme preparation were harvested at mid-log phase (1.5×10^6 cells/ml) to maximize DNA methylase yield; late-log or early stationary phase cells had significantly less enzyme activity which was much more difficult to dissociate from chromatin.

(Methyl-^{3}H)-2'-deoxythymidine (6.7 Ci/mMole) was from New England Nuclear Co. S-adenosyl-L-(methyl-^{3}H)methionine (5 to 9.5 Ci per mMole) was from Amersham-Searle Co.; paper chromatographic analyses gave assurance that the product contained negligible S-adenosylhomocysteine. Unlabeled S-adenosylmethionine, 5-bromo-2'-deoxyuridine, ethionine, nuclease-free Pronase, and highly polymerized DNA from calf thymus and *E. coli* were all products of CalBiochem, Inc. Pancreatic DNAase (E.C. 3.1.4.5.) was from Miles Labs. The homopolymer duplex $d(G)_n:d(C)_n$ was from Miles Labs. whereas the defined copolymer duplex $d(G\text{-}C)_n:d(G\text{-}C)_n$ was the generous gift of Dr. R. Wells of the University of Wisconsin. Highly polymerized DNA was obtained from Novikoff hepatoma cells by a previously described technique[18]. Routine enzyme assays initially used highly polymerized *Micrococcus luteus* DNA from Miles Labs. which had been further treated with RNAase and deproteinized and precipitated from isopropanol repeatedly before use. Subsequently, *M.luteus* DNA was prepared in this laboratory from frozen *M.luteus* cells (A.T.C.C. 4698) from P-L Labs., Milwaukee, Wisconsin.

In vitro DNA methylation was assayed as follows: 0.1 ml of S-adenosyl-L-(methyl-^{3}H)methionine (2 uCi) and 0.1 ml of unlabeled S-adenosylmethionine (840 pmoles) were mixed with 0.1 ml of Tris-Cl buffer (pH 8.0) of sufficient strength to make the final Tris concentration 0.143 Molar

(including the Tris buffer added in the enzyme fraction); 60 ug of M. luteus DNA (heat-denatured at 100°C for 20 mins. then quick-cooled) was added in 0.2 ml of 0.015 M NaCl-0.0015 M trisodium citrate (pH 7.0); enzyme was added in 0.1 ml in Tris buffers (pH 8.0) of varying strengths and containing 0.005 M dithiothreitol; final reaction volume was 0.6 ml. After 30 or 60 mins. incubation at 37°C, 0.1 ml of nuclease-free Pronase (2mg/ml H_2O) was added and the tube reincubated 20 mins. at 60°C. Following Pronase-treatment, 1.0 ml each of 0.075 M sodium pyrophosphate and a carrier salmon sperm DNA-bovine serum albumin solution (1.0 mg each per ml H_2) were added followed by 3 ml of cold 10% (w/v) trichloracetic acid (TCA). After centrifugation and a cold 5% TCA wash, the pellet was redissolved in 0.5 ml of 5 N NaOH, diluted to 5 ml with H_2O, incubated 20 mins. at 60°C, chilled, precipitated with 1.0 ml of cold 70% TCA and washed with cold 5% TCA. The pellet was then ground into a fine suspension in 0.7 ml of 5% TCA, heated at 100° for 20 mins., chilled, centrifuged, and a 0.5 ml aliquot of the supernatant added to 15 ml of Bray's counting fluid for liquid scintillation spectrometry at a tritium efficiency of 31%. Control studies verified that this procedure measured solely DNA methylation: formic acid hydrolysis and two dimensional paper chromatographic analysis[21] of the reaction product showed all of the labeled material to be 5-methylcytosine. About 90% of the labeled material was recovered as 5-methyl-2'-deoxycytidine after enzymatic hydrolysis of the reaction product and one dimensional paper chromatography using a borate buffer system[22]..

DEAE-cellulose was DE-52 from Whatman Biochemicals. 0.9 X 15 cm columns were prepared by gravity settling and rinsed with at least ten column volumes of buffer (0.05 M Tris-Cl (pH 8.0)-0.005 M EDTA-0.005 M dithiothreitol) prior to sample adsorption. Columns were developed using a linear salt gradient from 0 to 0.25 M in NaCl made with column equilibration buffer; total gradient volume was 100 ml; average flow rates were 0.3 ml/min. Agarose gel column chromatography was performed using BioGel A-1.5M (100-200 mesh) from BioRad Labs. After equilibration of the gel in 0.05 M Tris-Cl (pH 8)-0.005 M EDTA-0.005 M dithiothreitol, 0.9 X 15 cm or larger columns were prepared by gravity settling; the columns were developed by reverse flow using a Mariotte flask containing the running buffer and at an average flow rate not exceeding 0.1 ml/min.

Polyacrylamide gel electrophoresis was performed with 6% gels cast in 0.37 M Tris-Cl buffer (pH 8.8); the electrode buffer was 0.05 M sodium borate (pH 9.2)[23]; electrophoresis was carried out at 3.5 milliamps per

tube at 4°C. The gels were subsequently cut into 1.0 mm slices; each slice was homogenized in 0.2 ml of 0.5 M Tris-Cl buffer (pH 8), allowed to stand overnight at 4°C, centrifuged, and an aliquot of the supernatant assayed for DNA methylase activity. SDS-polyacrylamide gel electrophoresis was also performed[24].

RESULTS

Partial purification of enzyme: As previously reported[20] our first efforts to purify DNA methylase from Novikoff rat hepatoma cells met with an initial inability to solubilize enzyme activity from purified nuclei, inactivation of enzyme activity when using streptomycin sulfate to remove free nucleic acids, and an irreversible loss of enzyme activity when enzymatically active ammonium sulfate fractions were transferred to low ionic strength media. These problems have been overcome by the following method: for a single enzyme preparation twenty 400 ml suspension culture flasks at mid-log phase were harvested by centrifugation, the cell pellet rinsed once in isotonic NaCl, and resuspended in 65 ml of 0.01 M Tris-Cl (pH 7.5)-0.01 M NaCl-0.0015 M $MgCl_2$ (TSM buffer). The cells were swollen in this hypotonic medium for 30 mins. then homogenized with 100 strokes of a Teflon:glass Potter-Elvehjem homogenizer. The homogenate was made 0.5% (w/v) in Triton X-100 and the nuclei harvested by centrifugation. The nuclear pellet was rinsed once in TSM, resuspended in 30 ml of 0.5 M Tris-Cl (pH 8)-0.005 M dithiothreitol, and subjected to ten 5 second treatments at 4°C with a Polytron (type PT10; Kinematica GMBH, Lucerne, Switzerland) at full voltage setting. Following centrifugation at 50,000 X g for 30 mins. at 4°C, the supernatant (termed nuclear supernatant) was decanted and stirred for 20 mins. at 4°C with 15 ml of a 1:1 slurry of DE-52 in 0.5 M Tris-Cl (pH 8)-0.005 M dithiothreitol to remove free nucleic acids. At this ionic strength DNA methylase activity does not adsorb to the DEAE-cellulose. The DEAE-cellulose was removed by centrifugation and the resulting supernatant (termed DEAE pass) subjected to ammonium sulfate precipitation. 75% of the enzyme activity precipitates between 30 to 40% saturation and 20% between 40 to 50% saturation. For routine preparations a 25 to 50% saturation cut containing approximately 95% of the enzyme activity was employed.

Aggregation of enzyme activity in ammonium sulfate fraction: Although the enzyme activity in the 25 to 50% ammonium sulfate cut could be solubilized in 0.5 M Tris-Cl (pH 8)-0.005 M dithiothreitol, any attempt to lower the ionic strength of this medium prior to ion exchange chromatography resulted in irreversible loss of about 70% of the enzyme activity. Since one expla-

nation for this result was that tightly bound DNA was copurified with the enzyme, an attempt was made to dissociate this putative complex by preincubation of the solubilized 25 to 50% cut at 37 or 42°C and an ionic strength of 0.15 M salt (the ionic strength of the enzyme assay reaction mixture) and in the presence of unlabeled S-adenosylmethionine. Subsequent lowering of the ionic strength to 0.05 M no longer resulted in loss of enzyme activity but when the mixture was adsorbed onto a DEAE-cellulose column and eluted by a linear salt gradient (0 to 1.0 M NaCl), at least five peaks of enzyme activity were resolved. When each peak of enzyme activity from the DEAE-cellulose column was separately analyzed on agarose gel columns (BioGel A-15M), multiple species of enzyme activity were noted.

DNAase-treatment of the ammonium sulfate fraction: The plethora of enzymatically active peaks referred to above could represent a multiplicity of DNA methylases or differing states of association of enzymatically active subunits of DNA methylase or the incomplete dissociation of enzyme from variously sized fragments of copurified DNA. To test the last possibility, the 25 to 50% cut was redissolved in 0.5 M Tris-Cl (pH 8)-0.005 M dithiothreitol and mixed with 0.1 volumes of a pancreatic DNAase-$MgCl_2$ solution (550 units and 25.4 mg, respectively, per ml H_2O). Since the DNAase was only slightly active at this ionic strength and since the DNA methylase activity was lost if the ionic strength was lowered abruptly, the ionic strength of the mixture was step-wise lowered to 0.2M in Tris by the addition of H_2O over a two hour period. The mixture was incubated at 25°C for two additional hours followed by an 18 hour dialysis at 4°C against two 100 volume changes of 0.05 M Tris-Cl (pH 8)-0.005 M EDTA-0.005 M dithiothreitol. (The removal of Mg^{++} and the subsequent presence of EDTA totally inhibited DNAase activity such that substrate DNA in enzyme assay reaction mixtures was not degraded. EDTA alone at 0.001 M in the assay mixture had no effect on DNA methylase activity.)

Upon centrifugation of the retentate at 50,000 X g for 15 min., most enzyme activity was recovered in the supernatant. The supernatant was adsorbed to DEAE-cellulose and the column developed with a linear 0 to 0.25 M NaCl gradient. All enzymatic activity adsorbs to the DEAE-cellulose and is eluted at low ionic strength; very little enzymatic activity is associated with the bulk of the protein that is either not adsorbed or else elutes at higher ionic strength (Figure 1 and Table 1).

The enzymatically active fractions from the DEAE-cellulose column were pooled and precipitated by 90% saturation with ammonium sulfate. The precipitate was redissolved in 0.05 M Tris-Cl (pH 8)-0.005 M EDTA-0.005 M dithiothreitol

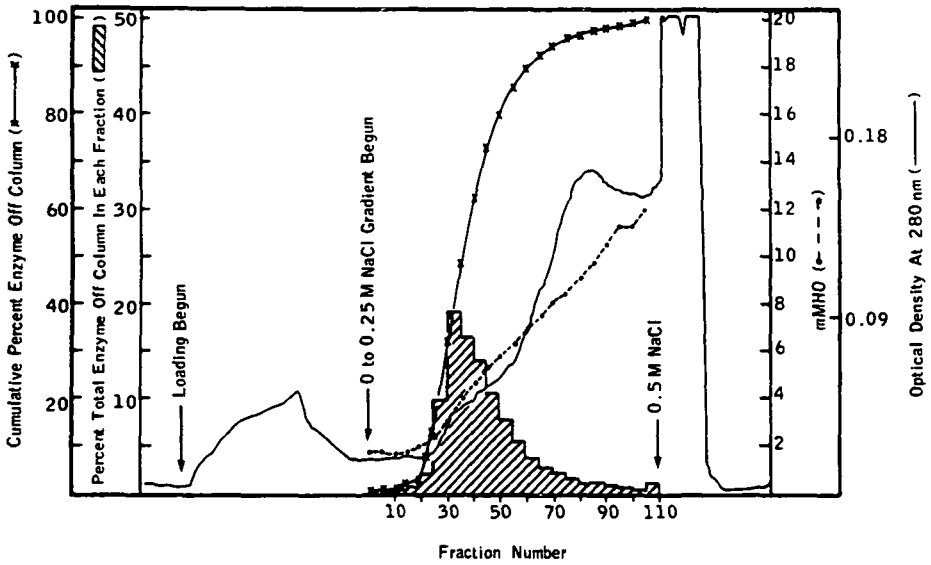


Figure 1. DEAE-cellulose chromatogram of the DNAase-treated 25 to 50% ammonium sulfate cut. See text for details. (——), optical density of effluent at 280 nm; (•---•), conductivity of effluent in millimhos; (x——x), cumulative percent of enzyme activity off column; cross-hatched bars, percent of total enzyme activity off column found in 5-tube pooled fractions.

Fraction	pmoles CH_3 transferred/mg protein per hour	Relative enzyme activity	% of homogenate activity
Homogenate	2.7	1.0	100.%
Nuclear supernatant	13.9	5.2	41.8
DEAE pass	22.3	8.3	63.7
25 to 50% cut	43.7	16.2	81.0
DNAase treatment:			
Pre-DEAE column	40.1	14.9	78.5
Not adsorbed to DEAE	10.0	3.7	4.5
Eluted at low ionic strength	144.6	53.7	43.8
Eluted at high ionic strength	0.5	0.2	0.6
Dialyzed 0 to 90% low ionic strength DEAE fractions	444.8	166.1	100.*
Supernatant from dialyzed 0 to 90% cut after centrifugation	2653.0	984.4	86.8*

Table 1. Enzyme activity comparison at different stages of purification. See text for details. (*) recovery data here are related to the enzyme activity pre- and post-centrifugation.

and dialyzed 18 hours at 4°C against two 100 volume changes of the same buffer. After centrifugation of the turbid retentate at 50,000 X g for 10 mins., 85% of the protein was found in the precipitate but almost all the enzyme activity remained in the supernatant resulting in a large increase in relative specific enzyme activity (Table 2).

DNA source	Heated ?	dpm/assay/60'	pmoles/mg DNA/60'
M luteus	no	8680	40.27
	yes	17270	80.09
E coli	no	1500.	2.08
	yes	8530.	11 87
Calf thymus	no	500	0.70
	yes	990	1 37
Mid-log Novikoff	no	260.	0.36
	yes	485	0.67
Synchronized Novikoff + methionine	no	170	0.79
	yes	295.	1.37
Synchronized Novikoff + ethionine	no	5210	30.75
	yes	1510.	8.91
$d(G)_n.d(C)_n$	no	32.	0.31*
	yes	148.	1.40*
$d(G-C)_n:d(G-C)_n$	no	840	6.14*
	yes	1610.	11.81*

Table 2. Methyl acceptance activity of various nucleic acid substrates using the 90% ammonium sulfate cut of the enzymatically active fractions from the agarose column shown in Figure 4. All incubations carried out for 30 mins. at 37°C and equivalent ionic strengths. Levels of nucleic acids used per assay: M.luteus, 60 ug; E.coli, 200 ug; calf thymus, 200 ug; DNA from mid-log phase Novikoff cells, 200 ug; DNA from synchronized Novikoff cells grown in methionine, 60 ug, DNA from synchronized Novikoff cells grown in ethionine, 47 ug; $d(G)_n:d(C)_n$, 0 03 OD units at 260 nm; $d(G-C)_n:d(G-C)_n$, 0.038 OD units at 260 nm. Heat-treatments were at 100°C for 20 mins. in 0.015 M NaCl-0.0015 M trisodium citrate (pH 7) followed by quick-cooling in an ice:water bath. The (*) refers to the fact that these data are expressed as pmoles transferred per OD unit per hour

The supernatant resulting from centrifugation of the dialyzed 0 to 90% cut of the low ionic strength DEAE fractions was analyzed on an agarose gel column (BioGel A-1.5M; 0.9 X 15 cm) using 0.05 M Tris-Cl (pH 8)-0.005 M EDTA-0.005 M dithiothreitol as the eluting buffer. The bulk of the enzyme activity elutes at or very close to the excluded volume of the gel column and two minor trailing peaks are observed (Figure 2).

The enzymatically active fractions from the agarose column were precipitated by 0 to 90% saturation with ammonium sulfate, redissolved in 0.05 M Tris-Cl (pH 8)-0.005 M EDTA-0.005 M dithiothreitol, and dialyzed 18 hrs. at 4°C against two changes of 100 volumes each of the same buffer. Aliquots of the centrifuged retentate were analyzed by polyacrylamide gel electrophor-

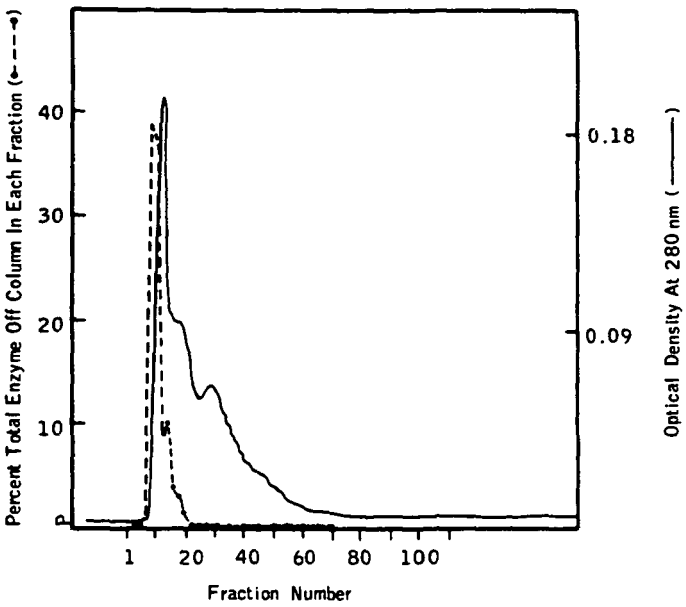


Figure 2. Agarose gel (BioGel A-1.5M) filtration of the supernatant resulting from centrifugation of the dialyzed 0 to 90% cut of the low ionic strength DEAE fractions. (——), optical density of effluent at 280 nm; (•---•), enzyme activity expressed as the percent of total enzyme activity off the column in each fraction.

esis using 6% gels under non-dissociating conditions and 6% SDS-gels. A "native" gel and a SDS-gel were stained and destained to localize polypeptide bands. A companion "native" gel was sliced, each slice eluted, and each eluate assayed for DNA methylase activity. As seen in Figure 3, three peaks of enzyme activity were detected. The companion stained electropherogram showed at least five stained bands which are represented at the top of Figure 3. By qualitative measure over 50% of the material loaded onto the "native" gel did not enter the gel yet *in vitro* assay of the first few slices containing this presumed high molecular weight material showed no detectable DNA methylase activity. All of the sample entered the SDS gel; there are at least thirty-four stained bands (Figure 4). Although molecular weight standards have been run on the "native" gel, the calculated values of about 300,000, 120,000, and 45,000 Daltons for the three peaks of enzyme activity are only approximations since a nondissociating gel was involved and the molecular weight standardization was not performed on the same gel as shown in Figure 3.

Some properties of the still heterogeneous enzyme: Since even the very

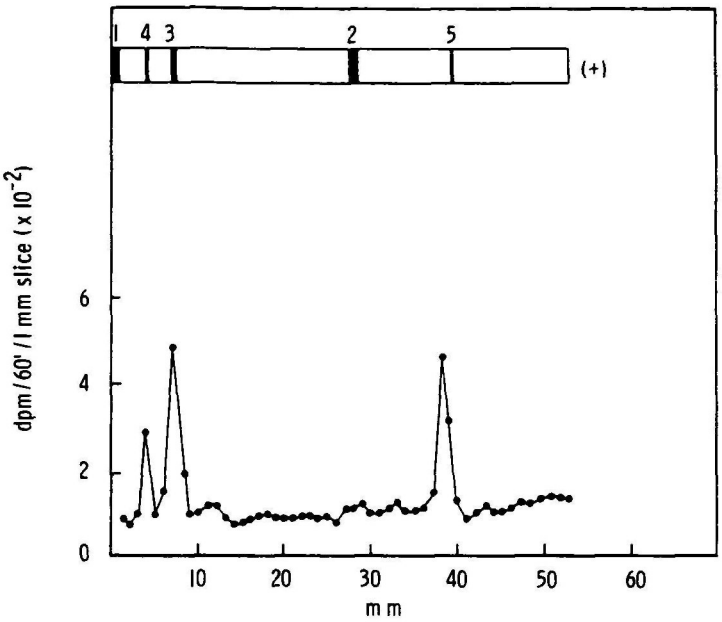


Figure 3. Schematic representation of electropherogram (top of chart) and enzyme activity in 1.0 mm slices of electropherogram (lower portion of chart) of 90% ammonium sulfate cut of enzymatically active fractions from the agarose column shown in Figure 2. See text for details. Numbers on top of the schematic electropherogram indicate a qualitative estimate of the relative staining intensity of the polypeptide bands.

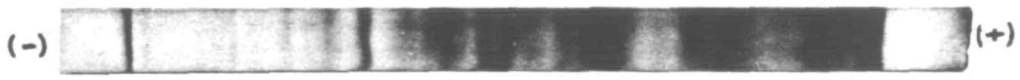


Figure 4. Photograph of SDS-6% gel electrophoretic analysis of the same material analyzed in Figure 3. Coomassie blue staining.

active enzyme preparation obtained to date is still contaminated with highly aggregable non-enzymatic proteins (cf. Figures 3 and 4), detailed studies on the kinetics and molecular mechanisms of action of the preparation are not yet warranted. The 90% ammonium sulfate precipitate of the enzymatically active fractions from the gel filtration of the DNAase-treated mixture has been analyzed with respect to pH optima, linearity of reaction with respect to time of incubation, and the ability to transmethylate various nucleic acid substrates.

When using native M.luteus DNA as substrate, a broad enzyme activity versus pH curve is obtained with peak activity at pH 6.75 to 7.0 but a pronounced shoulder at pH 7.5. When using heat-denatured M.luteus DNA, two distinct optima are noted at pH 6.75 to 7 and at pH 7.5. The pH optima for both the unfractionated nuclear supernatant and the redissolved 25 to 50% ammonium sulfate cut was approximately pH 8.0 with both native and denatured Micrococcus luteus DNA. A preliminary isoelectric focusing study gave indications of two peaks of enzyme activity with isoelectric points of approximately 9.3 and 8.5 (data not shown).

The time course of the in vitro transmethylation of native and heat-denatured DNA is shown in Figure 5 which represents a typical experiment. Complex biphasic kinetics are noted with both native and denatured DNA. The initial rates of reaction are linear for both substrates for about 45 mins. but the initial rate of reaction is about 1.8 times greater using denatured DNA. The cumulative methylation of the two substrates is approximately identical after 120 mins. of incubation although the methylation of native DNA has not yet attained a plateau.

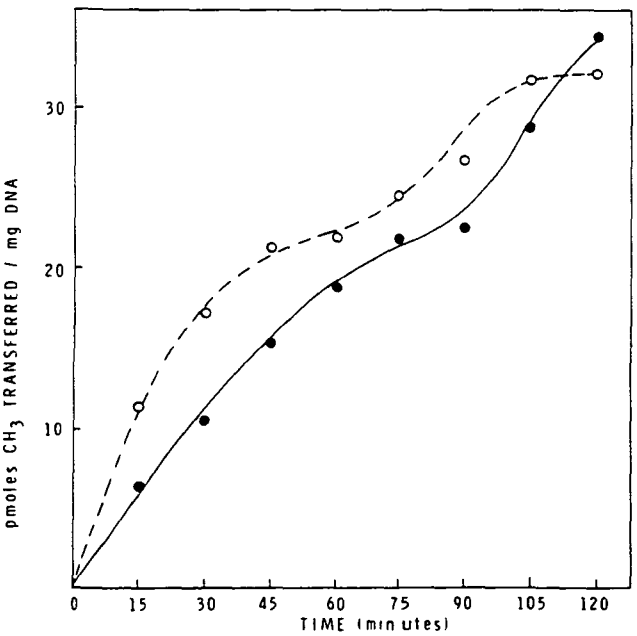


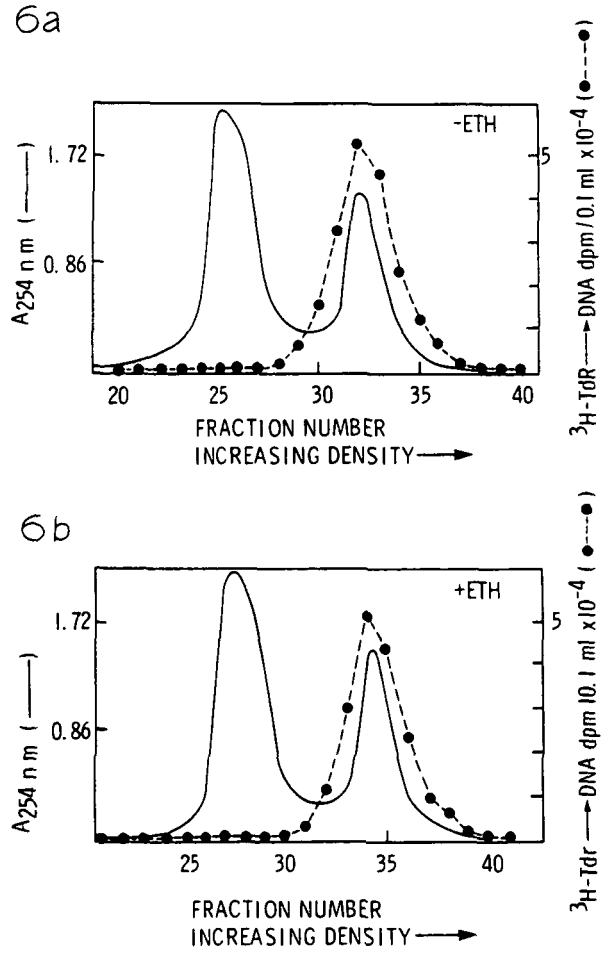
Figure 5. In vitro transmethylation of M.luteus DNA by a DNA methylase preparation versus time of incubation. 60 ug of either native (●——●) or heat-denatured (o---o) M.luteus were incubated at 37°C and aliquots of reaction mixture were removed and analyzed at the times indicated.

The methyl acceptance of various nucleic acid substrates using the most highly purified enzyme preparation is shown in Table 2. With one exception (discussed in the next section), heat-denatured DNAs were much better methyl acceptors than native DNAs. The differences in rates between native and denatured DNAs approximate the differences seen in Figure 5. The data in that Figure also indicate that the differences between native and denatured DNAs shown in the Table may disappear if the times of incubation were prolonged. Microbial DNAs were substantially better methyl acceptors in vitro than calf thymus DNA or homologous DNA isolated from mid-log phase Novikoff hepatoma cells. The homopolymer duplex $d(G)_n:d(C)_n$ was a poor substrate whereas the defined copolymer duplex $d(G\text{-}C)_n:d(G\text{-}C)_n$ was a good methyl acceptor. Neither ribosomal RNA nor mixed transfer RNAs from Novikoff cells served as substrates in the in vitro reaction (data not shown).

Homologous undermethylated DNA as a methyl acceptor: An interesting exception to the greater efficacy of denatured DNA as a methyl acceptor is found with DNA isolated from synchronized Novikoff heptaoma cells grown in the presence of ethionine. Understanding of the mechanism of action and specificity of DNA methylase has been retarded by the fact that DNA is a poor in vitro acceptor of methyl groups from its homologous enzyme since presumably all methylatable cytosine residues have been methylated in vivo. We have made preliminary efforts to artificially produce non-methylated Novikoff cell DNA using ethionine. (This protocol was suggested by the studies of Wildenauer and Gross[25].) Suspension cultures of Novikoff cells were synchronized by sequential treatment with Colcemid and hydroxyurea[26]. The cells were released from hydroxyurea block by resuspension in methionine-free medium which was 10^{-4} M in ethionine and allowed to complete about one round of DNA synthesis. DNA was isolated and purified from these cells and used as a substrate in the in vitro Novikoff cell DNA methylase reaction. The purity of this DNA preparation was comparable to the purity of DNA prepared from synchronized Novikoff cells grown in the presence of methionine or from mid-log phase Novikoff cells viz. less than 2% protein and no detectable contamination with RNA.

To examine the effect of ethionine on DNA replication per se, this experiment was repeated with two cultures both of which contained additionally 10^{-4} M 5-bromo-2'-deoxyuridine and (methyl-^{3}H)-2'-deoxythymidine. However, one culture was resuspended in methionine-containing medium whereas the second culture was resuspended in methionine-free medium that was 10^{-4} M

in ethionine. The cells were harvested and DNA prepared after about 60% of DNA replication was completed. As seen in Figures 6A and 6B, the alkaline cesium sulfate density gradient centrifugation patterns are identical. (The tube shift between Fig. 6A and 6B is an artifact of collection; parental and filial peaks band at nearly identical densities for both growth conditions.) The specific radioactivities of parental DNA bands were 34.9 and 36.9 dpm/ug DNA and for filial DNA were 5296 and 5258 dpm/ug DNA for the ethionine-treated and methionine control cultures, respectively.



Figures 6A and 6B. Alkaline cesium sulfate density gradient centrifugation patterns of DNA isolated from synchronized Novikoff hepatoma cells grown in (A) methionine-containing medium or (B) methionine-free medium that was 10-4 M in ethionine. (——), optical density at 254 nm; (----), incorporation of (methyl-^{3})-2'-deoxythymidine into DNA.

When native and heat-denatured DNA from ethionine-treated synchronized Novikoff cells was used as substrate for the Novikoff cell DNA methylase preparation, the native DNA sample was about 3.5 fold better as a methyl acceptor than the heat-denatured sample (Table 2). Both native and denatured DNA from ethionine-treated Novikoff cells were much better substrates than native or denatured DNA from methionine control cells (cf. lines 9 and 10 with lines 11 and 12 of Table 2). These results have been repeated with two different enzyme preparations using the same DNA preparations. The absolute degree to which the DNA from the ethionine-treated Novikoff cells is undermethylated has not yet been determined.

DISCUSSION

Substantial progress has been made toward the preparation of a eukaryotic DNA methylase. The most active preparation obtained is about 980-fold purified compared to enzyme activity in whole cell homogenate. Nevertheless, even this preparation contains multiple species of contaminating non-enzymatic proteins which tend to aggregate/associate into high molecular weight complexes that complicate enzyme purification steps.

A second drawback is that substantial enzyme activity is lost during the preparation due to the lengthy procedure and to the time required to complete a laborious enzyme assay before proceeding to the next step. The estimated half-life of the enzyme activity at 4°C is 4 to 4.5 days. The enzyme activity is unstable to freezing (with or without added protective reagents) until the final step shown on the last line of Table 2. Even this fraction loses about 15 to 20% of its activity per month at -80°C. We are currently modifying the procedure to effect a rapid dissociation of enzyme activity from copurified DNA and/or non-enzymatic protein aggregates in the 25 to 50% ammonium sulfate cut by agarose gel filtration in high ionic strength medium; preliminary results indicate that this approach may be useful.

The multiplicity of DNA methylase activity peaks in enzyme preparations from Novikoff cells (ref.20) may be, to a large extent, artifacts generated by copurified DNA since DNAase-treatment greatly reduced their number. However, three peaks of enzyme activity were noted in the analyses shown in Figures 2 and 3 and two peaks of activity were detected in a preliminary isoelectric focusing study using a DNAase-treated preparation. Although the best enzyme preparation available in this lab remains heterogeneous, some of its properties are of interest in this regard. The two pH optima observed, the complex biphasic kinetics of the in vitro reaction catalyzed by the preparation, and the differing isoelectric points are all consistent

with -- but do not prove -- the interpretation that there may be several DNA methylases in the Novikoff cell. If these results can be corroborated with more purified preparations (and kinetic, immunochemical and substrate sequence specificity studies thereon), some support might be available for the hypothesis of Holliday and Pugh[27] that in a given organism different DNA methylases with differing substrate sequence specificities are involved in the control of differentiation. If this hypothesis -- which is a variant of a scheme originally developed by Scarano[28,29] -- is valid, one might not necessarily expect a large number of DNA methylase types in the highly de-differentiated Novikoff rat hepatoma cell. Purification and characterization of DNA methylases from a more highly differentiated yet rapidly dividing cell type is being pursued to examine this thesis.

The ability of the present enzyme preparation to catalyze the transmethylation of various DNA substrates is similar to that reported by others using cruder enzyme fractions *viz.* a higher rate of methylation when using heterologous denatured DNAs than when using native DNAs. The mechanistic significance of this observation is not clear, however, since the same enzyme preparation will cumulatively catalyze the methylation of native heterologous DNA to as great or greater extent than the same amount of denatured DNA if the reaction is carried out for a sufficient length of time. The finding that putatively undermethylated native DNA homologous to the enzyme source is methylated at a greater rate than when heat-denatured (Table 2) is intriguing since it indicates that study of DNA methylases using heterologous substrates may not be representative of the *in vivo* mechanism of action of DNA methylases. The *in vitro* reaction product using methyl-deficient DNA and its homologous enzyme has not been extensively characterized (e.g. by localization of methyl groups in pyrimidine isopliths). The rate of methylation observed in this study (31 pmoles/mg DNA/hour), although 85-fold higher than the rate using DNA from mid-log phase cells, is low compared to the calculated pmoles of methyl group needed for saturation of DNA completely devoid of methyl groups which is calculated to be on the order of 15,000 pmoles/mg DNA. The observed rate is artificially low, however, since the *in vitro* reaction was run at a pH (8.0) at which the rate of reaction is only 50 to 55% of maximum. Reaction conditions need to be optimized for the totally homologous system before the *in vitro* reaction rate can be used as a measure of the degree of undermethylation of the homologous DNA. Further refinements of the synchronization and ethionine-treatment protocol are in progress to produce a Novikoff cell DNA that is near totally devoid of methyl

groups in at least one strand of DNA.

Current progress points to the availability of homogeneous DNA methylase(s) from Novikoff cells in the near future; methyl-deficient homologous DNA is presently available. With these two tools, a detailed and physiologically relevant *in vitro* study of the mechanism and site specificity of of the reaction can be undertaken. Through this study -- and a search for specific inhibitors of the enzyme -- we hope to gain some insight into the biological function of eukaryotic DNA methylation.

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