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Purification and some properties of human DNA- O^6-methylguanine methyltransferase

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Abstract. DNA-O^6-methylguanine methyltransferase was purified from the nuclear fraction of fresh human placenta using ammonium sulphate precipitation, gel filtration, affinity chromatography on DNA-cellulose and hydroxyapatite. The methyltransferase preparation was approximately 1-2% pure based on specific activity, and was free of nucleic acids. The protein reacts stoichiometrically with O^6-methylguanine in DNA with apparent second-order kinetics. The human methyltransferase has a pH optimum of about 8·5, similar to that of the corresponding rat and mouse proteins. NaCl inhibits the reaction in a concentration-dependent fashion. The human protein, like the rodent and *E. coli* methyltransferases, needs no cofactor. While lmM $MnCl_2$, lmM spermidine, 5mM $MgCl_2$ and 10 mM EDTA individually do not significantly inhibit the initial rate of reaction, the protein is nearly completely inactive in 5 mM $A1C1_3$ or $FeCl_2$ or 10 mM spermidine. The initial rate of reaction increases as a function of temperature at least up to 42°. The reaction is inhibited by DNA in a concentration-dependent manner, with single-stranded DNA being more inhibitory than duplex DNA.

Keywords. DNA repair; DNA alkylation; O^6-methylguanine; human methyltransferase.

Introduction

A critical factor in cellular responses to chemical mutagens and carcinogens is the ability of the cells to repair damages in DNA resulting from its reaction with the xenobiotic agents or their metabolites. Among the DNA lesions produced by simple methylating agents, such as *N*-methyl nitrosamines, O^6-methylguanine is of particular interest because of its inability to form normal base-pairs with cytosine (Loveless, 1969) and its preferential base-pairing with thymine during DNA replication (Snow *et al.,* 1984). Lack of O^6-alkylguanine repair correlates with increased mutagenicity in *Escherichia coli* (Samson and Cairns, 1977; Jeggo *et al.,* 1977; Jeggo, 1979), and is a possible factor in the organ specificity of tumors induced by alkylating agents (Goth and Rajewsky, 1974; Nicoll *et al.,* 1975).

In both bacterial and mammalian cells, O^6-methylguanine in DNA is repaired by similar methyltransferases; these proteins transfer the added methyl group to a cysteine residue on their own amino acid chains (Olsson and Lindahl, 1980; Mehta *et al.,* 1981; Bogden *et al.,* 1981; Pegg *et al.,* 1982) thereby restoring the original guanine base in DNA (Foote *et al.,* 1980). DNA-O^6-methylguanine methyltransferase functions in a stoichiometric, rather than catalytic, manner, i.e., the protein accepts

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Abbreviations used: EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; αToSF, α-toluenesulphonyl fluoride.

only one methyl group and is irreversibly inactivated by the process (Schendel and Robins, 1978; Robins and Cairns, 1979; Foote *et al*., 1980; Pegg *et al.,* 1982).

Although the mechanism of this methyl transfer in O^6-methylguanine repair is the same in *E. coli* and mammalian cells, the methyltransferases from these sources differ structurally and functionally. The protein is induced by 10- to 100-fold following exposure of *E. coli* to alkylating agents (Schendel and Robins, 1978; Karran, *et al.*, 1979; Robins and Cairns, 1979; Foote et *al.,* 1980; Mitra *et al.,* 1982). In contrast, the mammalian methyltransferases are only slightly inducible, if at all, by alkylation treatment (Montesano *et al.*, 1979; Sklar *et al.,* 1981; Myrnes *et al.,* 1982; Karran *et al.,* 1982; Foote and Mitra, 1984), and in the liver appear to respond to damage and regeneration in general, rather than specifically to alkyl-DNA lesions (Cleaver and Kaufmann, 1980). In *E. coli,* the methyltransferase activity for both O^6-alkylguanine and O^4-alkylthymine in DNA is present in the 39 kDa Ada protein (McCarthy *et al.*, 1984). An 18 kDa cleavage product retains the methyltransferase activity for O^6-methylguanine (Teo *et al*., 1984); the Ada protein also contains a second cysteine residue which accepts alkyl groups from methyl phosphotriesters in DNA (McCarthy and Lindahl, 1985; Margison *et al.,* 1985). Methylation of this second cysteine acceptor is responsible for the induction of methyltransferase in *E. coli* (Teo *et al.,* 1986). However, unlike the *E. coli* protein, the size of mammalian methyltransferases is approximately 24 kDa, and the mammalian proteins display no repair activity for phosphotriesters and O^4-alkylthymine (Yarosh *et al.,* 1985; Pegg *et al.*, 1983; Dolan *et al.,* 1984).

Although the bacterial methyltransferase has been extensively studied, the mammalian methyltransferases in general, and the human enzyme in particular, have not been well-characterized. Human studies are, moreover, hampered by the relative difficulty of tissue acquisition. The enzyme has been purified from regenerating rat liver approximately 1300-fold, resulting in a 2% pure protein (Pegg *et al.,* 1983), and from normal rat liver with 3800-fold purification and at least 0·15% purity (Hora *et al.,* 1983). A 3-step purification of mouse liver yielding 86-fold purification has also been reported, with no estimate of purity level given (Bogden *et al.,* 1981). Yarosh *et al.* (1984) describe a partial purification of DNA-O^6-methylguanine methyltransferase from human placenta, but the resulting fraction was less than 0·002% pure.

In this paper we describe an approximate 66,000-fold purification of DNA-O^6-methylguanine methyltransferase from human placenta. An initial characterization of the partially purified protein is also reported.

Materials and methods

Materials

[CH_3-^{3}H]-*N*-Methyl-*N*-nitrosourea (4Ci/mmol) was purchased from Moravek Biochemicals Brea, California, USA. Calf thymus DNA was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. All other biochemical reagents were of reagent grade. Single-stranded DNA bound to CM-cellulose was prepared according to Potuzak and Wintersberger (1976).

Preparation of O^6-methylguanine-DNA substrate

DNA containing O^6-[^{3}H]-methylguanine was prepared by a modification of the procedure reported by Karran *et al.* (1979). Calf thymus DNA was treated with N-[^{3}H] -methyl-*N*-nitrosourea for 18 h at 37°C, precipitated with 2 volumes ethanol, and incubated with 200 mM Na-cacodylate buffer, pH 7·2, at 80°C for 16 h to release 7- and 3-methylpurines. The preparation was first dialyzed at 4° for 48 h against 50 mM Tris-Cl, pH 7·4, and 1 mM EDTA with 1 M NaCl and then for 48 h against the same buffer without added NaCl. The final substrate had a specific radioactivity of about 100,000 cpm per mg DNA, of which 5 5 % was present in O^6-methylguanine as determined by acid hydrolysis and chromatography of an aliquot (Foote *et al.*, 1983). The remaining radioactivity was present primarily in phosphotriesters with a small amount in methylated pyrimidines. Only O^6-methylguanine acts as a substrate for the methyltransferase.

Assay of DNA-O^6-methylguanine methyltransferase

Standard assay buffer, unless stated otherwise, contained 20 mM K-PO_4 (pH 7·5), 0·1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 mM dithiothreitol (DTT), and 0·2 mM α-toluenesulphonyl fluoride (αToSF). Protein samples were mixed with a solution of assay buffer containing 160 fmol O^6-methylguanine in DNA and other components as indicated; in a total reaction volume of 200 μl. The mixture was incubated at 37°C and the reaction was terminated at 1 h (unless otherwise noted) by the addition of sodium dodecylsulphate to a final concentration of 1%. Protein was degraded by the addition of 100 μg Proteinase K (EC 3·4·21·14) (EM Biochemicals) and subsequent incubation at 37°C for 2 h. DNA was then precipitated by the addition of 0·3 M sodium acetate, 50 μg calf thymus DNA as carrier, and 3 volumes of ethanol, followed by freezing in liquid nitrogen for 5 min. The precipitate was removed by centrifugation at 10,000 *g* for 5 min and the supernatant was assayed for radioactivity in the presence of 9 ml ACS scintillation fluid (Amersham). Incubations containing bovine serum album (1·2 μg) in place of methyltransferase were used to determine background radioactivity (Waldstein *et al.*, 1982).

Results and discussion

Purification ofDNA-O^6-methylguanine methyltransferase from human placenta

In preliminary experiments, we found that whole extracts off human placenta contain activity equivalent to approximately 10 μg of the protein (based on molecular weight of 24,000) per 100 g of tissue. Up to 90% of the activity could be recovered in a crude nuclear fraction with an approximate 10-fold purification. In order to accumulate sufficient material for extensive purification of the methyltransferase, we routinely processed fresh placentas (300-400 g) and stored the pelleted nuclear fraction at -80° until needed.

Table 1 summarizes the results of a typical purification. Placentas were obtained

Table 1. Summary of purification of DNA-O^6-methylguanine methyltransferase from human placenta.

Step (Fraction No.)	Total A280 units	Total Activity (10^6 cpm)	Fold purification	Yield (%)
Nuclear extract (I)	53,460	15	*10	[90*]
Polyethyleneimne precipitation (II)	3,380	9	94	54
DEAE cellulose with DNA cellulose (III)	111	1·5	470	8·7
0–70% $(NH_4)_2SO_4$ (IV)	103	1·8	610	11
Sephadex G-75 (V)	10·7	0·56	1,830	3·2
Hydroxyapatite (VI)	0·19	0·37	65,930	2·2

*Estimate of purification from the crude extract based on previous studies.

from local hospitals as soon after delivery as possible and were transported to the laboratory on ice. All procedures were carried out at 0–4°C. Afters removal of the membrane, the tissue was cut into several small pieces and washed with 20 mM Tris-Cl (pH 7·4), 10% glycerol, 1 mM EDTA, 1 mM *ß*-mercaptoethanol and 0·2 mM αToSF (bluffer A) to remove excess blood. The tissue was then homogenized in two volumes of buffer A using a Polytron homogenizer (Brinkman Instruments) at low speed. The homogenate was strained through cheesecloth and the retained material was rehomogenized in one volume of buffer A and filtered again. The process was repeated once more and the combined filtrate was centrifuged at 6000 *g* for 30 min to pellet the intact and fragmented nuclei. The pellet was resuspended in one volume of buffer A and recentrifuged. The crude nuclear pellet could be stored at – 80°C for up to several weeks without a significant loss of methyltransferase activity.

Several pellet fractions (equivalent to 2-3 placentas) were combined after suspension in an equal volume of buffer A (based on the original weight of tissue) and sonicated with 3 min bursts until no intact nuclei were detectable under the microscope. The extract was then centrifuged at 10,000 *g* for 30 min and the supernatants (Fraction I) was made up to 0·02% polyethyleneimine (BDH Chemicals). After stirring for 30 min, the precipitated nucleic acid was removed by centrifugation at 10,000 *g* for 30 min. The supernatants (Fraction II) was loaded at 2 ml/min on a DEAE-cellulose (DE52; 4·2 cm × 10 cm) column connected in tandem with a ssDNA–cellulose column (6·5 cm × 7·5 cm) pre-equilibrated with buffer A. After loading of the sample, the columns were washed with 2 litres of 50 mM NaCl in buffer A at 2 ml/min before the methyltransferase was eluted with 0·5 M NaCl in buffer A. Fractions of 7 ml were collected and assayed, and those containing methyltransferase activity were pooled (Fraction III). The protein was then precipitated by 70% saturation with ammonium sulphate and collected by centrifugation (10,000 *g*, 30 min). The pelleted protein was dissolved in buffer A to a final volume of up to 40 ml (Fraction IV) and applied to a Sephadex G-75 column (2·7 cm × 85 cm). The column was eluted with 0·2 M NaCl in buffer A (0·3 ml/min). Fractions of 3 ml were collected and those containing methyltransferase activity (eluting at 42–68% of the column volume) were pooled. The enzyme was then concentrated by ultrafiltration in an Amicon stirred cell using a YM 10 filter (Fraction V).

$MgCl_2$ was added to the concentrate to titrate EDTA (to 2 mM) and the solution

was loaded onto a hydroxyapatite (Bio-Rad HTP) column (0·5 cm × 4 cm). The column was washed with 10 mM K-PO_4 (pH 7·4), 01 mM EDTA, 10% glycerol, 1 mM DTT and 0·2 mM αToSF (buffer B) and the protein was eluted by a gradient of 10 mM-75 mM K-PO_4 in 13 ml of buffer B. Fractions of 1 ml were collected and methyltransferase activity was found to be eluted at 15–30 mM K- PO_4 (Fraction VI).

Protein concentration was estimated by absorbance values at 280 μm. Based on the ratio of absorbance at 260 and 280 μm, the enzyme preparation was completely free of nucleic acids after gel filtration. The hydroxyapatite fraction has an absorbance too low to be measured accurately but a rough calculation of the mass of methyltransferase, based on its activity and molecular weight of 24,000, and the concentration of protein, indicates that the final hydroxyapatite fraction was about 1-2% pure. Attempts to purify the protein further *via* additional methods including high performance liquid chromatography caused a drastic loss in its recovery. In any case, we believe that our methyltransferase is more pure than any other mammalian preparation reported in the literature so far.

Kinetic behavior of the methyltransferase

Crude extracts and partially purified DNA-O^6-methylguanine methyltransferase from mammalian cells and tissues demethylate O^6-methylguanine in an apparently stoichiometric rather than catalytic fashion, as was shown with the *E. coli* enzyme (Foote *et al.,* 1980; Bogden *et al.,* 1981; Yarosh *et al.,* 1984; Hora *et al.,* 1983; Pegg *et al*., 1983). However, the definitive evidence for a direct methyl transfer from O^6-methylguanine in DNA to the methyltransferase itself on an equivalent basis, as shown with the *E. coli* protein (Lindahl *et al.,* 1982), could not be provided without a homogeneous enzyme preparation. Nonetheless, we determined the rate of demethylation reaction with our purified enzyme (figure 1). The reaction follows apparent second-order kinetics (figure 1, inset) with a calculated rate constant of approximately $1{\cdot}5 \times 10^8$ litre mol^{-1} min^{-1} (at 37°). Figure 1 also confirms the stoichiometric nature of reaction in that the extent of reaction at the plateau level corresponds to the amount of protein added.

pH-Dependence of the reaction rate of the methyltransferase

As is evident fro figure 1, the rate of methyl transfer declines after the first 10 min of incubation. The reason for this may be that, in addition to the depletion of the alkylated base in DNA and the methyltransferase, all O^6-methylguanines in DNA are not equally susceptible to the methyltransferase, possibly because of the effect of neighboring base sequences (Topal *et al.,* 1986). In any case, we routinely used only the initial rate for further kinetic studies by determining the extent of methylation after 3 and 6 min of incubation. The pH-dependence of the initial rate of reaction (figure 2) shows an optimum pH of about 8·5 for the human methyltransferase. This is comparable to the values obtained with other mammalian methyltransferases (Hora *et al.,* 1983). The *E. coli* methyltransferase also reacts optimally in slightly alkaline pH. While the pK of the sulphydryl group of the acceptor cysteine residue on the protein is not known, these results are consistent with the idea that Cys-S^- form is the reactive species and that the activity curve reflects the pH-dependent ionization of Cys-SH which has a pK of 8-8·9 in the free form (Sober, 1968).

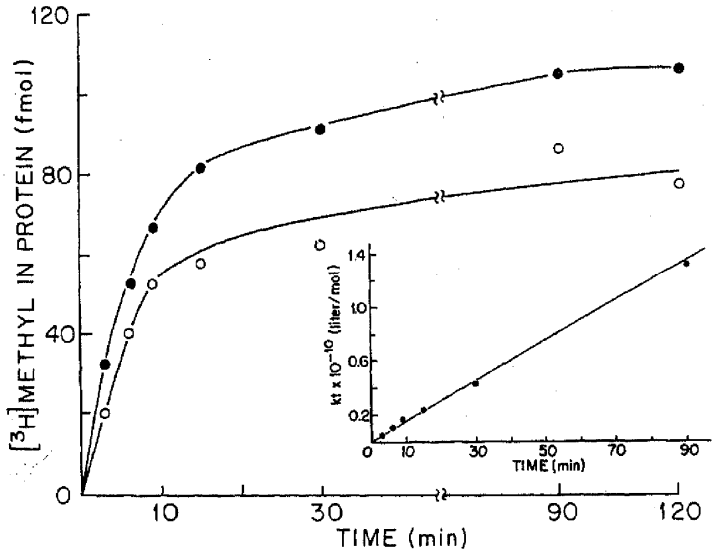
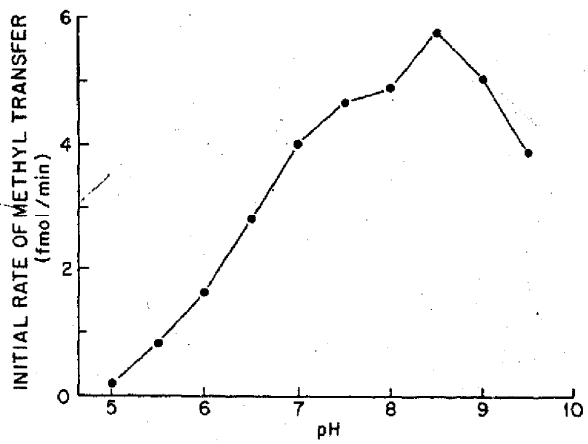


Figure 1. Kinetics of methyltransferase reaction at 37°. DNA substrate containing 160 fmol O^6-methylguanine was incubated with either, a 3 μl (O) or a 5 μl (●)aliquot of purified Fraction VII protein as described in 'materials and methods'. Duplicate samples were assayed for each time point. The inset shows the replot of data from the 5 μl reaction according to the second-order rate equation, $kt = 1/(a\text{-}b)\ \text{In}\ b(a\text{-}x)/(a(b\text{-}x))$ (Frost and Pearson, 1961), where a equals the initial concentration of O^6-methylguanine (160 fmol/200 μl = $8{\cdot}0 \times 10^{-10}$M), b equals the initial concentration of methyltransferase (~ 105 fmol/200 μl = $5{\cdot}25 \times 10^{-10}$ M) based on the plateau level of [^{3}H]-methyl protein, and x equals the amount reacted at time t.



Figured 2. Dependence of methyltransferase reaction rate on pH. One hundred fmol aliquots of methyltransferase were incubated with DNA containing 160 fmol O^6-methylguanine as described in 'materials and methods' except that 20 mM K-PO_4 was replaced by other buffers at 20 mM adjusted to different pH values as follows: Na citrate: (5·0 and 5·5); K- PO_4: (6·0, 6·5, 7·0, 7·5); Tris-Cl: (8·0, 8·5) and Glycine: (9·0 and 9·5).

Effect of ionic strength and polyvalent ions on methyltransferase activity

Figure 3 shows that the initial rate of the methyltransferase reaction is very strongly dependent on NaCl concentration. This is surprising because even in buffer of physiological ionic strength of about 0·15 M, only about 25% of the control activity was observed. In confirmation of earlier studies with *E. coli* and rodent proteins, we did not observe any cofactor requirement for the human methyltransferase. Low concentrations of $MgCl_2$, EDTA and spermidine do not alter the initial reaction rate of the protein. However, 10 mM spermidine, 5 mM $A1C1_3$ and 5 mM $FeCl_2$ individually abolish the activity nearly completely (table 2). It is possible that the high concentrations of polyvalent ions either bind the methyltransferase molecules or alter the conformation of the DNA substrate to make O^6-methylguanine inaccessible.

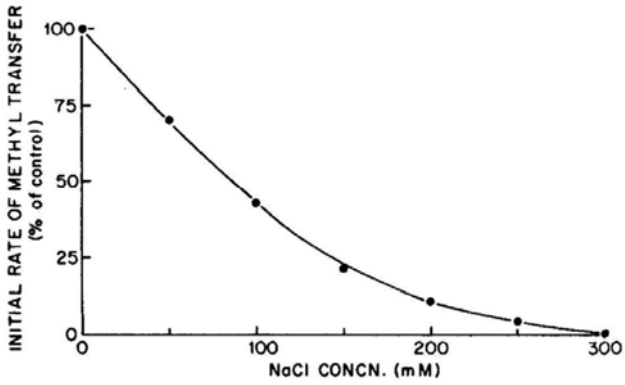


Figure 3. Effect of NaCl on methyltransferase reaction. In the presence of various NaCl concentrations, l00 fmol methyltransferase was incubated with the substrate and the activity measured as described in 'materials and methods'.

Table 2. Effect of polyvalent ions on methyltransferase activity (initial rate of reaction).

Addition	Activity (% of control)
5 mM $MgCl_2$	76
1 mM $MnCl_2$	70
5 mM $AlCl_3$	6·9
5 mM $FeCl_2$	<1
0·1 mM spermidine	100
1 mM spermidine	84
10 mM spermidine	<1
No EDTA	79
0·1 mM EDTA	(100)
1·0 mM EDTA	87
10 mM EDTA	82

Effect of temperature on the reaction rate of the methyltransferase

Figure 4 shows the effect of temperature on the initial rate of methyl-transfer reaction. The rate goes up continuously with temperature up to 42°C. In a separate experiment (not shown) we determined that the methyltransferase can be protected from heat inactivation at 50°C by the presence of duplex DNA. The inset in figure 4 shows the Arrhenius plot of the data; the energy of activation is calculated to be about 18 Cal. A comparison of the activation energy for the reaction of O^6-methylguanine methyltransferase with other O^6-alkylguanines may provide a basis for explaining the large differences in relative rates of dealkylation of O^6-methyl-, -ethyl, and -isopropylguanine in DNA by the protein (Morimoto *et al.,* 1985).

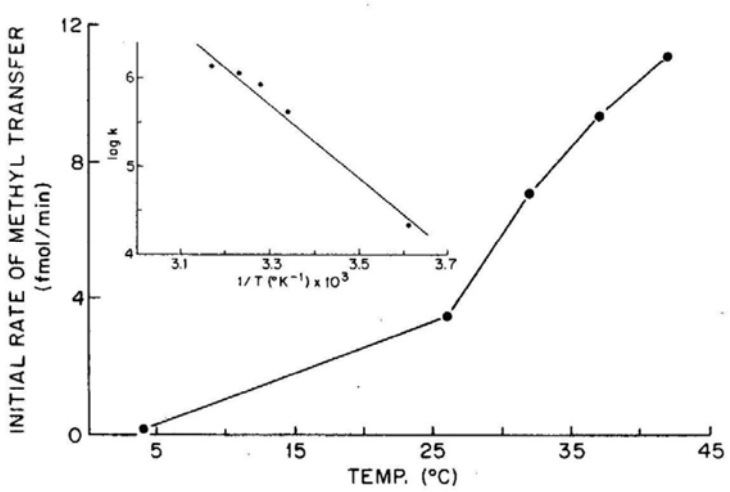


Figure 4. Effect of temperature on the reaction rate of methyltransferase. The initial rate of reaction was determined at various temperatures. The inset shows the Arrhenius plot of the data.

Inhibition of methyltransferase reaction by DNA

The *in vivo* rate of repair of O^6-methylguanine in DNA obviously depends not only on the number of methyltransferase molecules in a cell, but also on the accessibility of the lesion to the protein. Because the mammalian DNA is present in a highly organized chromatin structure and there is a vast excess of DNA compared to the few lesions, we investigated whether excess DNA affects the methyltransfer reaction. Figure 5 shows that the initial rate of the reaction is inversely related to the concentration of duplex DNA. It is unlikely that increased viscosity is responsible for this inhibition because denatured DNA, at the same concentration, though much less viscous, is twice as inhibitory as duplex DNA. Thus, it appears that competitive non-specific binding of the methyltransferase to DNA in the low ionic strength of the reaction mixture may reduce the rate of formation of the complex of protein and O^6-methylguanine, necessary for transfer of the methyl group.

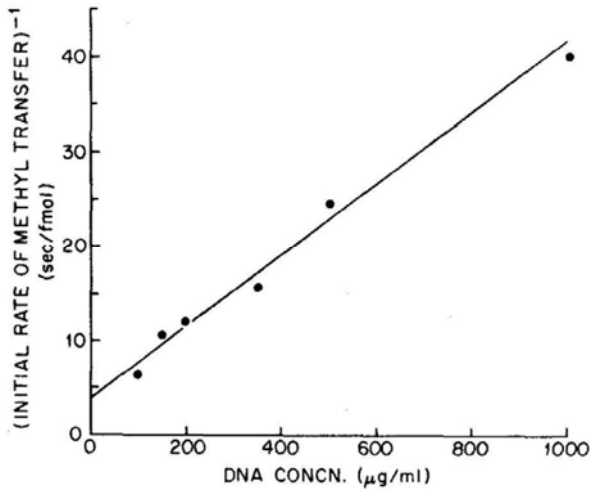


Figure 5. Effect of DNA on methyltransferase. The initial reaction rate of the enzyme was measured as a function of DNA concentration which was increased by the addition of untreated duplex DNA.

In conclusion, we have shown that partially purified DNA-O^6-methylguanine methyltransferase from human tissue is similar in its properties to the rodent methyltransferases. The initial rate of the methyltransferase reaction can be determined only by extrapolation to zero DNA concentration because of its inhibition by DNA. Aurther studies are necessary to determine whether the presence of histones and nonhistone chromosomal proteins affect the reaction rate. The strong inhibition of the reaction rate by salt is also surprising in view of the intracellular ionic strength. However, the inhibitory effects of excess DNA and physiological ionic strength can explain why the *in vivo* repair of O^6-methylguanine (Pegg *et al.,* 1984) is significantly slower than the rate of repair observed with the purified enzyme.

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