Lang, M. A., Juvonen, R., Jäivipärvinen, P., Honkakoski, P., & Raunio, H. (1989) *Arch. Biochem. Biophys. 271*, 139-148.
Lindberg, R., Burkhart, B., Ichikawa, T., & Negishi, M. (1989) *J. Biol. Chem. 264*, 6465-6471.
McKnight, G. S., & Palmiter, R. D. (1979) *J. Biol. Chem. 254*, 9050-9058.
Müllner, E. W., & Kühn, L. C. (1988) *Cell 53*, 815-825.
Negishi, M., Lindberg, R., Burkhart, B., Ichikawa, T., Honkakoski, P., & Lang, M. (1989) *Biochemistry 28*, 4169-4172.
Neupert, B., Thompson, N. A., Meyer, C., & Kühn, L. C. (1990) *Nucleic Acids Res. 18*, 51-55.
Nielsen, D. A., & Shapiro, D. J. (1990) *Mol. Endocrinol. 4*, 953-957.
Noshiro, M., & Negishi, M. (1986) *J. Biol. Chem. 261*, 15923-15927.
Noshiro, M., Lakso, M., Kawajiri, K., & Negishi, M. (1988) *Biochemistry 27*, 6434-6443.
Pasco, D. S., Boyum, K. W., Merchant, S. N., Chalberg, S. C., & Fagan, J. B. (1988) *J. Biol. Chem. 263*, 8671-8677.
Rouault, T. A., Hentze, M. W., Haile, D. J., Harford, J. B., & Klausner, R. D. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5768-5772.
Rouault, T. A., Tang, C. K., Kaptain, S., Burgess, W. H., Haile, D. J., Samaniego, F., McBride, O. W., Harford, J. B., & Klausner, R. D. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7958-7962.
Shaw, G., & Kamen, R. (1986) *Cell 46*, 659-667.
Silver, G., & Krauter, K. S. (1988) *J. Biol. Chem. 263*, 11802-11807.
Silver, G., & Krauter, K. S. (1990) *Mol. Cell. Biol. 10*, 6765-6768.
Silver, G., Reid, L. M., & Krauter, K. S. (1990) *J. Biol. Chem. 265*, 3134-3138.
Simmons, D. L., McQuiddy, P., & Kasper, C. B. (1987) *J. Biol. Chem. 262*, 326-332.
Soderkvist, P., Poellinger, L., Toftgard, R., & Gustafsson, J. A. (1988) *Cancer Res. 48*, 3045-3049.
Song, B.-J., Gelboin, H. V., Park, S. S., Yang, C. S., & Gonzalez, F. J. (1986) *J. Biol. Chem. 261*, 16689-16697.
Song, B.-J., Matsunaga, T., Hardwick, J. P., Park, S.-S., Veech, R. L., Yang, C. S., Gelboin, H. V., & Gonzalez, F. J. (1987) *Mol. Endocrinol. 1*, 542-547.
Song, B.-J., Veech, R. L., Park, S. S., Gelboin, H. V., & Gonzalez, F. J. (1989) *J. Biol. Chem. 264*, 3568-3572.
Squires, E. J., & Negishi, M. (1988) *J. Biol. Chem. 263*, 4166-4171.
Steward, A. R., Wrighton, S. A., Pasco, D. S., Fagan, J. B., Li, D., & Guzelian, P. S. (1985) *Arch. Biochem. Biophys. 241*, 494-508.
Voorman, R., & Aust, S. T. (1988) *Arch. Biochem. Biophys. 262*, 76-84.
Watkins, P. B., Wrighton, S. A., Schuetz, E. G., Maurel, P., & Guzelian, P. S. (1986) *J. Biol. Chem. 261*, 6264-6271.
Wong, G., Kawajiri, K., & Negishi, M. (1987) *Biochemistry 26*, 8683-8690.
Wood, A. W. (1978) *J. Biol. Chem. 254*, 5641-5646.

Effect of Cobalamin Derivatives on in Vitro Enzymatic DNA Methylation: Methylcobalamin Can Act as a Methyl Donor†

Annie Pfohl-Leszkowicz, Gérard Keith, and Guy Dirheimer*
Institut de Biologie Moléculaire et Cellulaire du CNRS and Centre de Recherche de l'Université Louis Pasteur, 15, rue René Descartes, 67084 Strasbourg, France

Received December 7, 1990; Revised Manuscript Received May 23, 1991

ABSTRACT: 5-Methylcytosine synthesis in DNA involves the transfer of methyl groups from *S*-adenosylmethionine to the 5'-position of cytosine through the action of DNA (cytosine-5)-methyltransferase. The rate of this reaction has been found to be enhanced by cobalt ions. We therefore analyzed the influence of vitamin B_{12} and related compounds containing cobalt on DNA methylation. Vitamin B_{12}, methylcobalamin, and coenzyme B_{12} were found to enhance significantly the de novo DNA methylation in the presence of *S*-adenosylmethionine for concentrations up to 1 μM, but at higher concentrations these compounds were found to inhibit DNA methylation. Methylcobalamin behaves as a competitive inhibitor of the enzymatic methylation reaction (K_i = 15 μM), the K_m for *S*-adenosylmethionine being 8 μM. In addition, the use of radioactive methylcobalamin shows that it can be used as a methyl donor in the de novo and maintenance DNA methylation reactions. Thus, two DNA methylation pathways could exist: one involving methylation from *S*-adenosylmethionine and a second one involving methylation from methylcobalamin.

The base 5-methylcytosine (m^5C) appears to be so far the only modified base that has been found in vertebrate DNAs. The extent and pattern of the genomic DNA methylation is not random, but is species- and tissue-specific (Gama-Sosa & Ehrlich, 1983). It occurs mostly in 5'-CpG-3' dinucleotides that are often clustered in the 5'-region of eukaryotic genes (Gardiner-Garden & Frommer, 1987). Many studies have shown that undermethylation of the transcriptional control regions of genes is correlated with the expression of these genes (Holliday, 1987; Cedar, 1988; Dynan, 1989; Michalowsky & Jones, 1989; Doerfler et al., 1990). In addition, de novo methylation is correlated with transcriptional inactivity (Gasson et al., 1983; Jahner & Jaenisch, 1984; Tasseron et al., 1989; Doerfler, 1990). Some authors have suggested that the inactivation of gene expression by CpG methylation could

† This work was partially supported by research grants from the Ligue Nationale de la Lutte contre le Cancer, Comité Départemental du Haut-Rhin, the Fondation pour la Recherche Médicale, and the Association de la Recherche contre le Cancer.

0006-2960/91/0430-8045$02.50/0

be explained by the inability of the methylated DNA to bind specific transcription factors (Iguchi-Ariga & Schaffner, 1989).

The DNA methylation is a process that is catalyzed by DNA (cytosine-5-)-methyltransferase (DNA methylase) (Borek & Srinivasan, 1966) and involves the transfer of methyl groups from *S*-adenosyl-L-methionine (SAM) to carbon 5 of cytosine residues. Its major function is the "maintenance" methylation of hemimethylated sites after replication, in order to preserve the DNA methylation pattern from one generation to the next. Nevertheless, the DNA methylase is able to transfer methyl groups to unmethylated sites in a de novo reaction. Since we have previously found that DNA methylase activities are stimulated by cobalt ions (Pfohl-Leszkowicz et al., 1987), we thought that several natural compounds that contain cobalt, such as cobalamin derivatives, could also have an action in these two enzymatic DNA methylation reactions. Three main cobalamin derivatives are found under natural conditions in man and animals: methylcobalamin (MeCbl), adenosylcobalamin (CoE B_{12}) and hydroxocobalamin (OHCbl). They derive all from cyanocobalamin (vitamin B_{12}), which is an essential nutritional factor for the maintenance of normal hematopoiesis [for a review see Stroinski (1987)]. The metabolism of vitamin B_{12} in mammalian cells involves the endocytosis of vitamin B_{12} bound to plasma transcobalamin. The corresponding complex is further degraded in the lysosome. Hydroxocobalamin enters the mitochondrion and is used to synthesize CoE B_{12}, which is required for activity of the mitochondrial enzyme methylmalonyl-coenzyme A mutase (Ellenbogen, 1979). Some of the cobalamin is also used to synthesize MeCbl in the cytosol (Ellenbogen, 1979), where it is required for the activity of the cytoplasmic enzyme methionine synthetase, which catalyzes the methylation of homocysteine into methionine (Langhlin et al., 1964; Mangum et al., 1969).

In this paper, we study the action of several cobalamin derivatives on the in vitro DNA methylation in presence of SAM and we show that MeCbl acts as a methyl donor in the in vitro enzymatic DNA methylation catalyzed by DNA methylase.

Material and Methods

Chicken erythrocyte DNA, dATP, dGTP, dCTP, dTTP, 5-methyldeoxycytidine 5′-triphosphate (m^5dCTP), and T4 polynucleotide kinase were from PL Biochemicals (Milwaukee, WI). *Micrococcus luteus* DNA, dithioerythritol (DTE), methylcobalamin (MeCbl), cyanocobalamin (vitamin B_{12}), deoxyadenosylcobalamin (CoE B_{12}), and hydroxocobalamin (OHCbl) were from Sigma (St. Louis, MO). Phenylmethanesulfonyl fluoride (PMSF), *S*-adenosylmethionine (SAM) and nuclease P1 were from Boehringer (Mannheim, FRG). [^{3}H]SAM, 15 Ci/mmol (555 GBq/mmol), [γ-^{32}P]-ATP, 5000 Ci/mmol, and [^{14}C]methyl iodide (CH_3I), 54 mCi/mmol (2.06 Bq/mmol), were from Amersham (U.K.). *Escherichia coli* DNA polymerase I was from Biolabs (Beverly, MA). Micrococcal nuclease and spleen phosphodiesterase were from Worthington Biochemicals (Freehold, NJ). Proteinase K was from Appligène (Strasbourg, France). DEAE-cellulose (DE-52) and carboxymethylcellulose were from Whatman (Maidstone, Kent, U.K.). Methylene chloride was from Aldrich.

Sprague-Dawley rats (120 g) were obtained from Sexal (Vigneul-sous-Montmédy, France).

DNA methylase was prepared from rat spleen and DNA methylation assays were performed at 37 °C as previously described (Pfohl-Leszkowicz et al., 1987). Briefly, the standard assay contained 50 mM Tris-HCl, pH 7.6, 0.2 mM DTE, 10 μM [^{3}H]SAM (2 μCi), 1 μg of DNA, and 10 μg of partially purified DNA methylase in 0.1 mL.

[^{14}C]Methylcobalamin was prepared according to Weissbach et al. (1963) by reacting [^{14}C]CH_3I with reduced cyanocobalamin as follows: 10 μmol of cyanocobalamin (13 mg) was dissolved in 3 mL of water, and nitrogen was allowed to bubble through the solution for at least 10 min. Then, 30 mg of sodium borohydride was added while nitrogen bubbling was continued. The solution turned gradually to brownish gray over a period of about 30 min. All subsequent steps were carried out in dim light. Labeling was performed by addition of 1 mCi (18 μmol) of [^{14}C]methyl iodide. The solution turned immediately to red when methyl iodide was added. After 15 min, the methylcobalamin in solution (3 mL) was extracted with a solution of phenol in methylene chloride (100:100 w/v). After rapid vortexing, the organic layer was separated by slight centrifugation for 1 min at 800 rpm in a Beckman J6 centrifuge. The aqueous layer was reextracted with successive aliquots (3 × 1 mL) of phenol/methylene chloride solution until no further color remained in the water layer. The combined organic layers were washed with 2 × 800 μL of distilled water and further diluted in methylene chloride to 5 times its initial volume. The methylcobalamin was finally extracted from the organic layer by distilled water (2 × 4 mL and 1 × 2 mL) until no color remained in the organic layer. The combined aqueous fractions were further washed with methylene chloride (3 times the volume of aqueous layer) to remove traces of phenol. The final purification of MeCbl was performed on a column of carboxymethylcellulose (20 × 5 cm) previously treated with 20 mL of 0.1 N hydrochloric acid and water until the washings became neutral. The methylcobalamin solution was then placed on the top of the column and eluted with distilled water in the dark. The major peak, corresponding to methylcobalamin as indicated by its absorption spectrum (Weissbach et al., 1963), was collected and was concentrated in a speedvac. About 8.2 μmol of MeCbl with a specific radioactivity of 140 μCi/μmol (based on a molar extinction coefficient of 8000 at 520 nm) was obtained.

Hemimethylated *M. luteus* DNA was prepared by nick translation (Hitt et al., 1988) of the native *M. luteus* DNA; a 5-mL reaction mixture containing 100 μg of DNA, 10 ng of DNase I, 440 units of *E. coli* DNA polymerase I, and 0.02 M each dATP, dGTP, dTTP, and m^5dCTP in 6.6 mM Tris-HCl, pH 7.4, and 10 mM DTE was incubated for 1 h at 20 °C. The hemimethylated DNA was deproteinized by 1 volume of Tris-HCl, pH 8, saturated phenol. The aqueous layer containing the DNA was treated by 1 volume of phenol/$CHCl_3$ and isoamyl alcohol (12:12 v/v), and the DNA was precipitated overnight by 2.5 volumes of cold ethanol at −20 °C. The DNA pellet was washed three times with 70% ethanol. In the above conditions at least 35% of the cytosines was replaced by 5-methylcytosine. Unmethylated double-stranded *M. luteus* DNA, used as a control, was synthesized similarly with use of dCTP instead of m^5dCTP.

To detect 5-methylcytosine (m^5C) in DNA, we used the method of Wilson et al. (1986) modified as follows: DNA (2 μg) was digested at 37 °C for 3 h with micrococcal nuclease (0.2 units) and spleen phosphodiesterase (6 units) in a reaction mixture (total volume 10 μL) containing 20 mM sodium succinate and 10 mM $CaCl_2$, pH 6.0. The digested DNA was diluted 60-fold. Aliquots (10 μL, corresponding to 0.16 μg of DNA) of the mixture of nucleoside monophosphates were converted to ^{32}P-labeled nucleoside 3′,5′-bisphosphates by incubation for 2 h at 37 °C with 2 units of T4 polynucleotide

Table I: Effect of Vitamin B_{12} and Related Compounds on de Novo and Maintenance DNA Methylation[a]

	cobalamin concentration												
	0.01 μM	0.05 μM	0.1 μM	0.3 μM	0.5 μM	1 μM	5 μM	10 μM	20 μM	50 μM	100 μM	200 μM	300 μM	400 μM
Native *M. luteus* DNA Substrate														
vitamin B_{12}	100	111	113	ND	123	119	100	98	ND	94	90	75.5	ND	63
OHCbl	100	104	102	ND	97	88	81	71	ND	39	37	33	ND	28.5
CoE B_{12}	100	114	130	ND	94	94	85	84	ND	44	35	32	ND	32
MeCbl	100	114	117	141	127	115	ND	88	55	47	26	21	7	0
Hemimethylated *M. luteus* DNA Substrate														
vitamin B_{12}	100	100	100	ND	100	100	100	100	100	100	100	100	ND	100
OHCbl	100	100	100	ND	100	100	100	100	88	55	50	35.5	ND	30
CoE B_{12}	100	100	100	ND	100	100	100	86	86	76	59	39	ND	30
MeCbl	100	100	98.5	ND	ND	97	93	87	89	84	54	44	ND	30

[a] A total of 1 μg of native or hemimethylated *M. luteus* DNA was incubated at 37 °C for 1 h in the reaction mixture as described in the Material and Methods section in the presence of 10 μM SAM with increasing amounts of vitamin B_{12}, OHCbl, CoE B_{12}, or MeCbl. Values are the average of triplicate assays and are expressed as percentages of control without cobalamins (standard deviation = 2%).

kinase and 10 μCi of [γ-^{32}P]ATP (5000 Ci/mmol) in 50 mM Tris-HCl, pH 9.0, containing 100 μM ATP, 10 mM $MgCl_2$, 1 mM spermidine, and 15 mM β-mercaptoethanol in a volume of 10 μL. After addition of 1 μL of mixture of dpAp, dpGp, dpTp, dpCp, and dpm^{5}Cp (4 μg of each) and potato apyrase (40 milliunits/μL), the reaction mixture was kept at 37 °C for 30 min. Labeled nucleoside 3′,5′-bisphosphates were then converted to nucleoside monophosphates by incubation for 2 h at 37 °C with nuclease P1 (2.5 μg) in 50 mM ammonium acetate, pH 4.5. Labeled 5′-monophosphates were separated by two-dimensional TLC using 20 × 20 cm glass cellulose plates. Unlabeled nucleotides were visualized under UV light (254 nm). Chromatography in the first dimension was carried out overnight with isobutyric acid/H_2O/NH_4OH (50:28.9:1.1 v/v/v), and chromatography in the second dimension was with 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/propanol (100:60:2 v/w/v). Quantification of individual nucleotides was carried out by scraping off the UV quenching spots and counting their radioactivity. The m^{5}dC content of DNA was calculated from the radioactivity found in m^{5}dCMP and dCMP by the equation

$$\% \text{ m}^5\text{dCMP} = \frac{\text{m}^5\text{dCMP}}{\text{m}^5\text{dCMP} + \text{dCMP}} \times 100$$

Results

Influence of Vitamin B_{12} and Related Compounds on in Vitro DNA Methylation in the Presence of S-Adenosylmethionine. The influence of vitamin B_{12}, OHCbl, MeCbl, and CoE B_{12} on partially purified rat spleen DNA methylase activities in the presence of SAM was tested. In order to measure the de novo DNA methylation activity, native *M. luteus* DNA, which was considered to contain no m^{5}dC, was used as a substrate. Maintenance DNA methylase activity was tested with the use of hemimethylated *M. luteus* DNA or chicken erythrocyte DNA, which is a suitable substrate for vertebrate maintenance DNA methylase (Pfohl-Leszkowicz et al., 1981, and 1987) probably because it contains a large number of hemimethylated sites (Adams & So, 1989).

Table I and Figure 1 show that vitamin B_{12} and MeCbl at concentrations ranging between 0.05 and 1 μM have a stimulatory effect on the de novo DNA methylation in the presence of SAM. Stimulations occur also with CoE B_{12} but only for the lower concentrations, 0.05 and 0.1 μM, whereas no stimulation could be observed for OHCbl. On the contrary, no stimulation was obtained for maintenance methylation.

At higher concentrations, ranging from 1 to 20 μM, depending on the cobalamin derivatives for the de novo methylation, and from 10 to 20 μM for the maintenance methylation, an inhibition of DNA methylation was observed except

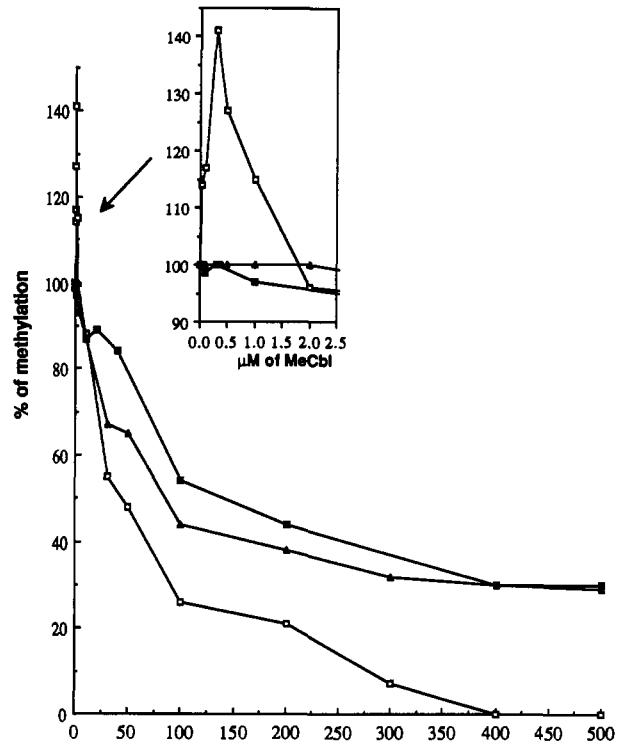

FIGURE 1: Effect of MeCbl on de novo and maintenance DNA methylation. A total of 1 μg of native (□) or hemimethylated *M. luteus* DNA (■) or native chicken erythrocyte DNA (△) was incubated at 37 °C for 1 h in a reaction mixture containing increasing amounts of MeCbl, as described in the Material and Methods section. The results are expressed as percentage of control DNA methylation without MeCbl and correspond to average values of triplicate assays.

for vitamin B_{12}, which has no effect on maintenance methylation. It is interesting to note (Figure 1) that MeCbl at a concentration of 400 μM inhibits completely the de novo methylation. In contrast, all other compounds studied display a residual methylation level even for high concentrations.

In this paper, we focused our studies especially on methylcobalamin since that compound was found to be very stimulatory for low concentrations and completely inhibitory for high concentrations. To elucidate how MeCbl inhibits the DNA methylation, we determined the initial methylation reaction velocity of DNA in the presence of variable amounts of SAM as well as in the absence or presence of MeCbl. Figure 2 shows that methylation time courses of native *M. luteus* DNA in the absence or presence of MeCbl are at least

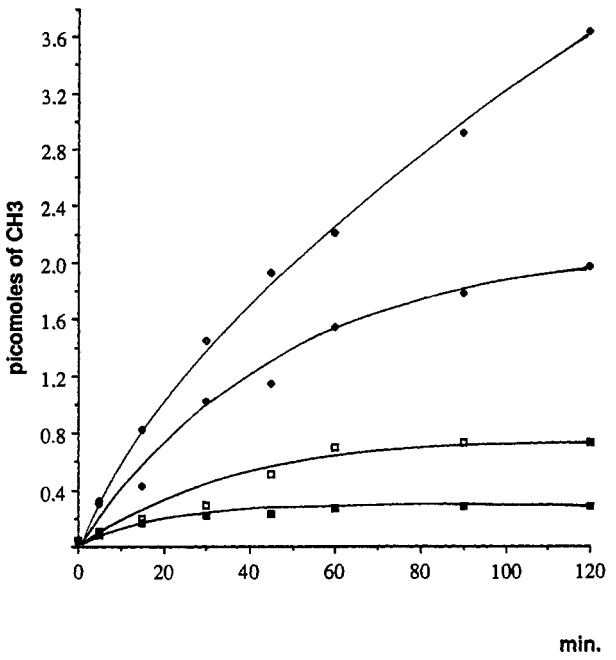

FIGURE 2: Time course of DNA methylation. A total of 1 μg of native (□) or hemimethylated (◇) *M. luteus* DNA was incubated at different time points in a reaction mixture without MeCbl (open symbol) or with 100 μM MeCbl (solid symbol) as described in the Material and Methods section. The results are expressed as picomoles of CH_3 incorporated into 1 μg of DNA and are an average of triplicate values.

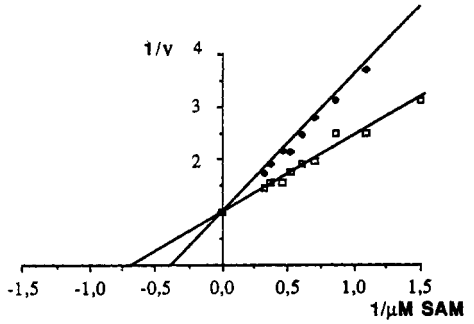

FIGURE 3: Lineweaver–Burk plot of inhibition of de novo methylation by MeCbl. A total of 1 μg of native *M. luteus* DNA was incubated for 20 min in the reaction mixture containing 40 μM MeCbl (◆) or no MeCbl (□) and increasing amounts of SAM as described in the Material and Methods section.

linear for 20 min. However, in the presence of MeCbl, the slope of the curve and the plateau were considerably reduced. With hemimethylated DNA in the absence of MeCbl no plateau is reached, at least at 120 min. (Figure 2). But, in the presence of MeCbl, the level of DNA methylation is noticeably reduced. Plotting $1/v$ versus $1/S$ gives a K_m for the SAM of 8 μM. With use of MeCbl (40 μM) the inhibition was found to be competitive (Figure 3). The corresponding K_i was calculated; it is 15 μM.

Influence of DNA Concentrations. With the use of increasing amounts of native *M. luteus* DNA in the standard assay, an inhibition by excess of substrate was found (Figure 4). On the contrary, no inhibition was obtained when 100 μM MeCbl was added to the reaction mixture. The concentration dependence in this case was hyperbolic. If one compares the methylation of native *M. luteus* DNA in the absence or in the presence of 100 μM MeCbl, with a high concentration of DNA (30 μg/100 μL) one obtains 0.47 pmol of CH_3 in the absence of MeCbl and 0.62 pmol of CH_3 in the presence of MeCbl.

When hemimethylated DNA was used, either in the absence or in the presence of MeCbl, the reaction was always inhibited

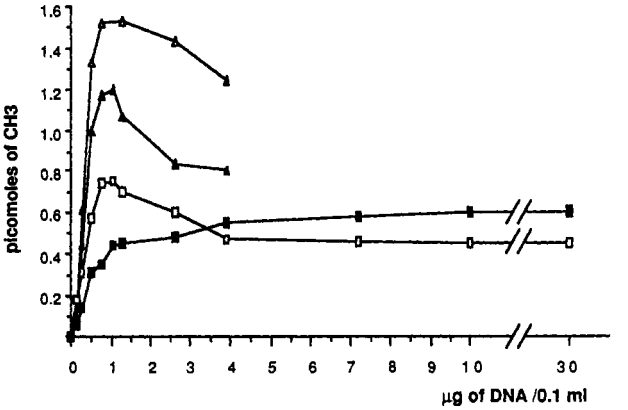

FIGURE 4: Influence of increasing DNA concentrations on DNA methylation. Increasing amounts of native (□) or hemimethylated (△) *M. luteus* DNA were incubated for 30 min in the presence (solid symbol) or absence (open symbol) of 100 μM MeCbl. The results are average values of duplicate assays and are expressed as picomoles of CH_3 incorporated into the DNA engaged in the reaction.

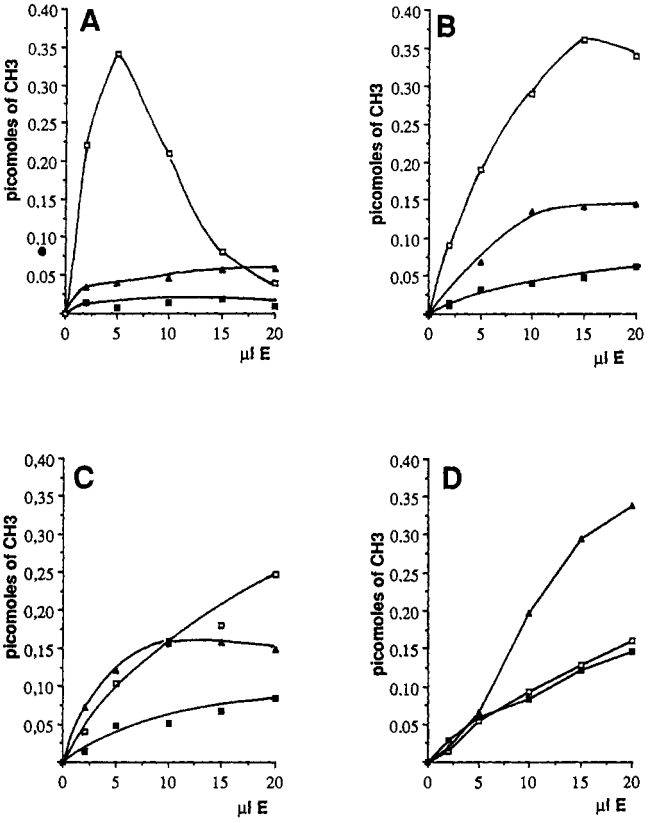

FIGURE 5: Influence of increasing amounts of DNA methylase on de novo DNA methylation. The reaction mixture contained increasing amounts of enzyme (0–20 μg) in addition to 0.25 μg (A), 1 μg (B), 3 μg (C), or 30 μg (D) of *M. luteus* DNA without MeCbl (□) or with 0.1 μM (▲) or 100 μM (■) MeCbl. Incubation time was 30 min. The results are average values of duplicate assays and are expressed as picomoles of CH_3 incorporated into the DNA engaged in the reaction.

by high DNA concentrations.

Influence of DNA Methylase Concentrations. (A) Methylation in the Absence of MeCbl. At a low DNA concentration (0.25 μg) and in the absence of MeCbl, the rate of both de novo (Figure 5A) and maintenance (Figure 6A) DNA methylations increased with increasing amounts of enzyme, whereas, at higher enzyme concentrations, above 5 μL for native *M. luteus* DNA and 10 μL for hemimethylated DNA, the DNA methylation level was markedly reduced. However, no inhibition by an excess of enzyme was found when higher

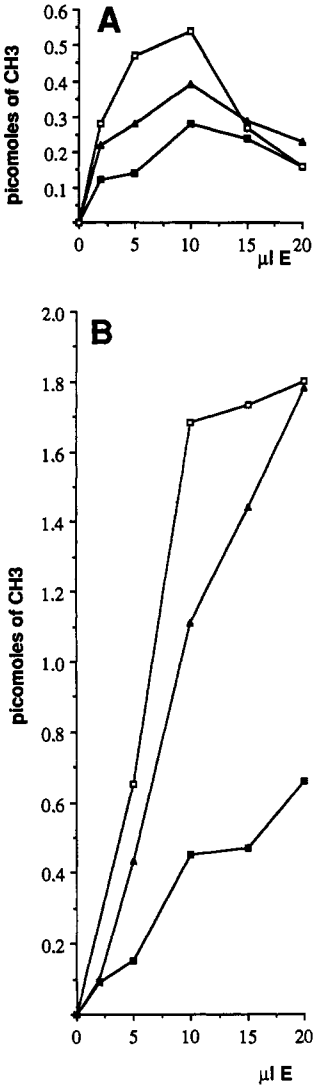

FIGURE 6: Influence of increasing amounts of DNA methylase on maintenance DNA methylation. The conditions are the same as in Figure 4 but hemimethylated *M. luteus* DNA was used, 0.25 µg (A) or 1 µg (B).

concentrations of DNA were used (Figure 5B,C,D and 6B).

(B) Methylation in the Presence of Variable Amounts of MeCbl. 0.25 µg of DNA: For a low DNA concentration, MeCbl, whatever its concentration [low (0.1 µM) or high (100 µM)], strongly inhibits the methylation reaction (Figure 5A), whereas the inhibition was less important with hemimethylated DNA (Figure 6A).

1 µg of DNA: MeCbl still inhibits the methylation reaction in a significant way (85%) for the high concentration (100 µM), but for the low concentration (0.1 µM) on the other hand, this inhibition is markedly reduced (only 65% of inhibition) (Figure 5B). But, with hemimethylated DNA the inhibition by 0.1 µM of MeCbl is less pronounced and disappears with 20 µL of enzyme.

3 µg of DNA: MeCbl at 100 µM still reduces the reaction rate, but for low concentration (0.1 µM of MeCbl) there is no influence or even a slight stimulation of the reaction, as long as small quantities of enzyme are used. For higher amounts of enzyme MeCbl becomes inhibitor (Figure 5C).

30 µg of DNA: For high concentrations of DNA and MeCbl (100 µM) the reaction was never inhibited (the two plots are identical). However, for a low concentration of MeCbl (0.1 µM) there is an important stimulatory effect (Figure 5D).

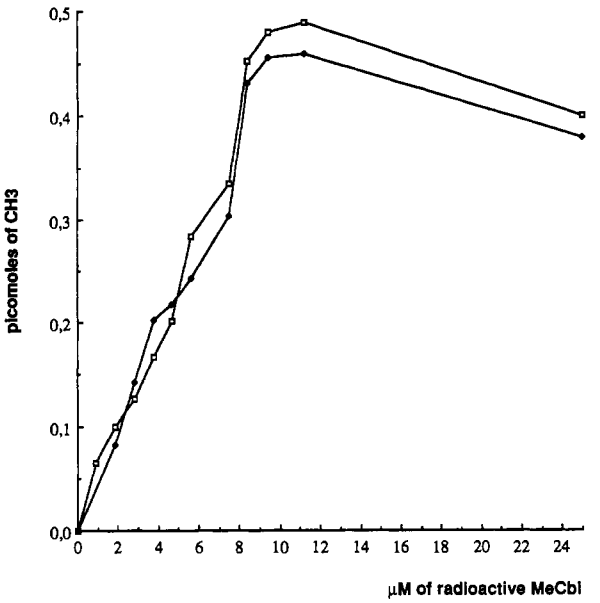

FIGURE 7: Methylation of DNA by radioactive MeCbl. A total of 1 µg of native (□) or hemimethylated (■) *M. luteus* DNA was incubated for 1 h in a reaction mixture containing increasing amounts of radioactive MeCbl (1–25 µM) and 10 µg enzyme but no SAM. The results are expressed as picomoles of CH_3 incorporated into 1 µg of DNA and are average values of duplicate assays.

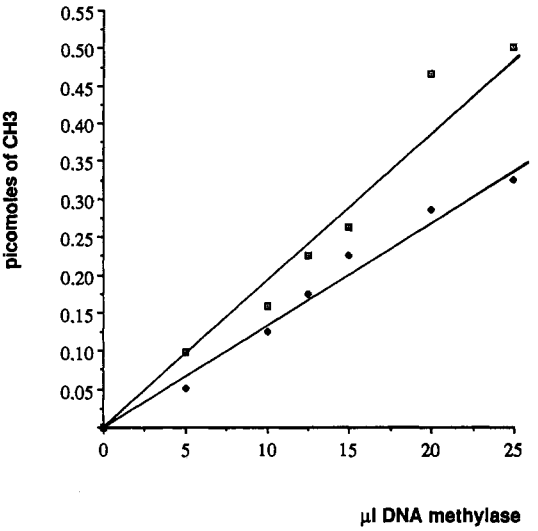

FIGURE 8: Effect of increasing amounts of enzyme in the presence of radioactive MeCbl. A total of 1 µg of native (□) or hemimethylated (◆) *M. luteus* DNA was incubated in a reaction mixture (0.1 mL) containing 100 µM MeCbl and increasing amounts of enzyme (0–25 µg). The values are an average of duplicate assays and are expressed as in Figure 4.

Is Methylcobalamin a Methyl Donor? In order to check if MeCbl could be a donor of methyl groups, native or hemimethylated *M. luteus* DNA was incubated in the absence of SAM but in the presence of increasing amounts of radioactive MeCbl. A linear increasing of CH_3-group incorporation into DNA was observed (Figure 7), but no significant difference was obtained between the two substrates. Thus, MeCbl could acts as a methyl donor. The result presented in Figure 8, where the reaction rate of enzymatic DNA methylation obtained with radioactive MeCbl increases linearly with increasing amounts of DNA methylase, confirms that MeCbl methylates DNA in vitro. In this latter case, hemimethylated *M. luteus* DNA is only slightly better methylated by radioactive MeCbl than native *M. luteus* DNA. The curves of de novo and maintenance-type methylation as a function of time are the same but

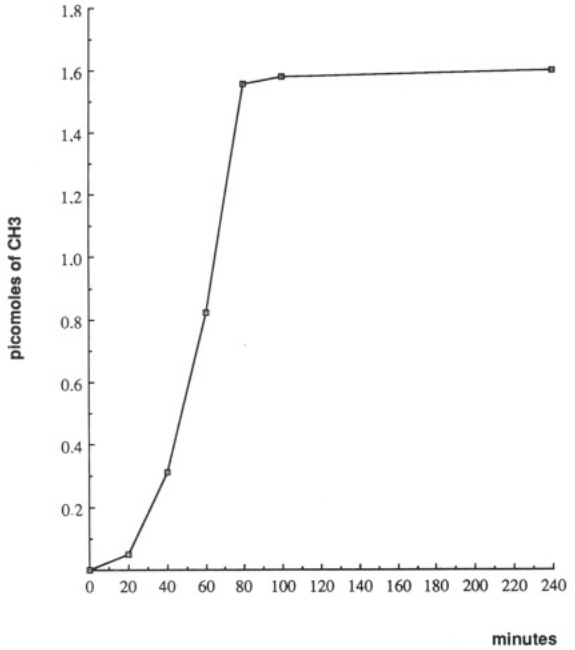

FIGURE 9: Time course of DNA methylation by MeCbl. A total of 1 μg of native or hemimethylated *M. luteus* DNA was incubated at different time points in a reaction mixture without SAM but in presence of 100 μM MeCbl. The results are expressed as picomoles of CH_3 incorporated into 1 μg of DNA and are an average of duplicate values.

are not of the classical type (Figure 9). It looks like as if the enzyme was activated by the substrate (MeCbl). Thus, there is no linear early reaction phase and we could not determine the K_m for MeCbl. SAM at concentrations up to 300 μM did not inhibit the methyl reaction in the presence of 80 μM MeCbl.

Identification of the Methyl Moiety Transferred to DNA. In order to characterize the methylated base formed by the action of MeCbl on DNA, we incubated nonradioactive MeCbl with DNA under the conditions given in the Material and Methods section. The DNA was isolated from the reaction mixture, and the m^5dCp was characterized by the ^{32}P post-labeling, as indicated in the Material and Methods section. The spot corresponding to m^5dC was scraped off and counted (Figure 10). The m^5dC content of *M. luteus* DNA was of 0.5%. After incubation for 1 h with 0.3 μM MeCbl, a content of 1.5% of m^5dC was obtained. No other methylated bases were detected.

Discussion

In the present paper, we show that cyanocobalamin (vitamin B_{12}) and three of its derivatives (CoE B_{12}; OHCbl; MeCbl) significantly influence the in vitro DNA methylation by rat spleen DNA methylase in the presence of 10 μM SAM. Indeed, low amounts of vitamin B_{12}, CoE B_{12}, and MeCbl significantly stimulate the de novo methylation, whereas no activation is observed with OHCbl. The increase of DNA methylation is highest with 0.3 μM MeCbl (47%) followed by 0.1 μM CoE B_{12} (30%) and 0.5 μM vitamin B_{12} (23%). Therefore, because the methylation measured in these experiments could only come from SAM, which is the only radiolabeled methyl carrier present in the reaction mixture, the three active cobalamin derivatives could have an allosteric interaction on the enzyme. However, these cobalamin derivatives do not activate the maintenance methylation. The above results, together with earlier results (Pfohl-Leszkowicz et al., 1987), suggest the existence of two different DNA methylases or the occurrence of different conformations of the

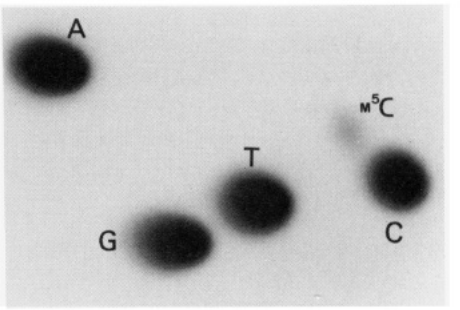

FIGURE 10: Detection of methylated nucleotides. An autoradiogram is shown of a developed TLC plate to which 0.16 μg of ^{32}P-postlabeled *M. luteus* DNA methylated in vitro by 0.3 μM MeCbl was applied with UV-detectable quantities of each of the nucleotides noted A = pdA, G = pdG, T = pdT, C = pdC, and m^5C = pm^5dC. Exposure to film was for 2 h.

enzyme as already suggested by Ruchiwarat et al. (1984) favoring either one or the other type of methylation and being sensitive or not to activation by cobalamins.

At concentrations between 1 and 20 μM, depending on the cobalamin derivatives, the de novo DNA methylation is inhibited. This inhibition is total in the presence of 400 μM MeCbl.

Maintenance DNA methylation is also inhibited by these compounds except vitamin B_{12}. However, the inhibition is less important than in the case of de novo methylation, and a remaining activity persists even in presence of 400 μM MeCbl (30%). In addition, MeCbl has been found to be a competitive inhibitor of SAM in the enzymatic DNA methylation reactions and its influence depends on the concentrations of DNA and of enzyme.

Several authors have previously shown that DNA methylase is irreversibly bound to DNA molecules and is inactivated by high concentrations of DNA (Predali-Noy & Weissbach, 1986; Palitti et al., 1987; Hitt et al., 1988). We confirm the above-mentioned inhibition in this study, and in addition, we show that the inactivation of the de novo DNA methylation by high concentrations of native *M. luteus* DNA is abolished by the presence of MeCbl. This may be due to a modification of the enzyme conformation favoring the dissociation of the enzyme–DNA–SAM complex. But this effect is not found with maintenance methylase.

Up to now, SAM was considered to be the universal methyl donor in DNA methylation (Borek & Srinivasan, 1966). In this paper, we demonstrate that MeCbl can also act as a methyl donor in in vitro DNA methylation. The rate of methylation in the presence of MeCbl and in the absence of SAM is similar with both native *M. luteus* DNA and hemimethylated DNA. Furthermore, SAM does not inhibit the incorporation of methyl groups incorporated by MeCbl. This result could indicate that SAM and MeCbl do not act on the same enzymatic sites. In fact, the nucleotide sequence of cloned mouse DNA methylase has been determined, revealing two domains: one binding SAM and another responsible for the methyl transfer (Bestor et al., 1988). This is in favor of this hypothesis. The inhibition of DNA methylation by SAM could be due to an allosteric effect or to the steric hindrance. An other explanation could be that in the enzymatic preparation we used, which is not completely pure, there is an enzyme working with SAM that is inhibited by MeCbl and another enzyme working with MeCbl that is not inhibited by SAM. At the present time, we cannot explain all the results that we obtained, particularly the intriguing effects of MeCbl when different DNA concentrations are used in the classical SAM-dependent methylation (Figure 4).

The question arises: does MeCbl act as a DNA methyl donor in vivo? The distribution of cobalamin derivatives in living organisms varies greatly depending on tissues and species (Schneider, 1987). For example, in rats the concentrations of total cobalamins are 0.1, 0.3, and 0.04 μM in liver, kidney, and spleen, respectively, whereas that of the methylcobalamin counterparts is 10% in kidney, 6.6% in spleen, and almost zero in liver as reported by Brazenas et al. (1979). These concentrations are far below the concentration of SAM in rat liver, which has been found to be 45 μM (Zappia et al., 1979). This difference could mean that the major DNA methylation process is the one using SAM as the methyl donor but does not exclude a secondary pathway that uses MeCbl as the methyl donor. However, the determinations of SAM and MeCbl were done on whole tissues and not separately in nuclei and cytoplasm, whereas DNA methylation obviously takes place in the nuclei. These two types of DNA methylation processes could coexist in living organisms. They could have in addition different specificities that have still to be found. They could even be catalyzed by different DNA methylases. Several hypotheses can be made: (i) MeCbl could be used for the DNA methylation at specific sequences of the DNA, different from those generally methylated by SAM; (ii) MeCbl could also act on specific sequences at different stages of the development of the cells in a so-called "fine-tuning DNA methylation" process whereas SAM acts in the major process.

References

Adams, R. L. P., & So, C. L. (1988) *FEBS Lett. 246*, 54–56.

Bestor, T., Laudano, A., Mattaliano, R., & Ingram, V. (1988) *J. Mol. Biol. 203*, 971–983.

Borek, E., & Srinivasan, P. R. (1966) *Annu. Rev. Biochem. 35*, 275–298.

Brazenas, G. R., Bartkeviciene, V. V., Aleksiene, A. M., Alioniene, A. J., Konoptkaite, S. J., & Zoryte, V. F. (1979) in *Vitamin B_{12}* (Zagalak & Friedriech, Eds.) pp 1113–1118, Walter de Gruyter, Berlin.

Cedar, H. (1988) *Cell 53*, 3–4.

Doerfler, W. (1990) *Phil. Trans. R. Soc. London 326*, 253–265.

Doerfler, W., Toth, M., Kochanek, S., Achten, S., Freisem-Rabien U., Behn-Krappa, A., & Orend, G. (1990) *FEBS Lett. 268*, 329–333.

Dynan, W. S. (1989) *Trends Genet. 5*, 35–36.

Ellenbogen, L. (1979) *Biochem. Nutr. 27*, 45–96.

Gama-Sosa, C. W., & Ehrlich, M. (1983) *Biochim. Biophys. Acta 740*, 212–219.

Gardiner-Garden, M., & Frommer, M. (1987) *J. Mol. Biol. 196*, 261–282.

Gasson, J. C., Ryden, T., & Bourgeois, S. (1983) *Nature 302*, 621–623.

Hitt, M. M., Wu, T. L., Cohen, G., & Linn, S. (1988) *J. Biol. Chem. 263*, 4392–4399.

Holliday, R. (1987) *Science 238*, 163–170.

Iguchi-Ariga, S. M. M., & Schaffner, W. (1989) *Genes Dev. 3*, 612–619.

Jahner, D., & Jaenisch, R. (1984) in *DNA Methylation Biochemistry and Biological Significance* (Razin, A., Cedar, H., & Riggs, A. D., Eds.) pp 189–219, Springer Verlag, New York.

Langhlin, R. E., Elford, H. L., & Buchanan, J. M. (1964) *J. Biol. Chem. 239*, 2888–2895.

Mangum, J. H., Murray, B. K., & North, J. A. (1969) *Biochemistry 8*, 3496–3499.

Michalowsky, L. A., & Jones, P. A. (1989) *Environ. Health Perspect. 80*, 189–197.

Palitti, F., Carotti, D., Grünwald, S., Rispoli, M., Whitehead, E. P., Salerno, C., Strom, R., & Drahovsky, D. (1987) *Biochim. Biophys. acta 910*, 292–296.

Pfohl-Leszkowicz, A., Salas, C., Fuchs, R. P. P., & Dirheimer G. (1981) *Biochemistry 20*, 3020–3024.

Pfohl-Leszkowicz, A., Baldacini, O., Keith, G., & Dirheimer, G. (1987) *Biochimie 69*, 1235–1242.

Predali-Noy, G., & Weissbach, A. (1986) *J. Biol. Chem. 261*, 7600–7602.

Ruchirawat, M., Becker, F. F., & Lapeyre, J. N. (1984) *Biochemistry 23*, 5426–5432.

Schneider, Z. (1987) in *Comprehensive B_{12}* (Schneider, Z., & Stroinski, A., Eds.) pp 175–223, Walter de Gruyter, Berlin.

Stroinski, A. (1987) in *Comprehensive B_{12}* (Schneider, Z., & Stroinski, A., Eds.) pp 335–353, W. de Gruyter Press, Berlin.

Tasseron-De Jong, J. G., Dendulk,, H., Van De Putte, P., & Giphart-Gassler, M. (1989) *Biochim. Biophys. Acta 1007*, 215–223.

Weissbach, H., Peterkofsky, A., Redfield, B., & Dickerman, H. (1963) *J. Biol. Chem. 238*, 3318–3324.

Wilson, V. L., Smith, R. A., Autrup, H., Krokan, H., Musci, D. E., Le, N. N. T., Longorias, J., Ziska, D., & Harris, C. C. (1986) *Anal. Biochem. 152*, 275–284.

Zappia, V., Carteni-Farnia, M., & Porcelli, M. (1979) in *Transmethylation* (Udsin, E., Borchardt, R. T., & Creveling, C. R., Eds.) pp 95–104, Elsevier North Holland.