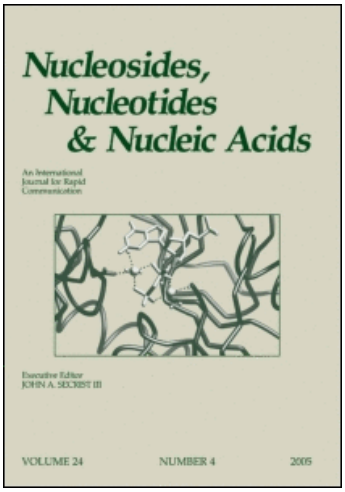

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Enzymatic Synthesis of Radioactive 5-Methyl-2'-Deoxycytidine 5'-Monophosphate by Using ^{32}P-Postlabeling

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ENZYMATIC SYNTHESIS OF RADIOACTIVE 5-METHYL-2'-DEOXYCYTIDINE 5'-MONOPHOSPHATE BY USING ^{32}P-POSTLABELING

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ABSTRACT - 5-Methyl-2'-deoxycytidine 5'-[^{32}P]- and deoxycytidine 5'-[^{32}P]-monophosphates were prepared from corresponding nucleotide homopolymers by using a ^{32}P-postlabeling procedure. The radioactive monophosphates obtained were well suited for biological and biochemical experiments.

5-Methylcytosine is the only modified or minor base in the DNA of higher eukaryotes. We have been interested in the possible salvage or reutilization of DNA 5-methyl-2'-deoxycytidine and its monophosphate (5MedCMP). For the study of metabolism and substrate behavior of 5MedCMP, we have synthesized a radioactive derivative employing the ^{32}P-postlabeling system developed by Randerath *et al.* (1) and recently modified by Wilson *et al.* (2) for the analysis of modified bases in DNA. This enzymatic procedure is easy to perform and may be applicable to synthesis of a variety of radioactive nucleoside monophosphates.

MATERIALS AND METHODS

Reagents and their storage - Polydeoxycytidylic acid and polydeoxy(5-methyl)cytidylic acid were from P-L Biochemicals (stored at 1 μg/μl in water, -20°C); micrococcal nuclease (*Staphylococcus au-*

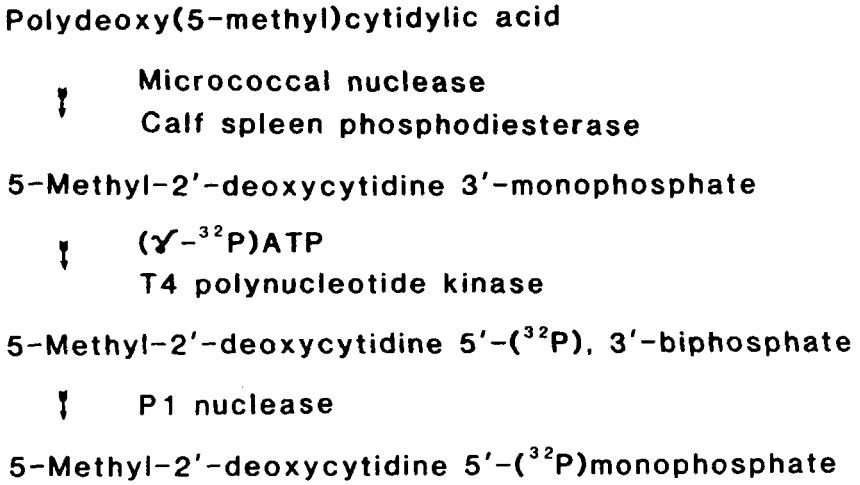

Figure 1. Enzymatic synthesis of 5-methyl-2'-deoxycytidine 5'-[^{32}P]monophosphate.

reus) from Sigma (1 U/µl, -20°C); phosphodiesterase (calf spleen) from Boehringer Mannheim (2 µg/µl or 4 U/ml, +4°C); T4 polynucleotide kinase from Boehringer Mannheim (10 U/µl, -20°C); P_1 nuclase (Penicillium citrinum) from Sigma (1 µg/µl or 0.3 U/µl in sodium acetate buffer, 30 mM, pH 5.3, +4°C); [γ-^{32}P]ATP (185 TBq/mmol, appr. 10 mCi/ml) from Amersham (-20°C). The chromatography (HPTLC) cellulose plates were from E. Merck.

^{32}P-postlabeling - The reaction scheme is outlined in Figure 1. The procedure involves minor modifications as compared to the original description by Wilson et al. (2). The additions were; 1 µg (1 µl) nucleotide homopolymer, 15.5 µl water, 5 µl reaction buffer (15 mM Tris-HCl, pH 8.8; 15 mM $CaCl_2$), 5 µl (5 U) micrococcal nuclease; 2.5 µl (5 µg) calf spleen phosphodiesterase. The reaction was allowed to proceed in a small Eppendorf tube for 2 hr at 37°C. 5 µl or 1/6 volume of the hydrolyzate was taken and the following additions were performed: 2 µl (20 µCi) [^{32}P]ATP, 2 µl reaction buffer (60 mM Bicine-NaOH, pH 9.5; 300 µM ATP, 60 mM $MgCl_2$, 10 mM

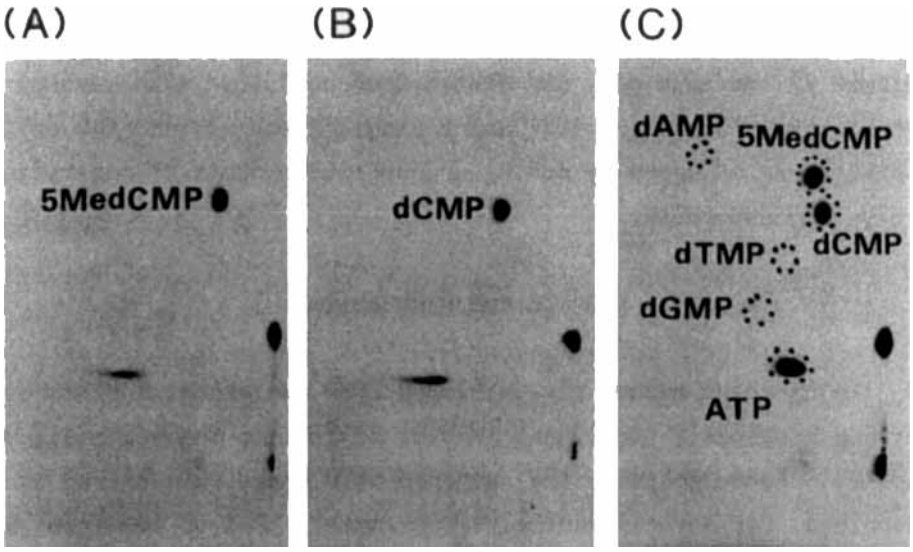

Figure 2. Autoradiography of a chromatographic resolution of enzymatically synthesized 5-methyl-2'-deoxycytidine 5'-[^{32}P] monophosphate (A), deoxycytidine 5'-[^{32}P]monophosphate (B), and both analogs (C). The first dimension (from bottom to top) was developed twice with isobutyric acid/water/NH_4OH (66/20/1), and the second perpendicular dimension (from left to right) with saturated ammonium sulfate/isopropanol/1 M sodium acetate (80/2/18). Conventional DNA deoxyribonucleoside monophosphate markers are illustrated in (C).

spermine, 60 mM dithiothreitol), 3 µl T_4 polynucleotide kinase (diluted 1/10 with water to make 2-5 U/3 µl immediately before addition). The reaction proceeded for a further 3 hr at 37°C. Then 10 µl P_1 nuclease (3 U) and 3 µl 10 mM $ZnSO_4$ were added and the reaction was allowed to proceed for an additional 60 min. The reaction was stopped by adding 2 volumes of ethanol. The proteins were precipitated for 2 hr at -20°C and the clear supernatant containing the nucleotides was saved after centrifugation; 10 000 x g for 15 min.

Separation of reaction products - The product nucleoside monophosphate was separated from radioactive ATP by HPTLC on cellulose plates using development with isobutyric acid/water/NH_4OH

(66:20:1). The reaction product was localized by autoradiography (Figure 2), scraped off, and eluted from cellulose with several water extractions. For biochemical and biological experiments the specific activity was adjusted by adding appropriate amounts of non-radioactive monophosphate.

RESULTS AND DISCUSSION

The present method was developed from analytical methods described previously (1,2), and is well suited for the synthesis of 5'-labeled ^{32}P-nucleotides. The reagents were good, when stored as described, for several months. Approximately 50 % of the label was incorporated into 5MedCMP and dCMP, when corresponding nucleotide homopolymers were used as starting material (Figure 2). The reaction product was exclusively the desired monophosphate. We have also applied this postlabeling system to other nucleotide polymers, such as calf thymus DNA, herring sperm DNA, and SPO-1 phage DNA. The latter Bacillus subtilis phage contains 5-hydroxymethyl-2'-deoxyuridine monophosphate instead of dTMP.

In conclusion, the ^{32}P-postlabeling system originally developed for analytical purposes (1,2) provides a convenient tool for synthesis of radioactive nucleoside monophosphates. The reaction product is easy to isolate, particularly if nucleotide homopolymers are used as starting material.

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