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Pronase-Based Assay Method for O^6-Methylguanine–DNA Methyltransferase

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A new, simple, and rapid assay method for O^6-methylguanine–DNA methyltransferase (MGMT) has been developed. When [*methyl*-^{3}H] DNA radiolabeled with *N*-[*methyl*-^{3}H]-*N*-nitrosourea was incubated together with tissue homogenate, [*methyl*-^{3}H] group was transferred to the enzyme, forming *S*-[*methyl*-^{3}H]cysteine. In contrast to the previous methods which determined the amount of [*methyl*-^{3}H] group removed from [*methyl*-^{3}H] DNA, the present method measured the amount of [*methyl*-^{3}H] transferred to the enzyme. This has been done by hydrolyzing the radiolabeled enzyme with pronase which is a proteolytic enzyme with a broad substrate specificity. On pronase digestion, [*methy*-^{3}H]-labeled enzyme becomes soluble in trichloroacetic acid. The method is very simple and rapid, and the only expensive equipment required is a scintillation counter which is a relatively routine piece of equipment at present. More than a dozen samples can be processed within 4–5 h without any difficulty. This new method has been employed in the studies on organ distribution of MGMT of rat and mouse.

Since the first discovery of the presence of O^6-methylguanine–DNA methyltransferase (MGMT)[1] in *Escherichia coli* in 1980, this enzyme has been one of the most extensively studied DNA repair enzymes (1). This enzyme repairs O^6-methylguanine in DNA, one of the alkylated DNA adducts formed by genotoxic alkylating agents and known to be the biochemical lesion for carcinogenesis. Thus, if not repaired, this lesion ultimately leads to mutagenesis and carcinogenesis by interfering with the GC hydrogen bonding in DNA (2–5).

This enzyme has an unusual characteristic in that it is not a catalytic enzyme in the strict sense. Although the methyltransferase is a protein in nature and removes the methyl group from damaged DNA, the enzyme itself becomes alkylated at its cysteine residue forming *S*-methyl cysteine. The alkylated enzyme consequently becomes inactivated (1, 6–10). Therefore, the reaction could be regarded as a "titration reaction." Thus, it is important for the organism to have an adequate number of MGMT to protect it from the consequences of mutagenesis and carcinogenesis caused by environmentally significant alkylating agents such as nitrosamines. In *E. coli*, it has been shown that the N-terminal region of the alkylated enzyme acts as an enhancer for transcription of the enzyme itself (11).

In view of the potential importance of MGMT in assessing the sensitivity of an individual toward alkylating agents, various assay methods for measuring MGMT activity have been developed. This includes direct measurement of repair of methyl-labeled synthetic oligonucleotides or DNA (4, 12, 13), the use of restriction endonuclease which specifically recognizes O^6-methylguanine residues (14, 15), or measurement of *S*-methyl cysteine in formed MGMT after acid hydrolysis (16). The above-mentioned methods are very time-consuming and laborious compared with our present method and require special apparatus in addition to routine laboratory equipment. The method described here is based on the fact that the [*methyl*-^{3}H]-labeled MGMT, the transmethylation product, is digested simply by pronase to yield free [*methyl*-^{3}H]cysteine or [*methyl*-^{3}H]cysteine-containing short peptides which become soluble in TCA. The radioactivity in the TCA supernatant is counted for estimating transmethylation repair activity. The method can handle a large number of samples in a 4- to 5-h period.

MATERIALS AND METHODS

Materials

N-[*methyl*-^{3}H]-*N*-Nitrosourea (specific activity, 20 Ci/mmol) was obtained from Amersham Corp. Histone II-

[1] Abbreviations used: MGMT, O^6-methylguanine–DNA methyltransferase; DTT, dithiothreitol; TCA, trichloroacetic acid.

0003-2697/96 $18.00

AS (a mixture of calf thymus histones), pronase E (from *Streptomyces griseus*), calf thymus and salmon sperm DNA, EDTA, and dithiothreitol (DTT) were purchased from Sigma. The rest of the chemicals were obtained from various commercial sources and of the highest purity available.

Preparation of [methyl-^{3}H] DNA

The [*methyl*-^{3}H] DNA was prepared according to the method described (1, 16). Calf thymus DNA (4 mg/ml) in 0.2 M sodium cacodylate–1 mM EDTA (pH 7.4) was incubated with *N*-[*methyl*-^{3}H]-*N*-nitrosourea at the ratio of 40 μCi/mg DNA for 4 h at 37°C in the dark. The treated DNA was precipitated with 2 vol of cold ethanol, washed once with ethanol, dissolved in the original volume of 0.1 M NaCl–10 mM sodium phosphate (pH 7.4), and then heated at 80°C for 17 h. This treatment is known to labilize most of the purines methylated at the N^7 and N^3 positions, but leave O^6-methylguanine residues intact (6). The heated DNA was precipitated with cold ethanol, dissolved in 10 mM Tris·HCl–1 mM EDTA buffer (pH 8.0), and dialyzed against the same buffer for 2 days at 4°C. DNA prepared in this manner contained 1.15×10^5 dpm/mg DNA with 42% of the radioactivity coeluting with the authentic O^6-methylguanine marker, 31% with N^7-methylguanine, and the rest was unaccountable when analyzed by HPLC C_{18} reverse-phase column (5 μm) (Fig. 5A).

MGMT Assay

The following condition was established for routine assay after a series of standardizations.

Transmethylation reaction (step 1). Ten percent tissue homogenate in buffer A [70 mM Hepes (pH 7.8), 0.5 mM EDTA, 5% glycerol, and 1 mM DTT] was prepared with electrically driven Teflon–glass homogenizer and the homogenate was passed through a double layer of cheesecloth. Ninety-five microliters of the buffer A, 70 μl of tissue homogenate, and 40 μl of water were preincubated at 37°C for 5 min. The reaction was initiated by the addition of 95 μl of [*methyl*-^{3}H] DNA (380 μg, 43,700 dpm), making the final volume 300 μl. The reaction was carried out for 1 h with intermittent shaking.

Pronase digestion (step 2). After 1-h incubation, 25 μl of unlabeled salmon sperm DNA (250 μg) and 20 μl of pronase (2 mg)[2] were added, and the final volume was made up to 350 μl with water. Unlabeled DNA was added in this step in order to suppress the effect of DNase activity during protein digestion (described under Discussion). The tubes were incubated for another 2 h[3] with occasional shaking.

Precipitation (step 3). After 2 h digestion of [*methyl*-^{3}H]MGMT with pronase, 40 μl of histone (25 μg) was first added to the mixture followed by the same volume of 30% TCA at 0°C. After 15 min, the mixture was centrifuged in a tabletop centrifuge, and the clear supernatant was counted for radioactivity. The addition of histone to the incubation mixture facilitates the precipitation of DNA by TCA.

The incubation mixtures which were treated with histone and TCA after 1 h initial transferase reaction (step 1 in the above) served as controls. The control values were subtracted from the assay values. The above reaction was carried out in duplicate and the values obtained were within ±5% of the averages. The results are expressed as picomoles [*methyl*-^{3}H] transferred per milligram tissue protein. Finally, protein concentration was determined by the method of BCA (Pierce Chemical Co., No. 23225) using bovine serum albumin as the standard.

HPLC Analysis of [methyl-^{3}H]DNA

In order to confirm the specificity of MGMT repair enzyme activity using this assay method, two incubation mixtures of 300 μl final volume were prepared. The assay tube contained rat spleen whole homogenate, whereas the control had water. Both were incubated at 37°C for 1 h (step 1 reaction), and 30 μl of 1 N HCl was added. The mixtures were further incubated at 80°C for 1 h in order to depurinate [*methyl*-^{3}H] DNA (18). Subsequently, the mixtures were centrifuged, and 260 μl of 0.04 M Tris–base and 42 μl of methanol were added into the clear supernatants. Fifty microliters of the mixture was analyzed on HPLC (Phamacia LKB) with Flow-one/beta detector (Radiomatic) attached. A C_{18} column (5 μm, 250 × 4.1 mm) was run with the mobile phase of solution A (50 mM KH_2PO_4, pH 5.6) and solution B (methanol:H_2O, 80:20). The flow rate was 1.0 ml/min and the Radiomatic (Flow-one/beta) detector splitter ratio was 50%.

RESULTS

Effect of [methyl-^{3}H] DNA concentration. Figure 1 illustrates the effect of [*methyl*-^{3}H]DNA concentration under the conditions used. When 1.3 mg protein of rat spleen homogenate was assayed, there was a plateau at around 250 μg of DNA. This represents approxi-

[2] In order to remove contaminating DNase, pronase E solution (20 mg/ml) was self-digested at 37°C for 2 h (17), lyophilized, resuspended as 100 mg/ml in a buffer [10 mM Tris–HCl (pH 7.5)–10 mM NaCl], and stored frozen until use. It was confirmed that DNase was completely eliminated.

[3] As shown later, pronase digestion is complete within 5 min under present assay conditions. However, 2-h incubation time was adapted for assay in order to assure complete digestion under unexpected conditions such as a large amount of MGMT present in the sample or pronase E preparation with low enzyme activity. This 2-h incubation time can be shortened depending on the situation.

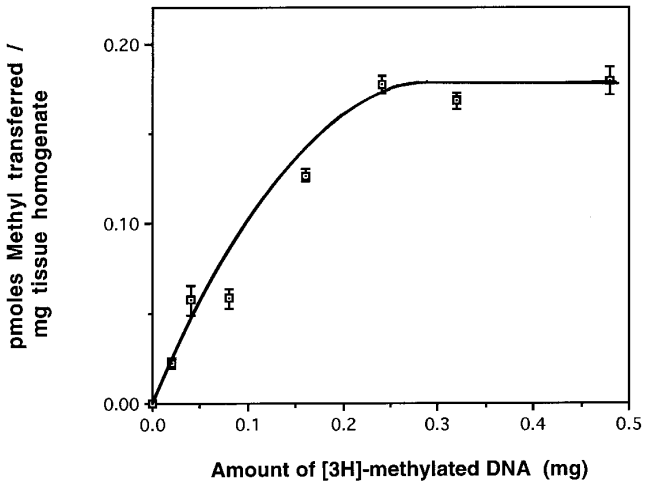

FIG. 1. Effect of [*methyl*-^{3}H] DNA concentration. Female rat, weighing 260 g, was used. Spleen whole homogenate (1.3 mg protein) and 380 μg of [*methyl*-^{3}H] DNA was used. The rest of the experimental conditions are described under Materials and Methods. The 41,780 dpm corresponds to 1 pmol of [*methyl*-^{3}H] transferred. The points in the figure represent averages of three independent determinations.

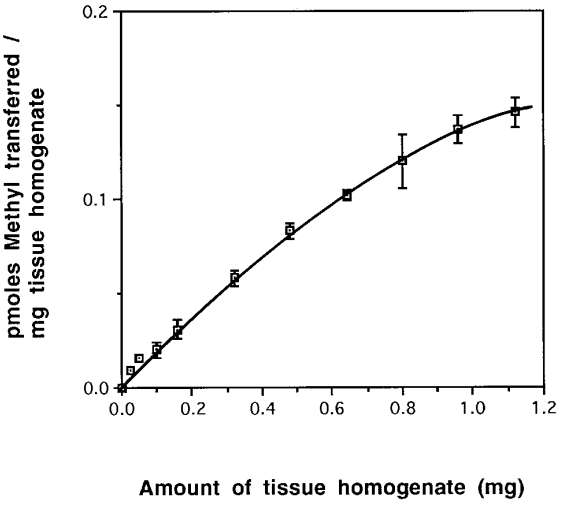

FIG. 2. Effect of enzyme (tissue homogenate) concentration. Female rat, weighing 500 g, was used. The rest of the conditions are the same as in Fig. 1. Different amounts of spleen homogenates were added in the total incubation mixture of 300 μl. The points in the figure represent averages of three independent determinations.

mately 0.19 pmol[4] of O^6- [*methyl*-^{3}H]guanine–DNA repaired. In order to safeguard complete saturation for MGMT, 380 μg of [*methyl*-^{3}H] DNA in 300 μl total incubation mixture was used in the following routine assay.

Effect of amount of MGMT. There is a smooth correlation between the amounts of MGMT (spleen homogenate) and of [*methyl*-^{3}H] transferred, only deviating slightly at higher tissue concentration (Fig. 2). It is apparent that about 0.14 pmol of [*methyl*-^{3}H] are transferred per milligram of tissue protein.

Time of incubation. Transfer of the [*methyl*-^{3}H] group from [*methyl*-^{3}H] DNA to MGMT is completed within 60 min of incubation (Fig. 3). Therefore, a 60-min incubation period was chosen for a routine assay. This time period has also been employed by others (16, 19). Here, 0.2 pmol of [*methyl*-^{3}H] group was transferred with 1 mg of tissue protein.

Incubation volume. Figure 4 shows that the total reaction volume (step 1) can be scaled down from 300 to 100 μl without any adverse effect. This is important in order to save expensive [*methyl*-^{3}H] DNA in a large number of assays in a clinical setting.

Effect of incubation time and amount of pronase to digest [*methyl*-^{3}*H*]*MGMT.* It was found in the present study that even 0.5 mg of pronase could solubilize all the [*methyl*-^{3}H]MGMT (data not shown). However, we did not attempt to find the minimum amount of pronase to digest all the MGMT. Instead, 2 mg of pronase was routinely employed.

Also, even 5 min of digestion with 2 mg of pronase was found to be sufficient to digest the [*methyl*-^{3}H]-MGMT under the conditions used (data not shown). However, 2 h was chosen for the incubation period with pronase (step 2).

Analysis of acid-hydrolysate of [*methyl*-^{3}*H*] *DNA.* In the present study, calf thymus DNA methyl-labeled nonenzymatically with *N*-[*methyl*-^{3}H]-*N*-nitrosourea

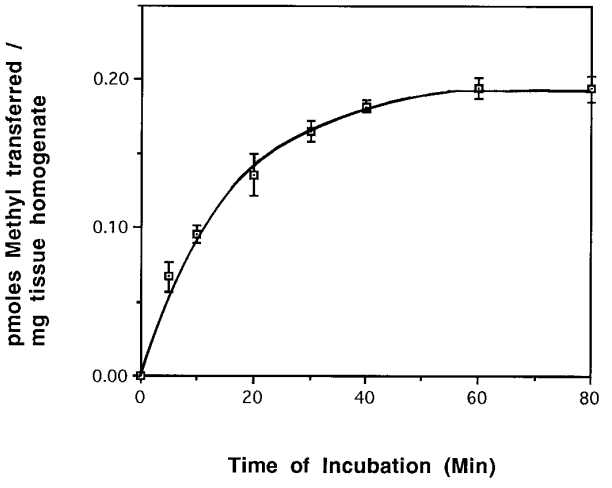

FIG. 3. Determination of incubation time. Spleen whole homogenate (1.14 mg) of female rat weighing 400 g was used. The rest of the experimental conditions are the same as in Fig. 1. The points in the figure are averages of three independent experiments.

[4] Since 43,700 dpm which was present in 300 μl incubation mixture is equal to total 1.05 pmol [*methy*-^{3}H] group or 0.56 pmol of O^6-[*methyl*-^{3}H]guanine [product analysis indicated that 53% of the total [*methyl*-^{3}H] incorporated resides in O^6-methylguanine (Fig. 5A)], 0.19 pmol [*methyl*-^{3}H] represents that approximately 35% of the O^6-[*methyl*-^{3}H]guanine-DNA was repaired (0.19/0.56 $\times$ 100).

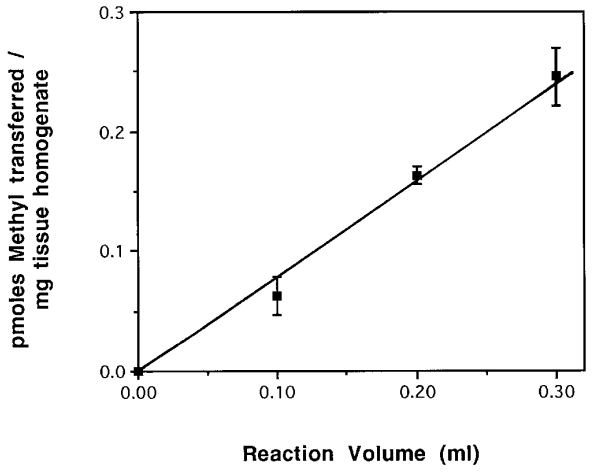

FIG. 4. Effect of incubation volume. Spleen whole homogenate (17 mg protein/ml) of female rat (body weight:400 g) was used. All the ingredients in the transferase reaction mixture (step 1) were equally scaled down from 300 to 200 or 100 μl incubation volume. However, amounts of pronase, histone, and TCA (step 2 and 3 reaction) were the ones used for 300 μl reaction volume. The points in the figure are averages of three independent determinations.

was employed as the substrate. Since it was nonenzymatic, specific labeling of O^6-methylguanine cannot be expected. In order to observe [*methyl*-^{3}H] labeling pattern, [*methyl*-^{3}H] DNA is hydrolyzed with 0.1 M HCl, and the hydrolysate was analyzed by HPLC with Flow-one/beta detector (Radiomatic) attached. As shown in Fig. 5A, two major radiolabeled peaks were identified: Approximately 53% of the total radioactivity incorporated was found to be in O^6-methylguanine and 17% as N^7-methylguanine, and the ratio of radioactivity in the O^6-methylguanine to that in N^7-methylguanine was 3.2. The rest of the radioactivity peaks could not be identified. As seen in Fig. 5B, on MGMT (spleen homogenate) treatment, the ratio of radioactivity in O^6-methyl- to N^7-methylguanine decreased to 1.4. This indicates that the amount of O^6-methylguanine greatly reduced, demonstrating an enzymatic removal of the [*methyl*-^{3}H] group from this compound. It has been demonstrated that N^7-methylguanine is not demethylated at DNA level (6).

Organ distribution of MGMT. This new assay method has been applied also to a study on the distribution of MGMT among some selected organs of rat and mouse (Table 1). It is noted that MGMT activity in rat liver is a little higher than in spleen, with no detectable activity in the brain. The absence of this enzyme in brain has been suggested as a reason for the prevalent occurrence of neoplastic growth in this organ after administration of methyl-*N*-nitrosourea (20, 21). Also, the absence of neoplastic growth in both spleen and liver has been suggested to be due to large amounts of this enzyme in these organs because O^6-methylguanine formed after administrations of methyl-*N*-nitrosourea should be rapidly repaired by this enzyme. It is also noted in Table 1 that mouse organs, in general, have only one-third of the MGMT activity of rat organs and that thymus, spleen, and liver have almost equal amounts of the enzyme.

DISCUSSION

In order to assess sensitivity of an individual to cancer risk, diagnosis, or progress of the disease, it is highly desirable to have a simple, but accurate measurement of some parameters. With this perspective in mind, MGMT has been the main focus of many intensive investigations (5, 18, 20). Indeed, it has been ob-

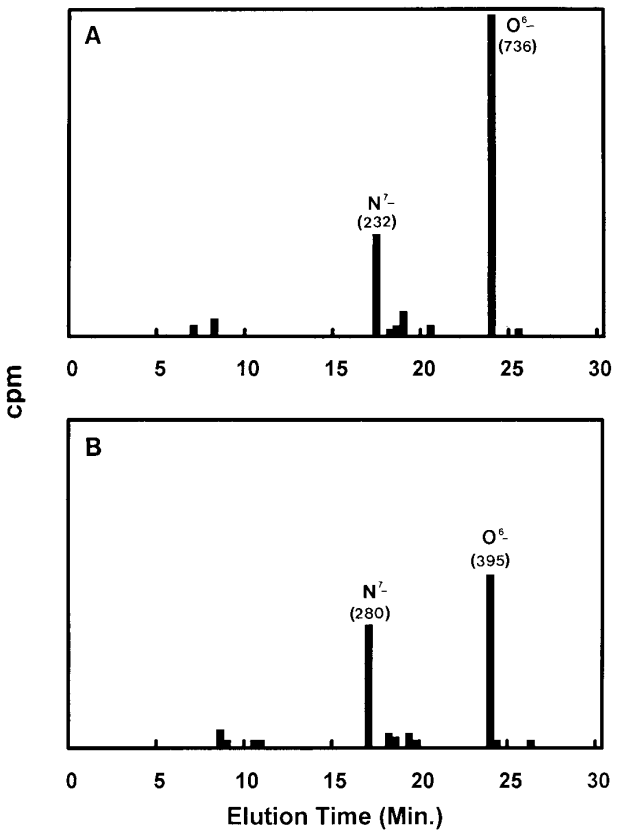

FIG. 5. Specific removal of radioactivity from the O^6-methylguanine, not from the N^7-methylguanine. Two incubation mixtures of 300 μl final volume were prepared: one assay tube contained rat spleen whole homogenate, whereas the other had water. Both were incubated at 37°C for 1 h (step 1 reaction) and depurinated with 1 N HCl. Subsequently, the mixtures were centrifuged, and the clear supernatants were analyzed on HPLC (Phamacia LKB) with Flow-one/beta detector (Radiomatic) attached. The numbers in parenthesis indicate cpm found in the peaks. N^7- and O^6- represent N^7-methylguanine and O^6-methylguanine, respectively. (A) HPLC profile of [*methyl*-^{3}H] DNA without tissue homogenate. The ratio of radioactivity present in O^6-methylguanine to that in N^7-methylguanine is 3.2. (B) Assay: HPLC profile of [*methyl*-^{3}H] DNA after the enzyme (spleen homogenate) reaction. The ratio of radioactivity found in O^6-methylguanine to that in N^7-methylguanine is 1.4.

TABLE 1

Distribution of MGMT among Some Selected Organs of Rat and Mouse

Animal	Organs	MGMT activity[a] (pmol [*methyl*-^{3}H] transferred/mg tissue protein)
Rat	Liver	0.155 ± 0.007
	Spleen	0.172 ± 0.037
	Brain	0
Mouse	Thymus	0.074 ± 0.019
	Spleen	0.061 ± 0.013
	Liver	0.059 ± 0.001
	Lung	0.036 ± 0.025

Note. Sprague–Dawley male rats, weighing approximately 500 g and A/J mice weighing 13.5 g were used. The total volume of reaction mixture was 300 μl. The rest of the experimental procedure is described under Materials and Methods.

[a] The values are averages ± SD of three separate experiments.

served that MGMT activity increased in many human tumor cells (21, 22).

Previously, Pegg and his co-workers studied the O^6-alkylguanine–DNA-alkyltransferase activity by analyzing the disappearance of O^6-methylguanine from [*methyl*-^{3}H] DNA or synthetic oligonucleotides on HPLC (4). This involved TCA precipitation and subsequent acid hydrolysis of [*methyl*-^{3}H] DNA after the enzymatic reaction. Recently, Wilson *et al.* reported a combined use of restriction endonuclease and a magnetic bead on which ^{35}S-end-labeled synthetic oligonucleotides were attached (15). After hybridization with complementary oligonucleotide containing O^6-methylguanine, the magnetic bead was incubated with alkyltransferase, followed by *Pvu*II restriction endonuclease treatment. Both of these methods measure the removal of methyl groups of O^6-methylguanine in DNA molecule.

In contrast to the above, Ro *et al.* determined the amount of [*methyl*-^{3}H] groups transferred to methyltransferase itself (16). They employed two steps hydrolysis: After incubating the [*methyl*-^{3}H] DNA with methyltransferase, ethanol precipitate was first hydrolyzed in 0.1 M HCl at 70°C to solubilize unreacted [*methyl*-^{3}H] DNA, and the TCA precipitate ([*methyl*-^{3}H]-labeled protein) was subsequently hydrolyzed to cleave peptide bonds in 20% formic acid–2 M HCl at 110°C. *S*-[*methyl*-^{3}H]Cysteine formed was analyzed by a small DOWEX50 (H^+) column.

Our assay method also determines the amount of methylation product, that is, the amount of [*methyl*-^{3}H] transferred to methyltransferase itself. Unlike the method of Ro *et al.*, however, we solubilize the [*methyl*-^{3}H]-labeled methyltransferase with pronase which has a broad specificity, so that prolonged digestion of a protein results in complete hydrolysis of protein to amino acids (23). Thus, the reaction mixture was first treated with pronase to solubilize [*methyl*-^{3}H] protein and the solubilized [*methyl*-^{3}H] protein hydrolysate was subsequently separated from unreacted [*methyl*-^{3}H] DNA with an aid of histone and TCA. This method does not require any expensive apparatus except a scintillation counter which is a relatively routine equipment nowadays. In our hands, a dozen incubation tubes can be easily processed in 4–5 h with duplicate values within ±5% variation. The most expensive item in this method is *N*-[*methyl*-^{3}H]-*N*-nitrosourea which is anyway required for the other methods mentioned above. As shown in Fig. 4, however, the incubation volume can be reduced below 100 μl, saving expenses.

Our method quantifies the amount of [*methyl*-^{3}H] transferred to protein (here, methyltransferase) from [*methyl*-^{3}H] DNA. Therefore, the method does not give any information on the nature of methylated bases repaired. As illustrated in Fig. 5A, the [*methyl*-^{3}H] in O^6-methylguanine constitutes approximately 53% of the total radioactivity of the [*methyl*-^{3}H] DNA and this is the major site for repair enzyme MGMT (Fig. 5B). As shown in Fig. 5B, N^7-methylguanine is not repaired by the MGMT pathway. It has been shown that the purified MGMT from *E. coli* failed to act on N-linked methyl groups of DNA bases, including N^6-methyladenine and N^7-methylguanine (24). The O^2-methylated pyrimidines are not repaired by a methyltransferase, but rather removed from DNA by an *N*-glycosylase.

At the early phase of our experiment, we encountered high control values [for example, approximately 0.25 pmol/mg tissue protein (10,300 dpm)]. Here, the control refers to the incubation tube in which only methyltransferase reaction was allowed (step 1 reaction), and represents the value found in TCA-soluble fraction without pronase digestion. This is the sum of radioactivity derived from [*methyl*-^{3}H] DNA preparation (about 0.02 pmol) and from digestion of [*methyl*-^{3}H] DNA by DNase present in the crude tissue homogenate during transmethylation reaction. On the other hand, the assay values were about 0.27 pmol/mg tissue protein, being only slightly identifiable over the control. Therefore, in order to suppress the DNase activity inherent in crude tissue homogenate, we increased EDTA concentration from 0.1 to 0.5 mM to remove Mg^{2+} which is cofactor for DNase activity, eliminated DNase present in pronase E preparation (see footnote 2), and added unlabeled DNA to the incubation mixture during pronase treatment. On these improvements, we routinely observed about 20–30% of the assay values as the control values [with rat spleen homogenates, the control values were 0.07 ± 0.02 pmol/mg protein (n = 12)], and this magnitude of the control values does not interfere with the accuracy of the present assay method.

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