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# STUDIES ON THE DISTRIBUTION OF $O^6$-METHYLGUANINE-DNA METHYLTRANSFERASE IN RAT

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**Abstract**—$O^6$-Methylguanine-DNA methyltransferase, a DNA repair enzyme which transfers the methyl group of $O^6$-methylguanine residue to a cysteinyl residue in the methyltransferase itself, was examined in rat organs by quantifying the *S*-methylcysteine formed in the methyl acceptor protein. Among the various organs examined, the spleen exhibited the highest enzyme specific activity followed by the thymus, liver, lung and testis. Brain had the lowest activity. The patterns of subcellular distribution of the methyltransferase in spleen and liver were different: while 75–80% of the activity was present in the nuclear fraction of the spleen, 54% of the activity in the liver was found in the nuclei and 35% in the cytosolic fraction. Forty-five and thirty-five percent of the total nuclear enzyme activity could be extracted with 1 M and 2 M NaCl solutions, respectively, indicating that the repair enzyme is not tightly bound to the nuclear matrix. When isolated nuclei were incubated with [*methyl*-$^3$H]DNA substrate and subsequently fractionated into histone and non-histone protein fractions, over 90% of the radioactivity was coeluted on a Bio-Rex 70 column with the non-histone fraction and only a negligible amount of radioactivity was found to be associated with the histone fraction. The molecular mass of the [*methyl*-$^3$H]methyltransferase in the non-histone fraction was determined to be 23,000, and its pI value was found to be 6.6 by two-dimensional polyacrylamide gel electrophoresis.

Alkylating nitroso compounds are capable of modifying several sites on pyrimidine and purine bases in DNA *in vivo* and *in vitro* [1]. Among these lesions, however, alkylation at the O$^6$-position of guanine strongly correlates with mutagenicity and carcinogenicity [2, 3]. Recently, a specific transmethylase to repair the lesion has been discovered in both *Escherichia coli* [4] and mammalian tissues [5, 6]. Unlike other known repair systems, such as DNA glycosylases which remove unnatural DNA bases [7], the transmethylation repair reaction merely removes the methyl group from the $O^6$-methylguanine ($O^6$-mG†) residue and regenerates the originally present guanine residue [8]. The removed methyl group has been shown to be transferred to a specific cysteinyl residue of the transferase itself, thereby inactivating any further activity of the enzyme [4, 7]. Recently, we have developed an *in vitro* assay method for $O^6$-mG-DNA methyltransferase by which *S*-methylcysteine formed in the transferase can be quantitatively determined [9].

In 1974, Goth and Rajewsky [10] demonstrated that the rate of $O^6$-ethylguanine removal from rat liver DNA was much greater than that from brain and kidney DNA, upon administration of *N*-ethyl-*N*-nitrosourea, suggesting the presence of an active $O^6$-ethylguanine DNA repair system in the liver. Subsequently, the repair activity was shown to be due to the presence of $O^6$-mG-DNA methyl(alkyl)-transferase in the liver [4–6].

In the present paper, we studied the tissue distribution of $O^6$-mG-DNA methyltransferase in several rat tissues by quantifying the *S*-methylcysteine yield in the methyl acceptor protein (methyltransferase itself). Unexpectedly, our results indicated that spleen exhibited the highest methyltransferase activity. Thus, subcellular distribution of the enzyme in spleen and liver as well as subnuclear localization of the enzyme were carefully studied. A preliminary report of some of this work has been presented at the meeting [11].

## MATERIALS AND METHODS

*Materials.* *N*-[*methyl*-$^3$H]-*N*-Nitrosourea (specific radioactivity, 3.5 Ci or 5.4 Ci/mmole) was obtained from the Amersham Corp. Calf thymus DNA, Dowex-50-X8 (200–400 mesh), and *S*-methylcysteine were purchased from the Sigma Chemical Co. Other chemicals were from various sources and of the best reagent grade available. Normal adult rats (CD-Fisher/344) were obtained from the Charles River Breeding Laboratories.

*Enzyme assay.* $O^6$-mG-DNA methyltransferase activity was assayed as described [9] by quantifying the *S*-[*methyl*-$^3$H]methylcysteine formed in the protein after incubating with [*methyl*-$^3$H]DNA substrate. [*Methyl*-$^3$H]DNA substrate was prepared as described [9, 12, 13]. The incubation mixture

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† Abbreviations: $O^6$-mG, $O^6$-methylguanine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; TPE buffer, 10 mM Tris HCl–1 mM PMSF–1 mM EDTA (pH 7.8); $A_{280}$, absorbance at 280 nm.

(2.0 ml), containing 70 mM HEPES-KOH (pH 7.8), 1 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol, 500 μg [*methyl*-$^3$H]DNA and tissue preparation, was incubated at 37° for 60 min. The reaction was terminated by the addition of 0.2 ml of 2.5 M sodium acetate and 4 ml of cold ethanol. The precipitates were collected and hydrolyzed in 0.1 M HCl at 70° for 45 min, followed by reprecipitation by 7.5% trichloroacetic acid. It was then dissolved in 20% formic acid–2 M HCl and hydrolyzed at 110° for 4 hr. The vacuum dried hydrolysates were then chromatographed on a column of Dowex-50($H^+$) (0.5 × 5 cm), which was equilibrated with 0.1 M sodium citrate, pH 2.7. One-milliliter fractions were collected. After washing the column with 8 ml of the citrate buffer, *S*-[*methyl*-$^3$H]methylcysteine was eluted with 0.1 M sodium citrate (pH 3.0) containing 0.4 M NaCl. A blank tube, which was not incubated but prepared identically, was run simultaneously. Methyltransferase activity measured in this manner with increasing amounts of rat liver whole homogenate showed a linearity up to 12 mg protein. In the present study, the methyltransferase activity in all of the fractions was measured with unsonicated preparations. Since recoveries of the enzyme activity in both spleen (86%) and liver (92.6%) were satisfactory (Tables 2 and 3), it can be surmised that nuclear membrane is not a limiting factor for the nuclear enzyme to interact with exogenously added DNA substrate during 1-hr incubations.

*Subcellular fractionation of tissues.* The subcellular fractionation of rat spleen was performed according to the method described [14] with slight modifications. Male rats (body weight 200–250 g) were decapitated by guillotine, and tissues were quickly removed and rinsed with cold 0.25 M sucrose solution. The spleen (about 1.5 g) was homogenized in 4 times its own volume of 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4), 4 mM 2-mercaptoethanol, and 0.1 mM EDTA. The homogenates were filtered through a double layer of cheesecloth and centrifuged at 600 *g* for 10 min in a Sorvall centrifuge. The supernatant fraction was withdrawn, and the bright red sediment was resuspended in about an equal volume of the sucrose solution and centrifuged. This washing procedure was repeated once more, and all the supernatant fractions were combined. The pellets were further fractionated in order to obtain clean nuclei as follows. They were dispersed in 24 ml of 0.25 M sucrose solution, and 8-ml aliquots of the suspension were layered over 20 ml of 0.34 M sucrose containing 10 mM Tris–HCl (pH 7.4), 4 mM 2-mercaptoethanol and 0.1 mM EDTA, and centrifuged at 2500 *g* for 30 min in an SW-25 rotor. All the pellets were combined into about 14 ml of 0.25 M sucrose solution and layered on 15 ml of the 0.34 M sucrose solution, followed by centrifugation at 400 *g* for 30 min in an SW-25 rotor. This last procedure of washing and layering was repeated once more. Finally, the pelleted nuclei were suspended in 0.25 M sucrose solution. The microscopic examination of the preparation showed no apparent unbroken cells nor membranous material.

The combined supernatant fractions at 600 *g* obtained from the earlier procedure described above (post-nuclear supernatant fractions) were centrifuged at 5000 *g* for 15 min. The pellets were saved, and the supernatant fraction was centrifuged again. Both precipitates were combined, resuspended in 5 ml of the 0.25 M sucrose solution, and centrifuged at 6600 *g* for 10 min to obtain a mitochondrial pellet. To isolate the microsomal fraction, the combined post-mitochondrial supernatant fractions were centrifuged further at 110,800 *g* for 60 min. The final supernatant fraction, usually 30–35 ml, represents the cytosol fraction.

For subcellular fractionation of rat liver, the liver was first perfused in 0.32 M sucrose–20 mM Tris–HCl (pH 7.4), and subcellular fractionation was carried out according to the method described by Lahav *et al.* [15]. The liver (3 g) was homogenized in 9 ml of 0.32 M sucrose containing 20 mM Tris–HCl (pH 7.4)–4 mM 2-mercaptoethanol and 0.1% phenylmethylsulfonyl fluoride (PMSF). Ten milliliters of the homogenate was added to an equal amount of 0.25 M sucrose containing the same concentrations of Tris–HCl, 2-mercaptoethanol and PMSF, and centrifuged at 600 *g* for 10 min. The pellet was suspended in 10 ml of the above 0.25 M sucrose solution, and centrifugation was repeated once more. The pellet was then resuspended in the sucrose solution and centrifuged at 900 *g* for 10 min to obtain the nuclear fraction. The postnuclear supernatant fractions from all the previous centrifugations were combined, and $MgCl_2$ was added to a final concentration of 3 mM. The mixture was centrifuged at 10,000 *g* for 20 min to obtain the mitochondria fraction. The post-mitochondrial supernatant fraction was further centrifuged at 105,000 *g* for 60 min to obtain the cytosolic fraction. The pellet is the microsome fraction.

*Fractionation of nuclear components.* The isolated nuclei were lysed by suspending them in an equal volume of 10 mM Tris (pH 7.8) containing 1 mM PMSF and 1 mM EDTA (TPE buffer), and incubated at 4° for 30 min followed by gentle homogenization. The lysate was then centrifuged at 700 *g* for 10 min. The pellet was washed twice with 2 vol. of TPE buffer, and the extracts were pooled (wash). The pellet was suspended in an equal volume of TPE buffer containing 1 M NaCl and vortexed vigorously. The mixture was centrifuged at 39,000 *g* for 10 min. This process was repeated once more. The supernatant fractions were pooled, and called "1 M salt extract". The resulting pellet was extracted twice with TPE buffer containing 2 M NaCl, as described above. The remaining insoluble pellet was called "matrix". The matrix was suspended in TPE buffer containing 4 mM 2-mercaptoethanol and 5% glycerol. The 1 M and the 2 M NaCl extracts were dialyzed overnight against the same buffer.

*Chromatographic and electrophoretic separation of* [methyl-$^3$H]*methyltransferase.* Isolated nuclei (20 mg protein) were incubated with 2.5 mg of [*methyl*-$^3$H] DNA substrate (146 pmoles of $O^6$-mG) in a total volume of 4 ml as described previously, and chromatin was prepared according to the method of Thibodeau and Verly [16]. Non-histone and histone fractions were then isolated according to the method of Levy *et al.* [17]. Briefly, the chromatin preparation was adjusted to 6 M urea–0.4 M guanidine-HCl–4 mM 2-mercaptoethanol–0.1 M sodium phosphate

(pH 7.2). DNA was sedimented by centrifugation at 300,000 *g* for 18 hr at 4° using a Ti 60 rotor in a Spinco model L2-50 ultracentrifuge. The supernatant fraction obtained was applied to a column of Bio-Rex 70 (1.5 × 25 cm) which was equilibrated with the above urea–guanidine buffer solution. The column was washed with the same buffer until $A_{280}$ of the eluate returned to a baseline value. The column was then eluted with the equilibrating buffer containing 4 M guanidine–HCl. Fractions (4 ml) were collected, and an aliquot from each fraction was counted for radioactivity (Fig. 1). The radioactive fractions (fractions 6–11) were pooled, dialyzed, and further fractionated on a column of DE-52 (2.0 × 22 cm) which had been equilibrated with 3 M urea–10 mM Tris-HCl–10 mM 2-mercaptoethanol (pH 8.5). The proteins were eluted using a NaCl gradient (250 ml of the 3 M urea buffer in the first chamber and 250 ml of the buffer plus 0.7 M NaCl in the second chamber) (Fig. 2). Two radioactive peaks were observed at 0.21 M and 0.45 M NaCl. The first peak (140–220 ml) was pooled, dialyzed against 10 mM Tris-HCl (pH 7.8)–1 mM dithiothreitol, lyophilized, and subjected to two-dimensional electrophoresis according to the methods of O'Farrell [18].

*Other analytical methods.* DNA content was estimated by rapid colorimetric methods described [19], using diaminobenzoic acid. Cytochrome *c* oxidase (EC 1.9.3.1) was measured according to the method of Wharton and Tzagoloff [20] following the rate of oxidation of reduced cytochrome *c* at 550 nm with a Coleman double beam spectrophotometer model 124. Cytochrome P-450 was determined from the CO-difference spectra of dithionite reduced samples using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ between 450 and 490 nm [21]. Lactate dehydrogenase (EC 1.1.1.27) was determined according to Bergmeyer and associates [22, 23] by following the rate of oxidation of NADH at 340 nm. Protein concentration was estimated by the method of Lowry *et al.* [24] using bovine serum albumin as the standard.

*Definition of unit and specific activity.* One unit of methyl acceptor is defined as the amount of protein which transfers one picomole of [*methyl*-$^3$H] to the methyl acceptor (methyltransferase). Specific activity is expressed as units of enzyme per milligram of protein. It should be noted that a time factor is not specified in defining the unit; because of the unusual property of the enzyme, in forming a dead-end complex [4, 25], a maximum activity is used.

## RESULTS

*Organ distribution of* O$^6$*-methylguanine-DNA methyltransferase.* Because of known differences in tissue susceptibility toward chemical carcinogens, we first measured the distribution of the methyltransferase activity in various rat organs (Table 1). Spleen exhibited the highest activity among the organs examined followed by thymus, liver and lung. Brain had only negligible activity. The highest activity in the spleen was unexpected, since traditionally the liver had been considered to have the most methyltransferase activity and has been widely studied for DNA repair enzymes [25–29]. Although the reason for this high activity in the spleen and thymus is not clear at present, it may be related to the biogenesis of lymphocytes which are known to have high $O^6$-mG-DNA methyltransferase activity [30, 31].

*Subcellular distribution of* O$^6$*-methylguanine-DNA methyltransferase.* Subcellular fractionation of the spleen, carried out by the 0.25 M and 0.34 M sucrose layering-differential centrifugation method [14], was found to be very efficient in removing

Table 1. Distribution of $O^6$-methylguanine-DNA methyltransferase in rat organs

| Organ | Enzyme activity* (pmoles $CH_3$-transferred/mg) |
|---|---|
| Spleen | 0.101 |
| Thymus | 0.076 |
| Liver | 0.062 |
| Lung | 0.024 |
| Testis | 0.018 |
| Kidney | 0.011 |
| Heart | 0.009 |
| Brain | 0.005 |

* Enzyme activity was determined as described in the text using 6 mg protein of tissue whole homogenate.

Table 2. $O^6$-Methylguanine-DNA methyltransferase, DNA and marker enzymes in subcellular fractions of rat spleen

| Fraction | Protein (mg) | DNA (mg) | Total enzyme activity* | | | |
|---|---|---|---|---|---|---|
| | | | $O^6$-mG-DNA methyltransferase | Cytochrome *c* oxidase | Protein methylase II | Lactate dehydrogenase |
| Whole homogenate | 176 | 13.0 | 20.8 (100)† | 550 | 387 | 75.7 |
| Nuclei | 40.6 | 14.7 | 15.4 (74.5) | 72.3 | 25 | 1.50 |
| Mitochondria | 11.0 | 0.15 | 0.11 (0.5) | 361 | 6.9 | 1.34 |
| Microsome | 22.4 | 0.39 | 0.29 (1.4) | 39.6 | 17.2 | 6.72 |
| Cytosol | 93.5 | ND‡ | 2.00 (9.6) | ND | 431 | 57.0 |

Cytochrome P-450 activity was not detectable in any fraction.

* Enzyme activities were defined as follows: picomoles of [*methyl*-$^3$H]methylcysteine formed in the methyl acceptor protein at 37° for $O^6$-mG-DNA methyltransferase; μmoles of cytochrome *c* oxidized per min at 38° for cytochrome *c* oxidase [20]; pmoles of methyl-group transferred to the methyl accepting protein for protein methylase II [32]; and μmoles of lactate formed per min at 25° for lactate dehydrogenase [23].

† Numbers in parentheses indicate percent activity.

‡ Not detectable.

Table 3. $O^6$-Methylguanine-DNA methyltransferase, DNA and marker enzymes in subcellular fractions of rat liver

| Fraction | Protein (mg) | DNA (mg) | Total enzyme activity* | | | |
|---|---|---|---|---|---|---|
| | | | $O^6$-mG-DNA methyltransferase | Cytochrome *c* oxidase | Cytochrome P-450† | Lactate dehydrogenase |
| Whole homogenate | 399 | 8.4 | 31.9 (100) | 71.8 | ND‡ | 818 |
| Nuclei | 101 | 7.95 | 17.3 (54.2) | | ND | 73.1 |
| Mitochondria | 91.2 | 0.48 | 0.757 (2.4) | 32.8 | | 57.5 |
| Microsome | 24.2 | ND | 0.387 (1.2) | 0.242 | 23.5 | 28.1 |
| Cytosol | 168 | ND | 11.1 (34.8) | ND | ND | 680 |

* The definitions of the respective enzymes are the same as in Table 2.

† Cytochrome P-450 was determined from the CO-difference spectra of dithionite reduced samples according to the method described in Ref. 21; activity is expressed as nmoles of cytochrome P-450 reduced.

‡ Not detectable.

contaminating red blood cells, and enabled us to obtain clean nuclear fraction. Histological examination by a phase contrast microscope of the nuclear preparation thus prepared did not indicate any discernible unbroken cells.

Purity of each fraction was ascertained by estimating DNA as well as various specific marker enzymes, and was shown to be satisfactory (Table 2). DNA was undetectable in the cytosol, and 66% of the cytochrome *c* oxidase activity (a marker enzyme for mitochondria) was present in the mitochondrial fraction. Also assayed were lactate dehydrogenase and protein methylase II (EC 2.1.1.24); both are primarily cytosolic enzymes [15, 32].

As shown in Table 2, the nuclear fraction contained 74.5% of the total methyltransferase activity. In another independent experiment, 80% of the activity was found in the nuclear fraction. The activity in the cytosol was only 9.6%; however, this figure may be maximal, since the enzyme may have leaked from the nuclei during preparation. Mitochondrial and microsomal fractions showed very little activity, namely, 0.5 and 1.4% respectively.

In contrast to the spleen, subcellular distribution of the methyltransferase in the liver exhibited quite a different profile. As shown in Table 3, a substantial amount of the methyltransferase activity (34.8%) was recovered from the cytosol and 54.2% from the nuclei, while mitochondria and microsome contained negligible amounts of activities. These results are in general agreement with the observations made by other investigators: 59% in the cytosol and 37% in the nuclei reported by Pegg *et al.* [25], and 26% in the cytosol and 62% in the nuclei by Renard and Verly [33]. It should be mentioned that the relative distribution of the methyltransferase in the liver nuclei and cytosol was found to vary from experiment to experiment. Thus, it is possible that liver nuclear membranes are "leaky", and thus redistribution of the methyltransferase occurs during isolation. Indeed, Thibodeau and Verly [16] reported earlier that, although chromatin is the primary site for the enzyme, the isolation method of the nuclei is critical in maintaining high repair activity.

Table 4. Relative distribution of $O^6$-methylguanine-DNA methyltransferase in subnuclear fractions

| Fraction | Methyl removed (pmoles/hr) | % Activity |
|---|---|---|
| Nuclei | 72.1 | 100 |
| Wash | 4.4 | 6.1 |
| 1 M NaCl extract | 30.0 | 41.6 |
| 2 M NaCl extract | 15.7 | 21.8 |
| Matrix | 7.2 | 10.0 |

About 500 mg of nuclei was extracted with 10 mM Tris–1 mM PMSF–1 mM EDTA (TPE buffer), TPE buffer + 1 M NaCl and TPE buffer + 2 M NaCl, as described in the text. Six milligrams protein from each fraction was used to determine $O^6$-mG-DNA methyltransferase activity.

*Subnuclear localization of* $O^6$*-methylguanine-DNA methyltransferase.* To further investigate localization of the methyltransferase in the nuclei, isolated liver nuclei were successively extracted with low and high salt solutions. As shown in Table 4, most of the activity was extractable, leaving only 10% of the total nuclear activity in the nuclear matrix. The low salt extractable fraction, "wash", contained only 6% of the activity. In a separate experiment when the "wash" was further centrifuged at 105,000 *g* for 60 min, the activity was further resolved into nuclear sap (3.2%) and membrane (1.7%). Over 60% of the activity was extracted with high salt solutions, indicating that the methyltransferase is not tenaciously associated in the chromatin.

*Fractionation of nuclear* [methyl-$^3$H]*methyltransferase.* To investigate further the kind of nuclear protein(s) which functions as the $O^6$-mG-DNA methyltransferase, the isolated nuclei were incubated with [*methyl*-$^3$H]DNA as a substrate and subsequently subjected to Bio-Rex 70 chromatography to separate chromosomal proteins. Most of the radiomethyl that was incorporated into the protein fractions coeluted with the non-histone protein fraction, while only negligible amounts of activity were found with the histone fraction (Fig. 1). The *methyl*-$^3$H-labeled non-histone fraction was further resolved into two peaks, using anion exchange chromatography (Fig. 2). The first peak eluted at 0.21 M NaCl, but the second peak was shown to be tightly bound to DE-52 and eluted only at 0.45 M NaCl, the salt concentration that elutes DNA [25]. Since the second peak was positive for the diaminobenzoic acid test which is specific for DNA [19], this radio-

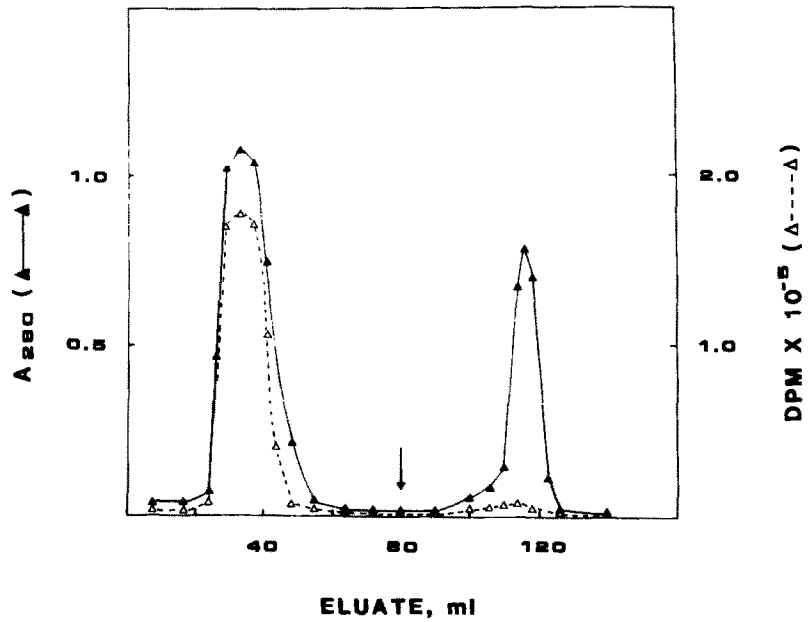


Fig. 1. Chromatographic separation of rat liver nuclear [*methyl*-$^3$H]protein on Bio-Rex 70. The nuclear protein (20 mg) incubated with [*methyl*-$^3$H]DNA was chromatographed on a column of Bio-Rex 70 (1.5 × 25 cm) which had been equilibrated previously with 6 M urea–0.4 M guanidine-HCl–4 mM 2-mercaptoethanol–0.1 M sodium phosphate, pH 7.2, as described in the text. The arrow indicates the buffer change to 6 M urea–4 M guanidine-HCl–4 mM 2-mercaptoethanol–0.1 M sodium phosphate, pH 7.2.

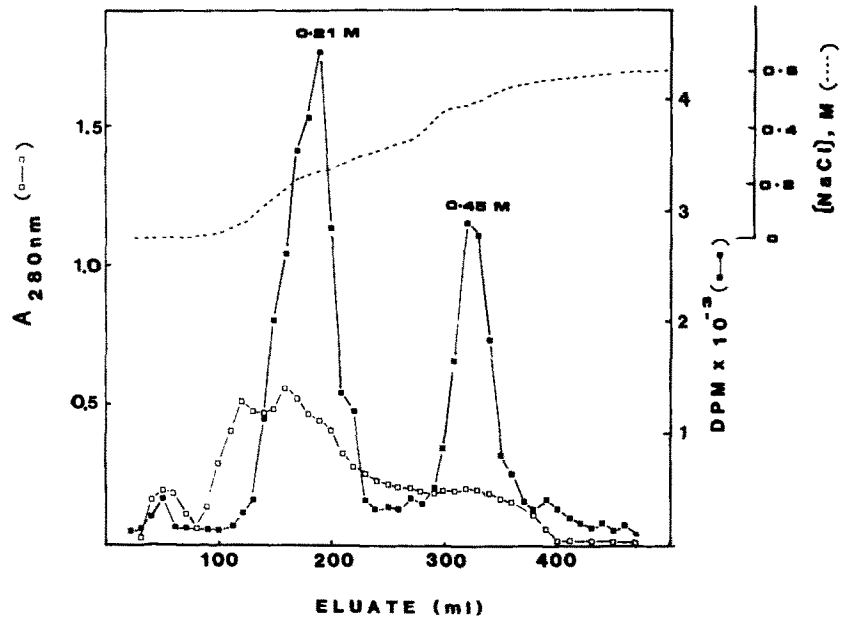


Fig. 2. Chromatogram of non-histone chromosomal [*methyl*-$^3$H]proteins on a DE-52 column. The radioactive fractions (24–44 ml) obtained from Bio-Rex 70 chromatography (Fig. 1) were pooled and dialyzed against 10 mM Tris–HCl (pH 8.5)–10 mM 2-mercaptoethanol containing 3 M urea. The sample was then applied to a column of DE-52 (2.0 × 22 cm) which was equilibrated previously with the same dialysis buffer. After eluting the column with 70 ml of the buffer, NaCl gradient elution was commenced (0–0.7 M).

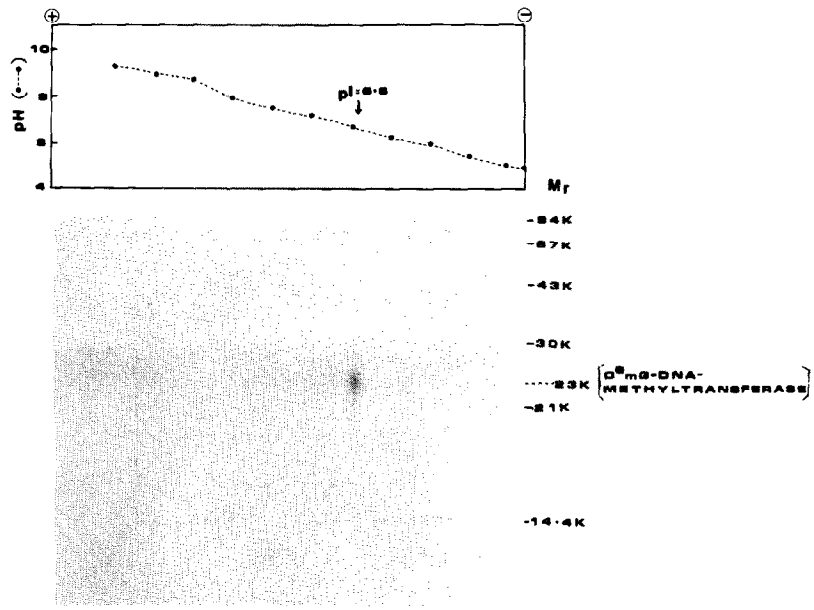


Fig. 3. Autoradiogram of the two-dimensional gel electrophoresis of [*methyl*-$^3$H]methyltransferase. Fractions of the first radioactive peak from DE-52 chromatography (Fig. 2) were pooled, dialyzed against 10 mM Tris–HCl (pH 7.8)–1 mM dithiothreitol, and lyophilized. A sample containing 7600 dpm (300 μg protein) was then subjected to two-dimensional gel electrophoresis according to the method of O'Farrell [18]. The first dimension of isoelectrofocusing gel electrophoresis was carried out in a glass tubing (130 × 2.5 mm inside diameter) containing 4% acrylamide, 9.1 M urea, 2% (w/v) NP-40, 5% 2-mercaptoethanol, 2% ampholine (mixed 4 parts of pH 5–8 and 1 part of pH 3.5–10 ampholines) and electrophoresed for 6 hr at 400 V. Two identical gels were prepared, one of which was used to measure the pH gradient as follows. The gel was cut into 5-mm sections and placed in vials containing 2 ml of degassed water. These vials were capped and shaken for a few hours before the pH was determined. The second dimension of the electrophoresis was carried out on a slab gel (12% acrylamide–0.1% SDS–0.375 M Tris-HCl, pH 8.8) at 60 V for 6 hr. At one end of the slab gel, a mixture of standard marker proteins was applied (phosphorylase *b* for 94,000; bovine serum albumin for 67,000; ovalbumin for 43,000; carbonic anhydrase for 30,000; trypsin inhibitor for 21,000, and α-lactoalbumin for 14,400). After the electrophoresis, the gel was treated for autoradiography with enhancer (New England Nuclear), dried, and enclosed with pre-exposed Kodak (X-Omat R) for 9 weeks at −70°.

activity may have been associated with DNA which carried over during the previous purification step. The first radioactive peak fractions were concentrated and subjected to two-dimensional gel electrophoresis. The autoradiogram shown in Fig. 3 indicates that the [*methyl*-$^3$H]methyltransferase possesses a molecular mass of 23,000 and a pI value of 6.6.

## DISCUSSION

Differential tissue susceptibility of experimental animals against alkylating chemical carcinogens has been well documented. Goth and Rajewsky [10] earlier classified rat organs as "high-risk" (fetal brain) and "low risk" (liver), based on the incidence of cancer after administration of *N*-ethyl-*N*-nitrosourea into the rat. Subsequently, "low-risk" organ was shown to have a higher capacity to repair $O^6$-mG residues in its DNA than the "high-risk", the presumptive carcinogenic site [1–3]. Thus, tissues and cells which lack this repair activity are postulated to be susceptible to carcinogenesis. Indeed, the lower level of activity in the brain (Table 1) correlated well with the *in vivo* study where this organ was shown to be highly susceptible to alkylating chemical carcinogen [10]. This repair mechanism was shown to be catalyzed by the methyltransferase reaction; the methyl group of the $O^6$-mG residue is transferred to the cysteine residue of the methyl acceptor protein forming *S*-methylcysteine [4–8, 25].

In the present paper, utilizing our newly developed assay method to measure the appearance of *S*-methylcysteine in the acceptor protein [9], we have investigated the levels of $O^6$-mG-DNA methyltransferase activity in several rat organs and found that spleen and thymus had the highest enzyme activity (Table 1). The relatively high methyltransferase activities in these organs are somewhat contradictory with the *in vivo* studies carried out in inbred mice where a single injection of *N*-methyl-*N*-nitrosourea yielded thymoma [34]. To explain this difference, differential formation of $O^4$-alkylthymidine in their respective target organs should also be considered, in light of a recent proposal by Swenberg *et al.* [35] that $O^4$-alkylthymidine can also be a carcinogenic site. In any event, the fact that a primary site for tumorigenicity in rat is brain [10] but not thymus, may indicate a species difference of target organ against the carcinogen (rat vs mice). Indeed,

the *in vivo* studies in mice using another carcinogen, namely *N*-methyl-*N'*-nitro-*N*-nitroguanidine, failed to yield thymic lymphoma at doses approximately 60% of the $LD_{50}$ values [36]. Thus, the significance of this pattern of enzyme distribution in rat is not clear at present.

Waldstein *et al.* [30] reported earlier that human lymphocytes have an active $O^6$-mG-DNA methyltransferase and the enzyme level varies greatly depending on the type of lymphocytes ($Mer^-$ or $Mer^+$) [31, 37]. Thus, the lymphoid tissues such as spleen and thymus may play an important role in expressing the level of methyltransferase during the lymphocyte differentiation and maturation.

The nuclei is the most likely site for a DNA repair enzyme to function. Studies on the subcellular distribution of the methyltransferase, however, indicate that the enzyme activity in the liver was present in both cytosolic and nuclear fractions (Table 3). Since total recovery of the enzyme activity was almost complete when assayed individually with each subcellular fraction, no apparent cofactors (activator or inhibitor) are involved in the liver. This is in good agreement with the conclusion made by Renard and Verly [33] that a cytoplasmic component is not necessary for the nuclear activity. Similar observations were also made with other DNA repair enzymes such as uracil glycosylases and AP endonuclease which were shown to be present in both the cytosol and the nuclear fractions of rat liver [16]. The questions of whether the methyltransferase present in these fractions functions with equal capacity to repair the damaged DNA *in vivo*, or whether the cytosolic species may be a precursor of the nuclear one, as suggested in the case of an AP endodeoxyribonuclease [16], are not answered at present. However, it is also possible that, since the methyltransferase appears to be loosely associated with chromatin components (Table 4), the enzyme in the chromatin may have been redistributed between cytosol and nuclear fractions, during its preparation.

Very recently, Craddock and Henderson [38] reported that the $O^6$-mG DNA methyltransferase activity is higher in the spleen than in the liver of rat when the activity is expressed on the basis of tissue weight, but not based on the amount of DNA. Thus, our result based on the amount of protein is in close agreement with the former expression of the activity by these investigators. It is obvious that, since a relatively large fraction of the total enzyme activity is present in the cytosolic fraction of rat liver (35%, Table 3 of this paper; 59%, Table 2 of Ref. 25; 72%, Table 1 of Ref. 28), only the amount of enzyme located in the nuclei rather than the total can be correctly related with the amount of DNA.

Analysis of the methyl-inactivated nuclear [*methyl*-$^3$H]methyltransferase under denaturing conditions has enabled us to determine some properties of the enzyme. The methylated methyltransferase was a non-histone chromosomal protein with a pI value of 6.6 (Figs. 1 and 3). Its molecular weight, 23,000 daltons determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 3), is in good agreement with that of human lymphoid cell methyltransferase (22,000 ± 1,500) [39] determined by gel filtration. Hora *et al.* [28] and Pegg *et al.* [25] also reported the molecular weight of rat $O^6$-mG-methyltransferase as 19,000 and 20,000 respectively. These values are within the limit of good agreement, since the sedimentation coefficient of the enzyme was not taken into consideration in all measurements. Furthermore, the molecular weight of the *E. coli* enzyme was reported to be 18,000 [13]. In view of the fact that this catalytically active *E. coli* methyltransferase is derived from the 37,000 precursor protein by proteolytic processing [40], it is quite possible that the presently described mammalian enzyme is similarly processed from a larger precursor protein.

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