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MGMT: A Personal Perspective

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The discovery of O^6-methylguanine-DNA methyltransferase (MGMT), first in *E. coli*, and subsequently in mammals, yeast and other eukaryotes, underscores the importance of the endogenous and induced formation of O^6-alkylguanine (O^6-alkylG) as well as the need for its repair to prevent mutations due to this mutagenic base adduct. Parallel and independent studies on the dual functions of alkylating agents that generate O^6-alkylG (and other alkylated base adducts), in the induction of carcinogenesis, as well as in the chemotherapy of cancer, led to an explosive interest in the study of O^6-alkylG, its formation and its repair, in the 80s. Studies on DNA methylation damage repair received a new boost with the more recent exciting discovery of a novel mechanism that repairs 1-methylA and 3-methylC both in single-stranded DNA and RNA by *E. coli* Alk B and its mammalian homologs ABH2 and ABH3. These enzymes are dioxygenases that act via oxidative demethylation (1–3) and this is reviewed elsewhere in this volume. This short review summarizes from my personal perspective, the early history of the discovery of O^6-alkylG repair in bacteria and mammals and its clinical implications.

Alkylating agents directly react with cellular macromolecules as electrophiles or generate electrophilic species after metabolic activation (reviewed in 4). These attack nucleophilic targets including DNA bases and phosphates, via S_N1 or S_N2 mechanisms (reviewed in 5). Lawley and Brooks pioneered studies of alkylating agents in the etiology of carcinogenesis more than forty years ago (6). Early studies by Lawley, Brooks, Magee, Singer and others demonstrated the formation of alkylated bases in RNA and DNA after reaction with alkylating carcinogens (6–8). The alkylation sites in DNA bases are now known to be the N1, N3, N7 atoms in purines, N3 and O^2 atoms in pyrimidines and the O^4 and O^6 atoms in T and G, respectively. Alkylation at N3 and N7 in G and A, and O^2 in C, destabilizes the glycosyl bond of the base and its spontaneous release leads to abasic (AP) sites. For a long time 7-alkylG was believed to be the key carcinogenic lesion (reviewed in 5). O^6-alkylation of G was not considered to be of critical significance because of its low abundance until Loveless proposed in 1969 in a seminal paper that O^6-alkylG should be mutagenic because of its mispairing potential with T (9). Even though the etiologic role of mutations in carcinogenesis was not established until much later, that mutations could lead to cancer was generally accepted at that time. Then Goth and Rajewsky (10) showed that O^6-ethylG but not 7-ethylG persists in the brain DNA of rats treated with N-ethyl-N-nitrosourea (ENU) which was consistent with the organotropism of N-nitrosamides in inducing CNS tumors in rodents (10). Kleihues and Margison independently showed that accumulation of O^6-methylG could be correlated with glial tumors induced by N-methyl-N-nitrosourea (11). These studies of Rajewsky and Kleihues and of Magee et al, are considered to be classic and led to increased interest in the mutagenic and carcinogenic properties of O^6-alkylG. Early studies on *in vitro* replication indicating that

O^6-methylguanine (m^{6}G) in a DNA template tends to incorporate T rather than C were consistent with Loveless's hypothesis about ambiguous base pairing of *O*^{6}-alkylG (12).

Evidence of mutagenic nature of O^6-methylG

One problem of studying the effect of O^6-alkylG in DNA is that alkylating agents alkylate multiple sites in bases as well as phosphate residues, among which O ^{6}-alkylG is a relatively minor base adduct. This makes it rather difficult to establish a causal linkage between a biological end point and a specific alkyl lesion, e.g., O^6-alkylG. We therefore decided to study m^{6}G as a unique base adduct in DNA. We generated O^6-methyldGTP (m^{6}dGTP) by chemical synthesis using established protocols, and were also able to label the C-8 position of G with [^{3}H] by starting with 8-bromo-*O*^{6}-methyldGTP and then reducing it with [$^3H_2$] (13). We collaborated with Warren Masker to examine the effect of incorporation of m^{6}dGMP in an *in vitro* T7 phage DNA replication assay (14). We showed that incorporation of m^{6}dGMP into replicated DNA, which was subsequently packaged *in vitro* for infectivity assays, significantly increased the reversion frequency of an amber mutation. This could be easily explained if incorporation of m^{6}G opposite T induced a transition mutation for AT→GC, as in the amber codon of mutation of T to C in TAG during replication (14). This was the first biological evidence of m^{6}G mispairing with T. Subsequently, John Essigman's lab, in an elegant study involving site-specific incorporation of m^{6}G into a plasmid, showed that this base was mutated to A upon plasmid replication (15).

Liza Snow, a Ph.D. student in our laboratory, then synthesized m^{6}G -containing oligodeoxynucleotides using terminal deoxynucleotidyl transferase which resulted in random incorporation of m^{6}G in polydeoxy(C, G, m^{6}G). She used various prokaryotic DNA polymerases to show that both T and C were incorporated during replication of the m^{6}G -containing template but not of a similar template that did not contain m^{6}G (16). This provided strong evidence for the ability of m^{6}G to pair with T. She also determined the kinetic parameters of DNA synthesis to conclude that m^{6}G inhibits DNA polymerase activity. Nevertheless, all DNA polymerases incorporated T when m^{6}G was present in the template. Her complementary studies examining incorporation of dm^{6}GMP opposite T in DNA were consistent with this conclusion (17). More recently, studies were carried out with well-defined templates with site-specific location of m^{6}G and replication with purified eukaryotic DNA polymerases (18). These studies confirmed that DNA polymerases pause at the m^{6}G residue before incorporation of C or T. Thus the DNA polymerases may have difficulty in aligning the complementary C or T opposite m^{6}G prior to their incorporation. Furthermore, the base pairing of m^{6}G with C or T using 2 H- bonds rather than 3 H-bonds as present in a G•C pair is weak. Our results showing increased turnover of C or T during replication of the m^{6}G -containing template supports this conclusion. Subsequent NMR spectroscopy and X-ray crystallography studies of m^{6}G -containing oligonucleotides provided direct evidence for the m^{6}G•T pair (19,20).

Adaptive response of E. coli to MNNG

While investigations on the contribution of O^6-alkylG to mutagenesis and carcinogenesis were continuing at a slow pace, a paradigm-shifting discovery established the concept of adaptive response in *E. coli* to alkylating agents which opened up a completely new approach to study repair of this mutagenic base lesion. Leona Samson in her Ph.D. dissertation project in John Cairns' laboratory was investigating the mechanism and kinetics of mutagenesis in *E. coli* by *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG), a classical agent used for generating *E. coli* mutants (21). MNNG is activated by thiols to generate methyldiazonium ions that alkylate DNA bases and phosphates via the S_N1 mechanism. Samson and Cairns observed that *E. coli* pretreated with submutagenic dose of MNNG became resistant to both mutagenesis and killing when challenged with a much higher dose of MNNG, and they named this phenomenon,

"adaptive response" (21). It should be noted that the mutagens are invariably cytotoxic and cytotoxicity and mutagenesis occur simultaneously. A similar phenomenon, (named the Weigel effect, after the discoverer), showing enhanced survival of UV-pretreated *E. coli* to a challenge dose of UV, was established many years earlier, and was subsequently linked to the SOS response (reviewed in 22). In this case, however, UV-pretreated *E. coli* developed resistance to UV, but simultaneously showed *enhanced* mutagenesis. It later became evident that UV (and as well as other genotoxic agents) activate the SOS response by inactivating the LexA repressor. The SOS regulon consists of multiple operons which encode not only repair proteins for UV damage but also specialized DNA polymerases for lesion by-pass synthesis, resulting in increased mutation frequency (22).

The Cairns Laboratory showed that the adaptive response is distinct from the SOS response in that MNNG pretreatment reduced both the toxic and the mutational effects of alkylating agents but not those of other genotoxic agents such as UV light (23). It is a testimony to the remarkable insight and ingenuity of John Cairns and his associates that they unraveled the molecular basis of the adaptive response. Subsequent studies showed that, by analogy with the SOS response triggered by UV light, the adaptive response corresponds to activation of the *ada* gene. The Ada polypeptide regulated several operons including that of *ada* itself, as in the case of the SOS regulon (24,25). However, unlike in the case of the SOS response, where regulation is negatively controlled by the LexA repressor, the Ada polypeptide acts as an *activator* of the genes involved in adaptive response which include *ada*, *alkA*, *alkB* and *aidB*, all of which were expected to be involved in methylated DNA repair or tolerance. However, the mechanism of activation is slightly different for each gene (26,27).

The Cairns group identified the functions of Ada, and established that Ada itself is responsible for repairing the principal mutagenic lesions induced by alkylating agents. Lindahl's group identified O^4-methylthymine among these as an Ada substrate. More in-depth studies on the mechanism of Ada regulation were subsequently carried out by Lindahl, Sekiguchi and their colleagues. Ada activates the *alkA* gene; AlkA excises O^2-methyl C and O^2-methyl T in the first step of their repair via the base excision repair (BER) pathway (reviewed in 28). AlkB, as already mentioned, restores A and C from 1-methyl A and 3-methyl C respectively via oxidative demethylation (1–3). Finally, Aid B's enzymatic role in methylation repair is still unclear (29). Cairns and his associates predicted from their *in vivo* data that (a) Ada acts stoichiometrically in the repair of mutagenic alkylated bases, and (b) AlkA acts catalytically (23). Subsequent studies confirmed these remarkable predictions.

Discovery of the MGMT reaction mechanism

The biochemical mechanism of action of Ada *in vitro* was independently discovered by us and by Lindahl and Olsson (13,30) using two different DNA substrates that provided complementary information (Fig. 1). In our efforts to elucidate the mutagenic mechanism of m^6G, we incorporated d[^{3}H]m^6GMP into a synthetic oligonucleotide, polydeoxy (C, G, or m^6G), as already described. It should be noted that our studies predate the era of chemical synthesis of oligodeoxynucleotides using phosphoramidite or phosphotriester chemistry. Our synthesis of polydeoxy (C, G, m^6G) was based on the rationale that with nearly equimolar amounts of C, and G together with m^6G, these polymers would contain a significant amount of duplex structure including the m^6G•C pairs that would be present in methylated DNA.

We incubated the DNA substrate with extracts of *E. coli* pretreated with MNNG for adaptation. After incubation, the radioactivity remained in DNA which we then isolated by phenol extraction and alcohol precipitation. After hydrolysis of DNA with DNase I, snake venom phosphodiesterase and alkaline phosphatase, followed by HPLC to separate the deoxynucleosides, we showed that some of the radioactivity in the incubated DNA had been

transferred to the G peak, while the control sample incubated with boiled extract contained [^{3}H] exclusively in the m^{6}G fraction. We concluded from this that (a) the m^{6}G repair enzyme acts on the DNA without degrading it, and (b) the repair involves *in situ* demethylation (13).

Olsson and Lindahl in their assay treated calf thymus DNA with [^{3}H]MNNG, which is known to generate m^{6}G along with 7-methylG (m^{7}G) and 3-methyl A (m^{3}A) as the major methylated base products (30). Based on the fact that m^{7}G and m^{3}A residues but not m^{6}G are readily hydrolyzed from DNA in neutral pH, they heated and precipitated the alkylated DNA to remove most of these methylated bases prior to incubation with extracts of adapted *E. coli*. They followed the transfer of radioactivity to proteins by hydrolysis of these proteins to amino acids and chromatographic separation and they showed that [^{3}H]-CH_3 was transferred exclusively to Cys residues (31). Thus they concluded that m^{6}G repair involves enzymatic transfer of methyl groups, presumably to the repair protein itself, which Lindahl named, "*O*^{6}-methylguanine-DNA methyltransferase," and we subsequently gave the acronym MGMT. The Enzyme Nomenclature Commission assigned the number EC 2.1.1.63 to Ada (and all MGMTs) and the MGMT was accepted as the formal gene name by the Human Gene Map Nomenclature Committee. Several alternative names have been given to this protein, among which O^6-alkylguanine transferase or AGT is also widely used (34).

Both Lindahl's group and we confirmed Cairns' prediction that the Ada protein acts stoichiometrically. Subsequently, with the discovery of mammalian MGMT, we and others confirmed the stoichiometric reaction of all MGMTs (31–34). Thus the MGMTs are not true enzymes and carry out a bimolecular reaction with a second order rate constant (32). *E. coli* Ada reacts at a very high rate, and human MGMT has a lower but still high rate constant of ~ 2×10^8 mol^{-1} min^{-1} (32,34). Cairns speculated that, while Ada's stoichiometric reaction is wasteful because it reacts only once, it may be necessary to repair m^{6}G rapidly in order to prevent mutagenesis due to the strongly mutagenic lesion in the replicating *E. coli* genome (23). Lindahl's studies also showed that Ada could not turn over because *S*-methylcysteine is extremely stable and there is no evidence for demethylation of methylated MGMT (28). Furthermore, m^{6}G (and other methylated bases) in DNA could be endogenously generated by chemical reaction with S-adenosyl- L-methionine (35).

Discovery of Ogt, a second MGMT in E. coli

Our MGMT assay based on m^{6}G demethylation is highly accurate not only because of the high specific activity of [^{3}H] m^{6}G but also because quantitation of MGMT is independent of the recovery of DNA as long as the number of m^{6}G residues present in excess in the reaction mixture is known (36). This reduced the error of quantitation of MGMT when recovery of DNA is low after incubation with a large amount of crude extract. Thus quantitation of radioactivity in G and m^{6}G eluted from the same column allows calculation of the fraction of radioactivity in G of the total radioactivity and provides a reasonably accurate measurement of the number of MGMT molecules/cell.

We calculated that unadapted *E. coli* contained about a dozen Ada molecules/cell and the number increased to several thousand in adapted cells (36). More importantly, *E. coli ada* mutants also contain a similar number of Ada molecules/cell which could not be explained at the time. The situation became clear later when Margison's lab showed that *E. coli* encodes a second, constitutive MGMT gene, which they named *ogt* (37). Thus the residual MGMT activity in the *ada* mutant of the MGMT activity in unadapted *E. coli* should be partly due to Ogt.

Detection and quantitation of MGMT activity in mammalian cells

The ubiquitous nature of MGMT was initially indicated by the presence of an Ada-like activity in *B. subtilis* (38). Leona Samson's laboratory cloned and characterized MGMT from the budding yeast (39–40). MGMT activity was later identified in a wide variety of organisms. Tony Pegg, in collaboration with us, identified and quantitated MGMT activity in rat liver extracts (41). Subsequent studies showed that mammalian MGMT similarly accepts the alkyl group from O^6-alkyl G to a Cys residue (34). The methyl acceptor Cys residue was identified after we succeeded in cloning the human and mouse MGMT cDNAs (42,43).

MGMT regulation in mammalian cells and its absence in Mer⁻/Mex⁻ cells

The significance of m^6G as a cytotoxic lesion in mammalian genomes and the tight regulation of its repair in some tumor cells was discovered by Bernard Strauss when he showed that some mammalian lymphoma cells that are hypersensitive to MNNG (and MNU) are deficient in repair of m^6G; he named these Mex^- (44). Rufus Day independently observed that some tumor cells are unable to reactivate MNNG-treated adenovirus and that they are deficient in m^6G repair. He named these Mer^- (45). We subsequently collaborated with Day and his associate, Dan Yarosh, on the quantitation of MGMT levels in various Mer^- cells (33). It became evident that the Mex^-/Mer^- cells have barely detectable level of MGMT (< 200–300 molecules/cells) compared to thousands to hundreds of thousands of MGMT molecules in various normal and tumor cells. Surprisingly, two independent clonal isolates of HeLa cells, named S3 and MR, have ~10^5 and < 200 MGMT molecules/cell respectively (46,47). MGMT is highly regulated, even in normal mammalian tissues (reviewed in 47).

The sensitivity of Mex^-/Mer^- cells to alkylating agents and their deficiency in m^6G repair implied the cytotoxic nature of the m^6G adduct in mammalian genomes, and this was investigated in great depth by many groups in more recent years (48,49). We showed earlier that transgenic expression of MGMT enhances alkylation resistance of naturally MGMT-deficient Chinese hamster cells (50). These observations confirmed earlier studies correlating MGMT level and resistance to methylating agents. Furthermore, a large variation in MGMT level in various normal and tumor cells indicated its tight regulation the teleological basis of which is not clear. More importantly, the lack of MGMT in Mex^- cells has profound clinical implications particularly in the treatment of glioblastoma for which the alkylating agents, including bifunctional *N*-chloroethyl-*N*-nitrosourea (CNU)-type drugs such as BCNU (carmustine) and monofunctional alkylating agents, e.g., procarbazine and temozolomide, have been extensively used (51). Although interstrand DNA crosslinks induced by CNU are the critical cytotoxic lesions, the initial adduct, O^6-chloroethylG is repaired by MGMT prior to formation of the crosslink (51,52). Tom Brent and others showed a correlation between MGMT levels and CNU resistance in various glioblastoma cells (52). While m^6G generated by monofunctional alkylating agents is not as cytotoxic, its persistence in MGMT-deficient cells results in cytotoxicity following DNA replication, as Peter Karran and his colleagues initially documented (53). The concept of futile mismatch repair-replication cycle of persistent m^6G•T pair as postulated by Karran suggests that unrepaired m^6G in Mex^- cells pairs with T during replication which then triggers the mismatch repair (MMR) process. Removal of T in the nascent strand is futile because of repeated incorporation of T opposite m^6G during repair synthesis. The persistent nascent strand gap leading to double-strand breaks triggers signaling for apoptosis (48,53,54). The resistance of MMR-deficient tumor cells to monofunctional alkylating agents provides strong evidence for the m^6G-initiated futile repair cycle (55,56).

The molecular basis for MGMT regulation thus became an important topic for subsequent investigation. We and others showed inducibility of the MGMT gene by genotoxic agents (57). We mapped the hMGMT promoter and identified several regulatory elements including

six putative Sp1 sites within the CpG island, two glucocorticoid response element (GRE), and two each of putative AP-1 and AP-2 elements (58). We investigated the potential function of each of the GRE and AP-1 sites in activation of MGMT expression (59,60). Moreover, we showed that remodeling of chromatin by recruitment of the histone acetyltransferase CBP/P300 activates the MGMT promoter (61). Several groups, including ours, explored the basis of MGMT extinction in Mer^{-}/Mex^{-} cells. The role of CpG methylation was indicated by the observed conversion of Mex^{-} into Mex^{+} cells after treatment with CpG demethylating agent 5-azacytidine (62). However, the total repression of MGMT in Mex^{-} cells appears to be due to extensive methylation of CpG in both the promoter and the transcribed sequences of the MGMT gene, which is unusually large (> 180 kb; ref. 63) relative to the small size(~ 1 kb) of the mRNA (64,65).

Cloning of mammalian MGMT cDNA

Our and others' initial efforts in cloning the MGMT gene by genomic complementation were uniformly unsuccessful, presumably because of the enormous size of the MGMT gene, as became evident later. On the other hand, cross-species phenotypic complementation turned out to be a powerful tool for cloning DNA repair genes in the form of cDNA. We predicted success in this strategy, at least for MGMT, because of the similarity of biochemical properties of *E. coli* Ada and partially purified mammalian MGMTs (32).

We succeeded in cloning the human MGMT cDNA using a phenotypic complementation assay by transforming MGMT-negative (*ada*, *ogt*) *E. coli* with a human cDNA library and screening for MNNG-resistant clones. We isolated several such clones and confirmed that the resistance was conferred due to expression of MGMT encoded by the plasmid (42). Sekiguchi, Karran and their collaborators subsequently cloned mammalian MGMT cDNAs using more conventional approaches (66,67). Purification of the recombinant human MGMT allowed us to identify the methylacceptor Cys residue and also to clone the mouse MGMT cDNA and purify the polypeptide. As expected, the human and mouse MGMT have extensive sequence identity and complete conservation of the sequence PCHRV which includes the alkylacceptor Cys145 residue in the human MGMT (and Cys149 in the mouse protein). This sequence is conserved in all other MGMTs including Ada (47). The mammalian MGMT is much smaller than the Ada protein which contains the N-terminal extension that folds into a distinct domain connected by a hinge sequence with the C-terminal MGMT domain (28). Samson's group published the cloning of the yeast MGMT gene using the same strategy a little earlier than when we cloned the human MGMT cDNA (39). We extended the cross species phenotypic complementation strategy to subsequently clone the cDNA of the human AlkA homolog which we named N-methylpurine-DNA glycosylase (MPG; 68); Samson's group independently named it 3-alkyladenine-DNA glycosylase (AAG).

Discovery of O^6-benzyl G as an MGMT pseudosubstrate

Early studies suggested that requirement of the presence of m^6G in an oligonucleotide to serve as the MGMT substrate. Pegg and Moschel discovered that O^6-benzylG (B^6G) is a potent inhibitor of MGMT because of its high affinity and ability to function as a pseudosubstrate (69). This was a seminal discovery with significant clinical implications because B^6G treatment sensitizes tumor cells and xenografts to BCNU (70,71). More recent studies of the biological effects of B^6G and of structural studies of MGMT to examine its binding to B^6G, are described in detail elsewhere in this volume.

Future Directions

Although the pace of research on MGMT might have slowed down somewhat in recent years, there are still many unanswered questions about regulation and fate of the methylated protein,

and also the possibility of additional repair-unrelated functions of this unusual protein. These should keep the investigators busy for some years to come. The translational aspect of MGMT, namely its tumor-cell specific inhibition with B^6G or some other small molecules for drug sensitization of tumors, along with ectopic MGMT-mediated protection of healthy tissues from drug toxicity, should also remain a challenging topic.

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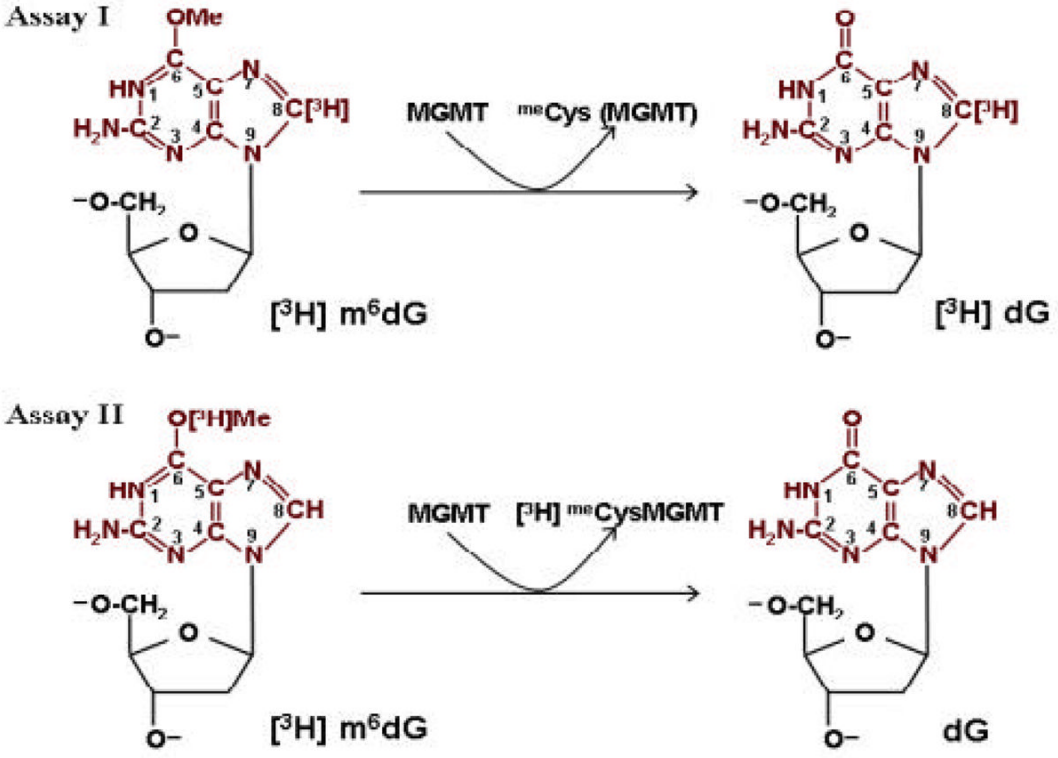

Fig. 1. MGMT Assay

Two complementary quantitative assays for MGMT were developed using [^{3}H]-labeled m^{6}G-containing DNA substrates. (Only the O^6-methyldeoxyguanosine moiety is shown here for convenience.) In (I), a synthetic oligodeoxynucleotide containing m^{6}G labeled with [^{3}H] at C-8 is used as the substrate. Demethylation of m^{6}G by MGMT generates [^{3}H]G which is then separated from [^{3}H]m^{6}G by HPLC. The amount of MGMT equals that of [^{3}H]G. In (II), DNA methylated with a [^{3}H]methylating agent is used as the substrate. MGMT is radiolabeled at the methyl-acceptor Cys during the methyltransferase reaction. After hydrolysis, [^{3}H]methylCys is quantitated.