*Mutation Research*, 198 (1988) 45–51
Elsevier



MTR 04529

# Gene expression in *E. coli* after treatment with streptozotocin

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(Received 9 April 1987)
(Revision received 12 August 1987)
(Accepted 25 August 1987)

*Keywords:* Streptozotocin; *N*-Methyl-*N*-nitrosourea; *N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine; Gene induction; Fusion mutants; (*E. coli*).

**Summary**

Gene induction by the methylating agents streptozotocin (STZ), *N*-methyl-*N*-nitrosourea (MNU), and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) was evaluated in *E. coli* fusion mutants. These mutants have fusions of the *lac* operon to genes induced by treatment with sublethal levels of alkylating agents and were previously selected from random insertions of the Mu-dl ($Ap^r$ *lac*) phage by screening for induction of β-galactosidase activity in the presence of methyl methanesulfonate or MNNG. The results demonstrate that STZ differs from MNNG and MNU in failing to induce *aidC* expression. Further, expression of *aidC* after exposure to MNU and MNNG occurs only in nonaerated cultures; aeration blocks the induction. Induction of *aidD, alkA, aidB,* and *sfiA* expression occurs with all 3 agents although at markedly lower concentrations of MNNG and STZ compared to MNU. *alkA* and to a lesser extent *aidD* mutants of *E. coli* strains were more sensitive to these agents, while no differences were evident between wild-type and *aidB* or *aidC* fusion mutants.

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An adaptive response is induced after exposure of *E. coli* to low, nonlethal levels of alkylators such as MNNG and MNU. The adaptive response ameliorates both the cytotoxic and mutagenic effects of these agents (Samson and Cairns, 1977; Jeggo, 1977). This repair mechanism is positively regulated by the *ada* gene (Jeggo, 1979; Sedgwick, 1983; Lemotte and Walker, 1985). Following the methylation of *ada* protein by methylphosphotriesters in alkylated DNA, methylated *ada* protein increases transcription of both *ada* and *alkA* (Teo et al., 1986). Ada protein abstracts methyl groups from $O^6$-methylguanine, thus preventing mutagenic events (Lindahl et al., 1982, McCarthy and Lindahl, 1984; Margison et al, 1985). *alkA*, on the other hand, specifies 3-methyladenine–DNA glycosylase II (Nakabeppu et al., 1986a,b). The release of cytotoxic lesions such as 3-methylguanine, 3-methyladenine, and 7-methylguanine from alkylated DNA is catalyzed by 3-methyladenine–DNA glycosylase II (Karran et al., 1982; Evensen and Seeberg, 1982).

In addition to *ada* and *alkA, ada* positively controls *alkB* and *aidB* expression (Kataoka and Sekiguchi, 1985; Volkert and Nguyen, 1984). The former is situated in close proximity to *ada* and

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may contribute to the repair of cytotoxic lesions, while the function of the latter is not known.

Alkylators also induce other genes in *E. coli* such as *aidC* (Volkert et al., 1986). This gene is not regulated by *ada* and its function is not known. In addition SOS repair is induced by alkylators and is not regulated by *ada*. SOS repair is initiated after activation of *recA* protein with cleavage of *lexA* repressor and enhanced transcription of about 17 genes (Little and Mount, 1982; Walker, 1984).

Streptozotocin (STZ) is a monofunctional nitrosourea with antineoplastic properties as well as a potent mutagen and carcinogen (Rakieten et al., 1971; Chick et al., 1977; Moertel and Hanley, 1979; Fram et al., 1986). Our prior studies with this agent have shown that cytoxicity by STZ is enhanced in *ada, alkA,* and *recA E. coli* mutants while not enhanced in *uvrA* mutants (Fram et al., 1986). Thus, both the adaptive response and post-replication recombinational repair contribute to survival after STZ treatment. Further, pre-mutagenic lesions, were, at least to some extent, repaired by $O^6$-alkylguanine–DNA alkyl transferase since *ada* mutants were hypermutable compared to *ada* mutants that also contained a multi-copy plasmid carrying the wild-type *ada* gene (Fram et al., 1986).

The current study was undertaken to analyze gene induction by STZ and to contrast the effects of this drug with those of other methylating agents. The structures of the agents tested are shown in Fig. 1. STZ resembles MNU except for the presence of a D-glucopyranoside group. MNNG is also structurally distinct from STZ. We wished to evaluate whether the effects of these methylating agents on gene induction were uniform or heterogeneous. An additional goal was to characterize differences in the induction of the various alkylator-inducible genes on the basis of structural differences in the methylating agents tested.

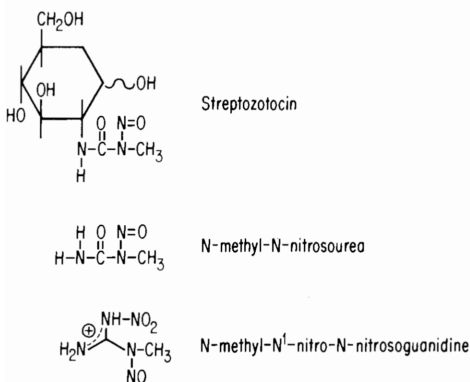


Fig. 1. Structures of streptozotocin, *N*-methyl-*N*-nitrosourea, and *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine.

## Methods

### *Bacterial strains*

The *E. coli* K-12 strains employed in the experiments are described in Table 1.

### *Reagents*

*N*-Methyl-*N*-nitrosourea (MNU), *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG), and streptozotocin (STZ), were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). A 10 mg/ml stock solution was prepared daily of MNU and MNNG in formamide and then diluted 1:10 in sodium acetate, pH 5. STZ (1 mg/ml) was dissolved in minimal medium.

### *Induction of β-galactosidase activity by alkylating agents*

Cells were grown overnight in minimal medium (E salts; glucose, 0.4%; Bacto casamino acids, 0.2%; thiamine, 0.2 μg/ml), diluted 1:50 and

TABLE 1

BACTERIAL STRAINS

| Strain | Relevant genotype |
|---|---|
| MV1161 [a] | Wild-type |
| MV1571 | *alkA51*::Mu-dl($Ap^r$ *lac*) |
| MV1563 | *aidB2*::Mu-dl($Ap^r$ *lac*) |
| MV1601 | *aidD6*::Mu-dl($Ap^r$ *lac*) |
| MV1608 | *aidC8*::Mu-dl($Ap^r$ *lac*) |
| MV1701 | *aidB2*::Mu-dl($Ap^r$ *lac*) ada *10*::Tn*10* |
| DM1931 [b] | *sfi*::Mu-dl($Ap^r$ *lac)Xcam* |

[a] All strains were derivatives of MV1161 and contain the following additional markers *argE3 his-4 leu-6 proA2 ara-14 galK2 lacY1 mtl-1 xyl-5 thi-1 rpsL3 supE44 tsx-33 rfa-550* (Volkert et al., 1986).

[b] DM1931 contains the following additional mutations del(*lac-pro*)X111 *hisG4 argE3 thr-1 ara-14 xyl-5 mtl-1 tsx-33 ilvts* su⁻.

regrown to $10^8$ cells/ml as determined by readings of the optical density. 1 ml of cells was incubated with the appropriate concentration of drug or no drug at 30 °C for 3 h. A 3-h incubation was employed since maximal gene induction by a variety of alkylating agents occurred after this interval (Volkert and Nguyen, 1984; Volkert et al., 1986). Cells were either aerated by shaking in a water bath or allowed to stand during drug treatment. β-Galactosidase activity was measured as previously described (Miller, 1972). Experiments were performed at least twice and representative results are shown.

*Cytotoxicity*

Cells were grown overnight as previously noted, diluted 1:100 and regrown to $10^8$ cells/ml. Cells were exposed to drug for 30 min at 30 °C without shaking, washed twice, and then dilutions plated onto L medium. After incubation overnight at 30 °C, colonies were counted, survival determined and expressed as percentage of untreated control. Experiments were performed at least twice and representative results are shown.

## Results

Gene fusions employed in this study consist of random insertions of the Mu-dl (Ap$^r$ *lac*) phage at the site of various alkylation-inducible genes. The insertion of the structural gene for β-galactosidase near the promoter of a gene results in the synthesis of β-galactosidase upon transcription. These fusions were previously screened for induction by methyl methanesulfonate (MMS) and MNNG as well as extensively characterized (Volkert and Nguyen, 1984; Volkert et al., 1986). The experimental approach provides a simple means for quantifying the extent of gene induction by the various agents tested.

Induction of the *aidC* mutant fusion is shown in Fig. 2. While both MNNG and MNU are potent inducers of this gene, expression of *aidC*::Mu-dl(Ap$^r$ *lac*) was not induced by STZ. Further, induction by MNU and MNNG occurs only when cells are not aerated. As with *aidB* (see below) and in keeping with prior studies, basal levels of expression of the *aidC* fusion were higher in cultures that are not aerated.

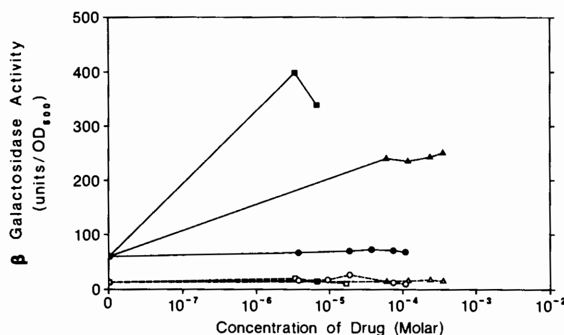


Fig. 2. Expression of *aidC8*::Mu-dl(Ap$^r$ *lac*). Cells were exposed to drug for 3 h at 30 °C with either vigorous shaking (open figures) or without shaking (closed figures). Symbols represent exposure to MNNG (■, □), STZ (●, ○), and MNU (▲, △).

Fig. 3 demonstrates that all 3 agents induced *aidD*::Mu-dl(Ap$^r$ *lac*) expression. While induction of the *aidD* fusion occurs after treatment by all 3 methylating agents, gene induction occurred at lower concentrations of MNNG and STZ than of MNU. There was no difference in the extent of *aidD* expression when cells were aerated or allowed to stand without shaking during drug exposure.

As with *aidD*, induction of *alkA*::Mu-dl(Ap$^r$ *lac*) occurred after exposure to all 3 agents (see Fig. 4). Peak expression also occurred at markedly

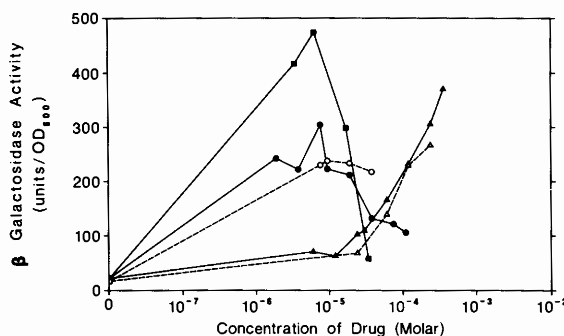


Fig. 3. Expression of *aidD6*::Mu-dl(Ap$^r$ *lac*). The culture was grown overnight, diluted 1:50 and grown with shaking to a cell density of $10^8$ cells/ml. Cells were exposed to drug for 3 h at 30 °C with either vigorous aeration (open figures) or without shaking (closed figures). β-Galactosidase activity was then determined. Symbols represent treatment with MNNG (■), STZ (●, ○), and MNU (▲, △). A representative experiment is shown.

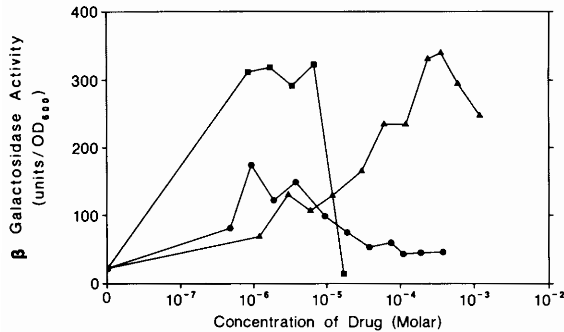


Fig. 4. Expression of *alkA51*::Mu-dl(Ap^r *lac*). An overnight culture was diluted 1 : 50 and cells were grown with shaking to a density of $10^8$ cells/ml. Cells were exposed to drug for 3 h at 30 °C without shaking. Symbols represent treatment with MNNG (■), STZ (●), and MNU (▲).

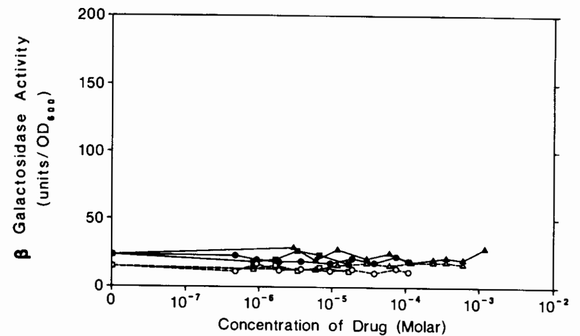


Fig. 6. Expression of *aidB2*::Mu-dl(Ap^r *lac*) *ada*::Tn10. Cells were exposed to drug for 3 h at 30 °C with either vigorous shaking (open figures) or without shaking (closed figures). Symbols represent exposure to MNNG (■, □), STZ (●, ○), and MNU (▲, △).

lower drug concentrations with MNNG and STZ than after treatment with MNU. Further, aeration of cells did not affect results (data not shown).

The induction of the *aidB* fusion by the various alkylators is shown in Fig. 5. In contrast to *alkA* and *aidD*, however, peak induction occurs at lower concentrations of STZ compared to MNNG and at markedly lower concentrations compared to MNU. Further, induction of *aidB*::Mu-dl(Ap^r *lac*) occurs to some extent when cells are incubated without shaking and prior to alkylator exposure (see Fig. 5). Thus, basal values of *aidB E. coli* cells are increased when cells are not aerated (see Fig. 5). Lastly, alkylator-induced gene expression was not enhanced in nonaerated cells when values were corrected by subtracting appropriate control values (see Fig. 5).

Since anaerobiosis might, to some extent, regulate *aidB* expression, the role of *ada* in regulating the induction of *aidB* by alkylators was evaluated in an *ada aidB* mutant. The results (see Fig. 6) demonstrate that induction of *aidB* by STZ, MNU, and MNNG requires a functional *ada* gene.

In order to evaluate SOS induction by the 3 agents, β-galactosidase expression in a *sfi*::Mu-dl(Ap^r *lac*) fusion mutant was analyzed. Fig. 7 reveals that all agents tested induce this gene. As with the other gene loci except for *aidC*, gene

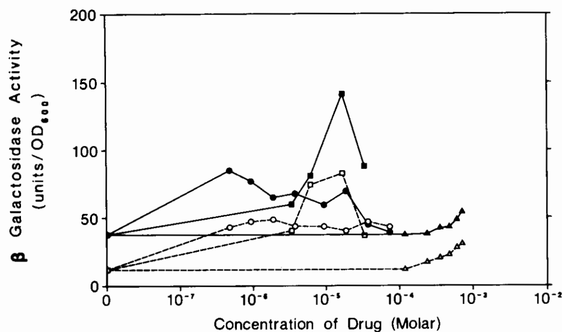


Fig. 5. Expression of *aidB2*::Mu-dl(Ap^r *lac*). Cells were exposed to drug for 3 h at 30 °C with either vigorous aeration (open figures) or without shaking (closed figures). Symbols represent treatment with MNNG (■, □), STZ (●, ○), and MNU (▲, △).

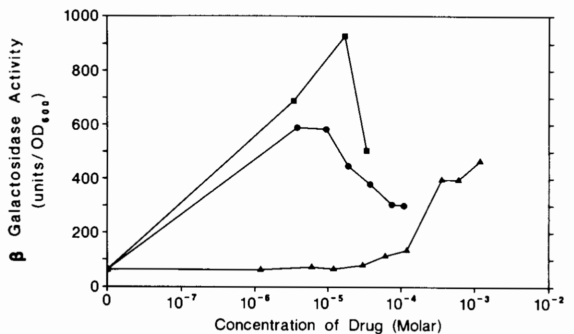


Fig. 7. Expression of *sfi*::Mu-dl(Ap^r *lacXcam*). Cells were exposed to drug for 3 h at 30 °C without shaking. Symbols represent exposure to MNNG (■), STZ (●), and MNU (▲).

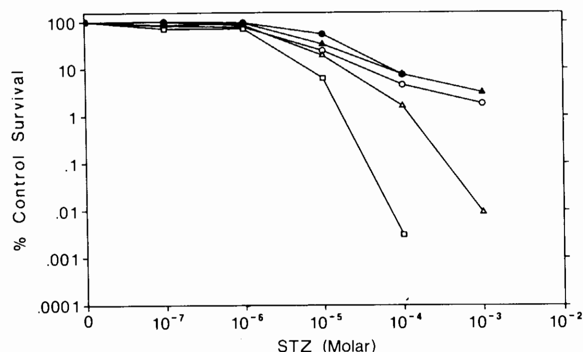


Fig. 8. Survival of *aid* mutants and their *aid*$^+$ parent strain after exposure to STZ. Overnight cultures were diluted 1:100 and grown to a density of $10^8$ cells/ml with aeration at 30 °C. Cells were treated with drug for 30 min at 30 °C without shaking, spun once, and washed twice. Cells were plated onto L plates at various dilutions. Symbols represent MV1161, *aid*$^+$ (○); MV1563, *aidB2* (▲); MV1571, *alkA* (□); MV1601, *aidD6* (△); and MV1608, *aidC8* (●).

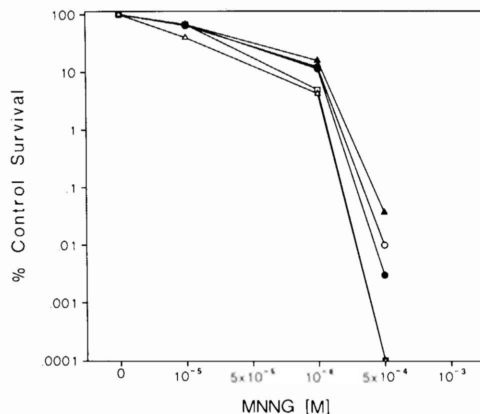


Fig. 10. Survival of *aid* mutants and their *aid*$^+$ parent strain after treatment with MNNG. The methods and symbols are the same as in Fig. 8.

induction occurs at markedly lower drug concentrations with MNNG and STZ compared to MNU.

Cytotoxicity by MNU, STZ, and MNNG was also evaluated in the various *aid* fusion mutants. Our goal was to analyze whether insertion of Mu-dl(Ap$^r$ *lac*) into the different alkylation-inducible genes affects cellular survival. Fig. 8 demonstrates that *alkA* and *aidD* cells are more sensitive that wild type to STZ. *aidB* and *aidC* cells, on the other hand, were no more sensitive than wild type. Fig. 9 reveals that MNU is a less potent cytotoxic agent than STZ on a molar basis. Further, as with STZ, *alkA* and to a lesser extent *aidD* cells, were more sensitive to MNU than wild type. A similar pattern of cytotoxicity was noted when cells were treated with MNNG (see Fig. 10).

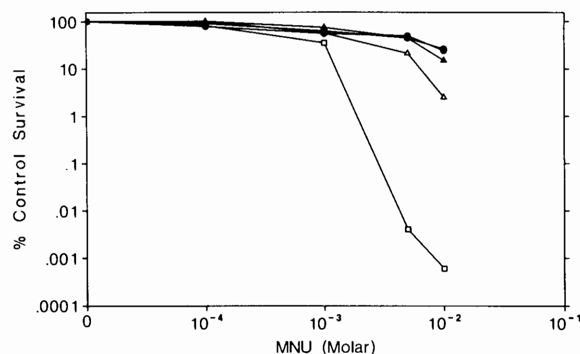


Fig. 9. Survival of *aid* mutants and their *aid*$^+$ parent strain after treatment with MNU. The procedure and symbols are the same as in Fig. 8.

## Discussion

Our results demonstrate that STZ, MNU, and MNNG induce *ada*. This finding suggests that STZ causes the formation of methylphosphotriesters in DNA, a lesion that induces *ada* (Teo et al., 1986). Further, the expression of *alkA* by these alkylators is a logical consequence based on the known regulatory role of methylated *ada* protein in *alkA* induction (Teo et al., 1986).

The basis for the marked differences in drug concentration required for expression of *ada* and *alkA* by STZ and MNU are unclear. Different quantities of a specific DNA lesion may be formed by STZ (for example, methylphosphotriesters) compared to MNU. On the other hand, intracellular concentrations of STZ may simply be higher at any given extracellular drug concentration. Since active transport of STZ occurs in some sensitive *E. coli* strains such a hypothesis is credible (Ammer et al., 1979; Lengeler, 1980a,b). Further, the tendency for gene expression at other loci (with the exception of *aidC*) to show a similar difference in sensitivity to STZ compared to MNU is also consistent with such an interpretation.

As in prior studies, the induction of *aidB* was enhanced in the absence of drug exposure when

cells were not aerated (Volkert and Nguyen, 1984; Volkert, unpublished data). While *aidB* is induced independently of *ada* in nonaerated cultures, alkylator-induced expression of *aidB* requires a functional *ada* gene. This conclusion is supported by the failure of alkylators to cause *aidB* induction in *ada* mutants.

Another possibility is that differing patterns of gene induction may result from the direct methylation and inactivation of $O^6$-alkylguanine–DNA alkyltransferase by the methylating agents tested. Recent experiments demonstrate that MNU and MNNG are more effective than STZ in directly inactivating human transferase, while all 3 agents are about equally effective in producing a DNA substrate that inactivates the transferase (Brent, 1986). Since methylated ada protein causes induction of genes regulated by *ada*, it seems unlikely that failure to methylate ada protein by STZ underlies induction of *alkA* and *aidB* at lower molar concentrations of STZ than of MNU. Lack of induction of *aidC* by STZ is also not likely explained by such an effect since *aidC* is not regulated by *ada* (Volkert, 1986).

The failure of STZ to induce *aidC* markedly contrasts with the effects of MNNG and MNU on *aidC* expression. These findings may suggest that structural differences between STZ and MNU may cause marked differences in gene expression. In this respect, STZ resembles MMS in its failure to induce *aidC* while inducing *aidB, ada*, and *alkA* (Volkert et al., 1986). Thus, STZ may, like MMS, form adducts at reactive oxygens in DNA less frequently than MNNG or MNU (Singer, 1982). Another possibility is that STZ exposure causes metabolic effects that underlie the inability of this agent to induce *aidC*. For example, an effect of STZ on the reducing environment of the cell might alter either the yield of reaction products necessary to cause gene expression or independently affect a regulatory mechanism that requires anaerobic conditions for induction by alkylating agents (see below).

In contrast to the other gene loci evaluated, expression of *aidC* occurs solely when cells are exposed to drug and not aerated. Qualitatively similar results are noted when gene induction in *aidC* mutant cells are analyzed when not shaken for 3 h or when grown under strictly anaerobic conditions of 95% nitrogen and 5% $CO_2$ (Volkert, unpublished data). Failure of STZ to induce *aidC* likely reflects fundamental differences in the regulation of *aidC* from that of *aidB, ada*, and *alkA*. While the latter loci are regulated by *ada, aidC* is not (Volkert et al., 1986).

In addition *aidC* is induced by alkylators that add large alkyl groups such as propyl *N*-nitro-*N′*-nitrosoguanidine, whereas *aidB* and *alkA* are not (M.R. Volkert, unpublished data).

Expression of the *sfi* mutant fusion mutant after treatment with STZ and MNU is not surprising in view of the known effects of MNNG in inducing SOS repair (Quillardet et al., 1982). As with all gene loci evaluated (except for *aidC*), far lower concentrations of STZ and MNNG were required to induce *sfi* than of MNU. At present it is unclear that these results occur because of differences in the types and quantity of DNA lesions induced by these agents or simply because intracellular levels of drug are lower at a given extracellular concentration of MNU. Experiments to quantify both the sites and extent of methylation by the agents examined would clarify this issue.

Our cytotoxicity results demonstrate a similar pattern in the sensitivity of the various *aid* fusion mutants to STZ, MNNG, and MNU. MNU was generally less toxic than the other agents. As with STZ and MNNG, however, both *alkA* and *aidD* fusion mutants were more sensitive to the agents evaluated. These findings are consistent with the presence of cytotoxic lesions such a *N*-3-methyladenine and *N*-3-methylguanine which are recognized by the inducible glycosylase specified by *alkA* (Karran et al., 1982; Nakabeppu et al., 1984). The significance of enhanced cytotoxicity in *aidD* mutants is unclear since the gene product specified by this gene is not known. *aidD* is presumably a fusion to the *ada alkB* operon that has apparently inactivated the *alkB* gene (Kataoka et al., 1983). Thus, *alkB* may contribute to repair of lesions induced by these alkylators.

Our studies emphasize the heterogeneity of the effects of alkylating agents. White STZ and MNU are structurally similar, the former fails to induce *aidC*. Both the molecular basis for these differences as well as the regulatory mechanisms that underlie the requirement for anaerobic conditions

for alkylator induction of *aidC* are areas for future investigation.

## Acknowledgements

Dr. M.R. Volkert is supported by N.I.H. Grants GM37052-01 and SO7-RR05712. This work was in part supported by the Diabetes Endocrine Research Center at the University of Massachusetts Medical School, N.I.H. Grant AM32520 (M.G.M. and R.J.F.).

The authors are grateful to Mrs. Dianne Sullivan for assistance in preparing the manuscript.

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