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THE ORIGIN OF DNA SINGLE STRAND BREAKS INDUCED BY ETHYLATING AGENTS IN MAMMALIAN CELLS

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Summary. - *Chinese hamster ovary (CHO) cells were treated with two ethylating agents, N-ethyl-N-nitrosourea (ENU) and diethylsulfate (DES), and the kinetics of DNA single strand break (ssb) induction and rejoining were determined in parallel with DNA adduct formation and removal. In the case of DES, DNA ssb as determined by alkaline elution (AE) were repaired very slowly with more than 50% of the lesions still present on DNA 3 h after treatment. In contrast, 45% of ENU-induced ssb were repaired within 10 min. From the relative concentration of the different ethylated products and their repair rates as measured by high performance liquid chromatography (HPLC) analysis of the ethylated DNA, a theoretical function was constructed that describes the number of ssb expected at each time point after exposure to the mutagen. DES-induced ssb are explained by excision repair processes active on the ethylated purines, mainly 3-ethyladenine (3-EtAde) and 7-ethylguanine (7-EtGua). On the same basis, the rapidly repaired ENU-induced ssb remain unexplained. These results are also discussed in relation to the sensitivity of the two techniques, AE and HPLC, for detecting DNA damage.*

Riassunto (Caratterizzazione delle rotture del DNA a singolo filamento indotte da agenti alchilanti in cellule di mammifero). - *Cellule di criceto, CHO, sono state trattate con due agenti etilanti, N-etil-N-nitrosourea (ENU) e dietilsolfato (DES), ed è stata studiata l'induzione e la riparazione delle rotture del DNA a singolo filamento parallelamente alla formazione e rimozione degli addotti al DNA. Nel caso del DES, le rotture del DNA, determinate con la tecnica dell'eluizione alcalina, vengono riparate molto lentamente con il 50% delle lesioni ancora presenti sul DNA dopo 3 ore dal trattamento. Molto diverso è quanto osservato dopo trattamento con ENU dove il 45% delle rotture viene riparato dopo 10 minuti. Conoscendo la concentrazione relativa dei diversi prodotti etilati determinati con analisi cromatografica (HPLC), è stato possibile costruire una funzione teorica che descrive il numero delle rotture del DNA atteso a tempi diversi dopo il trattamento. Le rotture del DNA indotte dal DES sono spiegate dai processi di riparazione per escissione attivi sulle purine etilate, principalmente la 3-etiladenina (3-EtAde) e la 7-etilguanina (7-EtGua). Le rotture del DNA indotte da ENU e riparate molto velocemente non trovano invece una facile spiegazione. Questi risultati saranno discussi anche in relazione alla sensibilità delle due tecniche usate per determinare il danno presente sul DNA, eluizione alcalina e cromatografia liquida ad alta pressione.*

Introduction

DNA single strand breaks (ssb) might arise in living cells *via* several different mechanisms: i) a fast chemical reaction following the absorption of energy from a charged particle or photon; ii) after a chemical attack by reactive species; iii) as transient expression of enzymatic reactions, in processes leading to excision of segments with various types of induced lesions.

In the case of alkylating agents, mammalian cells respond to the resulting damage either by directly removing the alkyl functionality, as is the situation with O^6-alkylguanines [1], or by excising the alkylation adducts from the genome, filling the resulting gaps, thereby restoring the original nucleotide sequence. For examples alkyl substitutions at the nitrogen atoms of purines in DNA, such as N-7 of guanine and N-3 of adenine, are substrates for a specific glycosylase that catalyzes the loss of alkylated purines by cleavage of the N-glycosydic bond [2]. The contribution of excision repair processes to removal of adducts from the alkylated DNA of mammalian cells is at present poorly defined. The enzymatic steps following the release of the alkylated base are believed to involve the formation of DNA nicks by apurinic/apyrimidinic (AP) endonucleases at the sites of base loss. AP sites are characterized by their ease of hydrolysis of adjacent phosphodiester bonds to yield ssb in

DNA; the half-life for hydrolysis is nearly 200 h under physiological conditions [3], although it is rapid in alkali.

The alkaline elution (AE) procedure provides a facile mean for detecting AP sites by virtue of their alkaline lability [4]. The number of DNA ssb at any given time after treatment with an alkylating agent should mainly reflect the chemical or enzymatic hydrolysis of the N-glycosidic bond of alkylated purine or pyrimidine bases to yield AP sites. Alkylation of the phosphate groups leading to phosphate triesters also generates alkali-labile sites, but they hydrolyze slowly at pH 12, which is commonly used in the AE procedure [5, 6].

In an attempt to understand the nature of the alkali-labile sites detected by AE, Chinese hamster ovary (CHO) cells were treated with different alkylating agents and the kinetics of DNA ssb induction and rejoining were determined in parallel with DNA adduct formation by chromatographic analysis. Two ethylating agents, N-ethyl N-nitrosourea (ENU) and diethylsulfate (DES), which react with DNA *via* different mechanisms, were selected for this comparison. ENU interacts preferentially with oxygen atoms (80% of the total alkylation), whereas DES has a strong preference for the highly nucleophilic centers on DNA such as nitrogen atoms.

Results

Suspension cultures of CHO cells were exposed to ^{3}H-labeled ENU and DES, and DNA adducts were measured by HPLC analysis as previously described [7]. In the same range of molar concentrations and at similar levels of total DNA alkylation, ENU and DES produced different relative amounts of each adduct (Fig. 1). As expected [8] the extensive alkylation at N-atoms of DNA bases was observed for DES, with 7-ethylguanine (7-EtGua) and 3-ethyladenine (3-EtAde) being the main adducts. ENU, in contrast, reacted preferentially at O-atoms [9] giving rise to relatively high levels of O^6-ethylguanine (O^6-EtGua), O^4-ethylthymine (O^4-EtThy), O^2-ethylthymidine (O^2-EtThy), O^2-ethylcytosine (O^2-EtCyt) (Fig. 1) and ethylphosphotriesters (data not shown; [10]). A linear relationship was observed between ENU and DES doses and the relative levels of the different DNA adducts.

The analysis of the dose-response curves for ssb induction as measured by AE shows that at equimolar concentrations, DES induces a greater number of ssb than ENU (the ratio of the linear slopes obtained for DES and ENU is approximately 1.6) (Fig. 2). This result is not surprising in view of the proclivity of DES to induce a high proportion of lesions such as 7-EtGua and 3-EtAde which give rise to alkali-labile sites. However the relative levels of N-alkylpurines induced by DES and ENU (e.g., 54 and 16 ethylations x 10^{-6} nucleotides respectively, at the dose of 4 mM) do not explain the difference in the rate of ssb induction. ENU induces a greater number of ssb than expected even when the amounts of O-ethylpyrimidines, which are possibly removed *via* a glycosylase [11], are taken into consideration. It is important to keep in mind that ssb as detected by AE are not a valid measure of total DNA damage, but rather are a dynamic measure of the DNA repair processes acting on alkylated bases. The number of ssb at a given time point corresponds to approximately 1% of the actual level of the ethylated products. Furthermore very different kinetics of ssb rejoining are observed after treatment with DES and ENU (Fig. 3). DES-induced damage was repaired very slowly with more than 50% of the lesions still present in DNA 3 h after treatment. In contrast, the kinetics of repair of ENU-induced damage show an initial

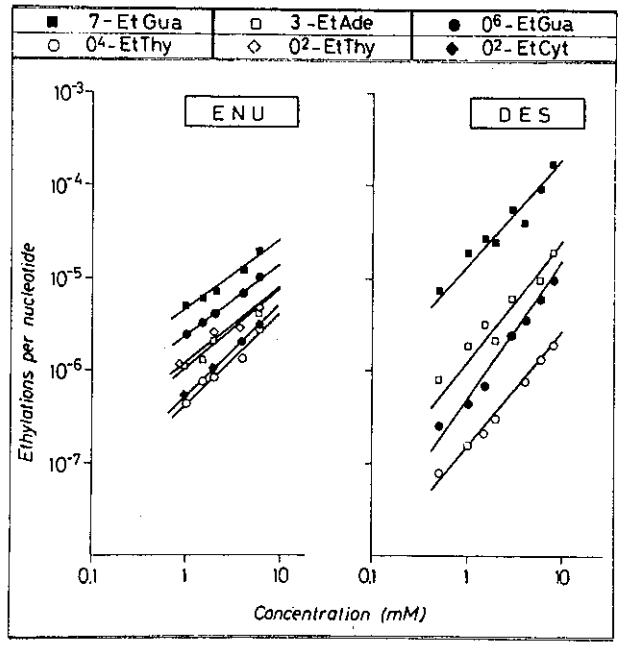

Fig. 1. - Frequency of several ethylated products in DNA of CHO cells as a function of the exposure concentration to DES and ENU. Each point represents the mean of three independent observations.

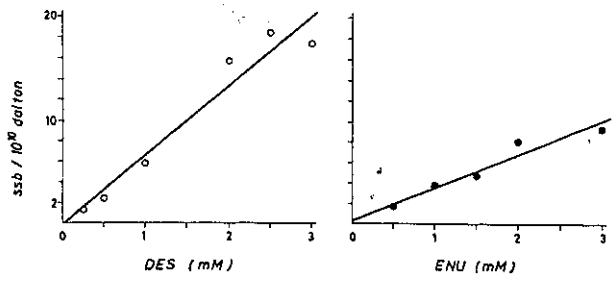

Fig. 2. - Induction of ssb as determined by AE after exposure to DES (open symbols) and ENU (closed symbols) for 30 min. Each symbol represents the mean of four independent observations. The number of ssb was determined by calibration with DNA of CHO cells exposed to a known dose of γ-rays with the assumption that 1 Gy of ^{60}Co-rays induces 2.5 ssb x 10^{10} molecular weight of DNA.

rapid rate of repair (45% of ssb repaired within 10 min) with no significant changes detectable after the first 30 min. A ratio of about 10 is observed between the time constants for ENU and DES exponential curves.

We therefore determined the kinetics of repair of the different DNA adducts by HPLC analysis of ENU-ethylated CHO DNA (Fig. 4). We excluded from this analysis O^6-EtGua, a lesion removed *via* the O^6-alkylguanine DNA alkyltransferase, which does not give rise to alkali-labile sites, as well as to phosphotriesters [5, 6]. The kinetics of removal of 7-EtGua, 3-EtAde, O^2-EtThy and O^2-EtCyt were described by an exponential curve ($p < 0.05$ or < 0.01). The N-alkylated purines were removed from CHO DNA with a half-life of 12 and 4 h for 7-EtGua and 3-EtAde, respectively, while almost 50% of O-alkylpyrimidines was still present on DNA after a 20 h post-treatment incubation time.

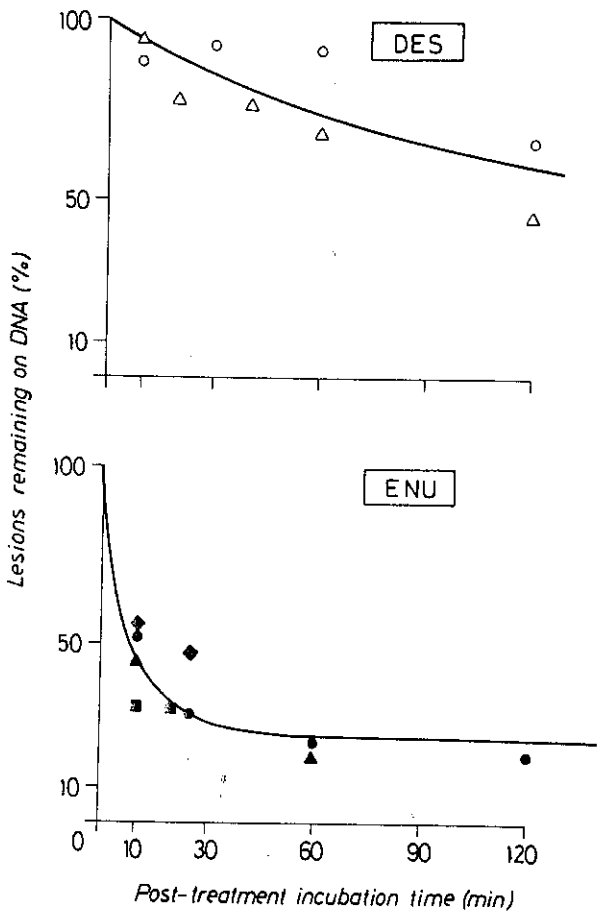

Fig. 3. - Rejoining of DNA ssb as measured by AE after different post-treatment incubation times following a 5 min exposure to 2 mM DES (open symbols) or 3 mM ENU (closed symbols). Each symbol represents the mean of two independent observations. The data are consistent with exponential functions:
$y_{(DES)} = 58.9\ e^{-.009t} + 41.1$
$y_{(ENU)} = 75.7\ e^{-.011t} + 24.3$
where t = min

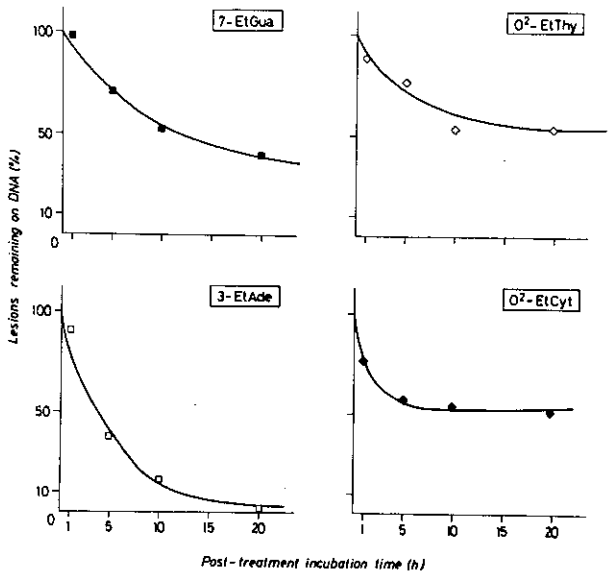

Fig. 4. - Removal of DNA ethylation products from CHO cells at increasing post-treatment incubation times after a 30 min exposure to 2 mM ENU. Each symbol represents the mean of at least two independent experiments. The data were fitted with exponential curves:
$y_{(7\text{-EtGua})} = 69.77\ e^{-.1t} + 30.2$
$y_{(3\text{-EtAde})} = 110\ e^{-.003t} + 1.1$
$y_{(O^2\text{-EtThy})} = 49\ e^{-.17t} + 51$
$y_{(O^2\text{-EtCyt})} = 46\ e^{-.67t} + 54$

From the relative concentrations of the different ethylated products and their repair rate it was possible to define a theoretical function describing the number of ssb as a function of post-treatment incubation time:

$$y_{(DES)} = (A)\ R_a + (B)\ R_b \qquad (1)$$

$$y_{(ENU)} = (A)\ R_a + (B)\ R_b + (C)\ R_c + (D)\ R_d \qquad (2)$$

where (A,B,C,D) are constants proportional to the concentrations of 7-EtGua, 3-EtAde, O^2-EtThy, O^2-EtCyt, respectively, and $R_{(a,b,c,d)}$, are functions describing the repair rate of the aforementioned bases (legend Fig. 3). The theoretical line for repair of DES-induced DNA damage fitted reasonably well with the experimental line (the two lines are not significantly different). However when the theoretical line for repair of ENU-induced damage was compared with the experimental one, a wide discrepancy was observed. In particular the initial fast decrease in the number of ssb was unexplained while the slow downward trend observed after 20 min was parallel to the theoretical curve (Fig. 5).

Discussion

DNA alkaline elution assay is widely used to assess DNA damage induced by a variety of chemicals with cytotoxic and mutagenic effects. The assay is sensitive

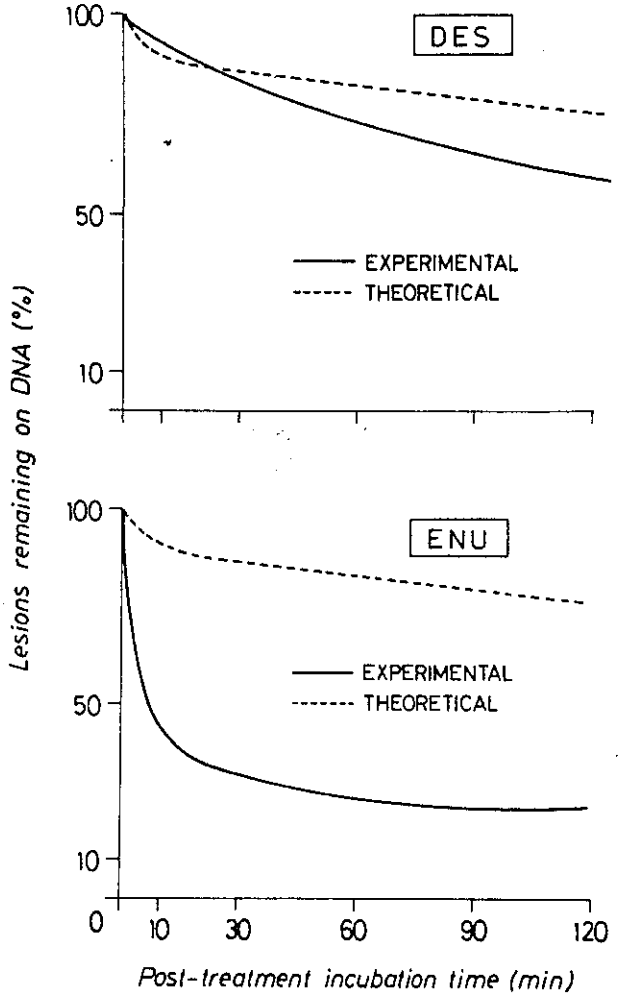

Fig. 5. - Kinetics of induction of ssb as a function of post-treatment incubation time. The data for the experimental curves (———) are shown in Fig. 3; the theoretical curves (------) were calculated according to the formula shown in "Results".

enough to detect DNA lesions at relatively low doses, and recent work has utilized this technique to investigate the factors that determine the sensitivities of different cell types to anti-cancer drugs [4].

It is well established that monofunctional alkylating agents produce DNA alterations that are converted to ssb during the exposure to the alkaline solutions used in filter elution assays. Assuming that alkali-labile sites represent AP sites arising mainly from alkylated purines, we constructed a theoretical function that describes the repair kinetics on the basis of HPLC data. In the case of DES, the overlap between the theoretical and the experimental fuction was in line with this assumption. However, in the case of ENU, DNA ssb detected by AE showed a very fast resealing that could not be explained on the basis of DNA adducts removal alone.

It is important to consider that ENU has a relatively high reactivity towards DNA as compared with DES. ENU reacts through the positively charged putative ethyldiazonium ion, whereas DES reacts as neutral molecule mainly by an SN_2 mechanism. The ethyldiazonium ions in the vicinity of DNA will attack sites according to their relative nucleophilicity and according to their steric accessibilities [12]. As a result, ENU gives rise to abundant alkylation of phosphodiester groups on the exterior of DNA double helix and ethylates O^2-pyrimidines at detectable levels. It is conceivable that repair processes commence as soon as the active ENU intermediate binds to DNA. The detection of ethylated bases by HPLC was performed after 30 min treatment time. We cannot exclude that, as a result of rapid removal of some alkylated products during the treatment time, we underestimated the actual number of lesions formed after exposure to ENU. However, because of the relatively low levels observed in *in vitro* experiments [7] of ethylated products that showed an initial rapid rate of removal (see O^2-ethylpyrimidines and 3-EtAde), these lesions alone cannot account for the removal of 50% of the total damage observed by AE analysis after 10 min post-treatment incubation time.

Another possibility is that ENU could induce DNA lesions that are not revealed by HPLC under our experimental conditions and that are repaired in a short time *via* AP site formation. Interestingly, cells treated with another SN_1-type alkylating agent, N-ethyl-N'-nitro-N-nitrosoguanidine (ENNG), reseal a fraction of DNA ssb very quickly by following the same kinetics observed after exposure to ENU [6]. Taken together, these data suggest that DNA damage induced by chemicals that interact preferentially with oxygen atoms in DNA is processed very rapidly by the cellular repair machinery.

Finally, an aspect that should be mentioned when considering mutagens which alkylate DNA at oxygen atoms is that this type of damage is not randomly distributed [13-16]. Persistence of O^6-methylguanine in the matrix DNA and rapid repair in active chromatin has been reported in different regions of rat liver chromatin DNA, while removal of N-alkylpurines has been shown to occur at a relatively uniform rate in different chromatin fractions [13]. A preferential repair of damage targeted at oxigen atoms is suggestive of disturbance of essential cellular functions because of its localization in specific regions of genome. Therefore a rapid removal of lesions induced by SN_1-type agents, such as ENU and ENNG, is not surprising.

Further studies on the nature of the lesions induced by alkylating agents are needed for a better understanding of mutagen and drug action mechanisms.

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REFERENCES

1. PEGG, A.E., WIEST, L., FOOTE, R.S., MITRA, S. & PERRY, W. 1983. Purification and properties of O^6-methylguanine-DNA-transmethylase from rat liver. *J. Biol. Chem.* **258**: 2327-2333.

2. SINGER, B. & BRENT, T. 1981. Human lymphoblasts contain DNA glycosylase activity excising N-3 and N-7 methyl and ethylpurines but not O^6-alkylguanines or 1-alkyladenines. *Proc. Natl. Acad. Sci. USA* **78**: 856-860.

3. LINDHAL, T. & ANDERSSON, A. 1972. Rate of chain breakage at apurinic sites in double stranded deoxyribonucleic acid. *Biochemistry* **11**: 3618-3623.

4. KOHN, K.W. 1979. DNA as a target in cancer chemotherapy: measurement of macromolecular DNA damage produced in mammalian cells by anticancer agents and carcinogens. *Methods Cancer Res.* **16**: 291-345.

5. SHOOTER, K.V. 1976. The kinetics of alkaline hydrolysis of phosphotriesters in DNA. *Chem. Biol. Interact.* **13**: 151-163.

6. DOGLIOTTI, E., LAKHANISKY, T., VAN DER SCHANS, G.P. & LOHMAN, P.H.M. 1984. Molecular dosimetry of DNA damage caused by alkylation. II. The induction and repair of different classes of single-strand breaks in cultured mammalian cells treated with ethylating agents. *Mutat. Res.* **132**: 41-49.

7. DOGLIOTTI, E., VITELLI, A., TERLIZZESE, M., DI MUCCIO, A., CALCAGNILE, A., SAFFIOTTI, U. & BIGNAMI, M. 1987. Induction kinetics of mutations at two genetic loci, DNA damage and repair in CHO cells after different exposure times to N-ethyl-N-nitrosourea. *Carcinogenesis* **8**: 25-31.

8. SUN, L. & SINGER, B. 1975. The specificity of different classes of ethylating agents towards various sites of HeLa cell DNA *in vitro* and *in vivo*. *Biochemistry* **14**: 1795-1802.

9. DEN ENGELSE, L., MENKVELD, G.J., DE BRIJ, R.J. & TATES, A.D. 1986. Formation and stability of alkylated pyrimidines and purines (including imidazole ring-opened 7-alkylguanine) and alkylphosphotriesters in liver DNA of adult rats treated with ethylnitrosourea or dimethylnitrosoamine. *Carcinogenesis* **7**: 393-403.

10. BIGNAMI, M., VITELLI, A., DI MUCCIO, A., TERLIZZESE, M., CALCAGNILE, A., ZAPPONI, G.A., LOHMAN, P.H.M., DEN ENGELSE, L. & DOGLIOTTI, E. 1988. Relationship between specific alkylated bases and mutations at two gene loci induced by ethylnitrosourea and diethylsulfate in CHO cells. *Mutat. Res.* **193**: 43-51.

11. MC CARTHY, T.V., KARRAN, P. & LINDAHL, T. 1984. Inducible repair of O-alkylated pyrimidines in *Escherichia coli*. *EMBO J.* **3**: 545-550.

12. SWENSON, D.H. & LAWLEY, P.D. 1978. Alkylation of deoxyribonucleic acid by carcinogens dimethyl sulphate, ethyl methanesulphonate, N-ethyl-N-nitrosourea and N-methyl-N-nitrosourea. *Biochem. J.* **171**: 575-587.

13. COX, R. 1979. Differences in the removal of N-methyl-N-nitrosourea-methylated products in DNase I-sensitive and -resistant regions of rat brain DNA. *Cancer Res.* **39**: 2675-2678.

14. BERKOWITZ, E.M.L. & SILK, H. 1981. Methylation of chromosomal DNA by two alkylating agents differing in carcinogenic potential. *Cancer Lett.* **12**: 311-321.

15. SUDHAKAR, E., TEW, K.D., SCHEIN, P.S., WOOLEY, P.V. & SMULSON, M. 1979. Nitrosourea interaction with chromatin and effect on poly ADP-ribose polymerase activity. *Cancer Res.* **39**: 1411-1413.

16. NEHLS, P. & RAJEWSKY, M.F. 1985. Differential formation of O^6-ethylguanine in the DNA of rat brain chromatin fibers of different folding levels exposed to N-ethyl-N-nitrosourea *in vitro*. *Cancer Res.* **45**: 1378-1383.

17. RYAN, A.J., BILLETT, M.A. & O'CONNOR, P.J. 1986. Selective repair of methylated purines in regions of chromatin DNA. *Carcinogenesis* **7**: 1497-1503.