Vol. 120, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 16, 1984 Pages 1-8

ENZYMATIC REPAIR OF O ALKYLATED THYMIDINE RESIDUES IN DNA : INVOLVEMENT OF A O^4-METHYLTHYMINE-DNA METHYLTRANSFERASE AND A O^2-METHYLTHYMINE DNA GLYCOSYLASE

Zahoor AHMMED and Jacques LAVAL

Unité de Biochimie et Enzymologie
Institut Gustave-Roussy, 94805 Villejuif Cedex, France

Received February 9, 1984

Alkylation of poly(dT) by N-[methyl-^{3}H](N-nitrosomethylurea) and subsequent annealing with poly(dA) yield a substrate containing O^2 and O^4-methylthymidine, 3-methylthymidine and phosphotriesters. In an *in vitro* assay using this substrate, cell extracts from *Escherichia coli* catalyse i) the transfer of the O^4-methyl present in O^4 methylthymidine to a protein which becomes alkylated; ii) the release of O^2-methylthymine by a glycosylase activity.
The two DNA repair activities described above appear to be involved in the adaptive response.

There is now considerable evidence showing that cellular lethality, mutagenicity and/or carcinogenicity result from a balance between the number and the structure of the induced DNA lesions and the efficiency of the cellular DNA repair mechanisms.
Loveless suggested (1) that O^6-methylguanine (O^6-MeG) could play an important role in mutagenesis. Physiological implications of the presence of O^6-MeG was shown in a variety of experiments reviewed in (2) and especially during investigations of adaptive response in *E.coli* (3) and in mammalian cells (2,4). Studies of the adaptive response at the molecular level have shown that O^6-MeG is repaired by an alkyltransferase which binds the alkyl group to a cysteine residue. This protein is present in bacteria and in mammalian cells (5-8).

In vitro replication experiments,using alkylation of poly(dAT) suggested that O^4MeT could miscode. Other experiments, using alkylated polynucleotides as template, indicate that besides alkylation at the O^6 position of guanine, substitution at the O^4 of the thymine miscodes during DNA (9-10) and RNA synthesis (11).Thymine methylated at the O^2 position, which is in the minor grove of DNA, miscodes to a lesser extent during DNA synthesis (12,10). Therefore,the O alkylated thymines may be potential harmfull lesions and should be

The abbreviations used are : MNU : N-[methyl-^{3}H] (N-nitrosomethylurea) ; O^2MedT : O^2-methylthymidine ; O^2MeT : O^2-methylthymine ; O^4MedT : O^4-methylthymidine ; 3-MedT : 3-methylthymidine ; O^6MeG : O^6-methylguanine. MNU (polydT.dA) : polydT alkyated with [^{3}H] MNU and subsequently annealed with polydA.

0006-291X/84 $1.50

eliminated by the cell. Investigations on the persistance of O^2-ethylthymine and O^4-ethylthymine in rat liver (13-15) suggest that they are enzymatically removed, although at a low rate.Other experiments suggest that O^4-ethylthymidine accumulates in DNA of hepatocytes from rats exposed continuously to diethylnitrosamine (16). Such experiments using methylated DNA are rendered more difficult due to the low level of alkylation of thymine (15-19). To overcome this drawback, we studied the repair of alkylated thymine by E.coli extracts using a specific substrate : poly(dT) alkylated with N-[methyl-$[^3H]$ (N-nitrosomethylurea) (MNU), purified, then annealed with poly (dA) to yield a double stranded deoxyribopolymer. We report that O^2-methylthymidine (O^2MedT) is repaired by a DNA glycosylase whereas O^4-methylthymidine (O^4MedT) is repaired by a transalkylase.

MATERIALS AND METHODS

Preparation of methylated Poly(dT).Poly(dA).

5 units of poly(dT) (P.L. Biochemicals Inc.) were dissolved in 0.5 ml of 0.1 M sodium perchlorate, 1 mM EDTA, (pH 8.0) ; 1 mCi of [methyl -^{3}H]MNU, (New England Nuclear, 1.5 Ci/mmole) was evaporated at 0°C to about 200 µl by blowing nitrogen, and mixed with 100 µl of 0.2 M Ammediol (Sigma) (pH 9.2). The poly(dT) solution was added immediately, and incubated at 20°C for 1 hour. The alkylated poly (dT) was dialyzed against 0.15 M NaCl 0.015 M Na citrate (pH 7.2) to eliminate the remaining unbound radioactivity and finally dialyzed against 10 mM Hepes-KOH (pH 7.5), 1 mM EDTA. In order to obtain double stranded substrate, the alkylated poly(dT) was annealed with 5 units of poly(dA) in 10 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA (pH 8.0), at 55°C for 10 min. then cooled to room temperature over a period of two hours. This substrate has a specific activity of 540 cpm per µg. It is abbreviated as MNU-poly(dT.dA).

Cell Extract.

Escherichia coli strain BS21 (20), a B/r mutant constitutive for adaptative response (obtained from B. Sedgwick) was grown at 37°C in L broth to late exponential phase. Bacteria were centrifuged, washed, resuspended in lysis buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 % glycerol) and stored at -70°C.
Bacteria were lysed as already described (21) by adding lysozyme (0.05 ml of a 4 mg/ml solution in 0.25 M Tris-HCl (pH 7.0) per ml of bacteria suspension), phenylmethane sulfonylfluoride and p-toluenesulfonylfluoride (Sigma) (1 mM each final concentration) and protease inhibitors : Aprotinin (Choay, Paris), Leupeptin and Antipain (Sigma) (2 µg/ml final concentration each). The cells were kept at 0°C for 30 min. then incubated at 37°C until they began to lyse, NaCl was added (400 mM final concentration) and the lysis was completed at 0°C. The lysate was sonicated at 0°C with a Branson Sonifier B-12 to reduce viscosity. It was clarified by centrifugation. All centrifugations described below are at 25,000 g for 15 min at 0°C. The supernatant (Fraction I) was collected.

Enzyme purification.

The cell extract (Fraction I) was precipitated by an equal volume of 1.6 % streptomycin sulfate (Rhone Poulenc, Paris) freshly dissolved in water, added dropwise under continuous stirring over a period of 30 min. The suspension was stirred for a further 45 min period and centrifuged. The supernatant (Fraction II) was precipitated by adding ammonium sulfate (Schwarz/Mann, Special Enzyme grade) to 75 % saturation under continuous stirring over a 45 min period. The precipitate was allowed to form for a further 20 min and collected by centrifu-

gation. The pellet was suspended in buffer A (50 mM Hepes-KOH, 1 mM EDTA, 1 mM dithiothreitol, 5 % glycerol, final pH 7.5). It was dialyzed for 2 hours against the same buffer (two changes of 250 ml) (Fraction III, contains 20 mg/ml of proteins). It was chromatographied on a column of DEAE cellulose (DE 52, Whatman) (1.5 cm X 8 cm) packed and equilibrated with buffer B(buffer A containing 0.25 M NaCl). Under these conditions,the active proteinswere eluted after the bulk of proteins. The most active fraction contained 1.2 mg per ml of proteins (Fraction IV) and was used unless otherwise stated.

Standard assay conditions.

MNU-poly(dT.dA) (2.19 µg) in 100 µl of assay buffer containing 50 mM Hepes-KOH, 1 mM EDTA, 1 mM DTT, 5 % glycerol, (pH 7.5) was incubated with E.coli purified proteins for 20 min. at 37°C. The reaction was terminated by chilling the mixture to 0°C. For analysis purpose at the nucleoside level, the incubation mixture was supplemented with deoxyribonuclease-1 (Sigma), snake venom phosphodiesterase (Boehringer, Mannheim, RFA), 4 µg each, bacterial alkaline phosphatase (Worthington) 10 µg, in the presence of 5 mM $MgCl_2$ and 1 mM $CaCl_2$, and incubated overnight at 37°C. The resulting nucleosides and phosphotriesters were supplemented with authentic O^4-MedT, O^2-MedT and 3-MedT cold markers (22-24) (obtained from Dr R. Saffhill and T. Lindahl) and analyzed by HPLC using either of the following systems.

1. "Resolve" 5µ Bondapak C18 Column (Waters Associates) was used as described by Saffhill and Fox (23) with a linear gradient of 30 min. between 10 %-40 % methanol in water (v/v) at pH 5.0 at a flow rate of 1 ml min^{-1}. The retention periods of the various markers were 5 min., 9.5 min., 12.5 min., 14.5 min. and 17.5 min. for T, O^2-MedT, O^2MeT, 3-MedT and O^4-MedT respectively. Fractions of 0.5 ml were collected and radioactivity measured.

2. Partisil M9 10/25 SCX (Whatman) column was eluted with a linear gradient of 20 min. between 20 mM ammonium formate (pH 4.0), 3 % methanol (v/v) and 40 mM ammonium formate (pH 4.0), 8 % methanol(v/v) at a flow rate of 4 ml min^{-1} with retention periods of 4 min., 6 min. and 9 min. for dT, 3-MedT and O^4-MedT respectively. This column does not allow the separation of O^2-MedT from 3-MedT. The disappearance of the radioactivity eluting at the position of O^4-MedT was taken as the measure of the O^4-methylthymine transferase activity. The appearance of the radioactivity eluting at the position of O^2-MeT was taken as the measure of the O^2-methylthymine glycosylase.

Gel filtration of the methylated protein in the denatured form

The methylated protein was characterized by separation from the substrate by gel filtration under denaturing conditions as described for the O^6 methylguanine-DNA methyltransferase (8), using a Sepharose 6B-CL column (0.9 X 78 cm) equilibrated with 6 M guanidine hydrochloride-1 mM EDTA (pH 5.0). Elution was performed at room temperature (6 ml per hour) and fractions of 1 ml were collected.

RESULTS

Figure 1 shows the HPLC analysis of MNU poly(dT.dA) hydrolysed to nucleosides by pancreatic DNase, phophodiesterase and phosphatase. Coelution with authentic markers allows the identification of three well resolved peaks as phosphotriesters, O^2MedT, and O^4MedT which elute with increasing times. The amount of N^3MedT wich appears in the third peak is higher than expected from previous experiments in wich poly(dAT) was used (15,20).The identity of the component(s) of this peak has to be resolved although it chromatographed with 3MedT in two different HPLC systems,thus nothing can be concluded regarding the

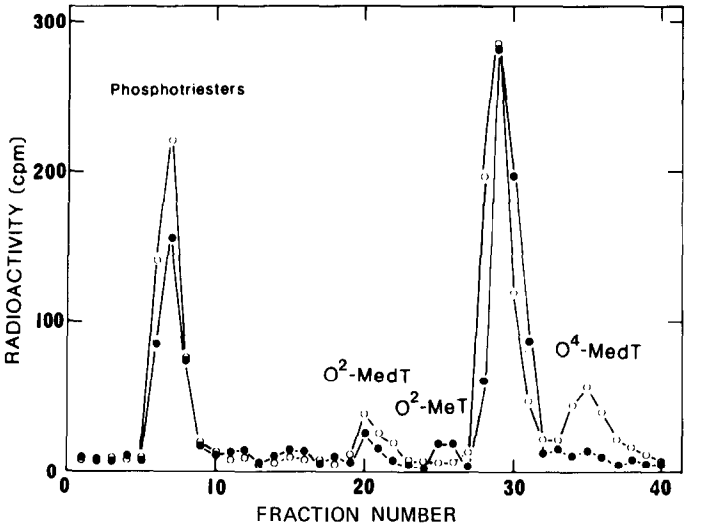

Figure 1 : HPLC analysis using a reverse phase column of the radioactive products released from MNU poly(dT.dA) incubated with (● - ●) or without (○ - ○) purified E.coli extracts then digested to nucleosides with DNase, phosphodiesterase and phosphatase. The substrate was incubated with 40 µg of E.coli proteins (Fraction III) for 20 min. The authentic markers were localized as ultraviolet-absorbing material. For details see Materials and Methods.

exact amount of the N^3MedT. The yield of the different alkylated products, excluding the peak wich coelutes with 3MedT relative to the total alkylation is 33 %, 5 % and 12 % for phosphotriesters, O^2MedT and O^4MedT respectively.

MNU poly (dT.dA) was incubated with extracts of E.coli (Fraction III), hydrolysed to nucleosides and analysed by HPLC. Figure 1 shows that compared to the untreated substrate, the peaks of phosphotriesters and O^2MedT are reduced, the peak of O^4MedT does not exist anymore, whereas the peak wich coelute with 3-MedT is unchanged. A new peak appears at the position of O^2-methylthymine. Under the chromatographic conditions used, dT, 3-methylthymine, O^2-methylthymine, and O^4-methylthymine elute in fraction 14, 18, 25, 28 respectively (data not shown). When the substrateis incubated with previously boiled enzyme, analysis at the nucleosides level shows a chromatogram similar to that obtained with the native substrate. The chromatographic characterization of the nucleosides and bases was confirmed using a cation exchange column as described in Material and Methods (data not shown).

The concomitant lowering of the O^2MedT peak with the appearance of a new peak at the position of O^2-methylthymine strongly suggests that this lesion is excised by a DNA glycosylase yielding a free base : O^2-methylthymine.

As shown in Figure 2A, the removal of O^4MedT is proportional to the amount of protein added. This protein is particularly heat resistant as 80 % of the activity remains after heating Fraction III for 2 min at 80°C. However the activity is lost by heating at 100°C for 5 min. The disappearance of O^4MedT

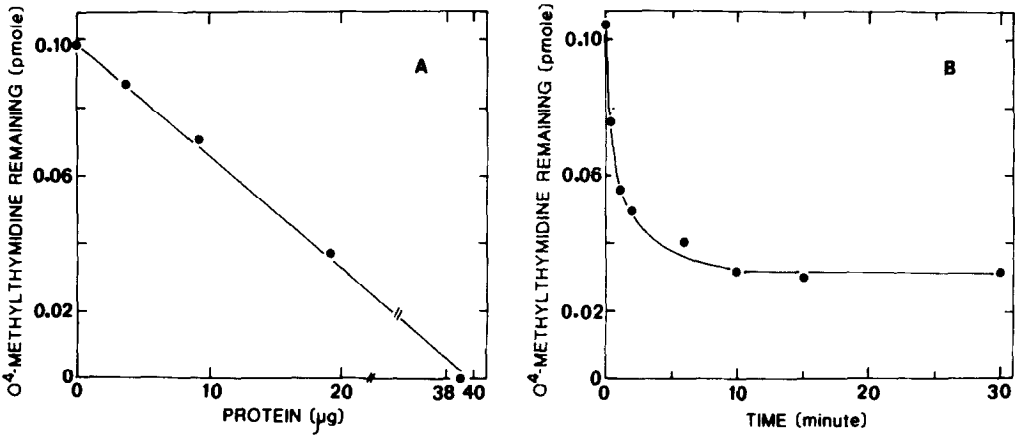

Figure 2A : Removal of O⁴MedT from MNU poly(dA.dT) by a partially purified cell extracts from E.coli. MNU poly(dT.dA) was incubated for 20 min. with increasing amounts of proteins (Fraction IV) then hydrolysed to nucleosides and analysed for remaining O⁴MedT by HPLC as described in Figure 1.

Figure 2B : Kinetics of removal of O⁴MedT from MNU poly(dT.dA) by purified E.coli BS21 extracts. Each assay contained 19 µg of proteins (Fraction IV). Analysis was performed as described in Figure 1

is not affected (less than 20 %) between pH 6.8 and 8.2 either in phosphate or in Hepes buffer. It is not affected neither by low concentration (1 to 5 mM) of $MgCl_2$ nor by the presence of EDTA 1 mM which was routinely used to inhibit contaminating nucleases. Kinetics of O⁴MedT removal as a function of time was measured and shows that the reaction is rapid and then levels off (Fig. 2B). It should be noted that the transferase activity can be separated from that of DNA glycosylase by the DEAE chromatography used to obtain fraction IV (data not shown).

The O⁴MedT appears to be repaired by a DNA-methyltransferase as no free base is detectable (O⁴MeT elutes in fraction 28,vide supra). If the methyl is removed by a DNA methyltransferase, a methylated protein should appear. In order to investigate the repair of O⁴MedT, MNU poly(dT.dA) was analysed by exclusion chromatography : 95 % of the material behave as high molecular weight material and 5 % is eluted at the position of bases or very small oligonucleotides (Fig.3). When MNU poly(dT.dA) is incubated with E.coli extracts (Fraction IV), then analyzed under the same conditions, (guanidine denaturation yielding unfolded proteins), the results (Fig.3) show that compared to the untreated substrate,the excluded peak of substrate is reduced whereas the peak of low molecular weight is unchanged. A new peak appears between the two already described,its molecular weight is in the range 20-25,000 daltons.

The specific activity of O⁴-methylthymidine-DNA-transmethylase from E.coli extracts was 20 times higher than that measured in the parental strain F26 (7.7 µg of proteins from the BS21 extract removed 140 fmoles of O⁴MedT whereas 160 µg of proteins from the F26 extracts or 180 µg of proteins from the AB 1157

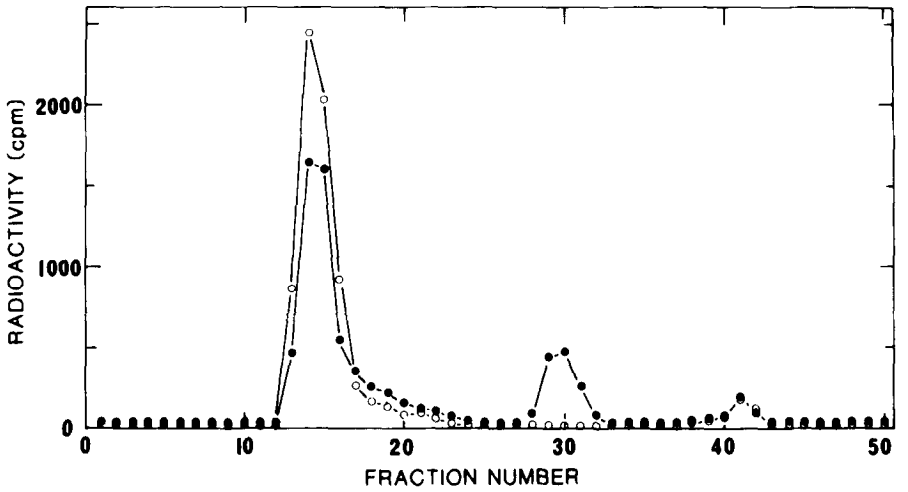

Figure 3 : Separation by gel filtration of MNU poly(dT.dA) from the E.coli [^{3}H] methylated protein in its unfolded form. Reaction mixture containing MNU poly-(dT.dA) was scaled up 7 times and incubated with 280 µg (●) or without (○) partly purified E.coli extracts (fraction IV), supplemented with guanidine hydrochloride and then chromatographied on a Sepharose 6B CL column. For details see Materials and Methods.

(25)extracts were needed to remove the same amount of O^{4}MedT). This property holds also true for O^{2}MedT-glycosylase. Since the BS21 mutant is constitutive for adaptive response this result strongly suggests that the two DNA repair activities described here are part of the adaptative response.

DISCUSSION

The repair of alkylated purines in DNA is now quite well documented. It depends so far upon DNA glycosylases (26-28) and a O^6-alkylguanine-DNA methyltransferase (5,6,7,8,29). Studies of the repair of methylated pyrimidines have been hampered by the scarcity of these lesions among the alkylated purines and phosphotriesters (17,20). The MNU poly(dT.dA) substrate used in our experiments obviates these drawbacks as it yields a higher amount of alkylated thymine than MNU alkylated poly (dAT.dAT) (15,20).

The O^4-methylthymine-DNA-methyltransferase described in this paper eliminates a clearly miscoding lesion (10) and seems to act in a same way as the transferase which repairs O^{6}MeG .It transfers the methyl from the O^4-methylated thymine to an acceptor protein. The unusual thermal resistance of this protein suggests that the transferase and methyl acceptor functions reside in the same heat stable protein as it has also been observed for O^6-methylguanine transferase from E.coli (5) and Raji cells (8). The biological properties exhibited by the O^{4}MedT and O^{6}MeG transferases are similar : the elimination of miscoding lesions. They are clearly part of the adaptive response as the constitutive

mutant has a much higher quantity of these activities than the parental strain.

The question of the specificity of these molecules arises. McCarthy et al. (30) have independently observed that O^4-MedT residues in MNU-treated E.coli are repaired by pure O^6-methylguanine-DNA-transalkylase. As their experiment was performed with the pure protein, it does not answer the question as to whether removal of the methyl from O^4MedT could be a side effect of the O^6MeG transferase: the real O^4-methyltransferase being an other protein P. Schendel (paper in press) has observed that E.coli extracts repair O^4MedT by trans-alkylase activities yielding alkylated proteins of 15, 18 and 20,000 molecular weight and that two of them are different from the O^6-methylguanine transferase (18,000 ± 1,000)(5).He also observed that phosphotriesters are repaired by a methyltransferase activity (MW 39,000). This observation fits with our finding that in HPLC analysis of MNU poly(dTdA) products, the peak of radioactivity eluting at the position of phosphotriesters is lower after incubation with E.coli extracts and that, therefore, it could also be part of the adaptive response. Repair of methylphosphotriesters would appear to be advantageous for the cell as they slow down DNA replication (31) and probably DNA methylation (32).

ACKNOWLEDGEMENTS

We thank Drs T. Lindahl for the gift of the methylated thymine and communicating manuscripts prior to publication, R. Saffhill for gift of methylated nucleosides and helpfull discussions for their separation,B.Sedgwick for bacterial strains and B. Singer for helpfull discussions.

This work was supported by the Centre National de la Recherche Scientifique (LA 147) and Action de Soutien à la Toxicologie Génétique, the Institut National de la Santé et de la Recherche Médicale (U140) and grant from the Association pour le Développement de la Recherche sur le Cancer.Zahoor Ahmmed was a recipient of a fellowship from the Association pour le Développement de la Recherche sur le Cancer, while on leave from the Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan.

REFERENCES

1. LOVELESS, A. (1969), Nature 233, 206-207.
2. MONTESANO, R. (1981) J. Supramol. Struct. Cell Biochem 17, 259-273
3. CAIRNS, J., ROBINS, P., SEDGWICK, B. and TALMUD, P. (1981) Prog. Nucleic Acid Res. Mol. Biol. 26, 237-243.
4. LAVAL, F. and LAVAL, J. (1984) Proc. Natl. Acad. Sci., USA. (1984), 81, 1062-1066
5. DEMPLE, B., JACOBSON, A., OLSSON, M., ROBINS, P. and LINDAHL, T. (1982) J. Biol. Chem. 257, 13776-13780.
6. RENARD, A. and VERLY, W.G. (1980) FEBS Lett., 122, 271-274.
7. PEGG, A.E., WIEST, L., FOOTE, R.S., MITRA, S. and PERRY, W. (1983) J. Biol. Chem. 258, 2327-2333.
8. HARRIS, A.L., KARRAN, P. and LINDAHL, T. (1983) Cancer Res. 43, 3247-3252.
9. ABBOTT, P.J. and SAFFHILL, R. (1979) Biochim. Biophys. Acta 252, 51-61.
10. SINGER,B.,SAGI,J.,and KUSMIEREK,J.T. (1983)Proc.Natl.Acad.Sci.USA,80,4884-
11. SINGER,B.,FRAENKEL-CONRAT,H. and KUSMIEREK,J.T. (1978) Proc.Natl. Acad. Sci., USA, 75, 1722-1726.

12. SAFFHILL, R. and ABBOTT, P.J. (1978) Nucleic Acids Res. 5, 1971-1978.
13. STEWARD, A.P., SCHERER, E. and EMMELOT, P. (1979) FEBS Lett. 100, 191-194.
14. SINGER,B., SPENGLER,S. and BODELL,W.J. (1981) Carcinogenesis 2, 1069-1073.
15. SINGER,B. and GRUNBERGER,D.,(1983)Molecular Biology of Mutagens and Carcinogens.Plenum Press.New York and London.pp 55-92,221-244.
16. SWENBERG,J.A., BEDELL,M.A., BILLINGS,K.C., HUH,N., KIRSTEIN,U. and RAJEWSKY,M.F.(1983)Proc.Am.Assooc.Cancer Res.24,68.
17. LAWLEY, P.D., ORR, D.J., SHAH, S.A., FARMER, P.B. and JARMAN, M. (1973) Biochem. J. 135, 193-201.
18. SINGER, B., KRUGER, M. and CARRANO, M. (1978) Biochemistry 17, 1246-1250.
19. JENSEN, D.E. (1978) Biochemistry 17, 5108-5113.
20. JENSEN, D.E. and REED, D.J. (1978) Biochemistry 17, 5098-5107.
21. SEDGWICK, B. and ROBINS, P. (1980) Molec. gen. Genet. 180, 85-90.
22. LAVAL, J., PIERRE, J. and LAVAL, F. (1981) Proc. Natl. Acad. Sci., USA, 78, 852-855.
23. SAFFHILL, R., and HALL, J. (1981) Carbohydrates, Nucleosides and Nucleotides 8, 573-583.
24. KUSMIEREK, J.T. and SINGER, B. (1976) Nucleic Acids Res., 3, 989-1000.
25. SAFFHILL, R. and FOX, M. (1980) Carcinogenesis 1, 487-493.
26. LAVAL, J. (1977) Nature, 269, 829-832.
27. LAVAL, J. and LAVAL, F. (1980) IARC Scientific Publication, eds Montesano, R., Bartsch, H., Tomatis, L. 27, 55-72.
28. LINDAHL, T., (1982) Ann. Rev. Biochem., 51, 61-87.
29. MEHTA, J.R., LUDLUM,D.B., RENARD, A. and VERLY, W.G. (1981) Proc. Natl. Acad. Sci., USA, 78, 6766-6770.
30. MCCARTHY,T.,KARRAN,T.,and LINDAHL,T.,(1984) EMBO J. in press.
31. MILLER, P.S., CHANDRASEGARAN, S., DOW, D.L., PULFORD, S.M. and KAN, L.S. (1982) Biochemistry 21, 5468-5474.
32. PFOHL-LESZKOWICZ, A., BOITEUX, S., LAVAL, J., KEITH, G. and DIRHEIMER, G. (1983) Biochem. Biophys. Res. Commun., 116, 682-688.