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Biochemistry

Human lymphoblasts contain DNA glycosylase activity excising N-3 and N-7 methyl and ethyl purines but not O^6-alkylguanines or 1-alkyladenines

(7-methylguanine DNA glycosylase/ethyl purine repair/analysis for alkyl bases by high-performance liquid chromatography)

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ABSTRACT **Cultured human lymphoblasts (CCRF-CEM line) have DNA glycosylase activities, the specificities of which were investigated by using high-performance liquid chromatography. In addition to 3-methyladenine, 3-ethyladenine, 7-methylguanine, 7-ethylguanine, 3-methylguanine, and 3-ethylguanine also were excised from alkylated double-stranded DNA and deoxypolynucleotides, but 1-methyladenine, 1-ethyladenine, O^6-methylguanine, and O^6-ethylguanine were not. The glycosylase activity was generally greater for the methylated than for the corresponding ethylated purines and was also greater toward 3-alkyladenine than toward 3-alkylguanine. 7-Methylguanine and 7-ethylguanine were excised to similar but low extents. However, in molar terms, the release of 7-methylguanine was similar to that of 3-methyladenine.**

Alkylating agents such as methyl methanesulfonate (MeMS), *N*-methyl-*N*-nitrosourea (MeNU), and *N*-ethyl-*N*-nitrosourea (EtNU) react with DNA and polynucleotides to form a variety of products. These include guanine alkylated at N-3, N-7, and O^6; adenine alkylated at N-1, N-3, N^6, and N-7; cytosine alkylated at N-3, N^4, and O^2; and thymine alkylated at N-3, O^2, and O^4, as well as alkyl phosphotriesters (1, 2). The *N*-nitroso alkylating agents react primarily with the oxygens, while the alkyl alkane sulfonates react primarily with nitrogens.

3-Methyladenine (m^3A), 3-ethyladenine (e^3A), O^6-methylguanine (m^6G), and O^6-ethylguanine (e^6G) have all been shown to be excised *in vivo* in both mammalian and bacterial DNA (3–8; for review, see ref. 9). 7-Methylguanine (m^7G) in *Escherichia coli* DNA appears to be lost *in vivo* by spontaneous chemical hydrolysis rather than by excision by an enzyme (4). Recent evidence suggests that the enzyme activities in mammalian cells differ from those in *E. coli* because, in mammalian cells, m^7G and e^7G are removed from alkylated DNA at a rate considerably greater than can be accounted for by chemical depurination (8, 10, 11). When *O*-ethyl pyrimidines are formed in mammalian cell DNA, these derivatives are also excised to a certain extent (8, 12).

Enzymes that specifically remove alkylated purines from DNA *in vitro* have been isolated from both bacterial and mammalian sources. The removal from DNA of free alkylated bases by a preparation of *E. coli* endonuclease II has been described by Kirtikar and Goldthwait (13), who found the predominant product to be m^3A. Since then, the active enzyme, 3-methyladenine-DNA glycosylase, has been extensively purified (14) and reported to be equally active toward e^3A, but inactive against m^7G, m^6G, and the analogous ethylguanines. A similar enzyme, identified and partially purified from *Micrococcus luteus* (15, 16), acted predominantly on m^3A but not at all on m^7G or m^6G. The crude *M. luteus* extract did, however, release m^3G (16).

We report a search for possible bases other than 3-methyladenine released by a DNA glycosylase activity present in an extract (fraction III) from human lymphoblasts (17). Methylated and ethylated DNA and double-standed deoxypolynucleotides were used as substrates, and the products of glycosylase action were identified by high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Preparation of Alkylated DNAs and Deoxypolynucleotides. Calf thymus DNA was treated with [^{14}C]MeMS (52–58 mCi/mmol; 1 Ci = 3.7×10^{10} becquerels) or with [^{3}H]MeNU (1 Ci/mmol) as described by Brent (17). [^{14}C]MeMS was also used to prepare methylated poly(dA·dT) and poly(dG·dC). Salmon sperm DNA and poly(dA)·poly(dT) were treated with [^{14}C]EtNU as described by Singer *et al.* (18). The maximum ethylation achieved (0.1%) was about 10% of the maximum methylation. The compositions of the purine fractions released by heating these alkylated polynucleotides in 0.1 M HCl at 37°C for 20 hr are given in Table 1.

DNA Glycosylase Assay. The enzyme preparation (fraction III) used was partially purified from CCRF-CEM cells by DEAE-cellulose chromatography and fractional precipitation with ammonium sulfate as described (17). This fraction was only 2- to 4-fold purified on the basis of specific activity; however, it was essentially free of nucleic acids and of 90% of the cells' apurinic endonuclease. Assays were performed in a buffer of 0.1 M NaCl/2 mM EDTA/0.05 M Tris/1 mM dithiothreitol (pH 7.5), and the enzyme preparation was stored in the same buffer.

A typical experiment used 40 μg of [^{3}H]MeNU DNA (which had about 5000 cpm of m^3A, 4000 cpm of m^6G, and 33,000 cpm of m^7G) in 0.2 ml of buffer and 0.2 ml of fraction III. After incubation at 37°C for 2 hr, the sample was cooled; denatured DNA was added as carrier (to a final concentration of 1 mg/ml), and the DNA was precipitated by using 2.5 vol 95% ethanol and 2 M sodium acetate to a final concentration of 0.1 M. After ≈1 hr at −20°C, the DNA was removed by centrifugation, and the supernatant was analyzed for free bases.

For the ethylated DNA and polymers, about 135 μg were used (about 1600 cpm of e^3A and 4800 cpm of e^7G). In these experiments, 0.2 ml of polynucleotide solution was mixed with 0.6 ml of buffer solution and 0.4 ml of enzyme. The incubation

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Abbreviations: MeMS, methyl methanesulfonate; MeNU, *N*-methyl-*N*-nitrosourea; EtNU, *N*-ethyl-*N*-nitrosourea; HPLC, high-performance liquid chromatography.

Table 1. Methyl and ethyl bases released from alkylated DNA and deoxypolynucleotides

		Purine, % of total					
		Adenine			Guanine		
Alkylating agent	Polynucleotide	N-3	N-7	N-1	N-7	N-3	O^6
MeMS	DNA	10			90		
MeNU	DNA	12			79	1	8
MeMS	Poly(dG·dC)				98	1.3	1
MeMS	Poly(dA·dT)	74	8	17			
EtNU	DNA	22		2	37	4	35
EtNU	Poly(dA)·poly(dT)	71	8	17			

Purines were determined by HPLC after acid treatment. Fig. 1 shows the elution time for numerous alkyl bases. The extent of alkylation was ≈0.1–1% of the total bases. For EtNU-treated DNA or polymers, about 50–60% of the total ethylation occurs on phosphates (18).

and ethanol precipitation were carried out as described above. Alkylated polymers were treated in the same manner as DNA.

The absolute amounts of the purines were determined by HPLC after depurination in 0.1 M HCl at 37°C for 20 hr. The solutions were then neutralized, and the depurinated polymers were separated from the free bases by ethanol precipitation.

Two types of control incubations were used. Alkylated DNA or polymers were incubated in buffer alone or in buffer containing heat-inactivated enzyme (100°C for 5 min).

HPLC of Depurinated Bases. Alcohol supernatants from glycosylase experiments were air-dried, redissolved in H_2O (maximum vol 0.1 ml), including added internal markers for bases. Samples were analyzed on a 250 × 4 mm Aminex HP-C cation exchange column (NH_4^+ form) (Bio-Rad) at 56°C using 0.4 M ammonium formate (pH 7) as solvent. The column was operated at a flow rate of 0.6 ml/min, and samples were collected directly into scintillation vials by using a Gilson fraction collector synchronized with the UV monitor and chart recorder (Bio-Rad). Depending on the separation desired, 0.6- to 3.6-min fractions were collected and assayed for radioactivity in Aquasol. When UV markers were added to experimental samples, the profiles of the markers were often displaced and, at times, split. This did not generally affect the separation of the alkyl bases, although the radioactivity profiles also reflected any anomaly of the marker. The results of the HPLC separations of a number of methyl and ethyl bases, including those pyrimidine nucleotides having a labilized glycosyl bond (19), are shown in Fig. 1.

RESULTS

Glycosylase Activity on Methylated DNA and Polymers. MeMS alkylated DNA primarily on the N-3 of adenine and N-7 of guanine, with virtually no reaction of the O^6 of guanine. When incubated with fraction III for 2 hr, most of the m^3A was removed (17). By using HPLC, which separates m^7G from m^3A clearly (see Fig. 1), we found that m^7G was also enzymatically cleaved, although the amount of m^7G released was low (Table 2). Neither buffer nor heat-inactivated enzyme treatment released substantial amounts of any of these derivatives. Because m^3A and m^7G are chemically depurinated at pH 7 [$t_{1/2} \approx 26$ and ≈155 hr (9), respectively], some depurination is expected on incubation alone.

MeNU methylated DNA to yield m^6G (in addition to m^7G and m^3A), which represented 8% of the total radioactivity in the purines (see Table 1). Enzyme treatment released m^3A and m^7G but not at all the m^6G (Table 2; Fig. 2A).

Model experiments using methylated double-stranded deoxypolynucleotides provided additional data, particularly with regard to glycosylase action on those derivatives that are present in only small amounts in methylated DNA. When [^{14}C]MeMS-

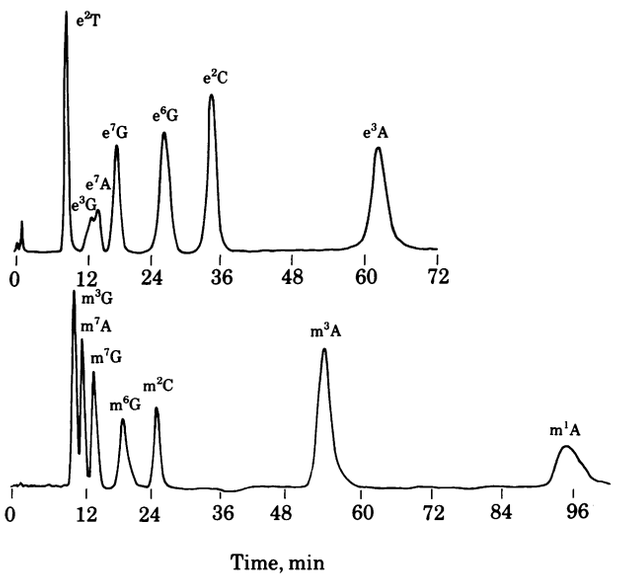

FIG. 1. HPLC separation of alkyl bases. (*Upper*) Ethyl bases. (*Lower*) Methyl bases. e^1A, which is not shown, is retained about 120 min.

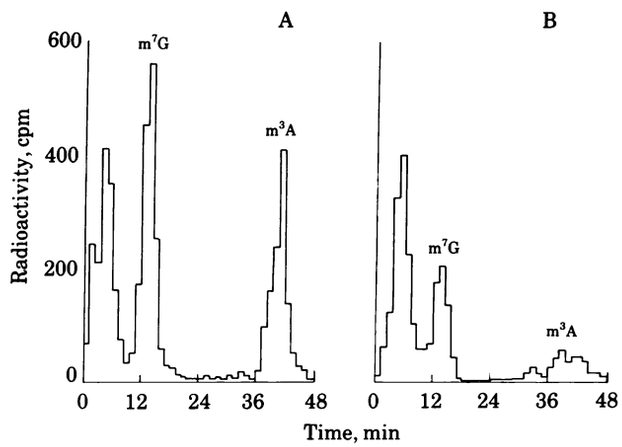

FIG. 2. Radioactivity profiles of HPLC separations of ethanol supernatants of [^{3}H]MeNU-treated DNA. (*A*) After enzyme incubation. (*B*) After buffer incubation. The net increases in A for m^7G and m^3A are 910 and 774 cpm, respectively. Excision of m^3A in this experiment is 35%. In similar experiments, the excision of m^3A is almost complete (see Table 2).

Table 2. Glycosylase activity in a partially purified extract from human lymphoblasts toward alkylated double-stranded deoxypolynucleotides

Alkylating agent	Polynucleotide	Alkylated purine, % of total*									
		m^3A	m^1A	m^7G	m^3G	m^6G	e^3A	e'A	e^7G	e^3G	e^6G
MeMS	Poly(dA·dT)	100 (9)	ND								
MeMS	Poly(dG·dC)			7 (0.7)	40 (4)	ND					
MeMS	DNA	100 (8)		6 (1)							
MeNU	DNA	82 (6)	ND	5 (2)		ND					
EtNU	Poly(dA)·poly(dT)						26 (3)				
EtNU	DNA						39 (11)	ND	5 (2)	26 (13)	ND

All analyses [except ethylated poly(dA·poly(dT)] were performed two or three times, generally on two separate alkylated preparations. Data shown are representative and not averages. ND, not detected, although present in an acid-depurinated sample.

* Values are expressed as percentages of the radioactivity in the equivalent HPLC peak after acid depurination. Values in parentheses represent the percentage of each derivative found with buffer or inactivated enzyme incubation alone. Published half-lives (9) of methyl purines in DNA incubated at pH 7 (m^3A, 18–40 hr and m^7G, 96–192 hr) are in reasonable agreement with controls for this table. The comparable half-life for m^3G has been determined in a single experiment to be >105 hr (4). m^7A is not given here because it has not been clearly established that the radioactivity corresponding to m^7A or e^7A in these HPLC analyses is actually due to these derivatives.

treated poly(dA·dT) was acid depurinated, of the total radioactivity in the purines, 74% was in m^3A, 17% was in m^1A, and 8% was in derivative that eluted with m^7A but has not been unequivocably established as m^7A (see Table 1). A complete experiment is illustrated in Fig. 3. The lower half shows the bases present after acid depurination, and the upper half shows the effect of enzyme depurination. The two small insets show the same polymer incubated with buffer only and with buffer plus heat-inactivated enzyme. Although m^3A was completely released, there was no release of m^1A. Incubation with inactive enzyme or buffer alone did not cause release of m^3A in excess of that caused by chemical instability of the glycosyl bond. However, the presumed m^7A has the most labile glycosyl bond of all the purines ($t_{1/2} \approx 2.8$ hr) (5) and was released to a great extent on incubation alone.

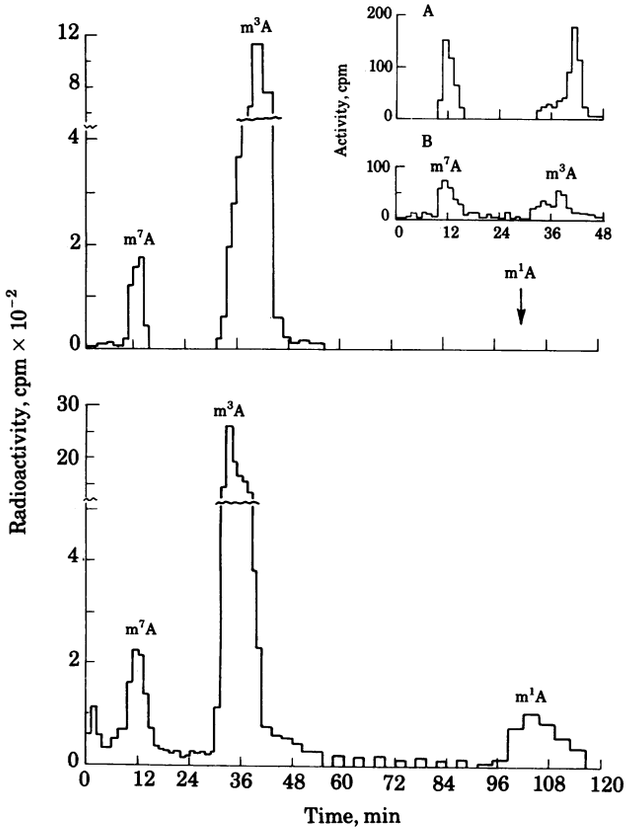

FIG. 3. Radioactivity profiles of HPLC separations of [^{14}C]MeMS-treated poly(dA·dT). (*Lower*) Ethanol supernatant after 0.1 M HCl depurination at 37°C for 20 hr. (*Upper*) Ethanol supernatant after enzyme incubation. (*Insets*) Ethanol supernatants after incubation with heat-inactivated enzyme (*A*) and buffer only (*B*). Positions shown are for authentic derivatives. Note that m^1A is not depurinated by the enzyme. All scales are the same up to the expanded segment.

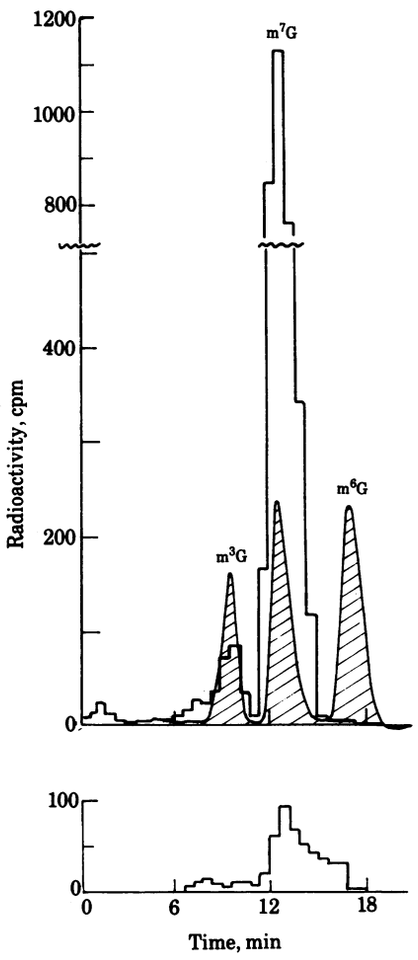

FIG. 4. Radioactivity and UV absorption profiles of HPLC separation of [^{14}C]MeMS-treated poly(dG·dC). (*Upper*) Ethanol supernatant after enzyme incubation. Open areas denote radioactivity added to the sample. (*Lower*) Ethanol supernatant of buffer-treated sample. Note that m^6G is not depurinated by the enzyme. Enzymatic release (enzyme depurination corrected for spontaneous depurination, compared with acid depurination) is 35% for m^3G and 6% for m^7G. Actual radioactivities are 224 and 3418 cpm, respectively.

When [^{14}C]MeMS-treated poly(dG·dC) was acid depurinated, of the total radioactivity in the purines, 1% was in m^6G and 1.3% was in m^3G, the remainder being m^7G (see Table 1). Although m^6G and m^3G were present in very low amounts, the HPLC separation shows that m^3G and m^7G are enzymatically released. In contrast, m^6G was not detected (Fig. 4).

Glycosylase Activity on Ethylated DNA and Polymers. EtNU ethylated DNA, yielding a large number of products. The distribution of purines after acid depurination of the sample used was 37% e^7G, 35% e^6G, 22% e^3A, 4% e^3G, and 2% e^1A (see Table 1; Fig. 5). Glycosylase activity on e^3A was clear, while activity on e^3G and e^7G was only slight and none was detectable on e^6G. Repeated experiments always showed that enzyme-mediated depurination of e^7G was at least 2-fold the spontaneous depurination on incubation alone.

Further information on the glycosylase activity toward e^3A was obtained by using [^{14}C]EtNU-treated poly(dA)·poly(dT).

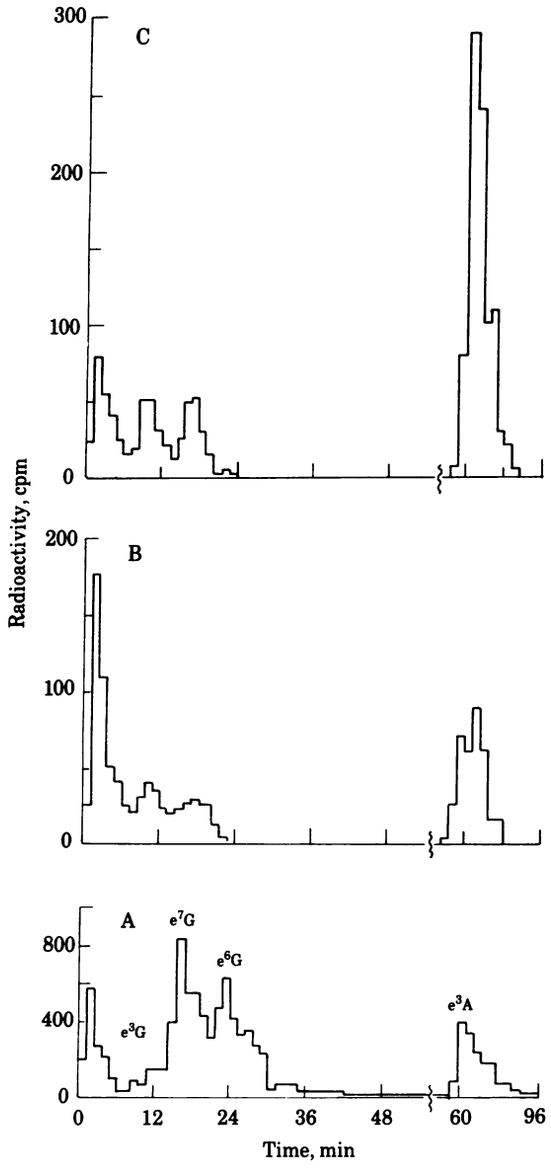

FIG. 5. Radioactivity profiles of HPLC separations of ethanol supernatants of [^{14}C]EtNU-treated DNA. (*A*) After depurination with 0.1 M HCl at 37°C for 20 hr. (*B*) After buffer incubation. (*C*) After enzyme incubation. The positions of authentic bases are given in *A*. (See also Fig. 1.) Release of derivatives corrected for control is 13% for e^3G, 3% for e^7G, and 28% for e^3A.

Although e^3A was depurinated, this occurred with a lower efficiency than found for m^3A (see Table 2).

Glycosylase Activity on Mixed Methylated and Ethylated DNAs. To assess whether the enzyme preparation acted preferentially on m^3A as a substrate rather than on e^3A, [^{14}C]MeMS-treated DNA and [^{14}C]EtNU-treated DNA were mixed in amounts such that m^3A would be approximately equivalent to e^3A (because ethylation is much less extensive than methylation, 27 times more ethylated than methylated DNA was used). The results of this experiment are shown in Fig. 6. Both m^3A

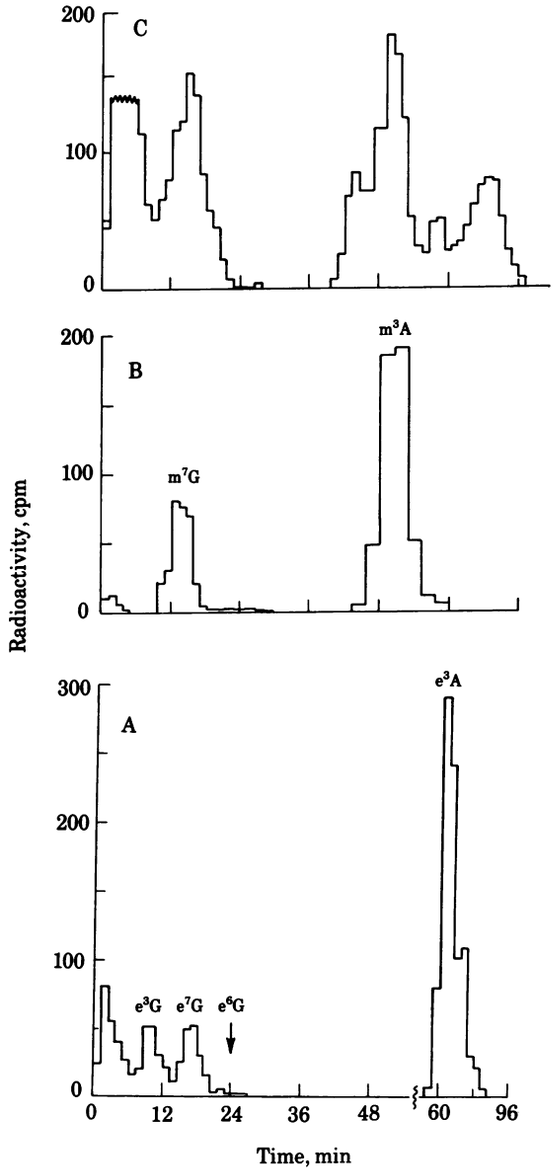

FIG. 6. Radioactivity profiles of HPLC separations. (*A*) Ethanol supernatant of [^{14}C]EtNU-treated DNA after enzyme incubation. (*B*) Ethanol supernatant of [^{14}C]MeMS-treated DNA after enzyme incubation. (*C*) Ethanol supernatant after enzyme incubation of mixed mixture of [^{14}C]EtNU-treated DNA and [^{14}C]MeMS-treated DNA. The amount of ethylated DNA was 27 times that of methylated DNA in order to have more e^3A than m^3A. Overloading of the HPLC column by the combined DNAs caused some peak splitting. Both m^3A and e^3A were released; after correction for the specific activity of the two DNAs, the molar amounts of m^3A and e^3A were similar, but less than found for each DNA incubated separately. Release of m^3A was essentially complete, but release of e^3A (corrected for spontaneous depurination) was only 12%.

and e^3A were recognized by the enzyme extract. The amounts of m^3A and e^3A excised were approximately equal in molar terms but somewhat less than released from the individual DNAs. Although the separation of m^7G and e^7G was not sufficient to allow an accurate determination of each, it was clear from the broad peak and position of radioactivity that both were present in amounts slightly less than found for the separate DNAs.

DISCUSSION

It has been assumed that bacteria cannot excise m^7G but are efficient in excision of m^3A (4, 20). On the other hand, there are considerable data showing that, in mammalian systems, m^7G is excised (8, 10, 11, 21), although this excision has not been emphasized because the rapid *in vivo* removal of m^3A predominates.

The enzyme(s) responsible for m^3A excision have been isolated from a number of sources. However, mammalian glycosylase activity has been shown only by Brent (17) and Ishiwata and Oikawa (22). All of these glycosylases have been termed 3-methyladenine-DNA glycosylase, and their activities have usually been studied only in terms of depurination of m^3A from methylated DNA. Riazuddin and Lindahl (14) indicated that e^3A is excised equally well by the purified *E. coli* enzyme, but no comparative data are shown. Shackleton *et al.* (16) concluded that a partially purified glycosylase from *M. luteus* excised not only m^3A but also m^3G, although this enzyme fraction did not remove m^7G from methylated DNA.

From what is known of the stability of alkylated purines and pyrimidines in mammalian cells, it is evident that the specificities of the various enzymes studied *in vitro* do not account for the observed loss of many derivatives *in vivo* (9). Moreover, none of the bacterial glycosylases have been active on m^6G or e^6G. The mechanism of removal of *O*-alkyl pyrimidines that occurs *in vivo* has never been investigated.

We attempted a systematic study of the range and limits of glycosylase activity in partially purified extract from human lymphoblasts [fraction III (17)]. In contrast to earlier work with a purer preparation [fraction IV (17)], fraction III showed glycosylase activity not only for m^3A but also for m^7G, m^3G, e^3A, e^7G, and e^3G, suggesting that the N-3 and N-7 substituents could be depurinated to varying extents. It is likely that m^7A and e^7A are also substrates, but this could not be demonstrated due to the chemical instability of their glycosyl bonds. This range of substrates is not necessarily recognized by a single enzyme, because the preparation used in this work was comparatively crude. It should be noted that glycosylase activity toward m^7G has also been found in bacteria and in rodent liver (23, 24).

Neither the *O*-alkyl pyrimidines nor O^6-alkylguanine nor 1-alkyladenine were detected as the free base after enzyme treatment of alkylated DNA or deoxypolynucleotides. The failure to find release of free O^6-alkylguanine is consistent with the absence of such glycosylase activity in bacterial enzyme preparations (14, 16) and with recent evidence that the repair of this derivative in bacteria (25) and rat cells (26) may be mediated by a methyltransferase reaction.

We conclude that, although glycosylase activities in human lymphoblasts are not restricted to excision of m^3A only, there are also definite limits. Ethyl purines are removed less efficiently than methyl purines, and such differences in extent or rate of repair may be a factor in the often observed greater carcinogenicity of ethylating agents as contrasted to analogous methylating agents.

There must also exist additional enzyme activities in mammalian cells specifically involved in repair of the *O*-alkyl pyrimidines and guanine. Current work (unpublished observations) suggests that these derivatives formed in the DNA from liver of EtNU-treated rats are rapidly removed ($t_{1/2} \approx 15$–50 hr).

It may be speculated that the glycosylase activities in mammalian cells are natural constituents originally evolved to repair certain improper biological methylations but also able to a lesser extent to recognize the larger ethyl substituent.

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1. Singer, B. (1975) *Prog. Nucleic Acid Res. Mol. Biol.* **15**, 219–284; 330–332.
2. Singer, B. (1976) *Nature (London)* **264**, 333–339.
3. Roberts, J. J. (1978) *Adv. Radiat. Biol.* **7**, 211–436.
4. Lawley, P. D. & Warren, W. (1976) *Chem. Biol. Interact.* **12**, 211–220.
5. Lawley, P. D. & Orr, D. J. (1970) *Chem. Biol. Interact.* **2**, 154–157.
6. Goth, R. & Rajewsky, M. F. (1974) *Z. Krebsforsch.* **82**, 37–64.
7. Goth-Goldstein, R. (1977) *Nature (London)* **267**, 81–82.
8. Bodell, W. J., Singer, B., Thomas, G. H. & Cleaver, J. E. (1979) *Nucleic Acids Res.* **6**, 2819–2829.
9. Singer, B. (1979) *J. Natl. Cancer Inst.* **62**, 1329–1339.
10. Warren, W., Crathorn, A. R. & Shooter, K. V. (1979) *Biochim. Biophys. Acta* **563**, 82–88.
11. Brent, T. P. (1979) *Biochem. Biophys. Res. Commun.* **91**, 795–802.
12. Steward, A. P., Scherer, E. & Emmelot, P. (1979) *FEBS Lett.* **100**, 191–194.
13. Kirtikar, D. M. & Goldthwait, D. A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2022–2026.
14. Riazuddin, S. & Lindahl, T. (1978) *Biochemistry* **17**, 2110–2118.
15. Laval, J. (1977) *Nature (London)* **269**, 829–832.
16. Shackleton, J., Warren, W. & Roberts, J. J. (1979) *Eur. J. Biochem.* **97**, 425–433.
17. Brent, T. P. (1979) *Biochemistry* **18**, 911–916.
18. Singer, B., Bodell, W. J., Cleaver, J. E., Thomas, G. H., Rajewsky, M. F. & Thon, W. (1978) *Nature (London)* **276**, 85–88.
19. Singer, B., Kröger, M. F. & Carrano, M. (1978) *Biochemistry* **17**, 1246–1250.
20. Warren, W. & Lawley, P. D. (1980) *Carcinogenesis* **1**, 67–78.
21. Margison, G. P., Margison, J. M. & Montesano, R. (1976) *Biochem. J.* **157**, 627–634.
22. Ishiwata, K. & Oikawa, A. (1979) *Biochim. Biophys. Acta* **563**, 375–384.
23. Laval, J. (1981) *Proc. Natl. Acad. Sci. USA* **78**, in press.
24. Margison, G. P. & Pegg, A. E. (1981) *Proc. Natl. Acad. Sci. USA* **78**, in press.
25. Karran, P., Lindahl, T. & Griffin, B. (1979) *Nature (London)* **280**, 76–77.
26. Pegg, A. E. (1978) *Biochem. Biophys. Res. Commun.* **84**, 166–173.