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COMMENTARY

MONOFUNCTIONAL ALKYLATING AGENTS AND APURINIC SITES IN DNA

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MONOFUNCTIONAL alkylating agents alkylate DNA at two sites: bases and phosphates.

Lawley and Brookes[1] have shown that the alkylation of the bases is quantitatively more extensive; when using double-stranded DNA, the alkylation occurs mainly at the N-7 of guanine and the N-3 of adenine. Those alkylating agents that can react by a SN1 mechanism also alkylate the O-6 of guanine; this alkylation may be responsible for the mutagenic effect of alkylating agents in bacteriophages.[2]

Alkylated purines are lost by hydrolysis of the glycosidic linkage, a process known as depurination. Lawley and Brookes[1] showed that the N_3-alkyl-adenine is hydrolyzed away much faster than the N_7-alkyl-guanine; in our work with the ethyl methanesulfonate,[3] we found, at 37° and pH 7, that the half-life in DNA of ethyl-adenine is 5 hr, while that of ethyl-guanine is 177 hr.

The hydrolysis of the glycosidic linkage leading to the loss of the alkylated purine leaves an apurinic site. The exposed aldehyde function may be the origin of an interstrand crosslink; we have shown[4] that, at neutral pH, about 1 crosslink is formed per 140 apurinic sites in DNA of T7 coliphage.

At alkaline pH, the apurinic site is quickly converted into a single-strand break by hydrolysis of a neighbouring phosphodiester bond.[5] The resistance of apurinic sites at neutral pH is highly dependent on the composition of the incubation medium. Using a 0·01 M phosphate buffer, Laurence[6] had found a half-life of several months at pH 7 and 37°. Lindahl and Andersson[7] underlined the influence of the magnesium ion concentration: with 0·01 M Mg^{2+}, they found a half-life of 190 hr at pH 7·4 and 37°. Using a medium approximating the ionic composition of the cellular sap, Crine,[8] in our laboratory, has found a half-life of 20 hr at 37° and pH 7.

The extent of alkylation of DNA phosphates is a problem which has only recently been solved. Lett *et al.*[9] stated that alkylating agents react primarily with phosphates in DNA and presented spectrophotometrical evidence for transalkylation from triester phosphates to bases in the macromolecule; the transalkylation, however, did not occur after alkylation with ethyl methanesulfonate. By contrast, the results of Lawley and Brookes[1] indicate that phosphate alkylation is negligible. Bannon and Verly[10] have shown that, with ethyl methanesulfonate (EMS), 15 per cent of the DNA alkylation is on the phosphates, while with methyl methanesulfonate (MMS), only 1 per cent is on the phosphates. In the case of MMS, this minor alkylation may

occur only on terminal monoester phosphates which are more readily alkylated than diester phosphates; it may be that alkylation of diester phosphates, exactly as alkylation of O-6 of guanine, requires an alkylating agent that can ionize and react by the SN1 mechanism (such as EMS but not MMS). Bannon and Verly[10] have also shown that the triphosphates resulting from an alkylation of DNA by EMS are stable at neutral pH.

INACTIVATION OF THE T7 COLIPHAGE BY MONOFUNCTIONAL ALKYLATING AGENTS

To investigate the molecular basis of the toxicity of monofunctional alkylating agents, their effect on the viability of the T7 coliphage has been studied. The inactivation of a phage particle is the loss of its ability to give a progeny when added to cells of the host bacteria.

In a typical experiment, T7 phage particles are incubated 2 hr at 37° with various concentrations of labelled EMS in a buffered medium at pH 7. When tested on *Escherichia coli* immediately at the end of the treatment, part of the phages is found inactivated; this corresponds to the *immediate inactivation*. If, after removing the alkylating agent, the phages are further incubated at 37° before being tested on *E. coli*, there is an increase of the inactivated fraction; this increase, after the end of the treatment, is called *delayed inactivation*.

The immediate and the delayed inactivations are one-hit processes: the logarithm of the surviving fraction is a linear function of the alkylation dose (number of alkyl groups per DNA molecule.)[11] The inactivation phenomenon thus appears uncomplicated by host cell reactivation; indeed, the inactivation kinetics are the same with *E. coli* B_{s-1}, *E. coli* B/r or the wild strain *E. coli* B. The exact nature of the efficient hits for immediate inactivation and for delayed inactivation remains to be elucidated.

Delayed inactivation. Lawley and Brookes[1] proposed that the delayed inactivation of phages produced by monofunctional alkylating agents[12] is due to the process of depurination. With the T7 coliphage, Lawley *et al.*,[13] using the half sulfur mustard, and Brakier and Verly,[11] using EMS, found a rigorous correlation between depurination and delayed inactivation: seven or eight depurinations are needed to have one delayed inactivation hit.

Why are those depurinations lethal? Is it because they may lead to interstrand crosslinks preventing DNA replication? We have indeed shown, using nitrogen mustard (a difunctional alkylating agent), that a DNA interstrand crosslink is always an inactivation hit for the T7 coliphage.[14] Crosslinks between DNA strands do not appear, however, as a significant cause of delayed inactivation with monofunctional alkylating agents: at pH 7, 1 crosslink is formed per 140 apurinic sites,[4] while seven or eight depurinations correspond to one inactivation hit. We tend to think that the depurination itself is the inactivation hit. Because the inactivation is a one-hit process and an average of seven or eight depurinations is necessary to inactivate a phage particle, it is concluded that the target in which the depurination must occur is only 1/7 or 1/8 of the phage genome. We do not expect that an apurinic site localized in only one strand can interfere directly with the DNA replication, but rather think that depurination must exert its toxicity either at the transcriptional or at the translational level. We propose that the DNA target is the *r*-strand (i.e. the transcribed strand) of those genes coding for proteins necessary for the replication of the phage

DNA, like gene 1 which codes for the T7 RNA polymerase (necessary for the transcription of the T7 late genes) and gene 5 which codes for the T7 DNA polymerase.[15]

How could an apurinic site be toxic? At the transcriptional level, one could suppose that the apurinic site stops the progression of the RNA polymerase along its template. My colleague, Mrs. M. Mamet,[16] has indeed shown *in vitro* that apurinic sites in T7 coliphage DNA reduce the amount of RNA synthesized by *E. coli* RNA polymerase without alteration of the initiation specificity. One could also suppose that deletion of one nucleotide in the synthesized RNA would lead to a frameshift at the translational step.

Even if apurinic sites in DNA can be shown to be "toxic" *in vitro* (i.e. to prevent the elaboration of a functional protein in a system capable of RNA and protein syntheses), the situation might be different *in vivo*. The apurinic site might lead to a single-strand break responsible for the premature release of an unfinished RNA leading to a non-functional protein. To invoke such a mechanism, it is necessary to postulate the existence of a nuclease for apurinic sites in the host bacteria, *E. coli* B: spontaneous hydrolysis of a phosphodiester bond adjacent to the apurinic site is too slow at neutral pH to be of any consequence as far as the delayed inactivation is concerned.

A nuclease for apurinic sites has been found in *E. coli* B by Verly and Paquette.[17,18] The enzyme is strictly specific for apurinic sites, having no action on normal DNA, either native or denatured, or on alkylated sites. Endonuclease II has been described by Friedberg and Goldthwait[19] as an enzyme acting on alkylated DNA; more recently, Hadi and Goldthwait[20] found that their enzyme also acts on depurinated DNA. Our nuclease for apurinic sites may be identical to endonuclease II; it also acts on alkylated DNA, but we have shown[21] that its action is restricted to the apurinic sites resulting from the spontaneous loss of alkylated purines. If indeed this enzyme plays a role in the delayed inactivation of phages by monofunctional alkylating agents, it is important that it acts only on apurinic sites and not on alkylated sites: the delayed inactivation results from the replacement of alkylated sites by apurinic sites.

Papirmeister *et al.*[22] have presented evidence that the toxic action of monofunctional alkylating agents is correlated with adenine alkylation as opposed to guanine alkylation, and that *E. coli* possesses an enzyme which acts on such alkylated adenine to produce single-strand DNA breaks. The correlation observed by Papirmeister may be due to the faster loss of alkylated adenines as compared with alkylated guanines and the postulated enzyme might be our nuclease acting on the resulting apurinic sites.

Immediate inactivation. The following chemical alterations will be examined as possible causes for the immediate inactivation of the T7 coliphage treated by a monofunctional alkylating agent: alkylation of the bases, depurination, alkylation of the phosphates, single-strand breaks, alkylation of the protein coat.

The number of apurinic sites and single-strand breaks present in the T7 phage DNA at the end of 2 hr treatment by EMS or MMS has been determined either by sedimentation velocity in an alkaline sucrose gradient,[8] or by using polynucleotide kinase after a treatment with NaOH and alkaline phosphatase.[23] Assuming that seven or eight depurinations or single-strand breaks are necessary to have one inactivation hit (data for delayed inactivation), the results show that these two causes cannot explain more than 1/2 of the immediate inactivation.

Comparing MMS and EMS, Bannon and Verly[23] found a proportionality between DNA total alkylation and immediate inactivation; one inactivation hit corresponds to about 100 alkylations. We hesitate however to conclude from these results that DNA alkylation is the prominent cause of immediate inactivation. Alkylation is far less toxic than depurination: delayed inactivation results from the replacement of alkylated sites by apurinic sites. Some alkylations might be lethal mutations; but* the lethal mutation would be present in only half of the progeny in the first generation and would go unrecognized by our assay method; moreover MMS is reportedly non-mutagenic for phages in contrast with EMS which is highly mutagenic.[12]

In a similar way, we are unable to demonstrate any toxicity of the triester phosphates: at the same level of alkylation, EMS and MMS have the same toxicity, but the alkylation of the phosphates is 15 per cent of the total alkylation with EMS and only 1 per cent with MMS. When using a 2 hr incubation with EMS, there are 15 triester phosphates for one immediate inactivation hit;[23] this seems to preclude the possibility that the host bacteria might contain an enzyme converting inocuous triester phosphates into toxic single-strand breaks. The chemical stability of the triester phosphates in DNA[10] also seems important if they are really inocuous.

Papirmeister[24] suggested that alkylation of the coat protein is a cause of toxicity of the alkylating agents for coliphages. We are presently measuring the fraction of phage particles which, after EMS or MMS treatment, are incapable of injecting their DNA in the host bacteria.

In conclusion, we are as yet unable to assess the relative importance of each kind of damage undergone by the T7 coliphage particle during immediate inactivation.

THE ENDONUCLEASES FOR APURINIC SITES

Using the method followed by Friedberg and Goldthwait[19] for endonuclease II, Paquette, Crine and Verly[21] have purified the nuclease for apurinic sites from *E. coli* B41, a mutant lacking endonuclease I:[25] after sonication of the bacteria and centrifugation, the nucleic acids were removed from the supernatant by precipitation with streptomycin sulfate and the proteins fractionated with ammonium sulfate; the fraction precipitating between 45 and 80 per cent saturation in ammonium sulphate, which contained the activity, was then chromatographed successively on DEAE-cellulose and phosphocellulose.

The purified enzyme needs Mg^{2+} for activity and has a pH optimum at 8·5 in Tris–HCl buffer. The purified enzyme has no activity on intact strands in native or denatured DNA. It acts at apurinic sites, forming single-strand breaks, only if the substrate DNA is double-stranded. That is has no action on alkylated sites was proved in the following way: labelled DNA containing many alkylated sites was incubated at 37° with the enzyme while the control contained no enzyme; aliquots were removed at different times from both incubation media and treated with NaOH to hydrolyze any apurinic sites before measuring the acid-soluble radioactivity. At any time, the acid-soluble radioactivity was the same whether the alkylated DNA had been treated by the enzyme or not; this shows that enzyme and NaOH cut the DNA strands at the same sites, i.e. the apurinic sites produced by loss of alkylated purines during incubation, and that the enzyme therefore does not attack alkylated sites. Although

* Some alkylations might however induce mispairing at the transcriptional level leading, at the translational level, to the synthesis of an inactive protein needed for the phage DNA replication.

both NaOH and enzyme break the DNA strand carrying the apurinic site, reduction of the aldehyde function with $NaBH_4$ suppresses the action of NaOH while still allowing strand cleavage by the enzyme. NaOH and enzyme cut the DNA strand near the apurinic site leaving a terminal 5′-phosphate.

Although we have not seen any host cell reactivation of the T7 coliphage treated with EMS or MMS, *E. coli* is known to repair damages produced by monofunctional alkylating agents. One might suppose that the repair is directed not so much towards the non-toxic alkylated sites,* but rather towards the dangerous apurinic sites. The repair mechanism might be analogous to the excision repair described after u.v. irradiation where, at least, three enzymes act successively to restore an intact DNA strand: u.v. endonuclease, DNA polymerase I and ligase. When apurinic sites are concerned, the repair endonuclease would be the nuclease for apurinic sites.

To investigate this possibility, Verly, Gossard and Crine[27] have incubated native T7 coliphage DNA containing a few apurinic sites together with the purified bacterial nuclease, DNA polymerase I with the four deoxynucleoside triphosphates, and T4 coliphage ligase with its coenzyme ATP. After a 3 hr incubation at 37°, the DNA strands were restored to normality, contrasting with the situation in a control without the nuclease where incubation did not suppress the apurinic sites in spite of the presence of the two other enzymes.

Evidence has been presented by Lindahl and Nyburg[28] for a spontaneous depurination of DNA at pH 7 and 37°. In our laboratory, Crine[8] has confirmed their finding using labelled coliphage T7 DNA and the method of centrifugation in a neutral sucrose gradient after denaturation by formamide or NaOH: the rate of depurination is 0·02/hr per strand, at 37° and pH7, in a medium having the ionic composition of the cellular sap.

It thus seems that the integrity of the genetic information necessary for cell survival would require a maintenance system in each cell for the continuous repair of the apurinic sites appearing in DNA. From these premises, we deduce that, if the nuclease for apurinic sites is a part of the DNA maintenance system, it ought to be present in every cell.

We have indeed found[29] that rat liver contains a highly active nuclease for apurinic sites. The mammalian enzyme was purified following the same steps used for the bacterial enzyme. As the bacterial enzyme, the purified mammalian enzyme is specific for apurinic sites; it has no action on normal DNA strands or on alkylated sites.[30] Lindahl and Andersson[7] have described a similar enzyme in calf thymus.

With L. Thibodeau, we have also looked for the presence of the enzyme in plants. We found[31,32] that a homogenate of *Phasaeolus aureus* leaves has little action on normal DNA or alkylated DNA, but that its action was greatly increased when the alkylated sites were replaced by apurinic sites.

The nuclease for apurinic sites thus seems to be present in all cells and it is likely a vital enzyme. We are now assaying the enzyme concentration in the different tissues of the rat; we also plan to follow the enzyme activity as a function of ageing, and to see whether the enzyme still works in cancerous tissues. After purifying the bacterial, plant and animal enzymes, we will compare their catalytic efficiencies and the primary

* Lawley and Brookes[26] found that, in *E. coli*, after a treatment with a difunctional alkylating agent, the crosslinked bases were removed much more efficiently than the monofunctionally alkylated bases.

structure of their constitutive polypeptides. These enzymes may be homologous proteins coded for by genes deriving from a common ancestor, and thus be suitable for phylogenetic studies encompassing all cellular organisms.

REFERENCES

1. P. D. LAWLEY and P. BROOKES, *Biochem. J.* **89,** 127 (1963).
2. A. LOVELESS, *Nature, Lond.* **223,** 206 (1969).
3. W. G. VERLY, H. BARBASON, J. DUSART and A. PETITPAS-DEWANDRE, *Biochim. biophys. Acta* **145,** 752 (1967).
4. J. BURNOTTE and W. G. VERLY, *Biochim. biophys. Acta* **262,** 449 (1972).
5. C. TAMM, H. R. SHAPIRO, R. LIPSHITZ and E. CHARGAFF, *J. biol. Chem.* **203,** 673 (1953).
6. D. J. R. LAURENCE, *Proc. Roy. Soc., Lond.* **271** (Serie A), 520 (1963).
7. T. LINDAHL and A. ANDERSSON, *Biochemistry* **11,** 3618 (1972).
8. P. CRINE and W. G. VERLY, unpublished results.
9. J. T. LETT, G. M. PARKINS and P. ALEXANDER, *Archs Biochem. Biophys.* **97,** 80 (1962).
10. P. BANNON and W. G. VERLY, *Eur. J. Biochem.* **31,** 103 (1972).
11. L. BRAKIER and W. G. VERLY, *Biochim. biophys. Acta* **213,** 296 (1970).
12. A. LOVELESS, *Proc. Roy. Soc., Lond.* **150(B),** 497 (1959).
13. P. D. LAWLEY, J. H. LETHBRIDGE, P. A. EDWARDS and K. V. SHOOTER, *J. mol. Biol.* **39,** 181 (1969).
14. W. G. VERLY and L. BRAKIER, *Biochim. biophys. Acta* **174,** 674 (1969).
15. F. W. STUDIER, *Virology* **39,** 562 (1969).
16. M. D. MAMET-BRATLEY, *Ann. ACFAS* **40,** 9 (1973).
17. W. G. VERLY and Y. PAQUETTE, *Can. Fed. Biol. Soc.* **685,** (1970).
18. W. G. VERLY and Y. PAQUETTE, *Can. J. Biochem.* **50,** 217 (1972).
19. E. C. FRIEDBERG and D. A. GOLDTHWAIT, *Proc. natn Acad. Sci., U.S.A.* **62,** 934 (1969).
20. S. M. HADI and D. A. GOLDTHWAIT, *Biochemistry* **10,** 4986 (1971).
21. Y. PAQUETTE, P. CRINE and W. G. VERLY, *Can. J. Biochem.* **50,** 1199 (1972).
22. B. PAPIRMEISTER, J. K. DORSEY, C. L. DAVISON and C. L. GROSS, *Fedn Proc.* **29,** 2 (1970); see also *Edgewood Arsenal Technical Report* 4650, June (1972).
23. P. BANNON and W. G. VERLY, unpublished results.
24. B. PAPIRMEISTER, *Spec. Publ.* No. 2–45. U.S. Army Chem. Res. and Dev. Lab., Army Chemical Center, Md., U.S.A. (1961).
25. H. DURWALD and H. HOFFMANN-BERLING, *J. mol. Biol.* **34,** 331 (1968).
26. P. D. LAWLEY and P. BROOKES, *Nature, Lond.* **206,** 480 (1965).
27. W. G. VERLY, F. GOSSARD and P. CRINE, manuscript submitted for publication.
28. T. LINDAHL and B. NYBERG, *Biochemistry* **11,** 3610 (1972).
29. W. G. VERLY and Y. PAQUETTE, *Fedn Proc.* **31(2),** 918 (1972).
30. W. G. VERLY and Y. PAQUETTE, *Can. J. Biochem.* **51,** 1003 (1973).
31. W. G. VERLY, Y. PAQUETTE, C. TREMBLAY and L. THIBODEAU, *Can. Fed. biol. Soc.* **673** (1972).
32. W. G. VERLY, Y. PAQUETTE and L. THIBODEAU, *Nature, New Biol.* **244,** 67 (1973).