The Effects of DNA Covalent Adducts on *in Vitro* Transcription

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I. Introduction

A number of compounds affect the biological properties of nucleic acids at the molecular level through physicochemical interactions or by covalent binding.[1] For example, some cytotoxic drugs are believed to exert their activity through covalent binding to DNA, impairing many vital cellular functions. One such cellular function, DNA-mediated RNA synthesis, can be reproduced with high fidelity in cell-free systems. *In vitro* transcription of DNA treated with alkylating agents may elucidate the biological effects of such ligands when covalently bound to DNA.

The different sequential steps of the transcription process can be distinguished. It is possible to study the consequences of covalent binding of a ligand to DNA on a particular stage of the transcription. An insight into the nature of impairment caused by the adduct formation is thus obtained.

Compounds which covalently bind to nucleic acids differ widely both in chemical structure and in the mechanism of their interaction with DNA. We will focus our attention on anticancer drugs whose effects on *in vitro* transcription have been thoroughly studied. Chemically distinct classes of compounds are represented: nitrogen (**1**) and sulfur mustard (**2**) and

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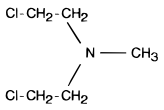
related compounds, platinum complexes (**3, 4**), furocoumarins (psoralen, **5**, and angelicin, **6**, derivatives), chlorpromazine (**7**), mitomycins (**8**), anthracyclines (adriamycin, **9**, and its cyanomorpholino derivative, **10**), nitracrine (**11**), and other nitroacridines. In addition the effects of some environmental mutagens and carcinogens such as benzo[*a*]pyrene (**12**), 2-aminofluorene (**13**), and its *N*-acetyl derivative (**14**) on *in vitro* transcription are similar and may provide meaningful and complementary information, although they elicit different consequences than anticancer drugs. A brief overview of such covalent DNA complexes will be presented, and the effects that these DNA modifications have on the different stages of transcription will be described.



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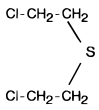

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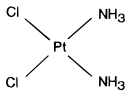

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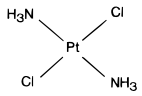

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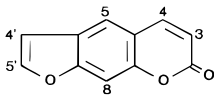

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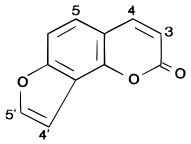

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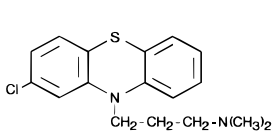

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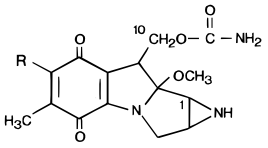

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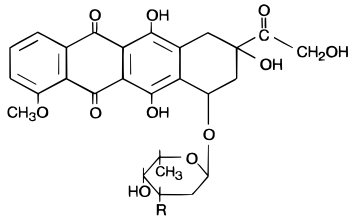
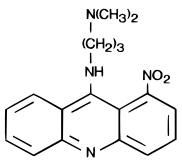


9 R = $-NH_2$



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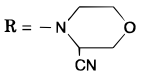
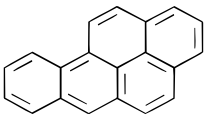
10 R = 



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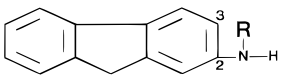
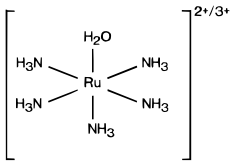


13 R = H

14 R = $-COCH_3$



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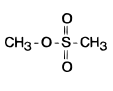

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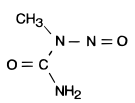

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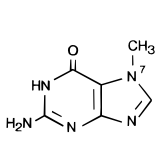
Another fundamental process in living cells which is dramatically affected by covalent adduct formation with DNA is replication. The information obtained by studying the effects of alkylating agents on *in vitro* DNA replication may complement the findings on *in vitro* transcription. Therefore, we will compare the results from both systems, if data become available.

II. Covalent Adducts

The structures of the DNA adducts formed by the compounds mentioned above have been thoroughly described in the literature. However, it may be useful to review some of their characteristics at this point.

Purine nucleosides, particularly G (see Figure 3a,b for the formulas of the nucleosides), represent the preferential target of alkylating agents. The N7 position is attacked by nitrogen mustards,[2,3] cisplatin (**3**),[4−6] pentaammineruthenium(III) **15** ions as well as other ruthenium complexes,[7,8] vinyl chloride and its metabolites,[9] and aflatoxin B_1.[10,11] Methyl- (**16**) and ethylmethanesulfonate, dimethyl sulfate, *N*-nitroso-*N*-methylurea **(17)**, and other small alkylating agents also react mainly with N7 of G (**18**). In addition the exocyclic O6 of G (**19**), O2 (**20**) and O4 (**21**) of T as well as the N3 of A and C can also be alkylated.[11−15] The exocyclic 2-amino group of G can be alkylated by mitomycins (**22**),[16,17] the benzo[*a*]-pyrene metabolite (**23**),[18] and possibly by adriamycin[19] as well as its cyanomorpholino derivative.[20] Aminofluorenes bind efficiently to the N2 (**24**) and C8 positions of G (**25, 26**).[11,21] It is assumed that chlorpromazine (**7**) is bound to position C8 of G (**27**).[22] Nitroacridines exhibit a less defined base specificity.[23,24] Furocoumarins bind to pyrimidines, usually to T.[25−27] In the presence of pyridine or tertiary amines osmium tetroxide which is used as a chemical probe of DNA structure form stable complexes with pyrimidine bases (**28**) reacting specifically with single-stranded and distorted double-stranded regions in the DNA.[28]

Depending on the structure of the ligand, the polynucleotide sequence, and the experimental conditions, alkylation can result in either monofunctional covalent complexes or inter- or intrastrand cross-links. Some compounds, such as methyl and ethyl methanesulfonate,[29,30] chlorpromazine,[31] aminofluorenes, and benzo[*a*]pyrene,[11,18] are capable of forming only one covalent bond with DNA whereas the binding of nitrogen mustard (**29**),[2,29,32] cisplatin (**30**),[5] furocoumarins,[27,33,34] mitomycins (**31**),[17,35−37] and, to a lesser extent, nitroacridines[24,38] can yield cross-links. Two major adducts of cisplatin forming intrastrand cross-links between adjacent purines (G-G and A-G) induce a distortion in the polynucleotide structure.[39−41] At G-C sites, cisplatin (**3**) forms interstrand cross-links between guanines on opposite strands, while its stereoisomer, *trans*-DDP (**4**), forms interstrand cross-links between G and the complementary C.[42,43] The two isomers can also form intrastrand cross-links between guanines in the G-T-G sequence.[44] However, due to steric reasons, only cisplatin (**3**) can form intrastrand cross-links on adjacent purines. Heat labile interstrand and intrastrand cross-links are formed by cyanomorpholinoadriamycin (**10**)[20,45−48] and probably also by adriamycin in the presence of Fe(III) ions.[19] Vinyl



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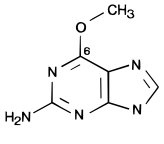

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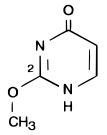

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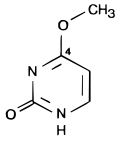

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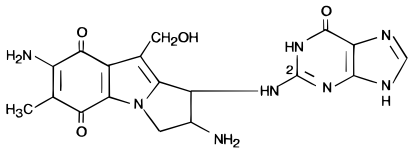

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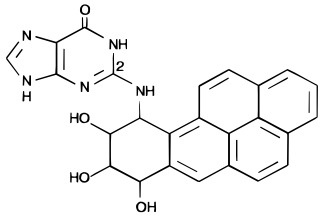

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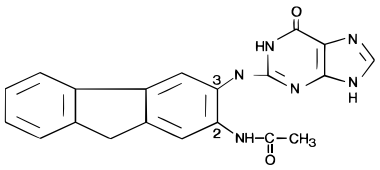

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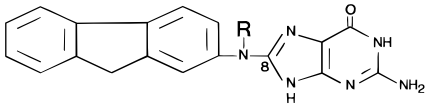
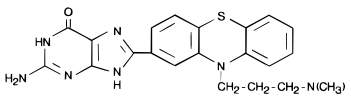


25 R = H; 26 R = $COCH_3$



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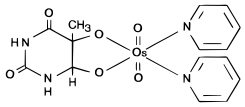

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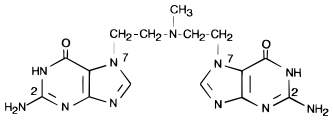

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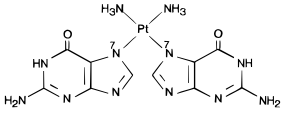

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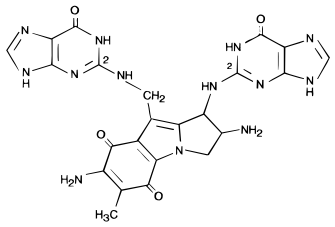

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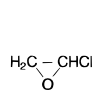
chloride metabolites (**32, 33**) form an additional ring on DNA bases (**34, 35, 36**).[9]

Alkylating compounds are converted into active species by different mechanisms. Nitrogen mustards (**1**) bear two chloroethyl groups which are highly reactive to nucleophilic substitution. Mitomycins (**8**)



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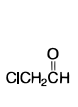

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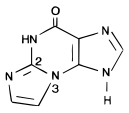

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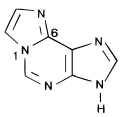

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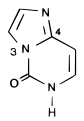

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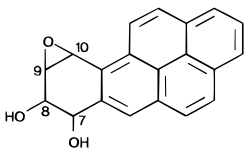
undergo an enzymatic reduction, which can be reproduced electrochemically using sodium dithionite or H_2/PtO_2, that first activates the C1 position and subsequently the C10 position of the drug.[17,49,50] Only C-G and not G-C sequences are cross-linked,[35,51,52] suggesting a strong sensitivity to the secondary structure of DNA as confirmed by Cera *et al.*[51,53] After UV irradiation or incubation with H_2O_2 in the presence of horseradish peroxidase, promazines (**7**) are activated to a radical cation, which intercalates in the double helix and binds covalently to G.[31] Adriamycin forms irreversible complexes with DNA after either reduction in the presence of Fe(III) ions and dithiothreitol[19,54,55] or enzymatic activation.[56] Activation of 1-nitroacridines to form alkylating species requires the presence of sulfhydryl compounds.[23,38,57,58] Metabolic activation of vinyl chloride to chloroethylene oxide (**32**) and chloroacetyl aldehyde (**33**),[9] of benzo[*a*]pyrene (**12**) to a diol epoxide (**37**),[11,18] and of aflatoxin B_1 to its epoxy derivative[10] yields potent DNA alkylating agents. Furocoumarins, structurally classified as psoralens (**5**) or angelicins (**6**), intercalate between the base pairs of DNA. UV irradiation causes them to form cyclobutan rings by a reaction between their furan (**38**) or pyrone (**39**) moieties and the 5,6 carbon atoms of T. The furan-side monoadduct (**38**) of psoralens can further covalently bind to an adjacent T (**40**) on the complementary strand[59] whereas for steric reasons angelicin monoadducts are unable to create cross-links.[26] Both A-T and preferentially T-A sequences can therefore be cross-linked.[60] Both the psoralen monoadducts and the cross-links induce a slight kink in the DNA backbone[27,61,62]



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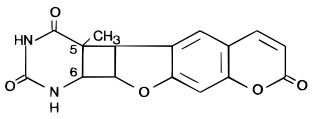

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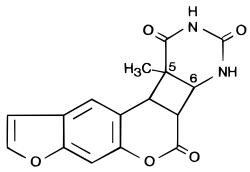

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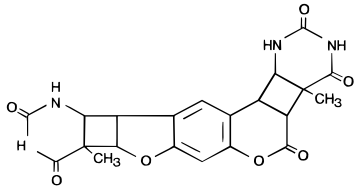

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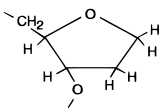

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When 2-aminofluorene derivatives bind to C8 of G in DNA they yield adducts (**25, 26**) which affect the

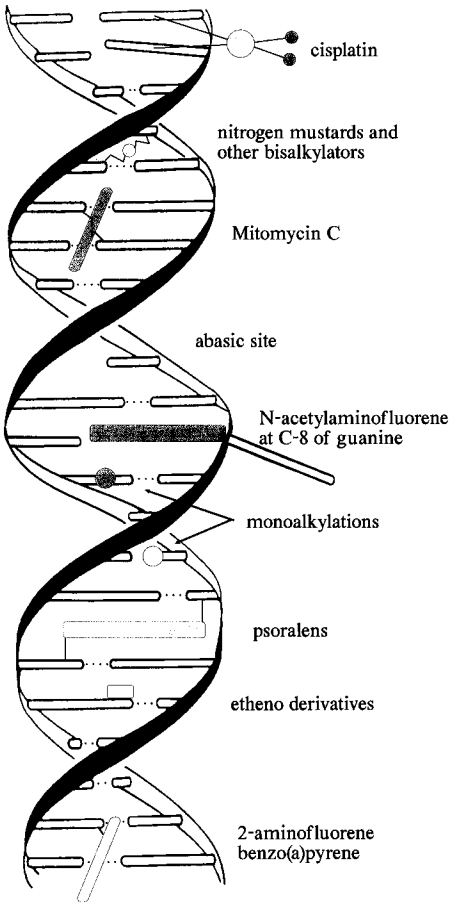


Figure 1. Schematic representation of some covalent adducts on the double helix. Some characteristic lesions are shown but many of them have been omitted, e.g. cisplatin forms intrastrand cross-links as shown, but also monoadducts and interstrand cross-links; mitomycins and furocoumarins form diadducts and monoadducts, the latter quantitatively prevailing.

polynucleotide conformation in different ways. The covalent complex between *N*-acetyl-2-aminofluorene and DNA (**26**) exhibits a dramatic conformational distortion for which an "insertion−denaturation" model has been proposed.[63−65] The less distorted 2-aminofluorene−DNA complex (**25**) has instead been ascribed to an "outside" binding mechanism.[21,66] NMR studies suggest that, in the latter, G is in the *syn* conformation and the adduct lies in the minor groove.[67,68] An equilibrium between "outside" binding and "inserted" complex may also exist.[69]

Alkylation of a base can lead to hydrolysis of the N-glycosidic bond and formation of an abasic (mostly apurinic) site. This lesion has been simulated in transcription studies by introducing tetrahydrofuran (**41**) into the polynucleotide chain.[70] There is a body of evidence that suggests that the majority of the structures described here which are formed in simple subcellular systems are also formed in experimental animals, or at the cellular level. In addition many have been demonstrated as occuring in humans.[11,21,29,58,71−73] In Figure 1 some lesions have been schematically presented.

III. In Vitro RNA and DNA Synthesis

Transcription is the process by which RNA pol reads the sequence of one strand of DNA, called the

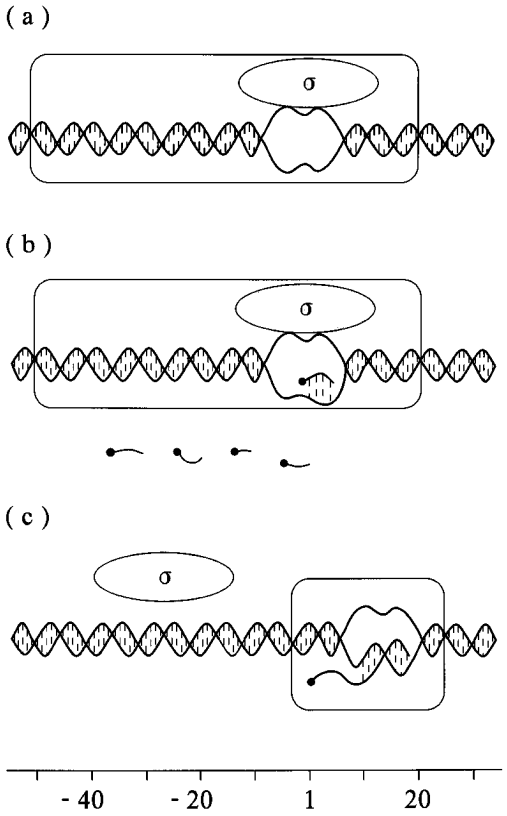


Figure 2. *E. coli* RNA pol−DNA initiation and elongation complexes on *lac*UV5 promoter.[78] (a) Open promoter complex. (b) RNA pol initiating RNA chains. Short RNA chains are released (abortive initiation). The abortive initiation may be experimentally observed if an incomplete set of the substrates is used and the complementary nucleoside triphosphate is lacking (see Figure 4). (c) The elongating RNA pol. The σ subunit has been indicated on scheme a and b just to show the fact that it dissociates from the elongating complex (c). The scale presents DNA length in base pairs relative to the initiation site (+1). The contours of the enzyme are drawn to present the length of DNA helix covered. It is considerably different for the open promoter complex (a) and at the elongation step (c). The scheme slightly modified was reproduced with kind permission of Dr. J. D. Gralla and the Publisher, Academic Press Ltd.

template strand, and synthesizes the complementary polyribonucleotide chain. An enzyme initiates the process by interacting with specific DNA sequences denoted as promoters. In eukaryotes, different enzymes are responsible for the synthesis of particular fractions of RNA, i.e. RNA pol I for most of ribosomal RNAs, pol II for messenger RNAs, and pol III for transfer and 5S rRNA.[74] The structural elements of the promoter are more complex than in prokaryotes. The interaction of other proteins such as transcription factors, and other regulatory proteins in addition to RNA pol are required for specific transcription to occur. On the contrary bacterial and phage enzymes do not always need the presence of such accessory factors.[75,76] Therefore the most commonly used enzymes in *in vitro* experiments are of prokaryotic origin: *Escherichia coli* DNA-dependent RNA pol,[75] a multimeric enzyme, and phage T7 or SP6 RNA pol which react as monomers.[76]

Once RNA pol has recognized and bound its specific promoter to form a closed complex, it induces unwinding and melting of the DNA helix ahead of the initiation site (Figure 2a) in a temperature-dependent

Table 1. Number of Adducts Molecules per 1000 Nucleotides which Reduce *in Vitro* RNA Synthesis to 37% (N_{37})

drug	DNA[a]	RNA pol	N_{37}[a]	semilog plot linear	n[a]	r[a]
nitracrine	CT	*E. coli*[96]	0.53	+	10	0.97
	T7	*E. coli*[b]	0.78	+	17	0.97
8-methoxypsoralen	CT	*E. coli*[b]	0.54	+	7	0.96
	T7	*E. coli*[97,98]	0.30	+	12	0.92
4,6-dimethylangelicin	T7	*E. coli*[98]	0.23	+	7	0.91
benzopsoralen	CT	*E. coli*[b]	0.49	+	7	0.98
	T7	*E. coli*[b]	0.64	+	6	0.98
benzo[*a*]pyrene	pDR100	T7[91]	0.22	−	2[c]	
	CT	*E. coli*[90]	8.8	+	6	0.99
	M13mp9t7	T7[152]	1.28		2[c]	
2-aminofluorene	pDR100	T7[91]	0.52	+	3	0.95
N-acetyl-2-aminofluorene	DR	*E. coli*[92]	11.1	+	4	0.91
	T7	*E. coli*[99]	0.62	+	3	0.90
	pDR100	T7[91]	0.55	+	3	0.96
nitrogen mustard	T7	*E. coli*[100]	1.13[d]	+	4	0.99
methyl methanesulfonate	T7	*E. coli*[94]	10[d]	+	12	0.90[d]
dimethyl sulfate	Ml	*E. coli*[101]	50	+	8	0.97
	CT	*E. coli*[101]	18	+	6	0.94

[a] CT= calf thymus; DR = duck reticulocyte; Ml = *Micrococcus lysodeicticus;* M13mp9t7 = M13 phage bearing T7 promoter. n = number of experimental points; r = correlation coefficient of linear regresion given by the equation $\log_{10} A = a + bN$, where N is the number of adduct molecules per 10^3 DNA nucleotides that reduces RNA synthesis to A percent of the uninhibited controls. [b] Tołwińska-Stańczyk and Gniazdowski, unpublished experiments. [c] Interpolated from two experimental points. [d] Data from refs 94 and 100. Other data were calculated for this review.

step forming an open complex. Subsequently, the first phosphodiester bonds are formed (initiation) and ribonucleotides are added according to the template sequence (elongation) until the RNA pol reaches the termination signal (termination) (Figure 2b,c). The newly synthesized RNA chain is then released and the enzyme starts the cycle again by binding to the promoter.

Interactions between proteins and nucleic acids in the transcription process have already been thoroughly reviewed (see refs 75, 77−81 and references therein). We will focus our attention on (i) the effects of covalent adducts on the total RNA synthesis, (ii) the fidelity of transcription, (iii) the binding of RNA pol to the promoter, (iv) the initiation step, and (v) the elongation step.

DNA pols are the key enzymes involved in replication (for reviews, see refs 82−84) although they usually need accessory proteins for "genuine" replication to occur both in prokaryotic and eukaryotic systems. In contrast to RNA pols, DNA pols cannot initiate polydeoxynucleotide chains *de novo* on a template but can extend the 3′ end of RNA or DNA primers. Primers are short oligonucleotides complementary to the template DNA strand and are synthesized by specific enzymes, called primases, which can be tightly associated with DNA pol (calf thymus DNA pol α[84]) or just loosely interacting with it (*E. coli* pol I and III[83]). In most of experiments described here the primers are added to the template in order to assign the starting point. Particular DNA pols have different roles; for example bacterial pol I is involved in DNA repair. Bacterial pol I and III exhibit 5′ → 3′ exonuclease activity,[82,83] allowing these enzymes both to initiate single-strand degradation starting at a nick in duplex DNA and to resynthesize DNA using the opposite strand as template (nick translation). *E. coli* DNA pols, eukaryotic pol δ and ϵ, and some preparations of pol α have 3′ → 5′ exonuclease activity, which can be used to remove misincorporated nucleotide (proofreading).[83,84] According to Singer,[85] the lack of exonuclease function in RNA pols renders these enzymes a better tool for studying the behavior of covalently modified templates.[86] To overcome the confusing results given by proofreading in *in vitro* replication, fragment of DNA pol devoid of exonuclease 5′ → 3′ activity ("klenow fragment" from *E. coli* DNA pol I) or devoid of both exonuclease 5′ → 3′ and 3′ → 5′ activities (e.g. sequenase from T7 DNA pol) are currently used in the laboratory.

In most of the experiments described here, *E. coli* DNA pol I, klenow, phage T7 and T4 DNA pols, sequenase, eukaryotic DNA pol α, and AMV RT (RNA dependent DNA pol) were used.

IV. Inhibition of Total RNA Synthesis

The decrease in the amount of RNA synthesized on a modified template is assessed by measuring the incorporation of radioactive ribonucleotides into the transcript. Transcription, as well as replication assays, were used to characterize the covalent interactions of furocoumarins,[25,87−89] benzo[*a*]pyrene,[90,91] 2-aminofluorene,[91,92] cisplatin and *trans*-DDP,[93] amminеruthenium complex,[8] and nitracrine[57] with DNA.

A straight line is generally obtained when the logarithm of the percentage of residual RNA synthesis vs the number of covalent adducts on the template is plotted. Mamet-Bratley[94] has pointed out that this relationship corresponds to a one-hit process[95] characterized by adduct densities decreasing RNA synthesis to 37% of the controls. This relationship may reflect a general phenomenon that adducts are strong barriers for elongation, with bypasses occurring infrequently. In Table 1 the number of covalent modifications per 10^3 DNA nucleotides that reduce RNA synthesis to 37% (N_{37}) are reported for some alkylating agents. Although these data have been obtained under different experimental conditions, the conclusions are still relevant. Different covalent adducts inhibit RNA synthesis to a different extent.

While covalent binding of 0.2−0.8 drug molecules of nitracrine,[96] furocoumarin,[97,98] or other large aromatic compounds[91,99] per 10^3 bases are sufficient to reduce RNA synthesis to 37%, a higher degree of template modification by nitrogen mustard[100] (1.1 drug molecules per 10^3 bases) or methylating[94,101] and ethylating[94] agents (10−50 modified bases per 10^3 bases) is required in order to achieve the same result. Generally, bulky adducts inhibit RNA synthesis to a greater extent than small alkylating species. Another factor which influences inhibitory effects is a length of the transcribed sequences. When the sequence is shorter the probability of a ligand hitting the target is lower.[102]

Covalently binding drugs inhibit *in vitro* transcription considerably more than any noncovalently interacting ligands.[103] This finding is in agreement with the difference in biological activity exhibited by these two types of compounds.[104] Transcription assays can reveal new ligand−DNA interactions. Covalent binding of aflatoxin B_1 to C was first deduced by the inhibition of RNA synthesis on poly [d(I-C)].[105,106] The formation of ammineruthenium complexes (**15**) with DNA has been first inferred by their inhibitory effect on RNA synthesis in isolated mouse liver nuclei.[7,8] Sometimes, however, structurally different covalent complexes, such as *N*-acetyl-2-aminofluorene (**26**) and 2-aminofluorene−DNA (**25**) adducts, inhibit transcription to a comparable extent.[91] No dramatic differences are observed between 8-methoxypsoralen, a cross-linking drug, and monofunctionally binding furocoumarins, such as 4,6-dimethylangelicin[98] and benzopsoralen[107] (Table 1).

Experiments with *E. coli* DNA pol I indicate that replication is more sensitive to psoralen cross-links (**40**) than to furocoumarin monoadducts (**38, 39**).[25,108] Attenuation of replication upon covalent binding of both cisplatin and *trans*-DDP to the template has been observed with human DNA pol α and *β*.[109]

V. Fidelity of Transcription and Replication on Covalently Modified DNA

Theoretically, errors in the transcript sequence may be generated by either alteration of specific DNA−RNA base-pairing properties or by template strand switching by RNA pol. The latter mechanism has been excluded by experiments on the complementarity of the RNA chains to the transcribed strand of DNA (transcript asymmetry). Boulé-Charest and Mamet-Bratley found that when *E. coli* RNA pol with T7 DNA bearing two nitrogen mustard (**1**) molecules per 10^3 bases was used, the total RNA synthesis decreased by 70%. But the transcript specifically hybridized with the template strand, behaving in the same way as the control RNA synthesized on the unmodified DNA.[110] This result indicates that *E. coli* RNA pol is able to transcribe the correct strand even with a high degree of covalent modification.

DNA−RNA base-pairing properties can be altered either by changes in the tautomeric equilibrium of the modified bases or by the formation of abasic sites. Monoadducts can also cause a shift in the tautomeric equilibrium of the base and/or a substitution of the hydrogen donor groups. Most likely this alters the ability of the base to form hydrogen bonds, thus potentially affecting coding fidelity (Figure 3).

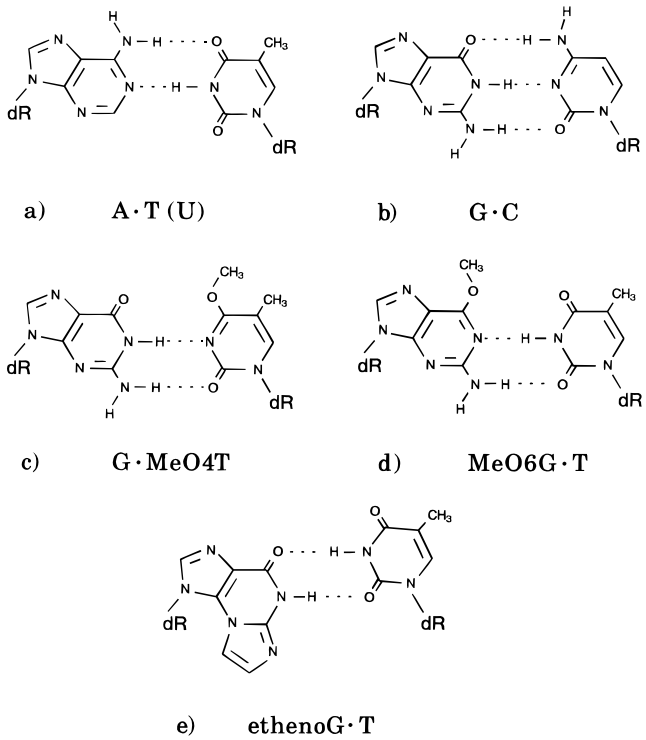


Figure 3. Watson−Crick base pairs A·T (a) and (b) G·C, and mispairing due to methylation of (c) T at O4 (MeO4 T·G), (d) G at O6 (MeO6 G·T) and (e) formation of ethenoguanosine, etheno G·T base pair. dR = deoxyribose.[11]

Table 2. Miscoding Properties[a] of Modified Bases as Revealed by Transcription and Replication *in Vitro* and Studies at the Cellular Level

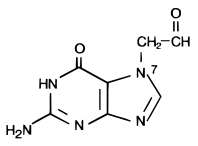
modified base in template	miscoded base(s)		
	in vitro incorporation by:		in the cell
	RNA pol	DNA pol	
O6 Me G	U > A[14,113,115]	T[14,117]	T[11,b]
O6 Et G	U > A[14]	T[14]	
O2 Me T	G[14]	G[14]	
O2 Et T	G[14]	G,[14] T[122]	
O2 Me U	G[15,116]		
O4 Me T	G[14]	G[14,15,114,116]	G[132]
O4 Et T	G[14]	G[14]	
O4 Me U	G[15,116]		
abasic site	A[68,128]	A > G[129]	A[131]
etheno G	U > A[123,124]	T[c,125]	
etheno C	U[9]	A > T[127]	A > T[130,133]

[a] Miscoding potency of the modified bases depends on the sequence context, concentration of a normal substrate and concentration of on erronously incorporated one. [b] Loechler, E. L.; Green, C. L.; Essigmann, J. M. *Proc. Natl. Acad. Sci. U.S.A.* **1984**, *81*, 6271. [c] AMV RT.

The effects of smaller substituents on base pairing in *in vitro* transcription and replication have been investigated (Table 2). Most of these modifications are a result of monoalkylating agents[14,111−122] (methyl and ethyl methanesulfonate, *N*-nitrosoureas, etc.). Alkylation of the exocyclic oxygens of T and G generally leads to miscoding (Figure 3c,d). In addition to expected A, G is incorporated opposite O4- (**21**) and O2-methylthymine (**20**) by RNA pols. O6-Methylguanine (**19**) miscodes for U in *in vitro* transcription[15,113,115,116] and for T in replication (Figure 3d).[14,15,114,117] Experiments with oligonucleotide con-

taining a specifically located O2-ethylthymine site reveal that T7 DNA pol may incorporate T opposite the lesion.[122] Methylation at N3 of C leads to incorporation of U and A by *Micrococcus lysodeicticus* RNA pol.[111,112] Methylation of G at N7 (**18**), a major alkylation site, does not alter base coding properties in the *in vitro* RNA synthesis system.[13,113,120,121] Similarly, methylation and ethylation[14,113] or even oxoethylation (**42**)[119] products at N7 of G do not miscode when copied by DNA pols.

Transcription fidelity depends on sequence context. DNA bases alkylated by vinyl chloride metabolites (**32, 33**) resulting in bases with an additional ring (**34**−**36**) is an example.[9,121] Ethenoguanine (**34**) is read by *E. coli* RNA pol and AMV RT as A or G between C residues but almost exclusively as A when inserted in poly(A) stretches.[123,124] Ethenoguanine miscodes for T (Figure 3e) with *E. coli* and *Drosophila melanogaster* DNA pol α[125] and with AMV RT.[126] U is misincorporated opposite ethenocytosine (**36**) by RNA pol,[9] while A and, to a lesser extent, T are incorporated by *E. coli* DNA pol I at the same lesion.[119,127] As previously mentioned (section II), alkylation can lead to the formation of abasic sites. *E. coli*, SP6 and T7 RNA pol,[70,128] *E. coli* DNA pol I, T4 pol, or AMV RT insert purine, usually A opposite an abasic site.[129−131] Effects of these modifications have been confirmed at the cellular level (Table 2).[130−133] Rabkin and Strauss[134] studies with eukaryotic DNA pols and *E. coli* DNA pol I reveal that fidelity of replication also depends on the divalent ion present in the buffer. In the presence of Mg^{2+}, C is correctly incorporated opposite the acetylaminofluorene bearing G, while in the presence of Mn^{2+}, *E. coli* DNA pol I incorporates A. Pol α from calf thymus or human lymphoma cells inserts any base with preference for A or C.[134] Misincorporation and inhibition of polynucleotide synthesis by *E. coli* DNA pol I and mammalian DNA pol α on poly [d(G-C)] and poly [d(A-T)] modified with *N*-acetyl-2-aminofluorene (**26**) has also been observed.[135] According to Shibutani *et al.*, stereoisomers of dihydroxyepoxybenzo[*a*]pyrene bound to G cause incorporation of A in replication.[136] When the A or G adducts of another polycyclic hydrocarbon, benzo[*a*]anthracene, were studied in replication with sequenase, A was inserted opposite the lesion.[137] This adduct may also induce misincorporation at the base preceding the lesion.[138,139]



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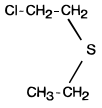

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The kinetics of elongation upstream, at, and downstream the 2-aminofluorene (**25**) adducts on G has been studied for reaction times ranging from 20 ms to 45 min.[140] Lindsley and Fuchs[140] used primers of different length to determine the start of replication few bases or one base upstream the modified G or at the opposite C. Correct incorporation of C is 5×10^4-fold slower on alkylated than on unmodified template. Similar experiments with *N*-acetyl-2-aminofluorene (**26**) yield a 4×10^6 slower incorporation. However, misincorporation of A is faster than the correct incorporation of C.[140]

VI. Early Steps of RNA Synthesis

A. Affinity of RNA Polymerase for Covalently Modified Promoters

Studies reported here indicate that covalent modification of the promoter does not dramatically affect enzyme binding. RNA pol seems to be more tolerant of promoter modifications than specific transcription factors.[141] Electron microscopy measurements showed that T7 RNA pol binds to specific promoters on T7 DNA bearing three to six methylated or ethylated bases per 10^3 nucleotides.[142] It was concluded that these covalent modifications do not alter the ability of RNA pol to recognize and bind the correct sequences.

Kornprobst *et al.*[101] assayed poly(G) synthesis with *E. coli* RNA pol on poly(dC) with calf thymus DNA as the competitor. A reduction of poly(G) synthesis was observed as a result of RNA pol partition between template and competitor. Interestingly, DNA in which 5% of the bases were methylated proved a better competitor than unmodified DNA, suggesting that the enzyme has a higher affinity for the alkylated competitor. This finding was corroborated recently by Gray and Phillips[143] who showed that when the UV *lac* promoter is cross-linked by nitrogen (**1**) or sulfur (**2**) mustards, it forms a complex with *E. coli* RNA pol that is more stable than that formed with the unmodified template. The monofunctionally binding analogue (**43**) exhibits only a slight stabilizing effect. These results could be due to one of the following: enzyme−DNA covalent binding by cross-linking agents, a change in DNA conformation, or a different charge distribution in the major groove induced by alkylation.[143] Depurination at the promoter region impairs the binding of the enzyme.[144]

Data on the binding properties of promoters bearing 20 nitracrine molecules per 10^3 nucleotides were obtained from competition experiments with calf thymus DNA[145] and from filter-binding assays with T7 DNA.[97] Results from these experiments indicate that both the amount of enzyme saturating the template and the binding kinetics are similar for free and covalently modified DNA. However, the RNA pol complex with modified T7 DNA exhibits lower stability at high ionic strength than that of the complex with the unmodified promoter. Similar experiments using T7 DNA containing six 8-methoxypsoralen-modified bases per 10^3 nucleotides did not show any change in the RNA pol−promoter complex formation or complex stability.[97]

According to Chan *et al.*, osmium tetroxide adducts (**28**) located at T 10 or 12 bases upstream from the initiation site in the bacterial promoter, enhance open promoter complex formation. Most likely this induces a local melting of the double helix at the adduct site that indirectly favors RNA pol binding[146] but does not alter the initiation and elongation steps. Corda and co-workers[147] have shown that with bacterial RNA pol and sequences bearing cisplatin intrastrand

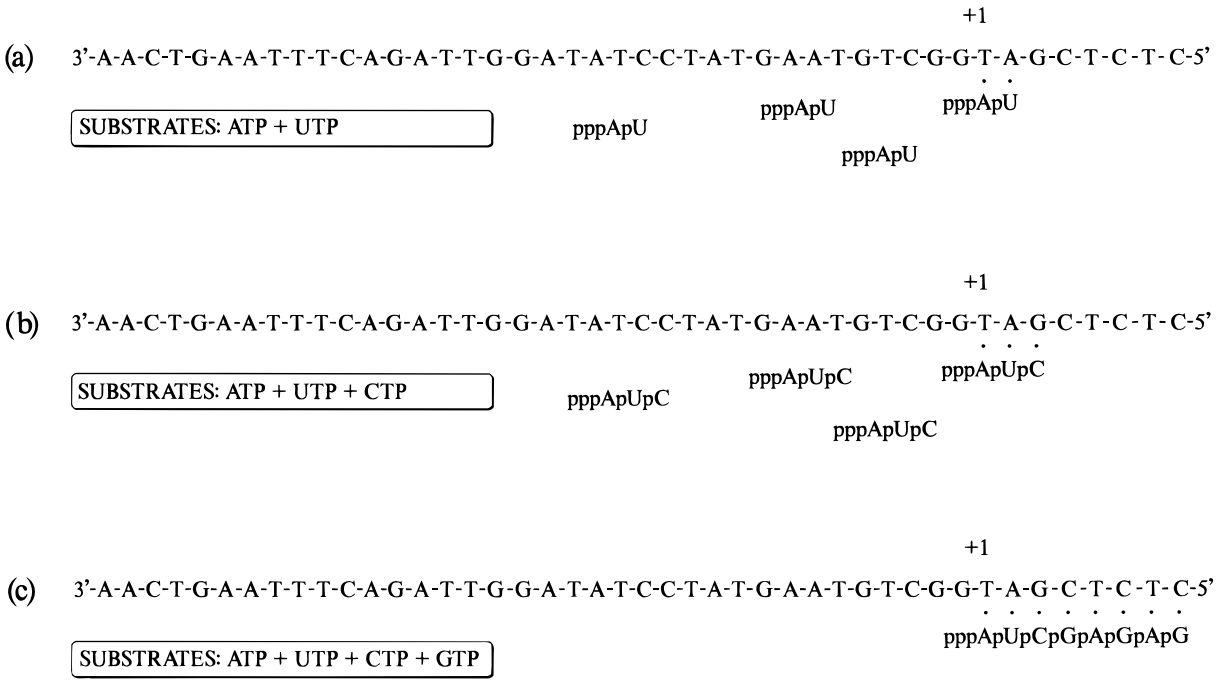


Figure 4. Abortive and productive initiation on A1 T7 promoter by *E. coli* RNA pol. Depending on the set of substrates used, either the dinucleotide (a) or the trinucleotide (b) is synthesized, or productive synthesis on A1 phage T7 promoter occurs (c); σ factor is released then (Figure 2c), and the enzyme enters the elongation step. (For sequence of the promoter, see: Dunn, J. J.; Studier, F. W. *J. Mol. Biol.* **1983**, *166*, 477.)

cross-links at either G-G (**30**) or A-G (see Figure 5) the enzyme recognizes these two covalent modifications differently. In fact, only the former lesion causes a decrease in the binding affinity of RNA pol for the promoter.

B. Initiation of Polyribonucleotide Chains

Initiation events can be analyzed by measuring the number of abortively or of stably initiated RNA chains. The substrates used to measure abortive initiation are the ribonucleotides corresponding to the first few bases of the RNA sequence. For example, when only ATP and UTP are added to *E. coli* RNA pol and T7 DNA, the enzyme repeatedly synthesizes pppApU on its promoters (Figure 4).[148,149] In some experiments, ribodinucleotides corresponding to the first two bases of the RNA chain are used as initiating substrates[150] (see Figure 5). The number of initiated RNA chains is measured by using γ (or β) ^{32}P-labeled nucleoside triphosphates. If γ-^{32}P- and ^{3}H- or ^{14}C-labeled NTP are used in an experiment, the average transcript length can be calculated by dividing the total number of bases (amount of ^{3}H or ^{14}C) by the number of chains (amount of ^{32}P).

Inhibition of abortive initiation was similar for both cross-linked DNA (8-methoxypsoralen) and that containing exclusively monoadducts DNA (angelicins). In these systems, inhibition of pppApU synthesis was considerably lower than the inhibition of total RNA synthesis.[97,98,151] This may be due to the presence of a relatively greater number of covalent modifications on long templates, compared to few adducts on the short initiation DNA region. Furthermore, the formation of the adduct at the initiation site (A-T) of T7 promoters (Figure 4) is less likely because of the furocoumarin binding sequence preference (see section II).[60] Hence, adducts located a few base pairs

Figure 5. Oligonucleotide repeating motives in polydeoxynucleotides used for the assay of cisplatin and transplatin adducts with *E. coli* and wheat germ RNA pol II.[44,147,153] (a) Two variants with either G-G or A-G in the center were used. The oligonucleotides were reacted with cisplatin as indicated and then polymerized. The initiation site was assigned using dinucleotides of the type N-N, which were complementary to the sequences on the platinated or the complementary strand as indicated by the horizontal bars. The length of the transcript was regulated by a suitable set of the substrates. For example if UTP is used with GpG, a trinucleotide is synthesised on the lower strand. Directions of the transcription are indicated. (b) Oligonucleotide motive bearing cisplatin or transplatin.[44]

away should be responsible for initiation inhibition.

Initiation and total RNA synthesis were compared in other systems. For methylated *Micrococcus lysodeikticus* DNA, total RNA synthesis is decreased by 65%, while initiation is decreased by only 10%. Methylation of calf thymus DNA does not change the number of initiated RNA chains,[101] whereas nitracrine (**11**) adducts on calf thymus DNA cause a decrease in the number of GTP- and to a lesser extent ATP-initiated transcripts.[145]

Interestingly, a decrease in the total RNA synthesis can be sometimes accompanied by an increase in the number of initiated chains. Using *E. coli* RNA pol to transcribe T7 DNA modified with *N*-acetyl-2-aminofluorene (**14**), Millette and Fink[99] observed a 40% increase of transcription initiation and a de-

crease to 15% of total RNA synthesis at an adduct density of 1.7 per 10^3 DNA nucleotides.[99] Using T7 RNA pol and plasmid DNA carrying T7 promoter, Nath *et al.* observed a 13% increase in initiation and a decrease to 25% of total RNA synthesis, with 1.4 *N*-acetyl-2-aminofluorene (**14**) adducts per 10^3 nucleotides.[91] No increase in initiation was observed using templates modified with 2-aminofluorene (**13**),[91] which induces less severe distorsion in DNA structure than the corresponding acetylated derivative (Figure 1).[63,66] In parallel experiments with template bearing 1.6 benzo[*a*]pyrene (**37**) molecules per 10^3 DNA nucleotides, a 50% increase in initiation was accompanied by a 87% decrease in total RNA synthesis.[91] This phenomenon was observed only when the ligand was bound to the template strand.[152]

Initiation and total RNA synthesis were studied by Corda and co-workers using different initiating dinucleotides from both strands of DNA bearing a cisplatin intrastrand cross-link on G-G or A-G[147,153] (Figure 5a). Data show that wheat germ and *E. coli* RNA pol can, to some extent, initiate RNA synthesis when the initiation site assigned by the dinucleotides is platinated or located immediately before the lesion. Initiation at sequences complementary to platinum adduct sites was considerably enhanced when compared to the unmodified template.[153] These experiments suggest that covalent modifications which interfere with elongation (see section VII) may enhance initiation events.

An increase in initiation may be ascribed either to rapid dissociation and reassociation of the RNA pol with the initiation site or to DNA conformational changes in the promoter region induced by the adducts. In summary, studies on DNA adducts and transcription suggest that modifications occurring only on one strand, and not resulting in intercalative adducts (Figure 1) (e.g. aminofluorenes, cisplatin, benzo[*a*]pyrene, osmium tetroxide), induce "quasi-melted" regions in DNA. They do not inhibit initiation, and on the contrary may stimulate it by facilitating open complex formation or by creating alternative initiation sites for RNA pol. On the other hand, both mono- and bifunctionally bound intercalating agents stabilize the double helix and decrease DNA "melting". In this case, initiation events are reduced rather than increased.

VII. Elongation and Termination

The DNA adducts presented below cause premature stops in elongation resulting in an accumulation of shorter transcripts. Molecular analysis of these aborted transcripts sheds further light on the role played by alkylating agents and the characteristics of their binding sites.[154] These aspects will be discussed for each group of drugs separately and, where data are available, compared to experiments with DNA pols.

A. Small Monofunctional Adducts

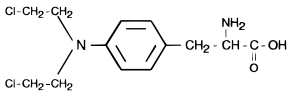
A difference between methylated and ethylated T7 DNA was reported by Mamet-Bratley in early studies.[94] Both these modifications inhibit total RNA synthesis to a similar extent (Table 1) but methylation causes a decrease in the average chain length of the transcript whereas ethylation does not.[155] The difference may be due to the distinct alkylation patterns of the agents used: methyl (**16**) and ethyl methanesulfonate. The former reacts mainly with N7 of G (**18**) and, to a lesser extent, N3 of A, while the latter causes ethylation of the oxygen atoms of G or phosphate residues.[155] However, DNA depurination occurring at the alkylation sites complicates the evaluation of data.[100,156] More recent experiments lead to the conclusion that N3 methylation of A affects T7 RNA pol although *E. coli* RNA pol is less sensitive.[157] Both initiation and total RNA synthesis are reduced to a similar extent, and no appreciable changes in RNA chain length are observed at low enzyme/template ratio. This result is ascribed to a slower movement of the enzyme along the template resulting in a decrease in reinitiations.[157] Neither methylation[158] nor monoalkylation with potentially bisalkylating drugs[159] of the N7 atom of G arrests T7 RNA pol, but both can cause DNA depurination and pausing of RNA pol at the abasic site.[158]

DNA pol appears to be more sensitive to alkylation. Using T7 DNA pol and an oligomer with a single ethylated T, Bhanot *et al.* observed that at low dNTP concentration 80% of chains are terminated just before the adduct and 14% at the adduct, and only 6% of chains are correctly elongated.[122] An increase in dNTP concentrations considerably enhances elongation past the lesion. Either A or T is incorporated opposite the monoadduct, but with ethylated T, A forms a base pair structure which is unsuitable for the addition of the next nucleotide. Therefore, only chains where T has been incorporated can be further elongated. These data suggest that DNA pol may elongate past an alkylated base adding next nucleotides to the mispaired nucleotide at the synthesized chain providing that the mispair retain a Watson−Crick-like alignment.[122]

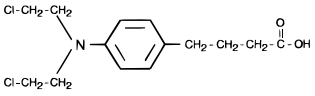
B. Nitrogen Mustard and Its Bis-Alkylating Congeners

E. coli RNA pol terminates transcription one base before G residues on nitrogen mustard modified DNA (**29**). The elongation stops can generally be ascribed to inter- and intrastrand cross-links, although some covalent lesions may be bypassed by the bacterial enzyme.[160] These results support the early hypothesis that nitrogen mustard intrastrand and interstrand cross-links are the major lesions responsible for *in vitro* transcription inhibition.[100] When the reaction is continued for up to several hours, the modified bases are hydrolyzed, and this in turn releases the transcription blockages.[144] The stops which are persistent under these conditions appear to correspond to interstand cross-links occurring between purines in the G-N-C/G-N-C or G-N-T/A-N-C sequences[144] (see also note 32). However, it has been reported that nitrogen mustard and other purine specific alkylating agents such as mephalan (**44**) and chlorambucil (**45**) induce transcription termination events that cannot be easily correlated with their sequence specificity.[161] Pieper *et al.* suggest that intrastrand cross-links can occur on A doublets and cause a clustering of transcription stops.[161,162] Recent

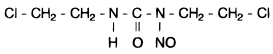
experiments with *N*,*N*′-bis(2-chloroethyl)-*N*-nitrosourea (**46**) with T7 and SP6 RNA pol indentify termination sites which correspond to interstrand cross-links between G and C.[159]



44



45



46

Taq DNA pol[163] have been used to analyze the sequence specificity of nitrogen mustards (**29**) and mephalan (**44**). These drugs terminated replication at $(G)_n$ ($n > 2$) sequences.[163] However, termination sites may correspond not only to the alkylated bases but also to the depurinated sites under the assay conditions.[163]

C. Cisplatin

E. coli, phage SP6 and T7, and wheat germ RNA pol II are used to characterize both cisplatin (**3**) and its inactive isomer *trans*-DDP (**4**), adducts.[42,44,153,159,164] A cisplatin intrastrand cross-link (Figure 5a) on the transcribed strand, but not on the non-transcribed strand, blocks bacterial, or wheat germ RNA pol II.[44,153] Although less efficient, transcription of sequences facing a cisplatin adduct has in fact been observed by Corda *et al.*[153] Bidirectional transcription with SP6 and T7 RNA pol has been used by Leng and co-workers to map cisplatin[165] and *trans*-DDP binding sites.[42] A different binding pattern at G-C sites (see section II) and a slower cross-linking rate for *trans*-DDP was revealed.[42] The cisplatin intrastrand cross-link at the G-T-G sequence (Figure 5b) induces transcription termination whereas the diadduct of *trans*-DDP may be bypassed by bacterial or wheat germ RNA pol.[44] Cullinane *et al.*[166] used bidirectional transcription to show that certain cisplatin derivatives, exhibiting a general reduction in interstrand cross-linking efficiency, form adducts that stop elongation only when located on the template strand.

In vitro replication with *Taq* DNA pol has also been used to map platination sites.[163,167−169] The monoadducts do not induce distinct replication stops.[6,170] Prokaryotic and eukaryotic DNA pols, like RNA pol, stop before cisplatin intrastrand cross-links on the template, leading to a similar termination pattern.[171,172] These stops are not absolute. Phage T7 and T4 and *E. coli* DNA pol I and III are capable of bypassing cisplatin adducts with different degrees of efficiency.[173] Phage T4 enzyme exhibits a high 3′ → 5′ exonuclease activity and bypasses cisplatin adducts very poorly.[173] *trans*-DDP intrastrand cross-link represents a weak barrier for both DNA[173] and RNA[44] pols, and bypassing is frequently observed. Bergnes *et al.* studied the 3′ → 5′ exonuclease activity of *E. coli* pol I on cisplatin- and *trans*-DDP-modified DNA and observed a release of monofunctionally platinated G.[174] The ability of DNA pol to bypass the lesion depends on the proofreading ability of the enzyme, the length of the template, the distance between the 3′ end of the primer and the lesion, and the sequence context of the adduct.[173] Klenow can sometimes bypass cisplatin cross-links on G-G, but not cisplatin cross-links on A-G.[175a] It was suggested by Bernges and Holler that cisplatin adducts may destabilize the complex between the template, the extended primer, and DNA pol, thus causing a halt to replication.[171]

D. Mitomycins

The effects of cross-linking by mitomycin C[54] and *N*-methylmitomycin A[175b] on DNA transcription have been studied with SP6 and T7 RNA pol. Elongation is interrupted at the interstrand cross-link[54] while only a few percentage of the monoadduct sites (namely, G residues) correspond to transcription stops. This could be due to a low yield of monoadduct formation or to the ability of the enzyme to bypass some of the monoadducts.[175b]

Replication past mitomycin C monoadducts is not observed with sequenase, klenow, *E. coli* DNA pol, and AMV RT. These enzymes terminate replication at the base preceding the lesion.[176] Bypass of the lesion by DNA pol can generally increase if Mg^{2+} is replaced for Mn^{2+} and the concentration of dNTP is raised.[130,134,173,177] Under these experimental conditions, however, incorporation of the nucleotide opposite the adduct occurs only if DNA pol lacks 3′ → 5′ exonuclease activity.[176]

E. Anthracyclines

Using *in vitro* transcription experiments, Cullinane and Phillips have studied the covalent interactions of anthracyclines with DNA.[54] Fe(III) ion mediated binding of adriamycin (**9**) to the template induces distinct elongation stops at the G residues in G-C sequences.[178] This adduct covers two base pairs on the template. This can be shown by comparing the stops induced on transcription from two promoters in opposite orientation. From sequence specificity of the stops the presence of interstrand cross-links on G[178] and covalent linking of both strands has been recently suggested.[93,179] Cyanomorpholinoadriamycin (**10**) binds to DNA by a different mechanism.[20,48,180] Its adducts induce distinct stops at both C-C and G-G sites and, to some extent, at G-C sequences. While the latter might be ascribed to interstrand cross-links, the stops at C-C and G-G are more likely to result from intrastrand cross-links. Interestingly, such intrastrand cross-links efficiently inhibit elongation even when located on the nontranscribed strand.[45,46]

F. Furocoumarins and Promazines

In vitro transcription studies using furocoumarins have been useful in determining the relative effect on transcription of monoadducts and interstrand cross-links. A priori, two possibilities should be considered: (i) the interstrand cross-link prevents the movement of RNA pol whereas the monoadduct is considerably less efficient; (ii) both cross-link and monoadduct are capable of stopping RNA pol. These possibilities have been addressed using the following

experimental approaches. Two products of the photoreaction of SV40 DNA with 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen were transcribed using *E. coli* RNA pol.[181] One template contained mainly monoadducts, the other one was heavily cross-linked. Elongation of the ribonucleotide chain by RNA pol was blocked two bases before the furocoumarin cross-link, and two bases before the T bearing the monoadduct. However, while monoadducts of promazine (**7**) derivatives assayed in the same study considerably decreased the number of initiated chains, they did not induce distinct stops in elongation.[181] It was concluded by Piette *et al.* that the promazine adducts are bypassed and inhibition is caused by single-stranded breaks in DNA.[182] Hearst and co-workers synthesized double-stranded polydeoxynucleotides containing *E. coli* RNA pol promoter and one 4′-(hydroxymethyl)-4,5′,8-trimethylpsoralen furan side monoadduct located 30 bases down from the initiation site either on the template or on the complementary strand.[183] The monofunctionally modified DNA was irradiated to generate an interstrand cross-link. In this case, the elongation by *E. coli*[183] and T7 RNA pol[184] was interrupted one base before the cross-link or the monoadduct located on the template strand. The lesions on the nontranscribed strand had no effect.[181,183]

The complexes of T7 and *E. coli* RNA pols and a polynucleotide bearing one 4′-hydroxy-4,5′,8-trimethylpsoralen molecule and the nascent transcript were assayed using footprinting with DNase I.[184−187] T7 RNA pol protects a region of 15 bases up from the adduct site on the template strand and about 10 bases on both sides of the lesion on the complementary strand, yielding identical protection patterns for the monoadduct on the coding strand and the interstrand cross-link.[184] At the interstrand cross-link, *E. coli* RNA pol protects a region of about 22 base pairs upstream and 15 base pairs downstream from the lesion.[185] The elongation complex arrested one base before the cross-link can then lose its RNA component while T7 RNA pol remains bound to the template for many hours.[186,187]

Psoralen monoadducts on a single-stranded template induce termination of *in vitro* replication. However, in nick translation experiments, *E. coli* DNA pol I only pauses at this lesion. A can still be correctly incorporated opposite the modified T.[188]

G. Carcinogens: 2-Aminofluorene, Benzo[*a*]pyrene, and Other Lesions

Premature transcription termination is also observed on DNA covalently modified with carcinogens. Polyacrylamide gel electrophoresis and average chain length measurements suggest that each *N*-acetyl-2-aminofluorene (**26**) adduct induces termination of transcripts synthesized by *E. coli* RNA pol on T7 DNA.[99] A transcription attenuation on calf thymus DNA bearing bulky benzo[*a*]pyrene adducts was shown by sucrose gradient analysis.[90] Nath *et al.* compared the effects of benzo[*a*]pyrene (**37**), *N*-acetyl-2-aminofluorene (**26**), and 2-aminofluorene (**25**) adducts on transcription of plasmid DNA with T7 RNA pol.[91] These three monofunctionally binding carcinogens induce discrete stops, but with different efficiency. Benzo(a)pyrene adducts block elongation of the T7 RNA pol[91] as well as rat liver RNA pol II in the short incubation time (10−45 min) usually used.[189] *N*-Acetyl-2-aminofluorene and, more often, 2-aminofluorene adducts can be bypassed by the enzyme.[70,91] The decrease of the average chain length is accompanied by an increase in initiation events (section VIB). The inhibitory effects of benzo[*a*]pyrene and aminofluorenes are strongly reduced when the adducts are located on the non-transcribed strand.[152,190] As in the prokariotic system, *N*-acetyl-2-aminofluorene and 2-aminofluorene adducts inhibit the RNA pol III transcribing 5S rRNA gene in the *Xenopus laevis* cell-free system, only when located on the coding strand.[190]

The effect on transcription of the four stereoisomers of benzo[*a*]pyrenediol epoxide (**37**) adducts located 16 bases downstream of the initiation site on double helical polynucleotide was studied by Choi *et al.* using T7 RNA pol.[191] The enzyme only pauses at the lesions since elongation stops disappear and the full length transcript is obtained by prolonging the incubation time. The efficiency of bypass differs considerably for stereoisomer adducts at N2 of G, the (+)-*trans* isomer being the most effective in stopping elongation. The synthesized oligonucleotides are terminated at the site three bases before the (−)-*cis* adduct. With any of the other three stereoisomers, the oligonucleotides are terminated just at the modified base.[191]

Hydrolysis of the glycosidic bond may lead to the loss of the modified base and formation of an abasic site. The enzyme pauses at this site. Hence this lesion may induce transcription termination or may be read as a purine (section V).[70,128,144,156−158] Recent studies indicate that when an abasic site is transformed into a single-strand break, T7 RNA pol may continue past the nick[70] and the product, one base shorter, is synthesized.[192] However *E. coli* and SP6 RNA pols are stopped at the nick.[128,192]

Carcinogens and mutagens also affect *in vitro* replication. Thrall *et al.* compared the effect of benzo[*a*]pyrene adducts on SP6 RNA pol and sequenase activities.[193] They concluded that adducts which arrest DNA pol do not efficiently block RNA pol. The effects of benzo[*a*]pyrene adducts on T7 DNA pol and T7 gene 4 protein (which unwinds DNA and synthesizes a primer) have also been studied.[194] The two proteins do not bypass the lesion, but whereas DNA pol dissociates immediately, gene 4 protein remains attached to the template at the lesion.

T4 and *E. coli* DNA pol I terminate replication one base before the *N*-acetyl-2-aminofluorene adduct but add one nucleotide opposite the 2-aminofluorene adduct.[195] In contrast, AMV RT correctly incorporates C opposite *N*-acetyl-2-(aminofluorenyl)guanine, but is unable to read G bearing a 2-aminofluorene adduct.[195]

E. coli DNA pol I is able to bypass, without pausing, 2-aminofluorene adducts on the template in nick translation experiments. G or A are incorporated opposite the lesion. Recent primer extension experiments indicate that 2-aminofluorene and to a lesser extent *N*-acetyl-2-aminofluorene adducts can be bypassed.[196−200] Generally, DNA pols with low 3′

Table 3. Effects of Adducts on Elongation in RNA Polymerase and DNA Polymerase Systems

drug	type of lesion[b]	base/sequence preference[c]	RNA pol			DNA pol		
			termination	bypass	references	termination	bypass	references
methyl and other small monoalkyl groups		G(A)	±	+++	158, 159	++±	±	122
nitrogen mustards	mono	G	±	+++	161			
	inter	G*-C/G*-C; G*-N-C/G*-N-C	++	+	143, 162, 164	+++	0	29, 163
	intra	G*-G*; G*-G*-G*; A*-A*	++	+	159, 161	+++	0	163
platinium	mono[e]	G(A)	±	+++	165, 166	±	+++	163, 167−170
cisplatin	inter	G*-C/G*-C	+++	−	44	++	+	173
	intra	G*-G*; A*-G*; (G*-N-G*)	+++	−	44, 153	++	+	167, 170
trans-DDP	inter	G*•C*	+	++	42	+	++	173
	intra	G*-N-G*	+	++	44	++	+	170, 173
mitomycins	mono	G	+	++	54, *d*	++	+	176
	inter	G/G	+++	−	54, *d*	+++	0	163
antracyclines								
adriamycin	inter ?	G*-C/G*-C	++	0	19, 54, 178			
CNMAdr	intra	G*-G*	++	+	20, 45, 46			
	inter	G*-C/G*-C	++	+	45, 46			
furocumarins	mono	T(C)	+++	−	181−187	+	++	188
	inter	T*-A/T*-A	+++	−	186	+++	±	188
fluorene								
AF	mono	G	+±	+±	68, 152	++	+	140, 141
AAF	mono	G	++	+	71, 90, 91, 99	++	+	140, 175, 196−201
benzo[*a*]pyrene	mono	G	++	+	91, 152	+++	−	194
			+	++	191			
abasic	sites		+	++	70, 128, 144, 156−158	++		163
	nick		++	+	192			

[a] CNMAdr = cyanomorpholinoadriamycin (**10**); AF = 2-aminofluorene (**13**); AAF = *N*-acetyl-2-aminofluorene. Terminations and bypasses are designed as not occurring (−), weak (+), moderate (++), strong (+++), if no data available (0). They are marked somewhat arbitrarily as the conclusions from different experimental approaches may be different. Generally a prolongation of incubation time, an increase of the substrates concentrations, and lowering in the case of DNA pol proofreading activity are factors favouring the bypasses. [b] Mono = monoadduct, inter = interstrand cross-link, intra = intrastrand cross-link. [c] G•C = base pair, G/G; G-C/G-C = bases or sequences located in complementary strands; a modified base in the sequence is denoted with an asterisk (*), N = any base, most often pyrimidine. [d] Cera and Crothers, personal communication. [e] Other platinum compounds resulting in monoadducts were often used.

→ 5′ exonuclease activity can incorporate a base opposite the lesion and continue replication.[199] The efficiency of the bypass depends on the sequence environment of the lesion.[200] The behavior of DNA pols at the modified site is carefully analyzed. Most DNA pols arrest replication one base before an *N*-acetyl-2-aminofluorene adduct, but klenow and sequenase add one base opposite the lesion.[176] Shibutani and Grollman compared the two adducts of *N*-acetyl-2-aminofluorene at C8 (**26**) and at N2 positions of G (**24**).[201a] The former is the major lesion formed but is easily repaired in the cell while the latter represents 5−15% of the total covalent modifications but is more persistent *in vivo*. C8 and, to a lesser extent, N2 adducts may be bypassed by klenow devoid of exonuclease activity. A is preferentially incorporated opposite the modified G.[201a] The results obtained by Belguise-Valladier *et al.* suggest that enzymes which exhibit 3′ → 5′ exonuclease activity remove the base incorporated opposite the lesion, whereas DNA pols devoid of proofreading function stall after incorporating the nucleotide opposite the lesion.[175a]

H. Bulky Substituents and Elongation

The data presented in this chapter suggest that the dimensions of DNA adducts play a role in inducing transcription termination. Bulky lesions on the template strand are more likely to stop elongation (Table 3). It is proposed that RNA pol tracking in either the major[201b] or minor groove[46] of double-helical DNA may be hindered by a bulky adduct. There are, however, structural requirements which are difficult to define for the covalent modifications necessary to arrest RNA pol. The monoadducts formed by promazine derivatives which do not induce distinct elongation stops in *in vitro* transcription[181] is but one example. A bypass of the other adducts particularly by smaller phage RNA pols can be observed. Usually the modifications on the complementary strand merely slow down the movement of RNA pol along the template. In replication experiments, DNA pols, particularly the enzymes devoid of 3′ → 5′ exonuclease activity, slowly incorporate a wrong base opposite the lesion and continue polynucleotide synthesis.

VIII. Concluding Remarks

This review has covered publications describing the effects of DNA alkylating compounds on the different stages of *in vitro* transcription. The experimental data indicate a general ability of covalent adducts to reduce the rate of transcription and generate RNA chains altered in size, sequences, or abundance.

Comparative data on the effects of covalent adducts on *in vitro* replication have also been reported. Interestingly, not every DNA covalent adduct that can arrest RNA pols blocks DNA pols. From the reported data, a few general comments can be made concerning the consequences of DNA covalent modifications on initiation, elongation, and fidelity of transcription.

RNA pol binding to the promoter is largely insensitive to the presence of covalent DNA lesions, and correct template-enzyme association is observed on covalently modified DNA. This may be due to the low incidence of adducts occurring in the relatively short promoter region. Some adducts (cisplatin, *N*-acetyl-2-aminofluorene, osmium tetroxide) can also induce structural distortions of the polynucleotide that may favor the binding of RNA pol, thus increasing the number of initiation events from preexisting promoters or inducing the formation of new initiation sites.

Both monoadducts and cross-links have a more dramatic effect on the elongation of transcription. Pausing of the RNA pol at the covalent adduct can lead to transcription termination. As proposed by von Hippel and Yager,[202] elongation and termination are competitive kinetic processes. Therefore RNA pol can pause and switch to a transcriptionally inactive conformation when the enzyme is stalled at the lesion, thus resulting in a stop in elongation. Specific adducts are discussed below.

Monoadducts, particularly bulky polycyclic substituents, are able to stop RNA pol when positioned on the template strand. Smaller monoalkylating agents do not usually cause transcription termination but can lead either to faulty incorporation of the base opposite the covalent modification, or a decrease in total RNA synthesis through slowing down the enzyme movement.

Premature termination of transcription is always observed before an interstrand cross-link, usually one or two bases before the lesion. As expected, the two DNA strands cannot be separated, and RNA pol is unable to read through the cross-linked site. Short transcripts accumulate; hence no functional RNA is produced. However, RNA pol can remain in a very stable elongation complex as shown by experiments with psoralen cross-linked DNA.[184] Hence conformational changes of the enzyme due to its transition from the initiation to the elongation phase can be followed (see Figure 2b,c).

Intrastrand cross-links located on the template strand cannot usually be bypassed by either RNA or DNA pols. The distortion in the polynucleotide structure caused by this lesion brings the transcription complex to a halt and the stalled RNA pol may be a signal for a repair of the lesion in the cell.[203] In both *in vitro* transcription and replication experiments, polynucleotide chains are terminated usually one base before the adduct.

Transcription fidelity is altered when RNA pol bypasses the covalent adducts and introduces errors in the transcript sequence. Miscoding can lead to the production of nonfunctional RNA, with dramatic consequences for the cell. Data on transcription fidelity can be compared with results from replication experiments using DNA pol devoid of proofreading activity. Base pair mismatches due to alkylation are observed in *in vitro* transcription and replication on modified templates and are confirmed as mutations at the cellular level.[14,15,66,73,204] Alkylation of exocyclic oxygens of bases by simple monofunctional compound formation of ethenobases or of abasic sites leads to miscoding. But methylation or ethylation at N7 of G, a common target for anticancer drugs, does not affect transcription fidelity.

The ability of covalent DNA adducts to alter RNA transcription has profound consequences for other cellular functions. There is evidence indicating that both the transcriptional control of the cell cycle[205−207] and the RNA content in the eukaryotic cell are closely related to the cell cycle kinetics.[208] These findings point to the importance of RNA synthesis inhibition in the mechanism of cytotoxicity of anticancer drugs. For nitracrine congeners, a close relationship between adduct-dependent inhibition of *in vitro* transcription and their cytotoxic effects has been observed.[38,209−212] It has been suggested that arrest in G_2 phase of the cell cycle by cisplatin is due to the inability of the cells to transcribe genes for mitosis.[213,214] Cisplatin is indeed a strong barrier for RNA pol[44] and, to lower extent, for DNA pol,[173,175a] while frequent bypasses of its ineffective isomer, *trans*-DDP, adducts are observed.[44,173] Actually a recent comparison of the effect of the two isomers on transcription in the cell[215] is consistent with the studies at the subcellular level.[44] These findings may provide a molecular basis for the different pharmacological properties of these stereoisomers. A relationship between DNA damage and alterations of RNA synthesis in cells treated with carcinogens has also been reported.[216,217] The inhibition of RNA synthesis by carcinogens leads to chromatin condensation in the cell.[218] The different effects of benzo[*a*]pyrene metabolites on *in vitro* transcription and replication are consistent with the different mutagenicity and carcinogenicity of the stereoisomers.[136,191]

In spite of all these reports, however, a direct relationship between *in vitro* data and *in vivo* activity is not always observed. The two systems differ in the density of DNA adducts, which is at least 10-fold higher in cell-free systems than in the cell.[102,219] Contribution of the effects on other multiple transcription factors[206,220] in the cells is not considered here. Nevertheless, most of the adducts discussed here are certainly formed in the cell,[71] resulting in similar sequence specificity[47,219] (see also Table 2). Experimental conditions as close as possible to the *in vivo* situation are needed to ensure the biological significance of the studies in cell-free systems. One possible approach would be the transfection of a modified template in cultured cells and analyses of the corresponding transcription products (e.g. refs 189 and 216).

A fundamental question which remains is how to target drugs onto active oncogenes, affecting the production of their mRNA and ultimately the expression of the oncoproteins, without altering the expression of normal functioning genes. Gene specificity has been reported for some alkylating agents: chlorpromazine preferentially inhibits the accumulation

of mRNA for lymphokines, interleukin 2, interferon-γ, tumor necrosis factor, and proto c-myc in human thymocytes.[221] Nitrogen mustard reduces the expression of amplified c-myc far more than that of constitutively activated genes in colon cancer cells.[222] Cisplatin preferentially decreases transcription from strong mammary tumor virus promoter in murine tumor cells.[223] The "antigene strategy" aims at targeting anticancer drugs by anchoring the reacting compound (psoralene[224] and azidoproflavine[225]) to an oligonucleotide whose sequence is complementary to a region of a specific oncogene.[226−229]

Some interesting questions remain to be answered. Are premature termination events responsible for antiproliferative property while interactions with promoters and initiation sites affecting regulation of genes induce mutagenic and cancerogenic effects? What are the structural requirements of the monoadduct in order that it induces a termination event? What is the mechanism of covalent lesion bypass and fidelity of transcription around the adduct? How does the enzyme select the base when misincorporation occurs? This review will hopefully contribute stimulating thought to future research which may lead to the answering of these questions.

IX. Abbreviations

A, adenosine; C, cytidine; G, guanosine; T, thymidine; U, uridine; N, any nucleoside; A·T or G·C, noncovalently hydrogen-bonded base pairs; A-C, covalently bound nucleosides with 5′(A) and 3′(C) free hydroxyl groups; NTP or pppN, nucleoside 5′-triphosphate; d (preceding a nucleoside symbol), deoxy, used when assignment of a nucleoside residue to deoxy or ribo series does not stem obviously from the text; DNA pol, DNA-dependent DNA polymerase; klenow = Klenow fragment of *E. coli* DNA pol devoid of exonuclease 5′ → 3′ activity; RNA pol, DNA-dependent RNA polymerase; RT, RNA-dependent DNA polymerase, reverse transcriptase; AMV, avian myeloblastosis virus; *E. coli*, *Escherichia coli*; DDP, diamminedichloroplatinium (II). Note that shortened forms of adduct names are used; hence benzo[*a*]-pyrene−DNA corresponds to the adduct formed by benzo[*a*]pyrene derivative benzo[*a*]pyrenediol epoxide, an ultimate carcinogenic form of this hydrocarbon.

X. Acknowledgments

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