Biochemical Pharmacology, Vol. 29, pp. 905–915.

0006-2952/80/0315–0905 $02.00/0

STUDIES ON THE EFFECTS OF THE ANTITUMOR AGENT CAMPTOTHECIN AND DERIVATIVES ON DEOXYRIBONUCLEIC ACID

MECHANISM OF THE SCISSION OF DEOXYRIBONUCLEIC ACID BY PHOTOACTIVATED CAMPTOTHECIN*

J. WILLIAM LOWN and HSIAO-HSIUNG CHEN
Department of Chemistry, University of Alberta, Edmonton, Alberta T6G 2G2, Canada

(*Received* 16 *May* 1979; *accepted* 25 *September* 1979)

Abstract—Aqueous solutions of the antitumor alkaloid camptothecin, its sodium salt, or a number of derivatives, when irradiated with 360 nm light in the presence of covalently closed circular DNA, produced single strand breaks in the DNA. The chromophore essential for the scission reaction consisted of intact rings A, B, C and D. The overall DNA breakage showed an inverse dependence on oxygen, and there is evidence for at least two reaction mechanisms. Photosensitization of the alkaloid may generate radicals which attack DNA or, in the presence of oxygen, generate hydroperoxy radicals. The photolytic reaction of camptothecin itself proceeded via formation of the photolabile camptothecin hemiacetal **4** which was isolated and characterized, and which suggests an alternative mechanism. In the anaerobic pathway, photodecarbonylation of the alkaloid may generate a diradical which can collapse to the racemized **4** or abstract hydrogen atoms from DNA leading to strand scission. In the presence of oxygen, the alternative aerobic pathway, in which hydroperoxy radicals are generated, can supervene leading to the generation of hydrogen peroxide and, then, the principal reactive species, the hydroxyl radical, which can then attack DNA. The intermediacy of these three species was proven unambiguously by (i) selective inhibition of scission with superoxide dismutase, (ii) selective inhibition with catalase, and (iii) spin-trapping and e.s.r. spectroscopy respectively. Certain antibiotics, which show a preference for binding to A,T rich regions of DNA, showed an enhancement in the extent of photoinitiated camptothecin cleavage. By contrast, antibiotics which have a preference for binding to G,C rich regions had a suppressing effect on such photodynamic DNA breakage. Natural negatively supercoiled CCC-DNA showed a greater extent of photoinitiated camptothecin breakage than DNA which had been relaxed with a topoisomerase. These results may indicate a weak intercalative interaction of the alkaloid into the G,C regions of DNA. The relative efficiencies of several synthetic analogues of camptothecin in promoting photoinduced DNA cleavage were compared.

Camptothecin **2** is a cytotoxic alkaloid derived from the the bark and stem wood of *Camptotheca acuminata* (family Nyssaceae), a tree native to China. The structure was elucidated by Wall *et al.* [1, 2]. Camptothecin inhibits the growth of a wide range of experimental tumors including murine leukemia L1210, Walker 256 rat carcinosarcoma, L5178Y, K1964 and P388 leukemias, Novikoff hepatoma *in vitro*, mastcell P815 sarcoma and reticulum cell sarcoma (A-RCS) [1, 3–8]. Preliminary clinical evaluations of the antitumor effects of camptothecin were encouraging [9, 10]. However, subsequent clinical trials demonstrated severe dose-dependent toxic side-effects [11–13]. It is clear that there is much room for improvement in its clinical effectiveness; this may come about by an understanding of its molecular mechanism of action. There is extensive evidence, including the rapid inhibition of nucleic acid synthesis [14–16], the observed fragmentation of DNA *in vitro* [14, 17], and the lack of effect of camptothecin on proteins including purified DNA polymerases and RNA polymerases [14], that nucleic acids are the principal cell target sites and probably underlie the cytotoxic effects of camptothecin [18]. There is no information on the molecular mechanism by which the observed degradation of DNA occurs. This prompted the present study of the effects of camptothecin and a number of derivatives on nucleic acids.

* Studies related to antitumor antibiotics—XIX.

EXPERIMENTAL

Materials

Camptothecin and the camptothecin sodium salt were supplied by Dr. Harry B. Wood, Jr. of the National Cancer Institute, Bethesda, MD. The synthetic camptothecin analogues **8–16** were supplied by Prof. C. R. Hutchinson, School of Pharmacy, University of Wisconsin, Madison. The synthetic camptothecin intermediates **17** and **18** were supplied by Prof. G. Stork, Department of Chemistry, Columbia University, New York. Olivomycin, chromomycin A_3 and NADPH were obtained from CalBiochem, San Diego, CA. Distamycin was obtained from Boehringer Mannheim Canada Ltd., Begin, St. Laurent, Quebec. Netropsin was a gift from American Cyanamid, Lederle Laboratories, Pearl River, NY. Ethidium bromide, disodium

EDTA, spermine and spermidine were from the Sigma Chemical Co., St. Louis, MO. Superoxide dismutase (EC 1.15.1.1; beef liver erythrocytes) was from Miles Laboratories, Elkhart, IN., and catalase (EC 1.11.1.6; beef liver) was from the Aldrich Chemical Co., Milwaukee, WI. PM2-DNA was prepared as described previously [19]; it contained at least 87–92% CCC form. Partially depurinated PM2-CCC-DNA was prepared by acid treatment, as described before [20], and its properties were confirmed by its characteristic behaviour with the apurinic site-specific endonuclease VI [21]. Calf thymus topoisomerase was prepared as described by Pulleyblank and Morgan [22]. The stock solution was stored in 50% glycerol at −20°. The activity was such that 2 μl of this solution were sufficient to completely relax 2.5 μg PM2-CCC-DNA in 15 min at 37°. Endonuclease VI was purified according to Verly and Rassart [21] from *Escherichia* BATCC 11303.

Melting points were determined on a Fischer–Johns apparatus and are uncorrected. The i.r. spectra were recorded on a Nicolet 7199 F.T. spectrophotometer; only the principal sharply defined peaks are reported. The n.m.r. spectra were recorded on Varian A-100 and Bruker 400 analytical spectrometers. The spectra were measured on approximately 10–15% (w/v) solutions in appropriate deuterated solvents with tetramethylsilane as standard. Line positions are reported in parts per million from the reference. Absorption spectra were recorded with a Beckman model DB spectrophotometer. Mass spectra were determined on an Associated Electrical Industries MS-9 double focussing high resolution mass spectrometer. The ionization energy in general was 70 eV. Peak measurements were made by comparison with perfluorotributylamine at a resolving power of 15,000. Kieselgel DF-5 (Camag, Switzerland) and Eastman Kodak precoated sheets were used for thin-layer chromatography. Preparative layer chromatography was performed with 20 × 20 cm, 1 mm thick plates coated with silica gel (E. M. Reagents, Darmstadt, Germany).

Methods

Fluorescence assay for detecting single strand breakage thermally or photochemically in DNA by camptothecin and derivatives. All measurements were performed on a G. K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to minimize fluctuations in the xenon lamp source. Wavelength calibration was performed as described in the manual for the instrument. One centimeter-square cuvettes were used. The excitation wavelength was 525 nm; the emission wavelength was 600 nm. The 100× scale of medium sensitivity was generally used; water was circulated between the cell compartment and a thermally regulated bath at 23°.

Reactions were performed at 21° in a total volume of 200 μl buffered with 50 nM potassium phosphate at pH 7.0. Reaction solutions had concentrations of 1.0 A_{260} units/ml of PM2-CCC-DNA (92% CCC), 1×10^{-5} M sodium camptothecin, and other components as indicated in the legends to the figures.

For the photochemical experiments the solutions were irradiated with a Sylvania black light at 360 nm, at an intensity of 17.50 ergs/mm^2/sec (determined by means of a 0.15 M potassium ferrioxalate chemical actinometer). In control experiments it was shown that light of this wavelength and intensity had no effect on DNA. Aliquots of 20 μl were removed at intervals and analyzed for the extent of strand breakage by addition to 2.3 ml of the standard pH 11.8 assay solution. This contained 20 mM potassium phosphate, pH 11.8, 0.2 mM EDTA and 0.5 μg/ml of ethidium bromide. The conversion of PM2-CCC-DNA to nicked circular DNA resulted in a 30 per cent increase in fluorescence in the alkaline ethidium assay solution. This contained 20 mM potassium after a heating and cooling cycle since the strands of DNA were then separable [19]. The fluorescence was measured using a blank without added sample which showed a 92 per cent return of fluorescence after the heating and cooling cycle. For the heat denaturation, the solutions were heated for 4 min at 96° on a Temp-Blok and cooled to 23°.

Preparative photolysis of camptothecin and sodium camptothecin. A 10 ml aqueous solution of sodium camptothecin (30 mg, 77 mM) was irradiated with a Sylvania black light at 360 nm for 16 hr at room temperature. A white precipitate formed which was collected and washed with cold water to give **4** [21 mg, (85% yield) m.p. 218° (dec)]. The hemiacetal **4** was comparatively pure but was further purified by preparative thick-layer chromatography using $CHCl_3$–CH_3OH (9:1) as eluant.

The mass spectrum showed mol. ion (calcd. for $C_{19}H_{16}N_2O_3$) 320.1161. Found 320.1160, 313 (M^+-OH), 291 (M^+-CH_2CH_3). The n.m.r. spectrum showed δ (pyridine-d_5 TMS), 0.82 (t 3H, CH_3 J = 7 Hz), 2.07 (m, eleven lines, 2H, -CH_2-CH_3), 5.00 (s, 2H, -CH_2-N), and 5.05 (ABq, 2H-CH_2O). Double irradiation at δ 2.07 gave a singlet at 0.82, and similar double irradiation at δ 0.82 gave an AB quartet at δ 2.07. In the infra-red spectrum the signal at 1750 cm^{-1}, characteristic of lactone stretching in the starting material, disappeared in the photolysis product hemiacetal.

The identical product **4** was obtained from the photolysis of camptothecin following the same procedure except that the solvent system was H_2O–pyridine (4:1).

Electron spin resonance. Electron spin resonance spectra were obtained on a Bruker ER-400 ESR spectrometer fitted with a Varian V3601 12 inch magnet with a VFR Hall effect controller. Hyperfine couplings were obtained by comparison with peroxylamine disulfonate (spacing 13.0 G), and g values were obtained by direct Fieldial measurement.

Spin trapping and e.s.r. detection of hydroxyl and formyl radicals from photolysis of sodium camptothecin and the camptothecin hemiacetal photolysis product **4**. A solution, containing 1.25×10^{-4} M sodium camptothecin, or compound **17**, 75 mM potassium phosphate buffer, pH 7.0, 6.0×10^{-2} M phenyl *N-tert*-butylnitrone, and 12.5% of methanol–acetonitrile (1:1), was photolyzed with a Sylvania 360 nm lamp for 2 hr at room temperature. The e.s.r. spectrum of the PBN·OH· nitroxide radical was recorded as a triplet of doublets with hyperfine splitting (hfs) $a^N = 16.0$ G, $a_\beta^H = 3.5$ G and g = 2.0061 (Fig. 4a) [23, 24].

A similar experiment was performed substituting the camptothecin hemiacetal **4** for camptothecin. The e.s.r. spectrum of the identical PBN·OH adduct was recorded but at a somewhat increased intensity relative to its generation from camptothecin.

A control experiment performed as above, but containing no alkaloid, showed that no radical species were generated.

A solution containing 1.25×10^{-3} M sodium camptothecin, 50 mM potassium phosphate buffer, pH 8.0, and 50 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was photolyzed with a Sylvania 360 nm lamp for 2 hr at room temperature. The e.s.r. spectrum of the DMPO·CO_2^- nitroxide radical was recorded as a triplet of doublets with hfs $a^N = 15.8$ G, $a^H = 19.0$ G and $g = 2.006$ (Fig. 4b). A control experiment performed as above but without photolysis of the sodium camptothecin showed that no radical species was produced.

Relaxation of supercoiling in PM2-CCC-DNA by calf thymus topoisomerase in the presence and in the absence of potential intercalating agents. The reactions were carried out on a 200 μl scale. The reaction mixture contained 10 mM Tris-hydrochloride buffer, pH 8, 1 mM EDTA, 0.2 M sodium chloride, approximately 1.0 A_{260} units/ml of PM2-CCC-DNA (92% CCC), and 1×10^{-5} M camptothecin or analogue. The control did not contain any alkaloid. Five microliter aliquots of calf thymus topoisomerase were added to both the sample and the control, and the mixture was incubated at 37°. Twenty microliter aliquots were withdrawn at intervals and assayed as before. The basis of the assay is that the topoisomerase relaxes the supercoiling of PM2-CCC-DNA by a nicking–closing mechanism which produces a 30 per cent decrease in ethidium fluorescence both before and after the heat denaturation–cooling cycle because of differences in topological constraints of the two forms of DNA [25]. Compounds which bind intercalatively to DNA will also untwist the duplex and remove the supercoils so that subsequent treatment with the topoisomerase will have no effect.

RESULTS

Solutions of sodium camptothecin show an absorption maximum at 370 nm. When a solution of 4×10^{-7} M sodium camptothecin was irradiated on the threshold of visible light at 360 nm in the presence of PM2-CCC-DNA, 15 per cent cleavage was observed in 60 min, as determined by the ethidium fluorescence assay method. The extent of DNA cleavage under these conditions increased with the concentration of the alkaloid: 1×10^{-6} M (25 per cent), 2×10^{-6} M (50 per cent), 5×10^{-6} M (70 per cent), 1×10^{-5} M (85 per cent) (Fig. 1) and 2×10^{-5} M (95 per cent). The DNA strand cleavage by photoactivated camptothecin showed a sharp wavelength dependence. A 2×10^{-5} M solution of sodium camptothecin gave, for different wavelengths, the following cleavage: 254 nm (0), 360 nm (75 per cent), 436 nm (0), 546 nm (0) and 577 nm (0). The observed extent of nicking caused by photoactivation of camptothecin or **17** at 360 nm showed an inverse dependence on oxygen concentration. Thus, an oxygenated solution of 1×10^{-5} M sodium camptothecin gave 37 per cent scission in 60 min, whereas careful degassing with argon resulted in enhanced nicking to 96 per cent in 60 min under photoactivation conditions (Fig. 2).

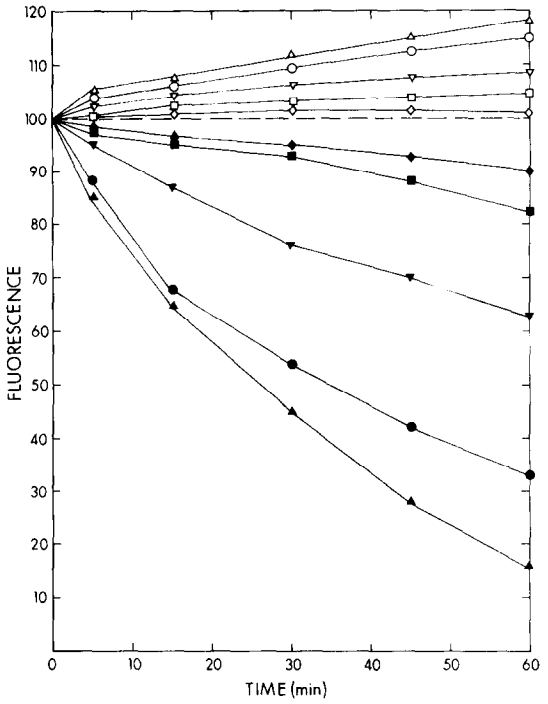


Fig. 1. Strand scission of PM2-CCC-DNA by sodium camptothecin under 360 nm irradiation and its selective enzymatic inhibition. Reactions were performed at room temperature in 50 mM potassium phosphate buffer, pH 7.0, and contained 1.0 A_{260} units/ml of PM2-CCC-DNA (92% CCC) and 1×10^{-5} M sodium camptothecin. The before heat denaturation ethidium fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation at 96° for 4 min followed by rapid cooling to 23°. Additional components were: (△ — △) none; (○ — ○) 5×10^{-6} g/ml catalase; (▽ — ▽) 5×10^{-5} g/ml superoxide dismutase; (□ — □) 1.5×10^{-2} M sodium benzoate; and (◇ — ◇) 0.6 M isopropyl alcohol.

Under aerobic conditions the breakage of DNA could be suppressed by selective inhibitors (Fig. 1). Catalase (5 μg/ml) resulted in 25 per cent inhibition, superoxide dismutase (50 μg/ml) gave a 55 per cent suppression, sodium benzoate (15 mM) an 80 per cent inhibition, and isopropyl alcohol (0.6 M) a 90 per cent inhibition.

The corresponding heat denatured enzymes at the same concentrations, however, resulted in no inhibition. Under the aerobic reaction conditions the singlet oxygen scavenger, histidine, had no effect on the observed extent of DNA breakage. The difficulty in removing the last traces of oxygen is indicated by the observed suppression of nicking that occurred with catalase and superoxide dismutase after extensive degassing with argon.

Preparative scale photolysis, at 360 nm, of solutions of either camptothecin or sodium camptothecin afforded cleanly the ring E modified hemiacetal **4** as a white solid (Fig. 3). The composition was established by accurate mass measurement at 320 as

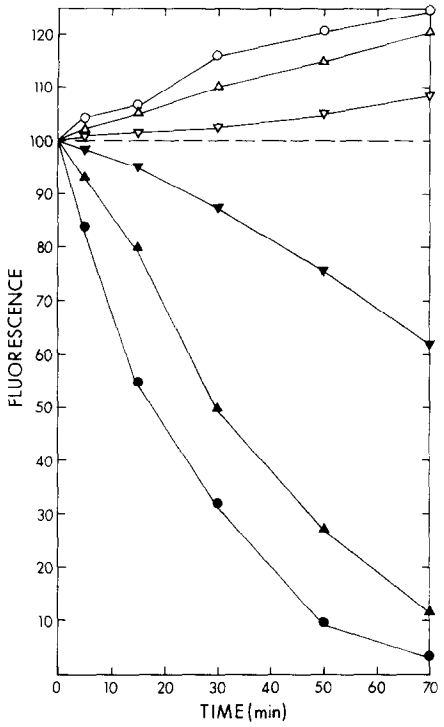


Fig. 2. Effects of oxygen on the rates of strand scission of PM2-CCC-DNA by sodium camptothecin under 360 nm irradiation. Reactions were performed at room temperature in 50 mM potassium phosphate buffer pH 7.0, and contained 1.0 A_{260} units/ml of PM2-CCC-DNA (92% CCC) and 1×10^{-5} M sodium camptothecin. The before heat denaturation ethidium fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after heat denaturation at 96° for 4 min followed by rapid cooling to 23°. Additional components were: (△ — △) none; (○ — ○) degassed with Ar; and (◇ — ◇) purged with O_2.

$C_{19}H_{16}N_2O_3$, using high resolution mass spectrometry. The infra-red spectrum shows OH at 3300 cm^{-1}, but no ketone carbonyl, in accord with the hemiacetal **4** rather than **6**. The 400 MHz n.m.r. spectrum showed a triplet at 0.82 δ which simplified to a singlet upon irradiating the 2.07 δ signal, and an eleven-line multiplet at 2.07 δ which simplified to a 7 Hz AB quartet upon irradiation of the 0.82 δ signal. There was no evidence of any additional coupling on the methylene protons which were clearly diastereotopic in accord with the proposed hemiacetal structure **4** in which ring E had been modified.

Further evidence in support of the suggested mechanism of formation of hemiacetal **4** from **2** is the observation that, whereas the chiral alkaloid **2** gives $[\alpha]_D^{22} = +33.8°$ in $CHCl_3$–CH_3OH, 4:1 (lit. $[\alpha]_D^{25} = +31.8$ [1]), by contrast the hemiacetal **4** was optically inactive in accord with its racemization via diradical **5**.

In addition, photolysis of sodium camptothecin **1** in the presence of DMPO afforded the stable DMPO·CO_2^- nitroxide radical, identified by its characteristic hyperfine splittings (hfs) of $a^N = 15.8$ G and $a^H = 19.0$ G (Fig. 4b) in accord with published values [26].

The purified compound **4** at 1×10^{-5} M, when irradiated at 360 nm in a solution of PM2-CCC-DNA containing 2.5% MeOH–CH_3CN (1:1), nicked the latter to the extent of 58 per cent in 60 min, i.e. closely comparable with that produced by the parent alkaloid or that of its sodium salt. The DNA scission produced by hemiacetal **4** was also subject to the oxygen effect. Thus, whereas irradiation of a 1×10^{-5} M solution of **4** containing oxygen induced 28 per cent cleavage of DNA, careful degassing with argon resulted, under comparable conditions, in 93 per cent DNA scission in the same time of 60 min. The DNA scission produced by **4** under these conditions was also selectively inhibited by catalase and

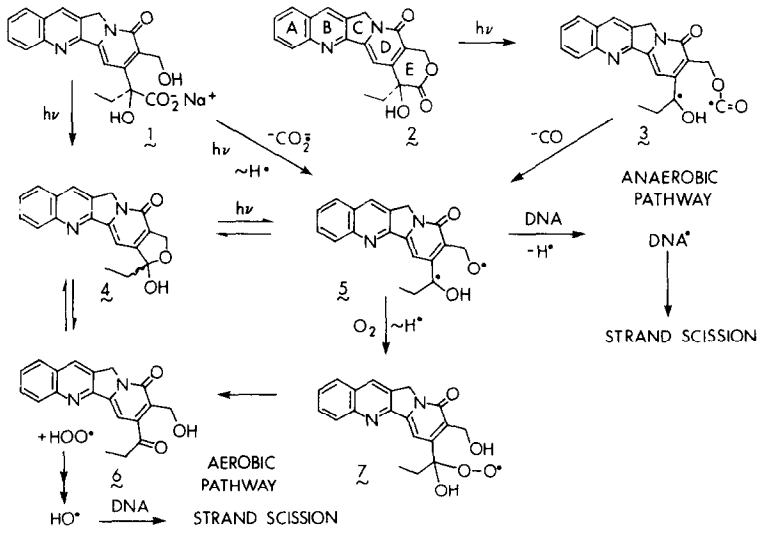


Fig. 3. Photolysis of sodium camptothecin **1** and camptothecin **2** at 360 nm to produce camptothecin hemiacetal **4** and, in the presence of DNA, strand scission by aerobic and anaerobic pathways.

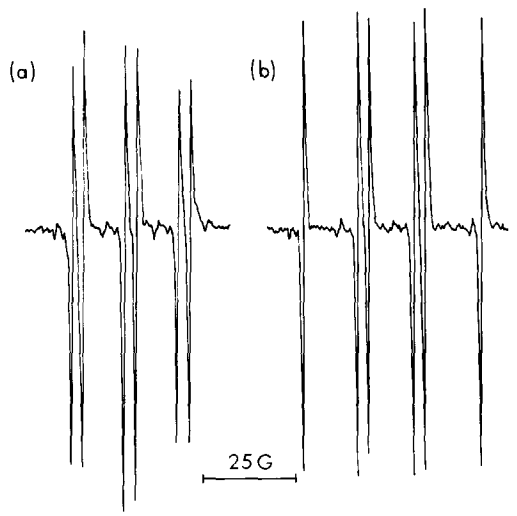


Fig. 4. Panel a: Electron spin resonance spectrum (microwave power 25 mW, modulation amplitude 0.2 G, scan time 1000 sec) of the PBN·OH· radical obtained by 360 nm photolysis of sodium camptothecin or compound **17** in the presence of 0.2 M PBN at pH 7.0. Hfs $a^N = 16.0$ G, $a_\beta^H = 3.4$ G and g = 2.0061. Panel b: Electron spin resonance spectrum (microwave power 25 mW, modulation amplitude 0.2 G, scan time 1000 sec) of the DMPO·CO_2^- radical obtained by 360 nm photolysis of sodium camptothecin in the presence of 50 mM DMPO at pH 8.0. Hfs $a^N = 15.8$ G, $a^H = 19.0$ G and g = 2.006.

by superoxide dismutase. Thus, the intermediacy of $O_2^{\cdot-}$ and H_2O_2 in the action of photoactivated camptothecin on DNA was unambiguously established by their selective enzymatic removal. While the generation of the hydroxyl radical and its direct role in degrading DNA were implicated by inhibition of scission by free radical scavengers, nevertheless, owing to the transient and reactive nature of the OH· species, more direct proof of its intermediacy was required. Irradiation of solutions of camptothecin or sodium camptothecin or of analogues **17** or **18** in methanol–acetonitrile (1 : 1), at 360 nm, in the presence of oxygen and of the spin-trapping agent phenyl *N-tert*-butylnitrone generated the six-line e.p.r. spectrum $a^N = 16.0$ G, $a_\beta^H = 3.4$ G and g = 2.0061 (Fig. 4a) characteristic of the PBN·OH· spin adduct [23, 24]. No such PBN·OH· spin-adduct was generated in the absence of the alkaloid.

The extent of 360 nm photodynamic campothecin-induced scission of DNA shows a dependence on the structure, especially of rings D and E, exemplified by the camptothecin analogues listed in Table 1. The essential structural feature required for photoinduced DNA cleavage was the moiety containing intact rings A, B, C and D which gave rise to the characteristic absorption in the 360 nm region. Thus, compounds which are not photolabile like camptothecin and sodium camptothecin, e.g. **15**—**18**, can nevertheless lead to DNA cleavage under 360 nm irradiation. Most of the camptothecin derivatives effected DNA scission to the extent of 54–92 per cent in 60 min under irradiation at 360 nm. The exception was compound **14** which bears a secondary alcohol moiety at C_5 and produced only 10 per cent cleavage.

In order to determine if camptothecin displays any DNA base preference, sequence specificity, or dependence on DNA topology, the effects of certain DNA sequence-specific binding agents on the camptothecin in photodynamic scission of DNA were examined. A solution of 1×10^{-5} M sodium camptothecin, when irradiated with DNA at 360 nm, showed a 60 per cent enhancement of nicking in 30 min in the presence of 1×10^{-4} M netropsin, the A,T specific binding antibiotic, compared with a similar experiment without netropsin. The structurally related A,T specific binder, distamycin, at 1.5×10^{-4} M showed a similar 30 per cent enhancement of nicking in 30 min by sodium camptothecin. By contrast, 5×10^{-5} M chromomycin A_3, which in the presence of an equivalent of $MgCl_2$ binds strongly and preferentially to G,C rich sites, caused a 67 per cent suppression of DNA cleavage in 2.5% aqueous acetonitrile in 30 min. The structurally related olivomycin at 5×10^{-5} M (also a G,C binder in the presence of an equivalent of Mg^{2+}), like chromomycin A_3, resulted in a 30 per cent suppression of DNA scission in 30 min in 2.5% aqueous acetonitrile, compared to controls. When aliquots of λ-DNA were added to netropsin in a pH 7.0 buffer, the absorption maximum of netropsin shifted from 322 nm to

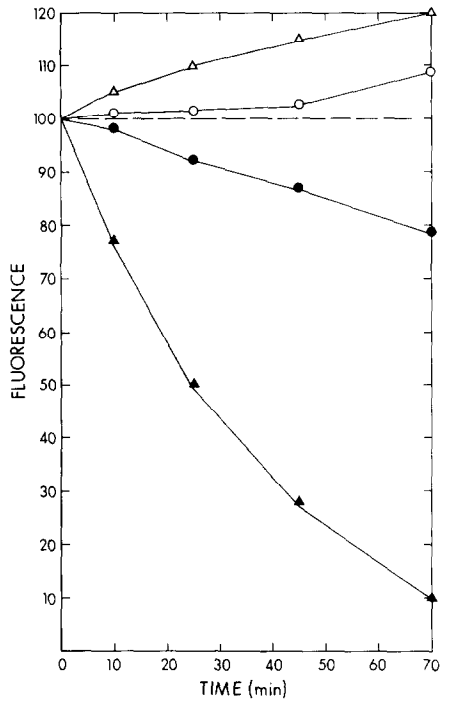
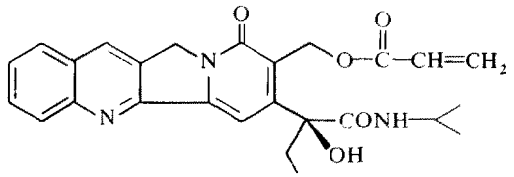
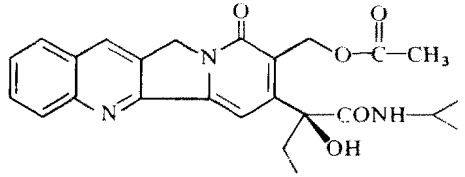
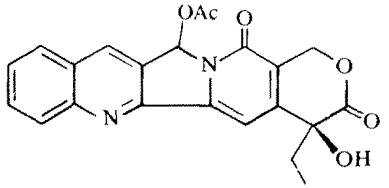
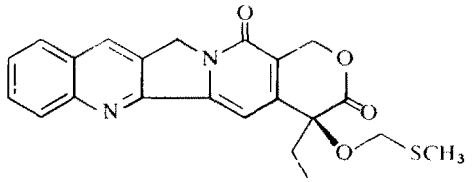
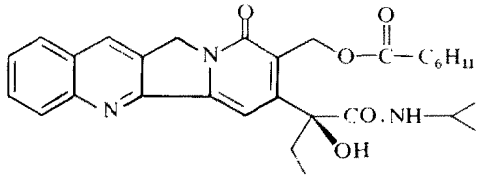
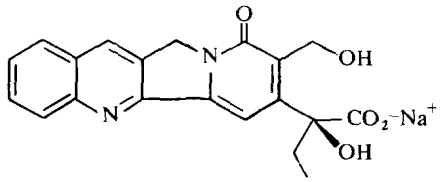
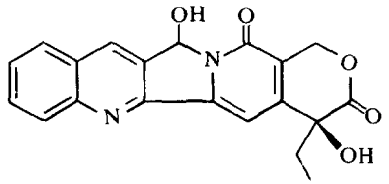
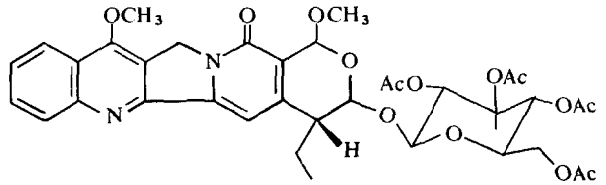
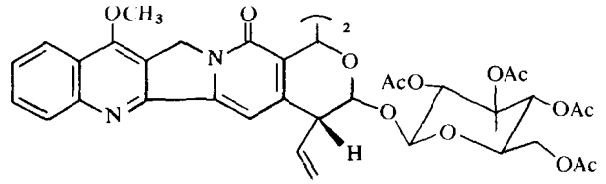
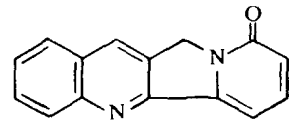
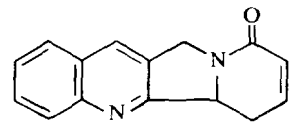


Fig. 5. Effect of the topological form of DNA on the rates of strand scission of DNA by sodium camptothecin under 360 nm irradiation. Reactions were performed at room temperature in 50 mM potassium phosphate, pH 7.0, and contained 1×10^{-5} M sodium camptothecin. The before heat denaturation ethidium fluorescence readings are shown as open symbols, and the closed symbols are fluorescence readings after heat denaturation at 96° for 4 min followed by rapid cooling to 23°. Additional components were: (△ — △) negatively supercoiled PM2-CCC-DNA (92% CCC); and (○ — ○) relaxed PM2-CCC-DNA (79% CCC) prepared by treatment with calf thymus topoisomerase and subsequent chromatography on A-15 M agarose.

Table 1. Cleavage of DNA photoinduced at 360 nm by camptothecin analogues*

		Scission (%)	λ_{max}	A
8		92	370	0.35
9		85	370	0.195
10		81	355	0.197
11		75	364	0.24
12		65	370	0.123

13		54	370	0.23
14		10	355	0.182
15		16	347	0.078
16		13	360	0.243
17		59	364	0.18
18		38	320	0.02

* Concentration of alkaloids, 1×10^{-5} M. Reaction time, 60 min. Reaction solvent, 2.5% CH_3CN in H_2O.

325 nm, indicative of binding, but at the concentration employed, 1×10^{-4} M, there was no significant absorption in the 360 nm region. Similarly, when aliquots of λ-DNA were added to 1×10^{-4} M distamycin at pH 7.0, the absorption maximum shifted from 332 nm to 338 nm and the absorption increased from 0.93 to 1.03, again indicative of binding, but there was no significant absorption at 360 nm at the concentrations employed in the camptothecin experiment. There are two absorption maxima for chromomycin A_3 at 408 nm and 320 nm. Addition of aliquots of λ-DNA caused the 408 nm peak to shift to 416 nm, while the 320 nm absorption remained unchanged. The intensity of the 408 nm peak increased from 0.34 to 0.377, while the intensity of the 320 nm peak remained unchanged. Olivomycin shows absorption maxima at 408 nm and 322 nm. Upon addition of aliquots of λ-DNA, the 408 nm absorption maximum moved to 418 nm with a concomitant increase of intensity from 0.34 to 0.36, while the 322 nm peak remained unchanged.

It is evident from the suppression of the fluorescence of ethidium in DNA in the presence of either netropsin or distamycin, compared to controls, that both of these antibiotics bind to DNA and in such a way as to remove potential ethidium intercalation sites. Subsequent treatment with calf thymus topoisomerase fully relaxed the negative supercoils of PM2-CCC-DNA, indicating that neither of the A,T specific binding agents intercalates into DNA. It may be noted that, while DNA-bound netropsin had no effect on the enzyme, free netropsin appeared to interfere with the nicking–closing action of the topoisomerase. Both olivomycin and chromomycin A_3 bind to DNA, as evidenced by ethidium fluorescence suppression, but the subsequent action of the topoisomerase on these DNA–antibiotic complexes relaxed the supercoils to a lesser extent.

In view of the results obtained on the effects of the sequence-specific binding agents on the efficiency of a camptothecin photoinduced DNA scission, the effects of the topological form of the DNA were assessed. Whereas a 1×10^{-5} M solution of sodium camptothecin with 360 nm irradiation gave 85 per cent nicking of PM2-CCC-DNA in 60 min, similar conditions using topoisomerase-relaxed PM2-CCC-DNA resulted in only 20 per cent nicking (Fig. 5). The difference observed was not due to the presence of the topoisomerase used to relax the DNA, since when the latter was removed by A-15M agarose chromatography the result on the extent of DNA cleavage was the same.

In addition, and in contrast to netropsin, neither camptothecin nor sodium camptothecin affected the combined endonucleolytic and ligase action of calf thymus topoisomerase. Solutions of 1×10^{-5} M sodium camptothecin irradiated at 360 nm cleaved PM2-CCC-DNA to the same extent, whether or not it had been depurinated previously by pulsed low pH treatment, when assayed by ethidium fluorescence at neutral pH. Neutral pH conditions were employed in this experiment because the apurinic sites are cleaved under strongly alkaline conditions.

In a parallel experiment, PM2-CCC-DNA was irradiated at 360 nm in the presence of 1×10^{-5} M sodium camptothecin until about 50 per cent of the DNA had been cleaved. The reaction was stopped and an aliquot was treated with endonuclease VI, an enzyme which specifically recognizes apurinic sites. No apurinic sites were formed during PM2-CCC-DNA cleavage by sodium camptothecin under irradiation at 360 nm.

DISCUSSION

Considerable evidence has been accumulated from biochemical and pharmacological studies that the mechanism of antitumor action of camptothecin is associated intimately with its effects on nucleic acids rather than on, for example, replication enzymes [14–18]. Thus, while the rapid inhibition of nucleic acid synthesis and the fragmentation of cellular DNA appear to underlie the cytotoxic effects of the alkaloid, the molecular mechanism by which the degradation of DNA occurs is not understood [18]. Provided the results are interpreted with caution, studies of the chemical modification of nucleic acids by antitumor agents *in vitro* can often permit a more detailed description of the molecular events associated with cytotoxity and represent a necessary first step in the examination of the action of these agents with DNA–histone complexes, nucleosomes, and then chromatin. Such studies have contributed to our understanding of the workings of such clinically important antitumor agents as bleomycin [27, 28], mitomycin C [29, 30], adriamycin [31], daunorubicin [32], maytansine [33] and nitrosoureas, e.g. 1,3.bis(2-chloroethyl)-1-metrosourea(BCNU) and (1,2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) [34, 35].

Progress toward a deeper understanding of the action of camptothecin has been hampered by the lack of a suitable *in vitro* method for studying its reactions with DNA. The present work was motivated by the desire to establish such a system and to determine the characteristics and structural dependence.

Camptothecin shows a sharply wavelength-dependent and selective photoactivation at 360 nm, near its absorption maximum, producing species which are capable of cleaving DNA. Under aerobic conditions the photoactivated alkaloid reacted with dissolved oxygen to generate reactive species including hydrogen peroxide, the superoxide anion and hydroxyl radicals (which are implicated in the single strand scission of DNA [36]); this was confirmed by the selective inhibition in the rate of DNA scission obtained by catalase and superoxide dismutase, and by general free radical scavengers, e.g. sodium benzoate, respectively. In addition, the fact that the heat-denatured enzymes catalase and superoxide dismutase, at the same concentration and under otherwise identical conditions, did not inhibit the cleavage demonstrates that the previous inhibition was not due to non-specific protein effects. The intermediacy of the hydroxyl radical in the scission process was confirmed by trapping it [37] as a PBM·OH· nitroxide spin adduct showing the characteristic six-line pattern with the correct hyperfine splittings. The results in Table 1 indicate that the structural moiety essential for photoinitiated DNA cleavage by camptothecin or its analogues was the

intact ABCD portion which gave rise to the characteristic absorption in the region of 370 nm.

For a given concentration of sodium camptothecin or of camptothecin intermediate **17**, the extent of photoinduced DNA scission increased as oxygen was removed from the system. This behaviour parallels that of the electron-affinic agents, e.g. metronidazole, misonidazole and *p*-nitroacetophenone [38, 39], when photolyzed in the presence of DNA, and indicates two distinct pathways are operative to cleave DNA, i.e. (i) photoactivation of camptothecin itself to generate DNA damaging species, and (ii) secondary reaction of the latter species to generate superoxide anions, hydrogen peroxide and hydroxyl radicals. The extreme sensitivity of the system to oxygen and the difficulty in removing the last traces of oxygen are illustrated by the fact that even after prolonged purging with argon the scission of DNA that accompanies 360 nm irradiation under these conditions may still be selectively inhibited by catalase and superoxide dismutase. There was no evidence that singlet-oxygen was produced during camptothecin irradiation. These results confirming the photoactivated generation of reactive oxygen species by camptothecin and its derivatives are analogous to recent reports that irradiation of photosensitizers such as rose bengal and other dyes in the presence of oxygen generates superoxide anions and other oxygen species [40, 41]. These results have been interpreted in terms of the following mechanism.

$$\mathrm{In} + h\nu \rightarrow \mathrm{In}^* \quad (1)$$

$$\mathrm{In}^* + \mathrm{R{:}H} \rightarrow \mathrm{In{\cdot}H} + \mathrm{R}^{\cdot} \quad (2)$$

$$\mathrm{In{\cdot}H} + \mathrm{O_2} \rightarrow \mathrm{In} + \mathrm{HO_2}^{\cdot} \quad (3)$$

The photoinitiator In (methylene blue, rose bengal, camptothecin, etc.), excited by absorption of a quantum of light, accepts a single electron from R:H which may be an organic solute or DNA (which then leads to strand scission) by reaction (2) under anaerobic conditions. In the presence of oxygen the initiator radical would be expected to relinquish its unpaired electron, yielding the perhydroxy radical. The latter may them lead to the detected species, superoxide anions, hydrogen peroxide and hydroxyl radicals (confirmed by the spin-trapping experiment), by the following steps. The generation of hydroxyl

$$\mathrm{HOO}^{\cdot} \rightleftharpoons \mathrm{O_2}^{\cdot-} + \mathrm{H}^+ \quad (4)$$

$$2\mathrm{O_2}^{\cdot-} + 2\mathrm{H}^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} \mathrm{H_2O_2} + \mathrm{O_2} \quad (5)$$

$$2\mathrm{H_2O_2} \xrightarrow{\text{catalase}} 2\mathrm{H_2O} + \mathrm{O_2} \quad (6)$$

$$\mathrm{DNA{\cdot}Fe^{3+}} + \mathrm{O_2}^{\cdot-} \rightarrow \mathrm{DNA{\cdot}Fe^{2+}} + \mathrm{O_2} \quad (7)$$

$$\mathrm{DNA{\cdot}Fe^{2+}} + \mathrm{H_2O_2} \rightarrow \mathrm{DNA{\cdot}Fe^{3+}} + \mathrm{OH}^{\cdot} + \mathrm{OH}^- \quad (8)$$

$$\mathrm{OH}^{\cdot} + \mathrm{DNA} \rightarrow \text{Strand breakage}$$

radicals in such systems was formerly attributed to the Haber–Weiss reaction (9) but this reaction has

$$\mathrm{H_2O_2} + \mathrm{O_2}^{\cdot-} \rightarrow \mathrm{H_2O} + \mathrm{OH}^{\cdot} + \mathrm{OH}^- \quad (9)$$

been shown recently to be quite slow ($k < 0.3\ \mathrm{M^{-1}\ sec^{-1}}$) and is unlikely to compete with other steps involving $\mathrm{O_2}^{\cdot-}$ [42–44]. It is now considered more likely that $\mathrm{OH}^{\cdot}$ radicals are generated by reaction of $\mathrm{O_2}^{\cdot-}$ and $\mathrm{H_2O_2}$ with traces of $\mathrm{Fe^{2+}}$ associated with DNA in steps such as (4) and (5) [45]. Hydroxyl radicals have been implicated in the direct degradation of DNA by attack primarily at the ribose sugar [36]. While the above mechanisms may account for the photoinitiated degradation of DNA by two distinct pathways under aerobic and anaerobic conditions for camptothecin derivatives **17** and **18**, there is evidence for the contribution of alternative pathways in the case of the parent alkaloid and derivatives involving modification to ring E.

The preparative scale photolysis of either camptothecin or sodium camptothecin afforded the hemiacetal **4** (Fig. 3) in excellent yield. Irradiation of **4** in the presence of DNA resulted in very efficient cleavage in a process which shows characteristics similar to those using the parent alkaloid, i.e. sharp wavelength dependence and evidence of two competing pathways, aerobic and anaerobic. These results may be rationalized as shown in Fig. 3. Irradiation of camptothecin **2** results in decarbonylation via species **3** to give the resonance stabilized diradical **5** which collapses to the hemiacetal **4** which was isolated and characterized. Irradiation of sodium camptothecin similarly affords hemiacetal **4**.

The intermediacy of diradicals **3** and **5** suggests an alternative mechanism for DNA scission. In the absence of oxygen, species **5** could abstract a hydrogen atom from the deoxyribose sugar of DNA leading to strand scission via an anaerobic pathway. In the presence of oxygen, an interaction between the intermediate diradical **5** and oxygen may produce the hydroperoxy radical. The overall result in the presence of oxygen may be interpreted by the aerobic pathway suggested in Fig. 3, although other detailed mechanisms leading to the formation of $\mathrm{OH}^{\cdot}$ may be envisaged.

The low observed extent of DNA cleavage of **14** (10 per cent) relative to **10** (81 per cent) may be mentioned. Compound **14** is the only derivative bearing a secondary alcohol grouping (at position 5) which, if photolysis is generating superoxide and hydroxyl radicals, could act as a radical scavenger and suppress the observed amount of DNA cleavage.

The effects of certain DNA sequence-specific binding agents on the photodynamic scission of DNA by camptothecin provide some insight into the influence of topological changes in the helix on the course of the reaction. Netropsin is an antibiotic that is though to bind preferentially to XTTTX sequences (where X is any nucleotide), i.e. A,T rich regions in the minor groove of bihelical DNA [46, 47]. Recent X-ray evidence on the characteristic shape of netropsin and models for its binding action support this contention [48]. The binding specificity of the structurally related antibiotic, distamycin A, is TTTTX, again in the minor groove of DNA [49]. Preincubation of the DNA with these two agents resulted in an enhancement in the extent of camptothecin-induced DNA scission compared with controls. By contrast, the complementary experiments with chromomycin A_3 and olivomycin, which are antibiotics that in the presence of an equivalent of $\mathrm{Mg^{2+}}$ bind preferentially to G,C rich regions in the minor groove of duplex DNA [50, 51], show sub-

stantial suppression of the photodynamic scission of DNA by camptothecin. Since none of the four antibiotics absorbed appreciably at 360 nm in the concentrations used, the observed effects are not due to preferential absorption of the incident light. The effects of the sequence-specific binding agents on the DNA scission by sodium camptothecin could be interpreted at this stage as showing either (i) a sequence preference for binding by the alkaloid and subsequent cleavage of DNA, and/or (ii) a topological change induced in the DNA by G,C selective binders which render the DNA less susceptible to the sodium camptothecin-induced cleavage and a different topological change induced by A,T selective binders which make the DNA more susceptible to cleavage. In support of the second interpretation it is clear that, whereas native negatively supercoiled PM2-CCC-DNA is readily cleaved, the relaxed form is much less susceptible to a camptothecin-induced scission. It is possible that the planar aromatic, camptothecin, intercalates with a short residence time into G,C rich regions of DNA. Since supercoiled DNA binds intercalators more strongly than relaxed DNA does [52], this may be sufficient to account for the observed differences due to topological form noted above. Such intercalation by camptothecin, if it occurs, must be relatively weak, since it could not be detected by the topoisomerase assay. Others have reported evidence of weak intercalative interaction of camptothecin [53].

In conclusion, it is evident that solutions of camptothecin or sodium camptothecin, when irradiated with near-visible light at their absorption maxima, can produce photodynamic cleavage of DNA. The reaction appears to take place by at least two distinct mechanistic pathways, aerobic and anaerobic, and proceeds via formation of an isolable but photolabile hemiacetal with the concomitant generation of radical species of sufficient reactivity to cleave DNA. The exploration of the characteristics of the photochemical mode of camptothecin degradation of DNA led to to the development of a thermal system for the degradation of DNA by the alkaloid. This procedure, more akin to the situation that obtains *in vivo*, will form the subject of a subsequent paper.

Acknowledgements—This research was supported by grants to J.W.L. from the National Cancer Institute of Canada, the Natural Sciences and Engineering Research Council of Canada, and by the Chemistry Department of the University of Alberta. The authors wish to thank Dr. C. R. Hutchinson for discussions pertaining to the subject of the paper.

REFERENCES

1. M. E. Wall, M. C. Wani, C. E. Cook, K. H. Palmer, A. T. McPhail and G. A. Sim, *J. Am. chem. Soc.* **88**, 3888 (1966).
2. A. T. McPhail and G. A. Sim, *J. chem. Soc.* 923 (1968).
3. J. M. Venditti and B. J. Abbott, *Lloydia* **30**, 332 (1967).
4. W. D. DeWys, S. R. Humphreys and A. Goldin, *Cancer Chemother. Rep.* **52**, 229 (1968).
5. T. R. Govindachari and N. Viswanathan, *Indian J. Chem.* **10**, 453 (1972).
6. S. Tafur, J. D. Nelson, D. C. Delong and G. H. Svolboda, *Lloydia* **39**, 261 (1976).
7. B. J. Abbott, *Cancer Treat. Rep.* **60**, 1007 (1976).
8. R. C. Gallo, J. Whang-Peng and R. H. Adamson, *J. natn. Cancer Inst.* **46**, 789 (1971).
9. J. A. Gottlieb, A. M. Guarino, J. B. Call, V. T. Oliverio and J. B. Block, *Cancer Chemother. Rep.* **54**, 461 (1970).
10. G. R. Pettit, *China Q.* **59**, 789 (1976).
11. J. A. Gottlieb and J. K. Luce, *Cancer Chemother. Rep.* **56**, 103 (1972).
12. C. G. Moertel, A. J. Schutt, R. J. Reitemeier and R. G. Hahn, *Cancer Chemother. Rep.* **56**, 95 (1972).
13. F. M. Muggia, P. J. Creaven, H. H. Hansen, M. H. Cohen and O. S. Selawry, *Cancer Chemother. Rep.* **56**, 515 (1972).
14. M. S. Horwitz and S. B. Horwitz, *Biochem. biophys. Res. Commun.* **45**, 723 (1971).
15. D. Kessel, *Biochim. biophys. Acta* **246**, 225 (1971).
16. D. Kessel, *Cancer Res.* **31**, 1883 (1971).
17. H. B. Bosman, *Biochem. biophys. Res. Commun.* **41**, 1412 (1970).
18. S. B. Horwitz, in *Antibiotics* III. *Mechanism of Action of Antimicrobial and Antitumor Agents* (Eds. J. W. Corcoran and F. E. Hahn), pp.48–57. Springer, New York (1975).
19. A. R. Morgan and D. E. Pulleyblank, *Biochem. biophys. Res. Commun.* **61**, 396 (1974).
20. J. W. Lown and L. W. McLaughlin, *Biochem. Pharmac.* **28**, 2123 (1979).
21. W. G. Verly and E. Rassart, *J. biol. Chem.* **250**, 8214 (1975).
22. D. E. Pulleyblank and A. R. Morgan, *Biochemistry* **14**, 5205 (1975).
23. J. R. Harbour, V. Chow and J. R. Bolton, *Can. J. Chem.* **52**, 3549 (1974).
24. C. S. Lai and L. H. Piette, *Biochem. biophys. Res. Commun.* **78**, 51 (1977).
25. J. S. Lee and A. R. Morgan, *Nucleic Acids Res.* **5**, 2425 (1975).
26. J. R. Harbour and M. L. Hair, *Can. J. Chem.* **57**, 1150 (1979).
27. E. A. Sausville, J. Peisach and S. B. Horwitz, *Biochem. biophys. Res. Commun.* **73**, 814 (1976).
28. J. W. Lown and S. K. Sim, *Biochem. biophys. Res. Commun.* **77**, 1150 (1977).
29. M. Tomasz, C. M. Mercado, J. Olson and N. Chatterjie, *Biochemistry* **13**, 4878 (1974).
30. J. W. Lown, A. Begleiter, D. Johnson and A. R. Morgan, *Can. J. Biochem.* **54**, 110 (1976).
31. M. F. Goodman, G. M. Lee and N. R. Bachur, *J. biol. Chem.* **252**, 2670 (1977).
32. J. W. Lown, S. K. Sim, K. C. Majumdar and R. Y. Chang, *Biochem. biophys. Res. Commun.* **76**, 705 (1977).
33. J. M. Lown, K. C. Majumdar, A. I. Meyers and A. Hecht, *Bioorg. Chem.* **6**, 453 (1977).
34. R. B. Brundrett, J. W. Cowens and M. Colvin, *J. med. Chem.* **19**, 958 (1976).
35. J. W. Lown, L. W. McLaughlin and Y. M. Chang, *Bioorg. Chem.* **7**, 97 (1978).
36. M. Dizdaroglu, C. von Sonntag and D. Schulte-Frohlinde, *J. Am. chem. Soc.* **97**, 2277 (1975).
37. E. G. Janzen, *Accts chem. Res.* **4**, 31 (1971).
38. L. Roizin-Towle and E. J. Hall, *Br. J. Cancer* **37**, 254 (1978).
39. G. E. Adams, *Br. med. Bull.* **29**, 48 (1973).
40. V. S. Srinivasan, D. Podolski, N. J. Westrick and D. C. Neckers, *J. Am. chem. Soc.* **100**, 6513 (1978).
41. I. Fridovich and P. Handler, *J. biol. Chem.* **235**, 1835 (1960).
42. J. J. van Hemmen and W. J. A. Meuling, *Archs Biochem. Biophys.* **182**, 743 (1977).
43. G. J. McClune and J. A. Fee, *Fedn. Eur. biochem. Soc. Lett.* **67**, 294 (1976).

44. B. Halliwell, *Fedn. Eur. biochem. Soc. Lett.* **72**, 8 (1976).
45. B. H. J. Bielski, in *Biochemical and Medical Aspects of Active Oxygen*, p.7. University Park Press, Tokyo (1977).
46. C. Zimmer, in *Progress in Nucleic Acid Research and Molecular Biology* (Eds. J. N. Davidson and W. E. Cohn), Vol. 15, pp.285–312. Academic Press, New York (1975).
47. C. Zimmer, K. E. Reinert, G. Luck, U. Wahnert, G. Lober and H. Thrum, *J. molec. Biol.* **58**, 329 (1971).
48. H. M. Berman, S. Neidle, C. Zimmer and H. Thrum, *Biochim. biophys. Acta* **561**, 124 (1979).
49. G. Luck, C. Zimmer, K. E. Reinert and F. Arcamone, *Nucleic Acids Res.* **4**, 2655 (1977).
50. G. F. Gause, in *Antibiotics* III. *Mechanism of Action of Antimicrobial and Antitumor Agents* (Eds. J. W. Corcoran and F. E. Hahn), pp.197–202. Springer, New York (1975).
51. M. A. Chirigos, F. J. Rauscher, I. A. Kamel, G. R. Fanning and A. Goldin, *Cancer Res.* **23**, 1646 (1963).
52. E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond and M. J. Waring, *The Molecular Basis of Antibiotic Action*, pp.200–205. John Wiley, New York (1972).
53. S. B. Horwitz, C. Chang and A. P. Grollman, *Molec. Pharmac.* **7**, 632 (1971).