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INTERACTIONS OF THE GLYCOPEPTIDE ANTITUMOR ANTIBIOTICS BLEOMYCIN AND TALLYSOMYCIN WITH DEOXYRIBONUCLEIC ACID *IN VITRO**

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Abstract—Tallysomycin (TLM), an experimental glycopeptide antitumor antibiotic related to bleomycin (BLM), at a concentration of 4.8×10^{-5} M sequesters Fe^{2+} and nicks circular DNA to 75–80 per cent in 45 min. The DNA scission reaction which requires oxygen and is suppressed by other divalent ions and by EDTA is pH dependent and shows optima at 9.6 and 11.2. BLM under comparable conditions shows three pH optima at 9.3, 10.6 and 11.2. TLM binds to DNA more strongly than BLM, especially at pH 7.0 to 4.7. The three intermediates, $O_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$, are implicated in the DNA scission by tallysomycin as indicated by (1) inhibition by superoxide dismutase, (2) inhibition by catalase, and (3) e.s.r. detection of the spin-trapped PBN·OH nitroxide radical, respectively. The A,T-specific DNA binding agents, netropsin and distamycin, enhance the TLM-induced cleavage by 7 per cent and 9.5 per cent, while G,C-specific agents, olivomycin and chromomycin-A_3, enhance the scission by 15 per cent and 16 per cent, respectively. TLM and BLM suppress the extent of psoralen-photo-induced DNA cross-linking by 24 per cent and 53 per cent, respectively. TLM with the spermidine moiety truncated by spermidine oxidase binds to DNA with less efficiency (5.5 per cent) than does the parent antibiotic (19 per cent). However, the modified TLM also sequesters Fe^{2+} and nicks DNA with comparable efficiency to TLM under similar conditions. This suggests (1) that the spermidine moiety is not involved in binding Fe^{2+} at the 'active site' of the antibiotic responsible for DNA cleavage, and (2) that in binding to DNA, at least of TLM·Fe^{2+}, intercalation by the bithiazole moiety is more significant than electrostatic attraction by the spermidine chain. The results are in accord with a mode of action in which TLM sequesters Fe^{2+}, binds to DNA and produces $OH^{\cdot}$ radicals close to the duplex to cleave the latter. A chemical mechanism is suggested for this process.

Tallysomycins (TLM) A and B (Fig. 1) are new glycopeptide antibiotics, produced by an unusual actinomycetes strain of *Streptomyces*-like morphology [1]. The structures of these antibiotics have been determined [2, 3]. They are closely related to bleomycin (BLM), differing only in the amino acid composition and by the fact that they contain an additional unique sugar, 4-amino-4,6-dideoxy-L-talose [2, 4]. The antitumor activities of TLM-A and -B have been reported [1, 5]. Both TLM-A and -B were highly active against B16 melanoma, sarcoma 180 ascites tumor and Lewis lung carcinoma and moderately active against P388 leukemia, but were inactive against lymphoid leukemia L1210. The antitumor activity of TLM-A was 2~3 times that of TLM-B and 3~17 times that of BLM. They exhibited significantly greater activity against a number of bacteria and fungi than did BLM [1]. Induction of lysogenic bacteria *Escherichia coli* W1709 (λ) by TLM occurred at concentrations 30-fold less than that for BLM [1]. On the other hand, TLM-A was about 1.5 and 4 times more toxic for mice than TLM-B and BLM, respectively [5]. However, the most common side effect in the clinical application of BLM is lung toxicity [6], and since TLM causes pneumonitis/fibrosis in only half as many test animals as does BLM at equitoxic doses [4], it shows promise in this regard. In common with BLM [6], TLM has the great potential clinical advantage of being only slightly myelosuppressive and immunosuppressive, due to high levels in bone marrow of the inactivating aminopeptidase enzyme bleomycin hydrolase [7].

The transition metal binding properties of both BLM [8, 9] and TLM [10] have been reported. TLM has been shown to have two metal binding sites whereas BLM has only one. One site is similar to that of BLM and sequesters Fe^{2+} or Cu^{2+} at the pyrimidine–imidazole portion of the molecule. A second thermodynamically less stable site utilizes the amino group of the L-talose moiety and the amino groups on the 'tail' portion of the antibiotic to bind metal ions such as Cu^{2+} and Ni^{2+} but not Zn^{2+} (see Fig. 10).

The affinity constants of BLM and TLM for salmon sperm DNA have also been determined [11]. They were 3.41 ± 0.42 (S.D.) $\times 10^4$ M^{-1} and $8.47 \pm 1.06 \times 10^5$ M^{-1} for BLM and TLM respectively. Thus, the affinity constant of TLM for DNA was significantly greater than for BLM.

BLM has been shown to degrade DNA both *in vivo* and *in vitro* [12–16], producing both single strand and double strand breaks. DNA cleavage is enhanced in the presence of reducing agents and by the addition of Fe^{2+} and requires dissolved oxygen [12, 16–19]. The DNA cleavage is inhibited by Cu^{2+}, Zn^{2+}, Co^{2+} [13], Mg^{2+} [20] and by EDTA. The chemical mechanism of this BLM-induced cleavage

* Studies related to antitumor antibiotics—XX.

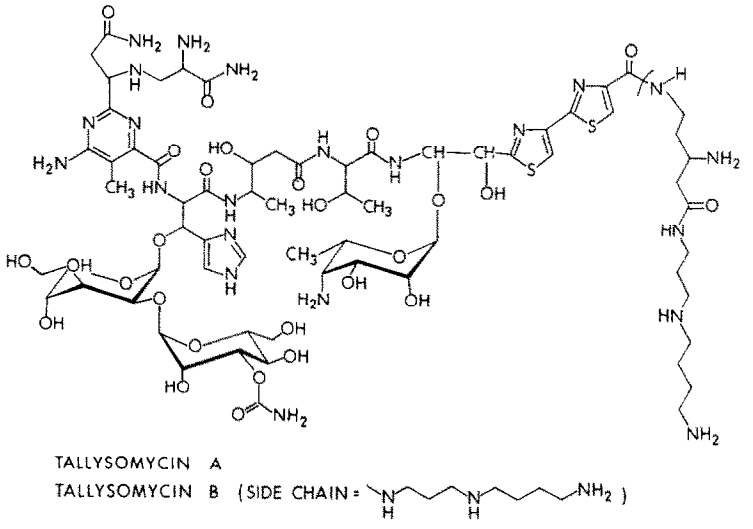

Fig. 1. Structures of the antitumor antibiotics TLM-A and TLM-B.

of DNA has been examined [18] by the ethidium fluorescence assay in conjunction with PM2-CCC-DNA [21–23]. It has been shown that the reaction involves the intermediacy of $O_2^{\cdot}$, H_2O_2 and $OH^{\cdot}$ produced by the low concentration of Fe^{2+} in the BLM and oxygen. Additional support for the above mechanism comes from the fact that DNA-cleavage is inhibited by (1) superoxide dismutase, (2) catalase (and more efficiently by the two enzymes together) and (3) free radical scavengers, e.g. isopropyl alcohol [18]. We decided to examine the reactions of TLM-A with PM2-CCC-DNA under a variety of conditions by the ethidium fluorescence assay with a view to elucidating the mechanism of its action and to establishing any similarities or differences between TLM-A and BLM in their reactions with DNA. While our work was in progress, a less extensive report on the cleavage of DNA by TLM-A employing alternative methods was published [11].

MATERIALS AND METHODS

BLM-A_2 and TLM-A were obtained from Bristol Laboratories, Syracuse, NY. TLM-E_{1a} (Cu^{2+} complex) and TLM-E_{1b} (Cu^{2+} complex) were supplied by Dr. H. Kawaguchi, Bristol-Banyu Research Institute, Meguro-ku, Tokyo. The free modified antibiotics were prepared by precipitation of the copper with hydrogen sulfide and chromatographic purification on Amberlite XAD-4. Solutions were prepared in deionized water. Superoxide dismutase (EC 1.15.1.1) was from Miles Labs, Elkhart, IN, and catalase (EC 1.11.1.6) (beef liver) from the Aldrich Chemical Co., Milwaukee, WI. T4-DNA ligase (EC 6.5.1.1) was obtained from P.L. Biochemicals, Milwaukee, WI. Phenyl *N-tert* butyl nitrone (PBN) was purchased from the Aldrich Chemical Co. Ethidium bromide and disodium EDTA were purchased from the Sigma Chemical Co., St. Louis, MO. NADPH was purchased from CalBiochem, San Diego, CA. Netropsin hydrochloride was from the American Cyanamid Co., Pearl River, NY. Distamycin A hydrochloride was obtained from Boehringer-Mannheim, West Germany. Olivomycin and chromomycin A_3 were purchased from CalBiochem. Psoralen (furo[3,2-g]-coumarin) was obtained from Dr. M. J. Ashwood-Smith, Department of Biology, University of Victoria, British Columbia, Canada. The λ and PM2-CCC-DNA (87 per cent CCC, 13 per cent OC) were prepared as described before [24]. The calf thymus topoisomerase was prepared as described by Pulleyblank and Morgan [25] and Herrick and Alberts [26]. The stock solution was stored in 50 per cent glycerol at −20°. The activity was such that 2 μl of this solution was sufficient to completely relax 2.5 μg of PM2-DNA in 15 min at 37°.

Endonuclease VI was purified according to the method of Verly and Rassart [27] from *E. coli* BATCC 11303; after the phosphocellulose chromatography, the enzyme was stored in 0.15 M NaCl–0.04 M sodium phosphate (pH 6.5) with an equal volume of glycerol and kept at −20°. For the experiment, this preparation was diluted with a suitable buffer.

Fluorescence determination of cleavage of PM2-CCC-DNA by BLM and TLM. The fluorometric method of measuring strand breakage of PM2-covalently closed circular DNA (CCC-DNA) and its inhibition by enzymes and free radical scavengers has been described [21–24]. By raising the pH to around 11.8, duplex DNA can still be detected very sensitively (as little as 0.1 μg) by the enhanced fluorescence of ethidium bromide which specifically intercalates duplex DNA. At these high pH levels any short intramolecular base pairing of denatured DNA is destabilized and it assumes a true single-stranded conformation. At this high pH, CCC–DNA gives a quantitative return of fluorescence (at a lower pH a denatured form of CCC–DNA is obtained), and this has been exploited as a sensitive assay for measuring breaks introduced into CCC-DNA by either nucleases or chemical means [24].

Ethidium bromide binds intercalatively to negatively supercoiled DNA and, in consequence, suffers an enhancement of fluorescence which is recorded. The conversion of PM2-CCC-DNA to nicked or open circular (OC) DNA results in the release of topological constraints allowing more ethidium to intercalate, and consequently a characteristic 30 per cent increase in fluorescence is observed in the pH 11.8 ethidium assay solution (see below). After heat denaturation (96°, 4 min) and cooling to 22°, since the strands are now separable a loss of fluorescence is observed in contrast to the control CCC-DNA which, we have seen, returns to duplex register. Therefore, at any given time the loss of fluorescence after the heating and cooling cycle, compared to the control, is proportional to the percentage of DNA suffering single strand scission [21, 24].

All measurements were performed on a G.K. Turner and Associates model 430 spectrofluorometer equipped with a cooling fan to reduce 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 and the emission wavelength was 600 nm. The 30× and 100× scales of medium sensitivity were generally used and water was circulated between the cell compartment and a thermally regulated bath at 22°.

The reaction mixtures were buffered to the appropriate pH with potassium phosphate. The reactions were carried out in a total volume of 100 μl at 37° in deionized water. The reaction solution contained approximately 1.50 A_{260} units of PM2-CCC-DNA (87 per cent CCC, 13 per cent OC), 50 mM buffer and the appropriate concentrations of BLM and TLM. At intervals, 10 μl aliquots were withdrawn and added to 2 ml of assay solution which contained 20 mM potassium phosphate (pH 11.8), 0.2 mM EDTA and 0.5 μg/ml of ethidium bromide [28]. The fluorescence was measured using a blank without added sample. The solution was then heat denatured at 96° on a Temp-Blok for 4 min and cooled rapidly in an ice-bath and then in a thermostated water-bath at 22° for 5 min and the fluorescence was read again. In a control experiment, it was shown that none of the components interfered with the ethidium fluorescence.

Effects of Fe^{2+} *or reducing agents on the cleavage of PM2-CCC-DNA by TLM.* The reactions were carried out on a 100 μl scale at 37° at the appropriate pH. The reaction mixtures contained approximately 1.50 A_{260} units of PM2-CCC-DNA (87 per cent CCC, 13 per cent OC), 50 mM potassium phosphate buffer, either 4.8×10^{-5} M or 1.92×10^{-5} M TLM, and either 4×10^{-6} M ferrous sulfate (reaction pH 7.0) or 1 mM NADPH (reaction pH 9.0) or 2 mM 2-mercaptoethanol (2-ME) (reaction pH 9.0). Aliquots of 10 μl were withdrawn at intervals and assayed for DNA scission at pH 11.8 as described above. In separate control experiments it was shown that NADPH separately had no effect on DNA, and 2-ME nicks ~12 per cent DM2-CCC-DNA under similar reaction conditions.

Assay for alteration of superhelical density of circular DNA by BLM or TLM binding. The reaction was performed on a 100 μl scale in deionized water. The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 1.50 A_{260} units of PM2-CCC-DNA, 0.2 M sodium chloride and 2.66×10^{-5} M BLM. The control did not contain any BLM. Both the sample and control were incubated at 37° for 30 min. Five microliters of the calf thymus topoisomerase were added to both the sample and the control, and incubation was continued at 37°. Aliquots (10 μl) were withdrawn at intervals and assayed as described before. The basis of this assay is that intercalation into DNA base pairs is accompanied by relaxation of supercoiled PM2-CCC-DNA to a non-supercoiled form. Topoisomerase also relaxes supercoiled PM2-CCC-DNA by a nicking–closing mechanism which produces a 33 per cent decrease in ethidium fluorescence because of differences in topological constraints of the two forms of DNA [29]. Thus, the extent of fluorescence decrease in the PM2-DNA produced by an agent, relative to the enzyme control, is a measure of the extent of intercalative interaction. An additional control was performed by determining any generalized reduction in ethidium fluorescence produced by the drug with linear calf thymus DNA, and using this value to correct the fluorescence change observed with PM2-DNA.

A similar assay was performed using 4.8×10^{-5} M TLM with 20 mM EDTA.

Endonuclease VI *assay for possible depurination of relaxed PM2-CCC-DNA by TLM or BLM at* pH 7.0 *and* pH 9.6. The basis of the assay is that the enzyme cleaves apurinic PM2-CCC-DNA specifically and thereby converts it into OC-DNA, which results in a change in ethidium fluorescence both before and after heat denaturation when measured at pH 8.0 [30]. The reaction mixture, which consisted of 1.50 A_{260} units of PM2-CCC-DNA in 50 mM potassium phosphate buffer, pH 7.0 or 9.6, and with either TLM or BLM at concentrations of 4.8×10^{-5} M and 6.66×10^{-5} M, respectively, was incubated at 37° for 3 hr. Aliquots (10 μl) of endonuclease VI were added and incubated at 37°. Further 10 μl aliquots were withdrawn at intervals and analyzed by the pH 8.0 ethidium fluorescence assay. In addition, control experiments were performed to determine the extent of cleavage by TLM and BLM using the pH 11.8 ethidium fluorescence assay. Conversion of depurinated PM2-CCC-DNA to PM2-OC-DNA by the endonuclease VI would result in a characteristic 30 per cent increase in fluorescence as a result of the release of topological constraints. After heat denaturation at 96°/4 min when the PM2-DNA is converted into single strands, followed by rapid cooling to 22°, the fluorescence was read again. A fraction active toward endonuclease VI is revealed by the loss of fluorescence after heat denaturation. The control for the assay consisted of a similar reaction substituting native PM2-CCC-DNA.

Assay for effects of DNA-sequence specific binding reagents and ethidium on the cleavage of PM2-CCC-DNA by TLM-Fe^{2+} *and BLM-*Fe^{2+}. The reactions were performed on a 100 μl scale. The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.0), 1.48 A_{260} units of PM2-CCC-DNA (87 per cent CCC, 13 per cent OC), 1 mM magnesium chloride for chromomycin A_3 and olivomycin, the

appropriate concentrations of netropsin, distamycin, chromomycin A_3, olivomycin or ethidium, 4.8×10^{-5} M of TLM or 2.66×10^{-5} M BLM and 4×10^{-6} M $FeSO_4$. The DNA solutions were incubated with the drugs for 10 min at 37° before the addition of TLM, BLM and $FeSO_4$. After the addition of $FeSO_4$, the solutions were stoppered and protected from light to prevent possible cleavage of the DNA by the drug [31], and incubated at 37°. Aliquots (10 μl) were withdrawn at intervals and analysed for the extent of single strand scission by the pH 11.8 ethidium fluorescence assay.

Effects of TLM and BLM on the photo-induced cross-linking of λ-DNA by psoralen. The reactions were carried out on a 100 μl scale. The reaction mixtures contained 1.50 A_{260} units of phage λ-DNA, 50 mM potassium phosphate buffer (pH 7.0), 4.80×10^{-5} M TLM or 6.66×10^{-5} M BLM and 2×10^{-4} M psoralen and 5 per cent (v/v) ethanol. The DNA solution was incubated with TLM or BLM for 10 min at 22° before adding psoralen. After the addition of psoralen, the solutions were irradiated at 22° 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 [32]). Aliquots of 10 μl were removed at intervals and analysed for the extent of cross-linking by the standard pH 11.8 ethidium fluorescence assay. After the heat denaturation and subsequent cooling, only covalently linked complementary (CLC)-DNA showed return of fluorescence since the covalent links provided a nucleation site for renaturation [23, 33]. The ratio of the fluorescence after heating to the fluorescence before heating gave the extent of covalent cross-linking of the DNA. In a separate experiment, the extent of covalent cross-linking of λ-DNA by psoralen in the absence of either TLM or BLM was determined under similar conditions as described before [34].

Effects of metal ions and EDTA on the cleavage of PM2-CCC-DNA by TLM-Fe^{2+}. The reactions were performed on a 100 μl scale in deionized water. The reaction mixtures contained 1.50 A_{260} units of PM2-CCC-DNA (87 per cent CCC, 13 per cent OC), 50 mM potassium phosphate buffer (pH 7.0), 1 mM metal ion (Cu^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+}) or EDTA (1 mM or 20 mM), 4.8×10^{-5} M TLM and 4×10^{-6} M $FeSO_4$. The solutions were incubated at 37° and analysed for the extent of single strand DNA cleavage by the standard pH 11.8 ethidium fluorescence assay.

Assay for inhibition of T4-DNA ligase by BLM, TLM and TLM-E_{1a}. The basis of the assay is that the action of the ligase on PM2-OC-DNA converts it to PM2-CCC-DNA with consequent introduction of topological constraints so that less ethidium intercalates and as a consequence the fluorescence value falls. In effect, the assay is the reverse of the nicking assay. The reactions were performed on a 50 μl scale at 30°. The solutions contained 20 M Tris–hydrochloride buffer (pH 7.6), 10 mM dithiothreitol, 1 mg/ml of gelatin, 5 mM adenosinetriphosphate, 10 mM magnesium chloride, 1.00 A_{260} units of PM2-OC-DNA (100 per cent nicked by S_1-nuclease from *Aspergillus oryzoe*), 2 units/ml of T4-DNA ligase and 0.64 mM BLM, TLM or TLM-E_{1a}. At 1-min intervals, 10 μl aliquots were withdrawn and added to 2 ml of the ethidium (pH 11.8) assay mixture described above. The fluorescence was read both before and after 96°/min heat denaturation. Ligation was evident from a fall in fluorescence before heat denaturation and a corresponding rise in fluorescence after heat denaturation. The control reaction which consisted of the above mixture without added antibiotics showed 67 per cent ligation of the DNA after 5 min.

Spin trapping and e.s.r. detection of radical species generated from TLM and BLM under atmospheric oxidation. Electron spin resonance spectra were obtained on a Bruker ER-400 ESR spectrometer fitted with a Varian V-3601 12 inch (1 inch = 25.4 mm) 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.

The reactions were carried out on a 315 μl scale. The solutions contained 1 mg (1.53×10^{-3} M and 2.12×10^{-3} M, respectively) of TLM or BLM, 80 mM potassium phosphate buffer (pH 8.0), 80 mM PBN, 1.5 mg (5.37×10^{-3} M) NADPH and 6.5 per cent (v/v) each of CH_3OH and CH_3CN in water. The solutions were maintained at room temperature with free access to atmospheric oxygen for 16 hr and the e.s.r. spectrum of the spin-adduct was recorded.

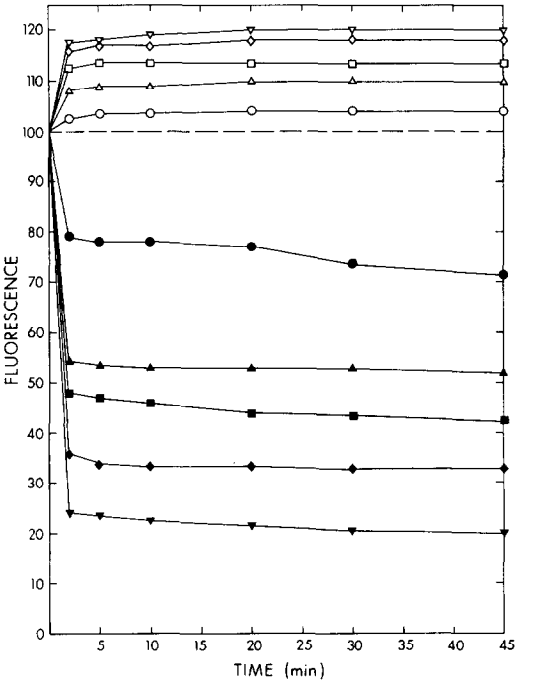

Fig. 2. Cleavage of PM2-CCC-DNA by various concentrations of TLM in the presence of Fe^{2+}. Reactions were performed in deionized water at 37° in 50 mM potassium phosphate buffer, pH 7.0, and contained 1.50 A_{260} units/ml of PM2-CCC-DNA (87 per cent CCC) and a constant concentration of 4×10^{-6} M $FeSO_4$. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Additional components were: (○) none, (△) 9.6×10^{-6} M TLM, (□) 1.92×10^{-5} M TLM, (◇) 2.88×10^{-5} M TLM, and (▽) 4.8×10^{-5} TLM, (----) control.

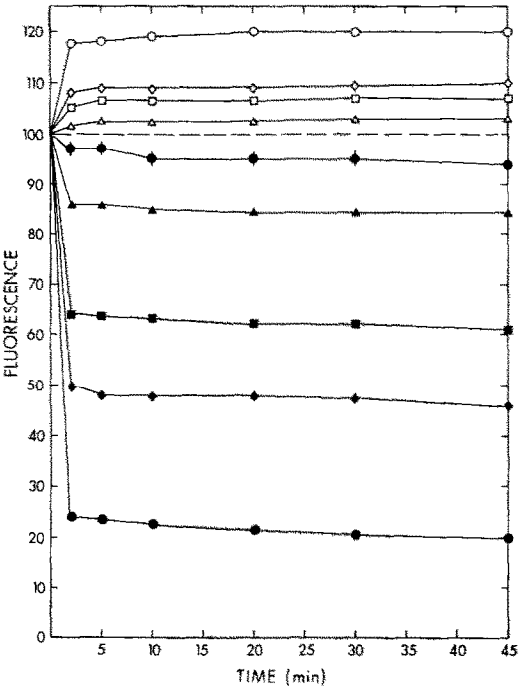

Fig. 3. Cleavage of PM2-CCC-DNA by various concentrations of $FeSO_4$ in the presence of TLM. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Reactions were performed using the conditions described in the legend for Fig. 2 and with a constant concentration of 4.8×10^{-5} M TLM but with the following additional components: (●) none, (the before heat denaturation readings are omitted for clarity), (△) 3.2×10^{-7} M $FeSO_4$, (□) 6.4×10^{-7} M $FeSO_4$, (◇) 9.6×10^{-7} M $FeSO_4$, and (○) 4×10^{-6} M $FeSO_4$, (----) control.

Inhibition of PBN·OH nitroxide radical generation by catalase and superoxide dismutase. The reactions were carried out as described before, but also contained 0.5 mg catalase and 1 mg superoxide dismutase. After keeping at room temperature overnight, the e.s.r. spectrum was recorded as described above.

RESULTS

A freshly prepared 4×10^{-6} M $FeSO_4$ solution nicked 28.5 per cent PM2-CCC-DNA (87 per cent CCC, 13 per cent OC) in 45 min at 37° and pH 7.0. But the presence of 4.8×10^{-5} M TLM enhanced the cleavage of DNA to 75–80 per cent within 45 min, whereas TLM alone was capable of nicking only ~5 per cent under the same reaction conditions. It was also found that at a constant concentration of $FeSO_4$ (4×10^{-6} M) the extent of cleavage of PM2-CCC-DNA was proportional to the concentration of TLM (Fig. 2). Similarly, at a constant concentration of TLM (4.8×10^{-5} M), the extent of cleavage of DNA was proportional to the concentrations of $FeSO_4$ (Fig. 3).

A freshly prepared 4.8×10^{-5} M solution of TLM in deionized water nicked 20–30 per cent PM2-CCC-DNA in 45 min at pH 9.0 and 37°, but only ~5 per cent at pH 7.0. Also, the drug lost its nicking properties on prolonged exposure to air. Addition of 4×10^{-6} M $FeSO_4$ to the inactivated TLM resulted in 96 per cent nicking of PM2-CCC-DNA in 45 min at 37° and pH 9.0. Alternatively, addition of 1 mM NADPH to 4.8×10^{-5} M TLM produced 78 per cent nicking of PM2-CCC-DNA in 45 min at 37° and pH 9.0, whereas NADPH separately had no effect on DNA under the same reaction conditions.

Similarly, addition of 20 mM 2-ME under the above conditions produced 82 per cent nicking, whereas 2-ME alone was capable of nicking only 12 per cent PM2-CCC-DNA under the same conditions (Fig. 4).

The efficiency of cleavage of DNA by both BLM and TLM depended on the pH of the medium. For BLM the efficiency of nicking increased from pH 4.7 (no nicking) to higher pH values. Three pH optima were observed. They were 9.3, 10.6 and 11.2. For TLM the same pattern was seen from pH 7.0 to higher pH values. In this case only two pH optima were distinguished, 9.6 and 11.2 (Fig. 5). It was also found that, at lower pH values (pH 7.0 and below), TLM binds strongly to DNA to form a complex causing a decrease in the ethidium fluorescence of DNA before heat denaturation due to steric hindrance of the approach of the ethidium to the DNA. This binding increased from pH 7.0 to pH 4.7. At pH 4.7, the complex precipitated. On the other hand, BLM caused no such reduction in the ethidium fluorescence of DNA.

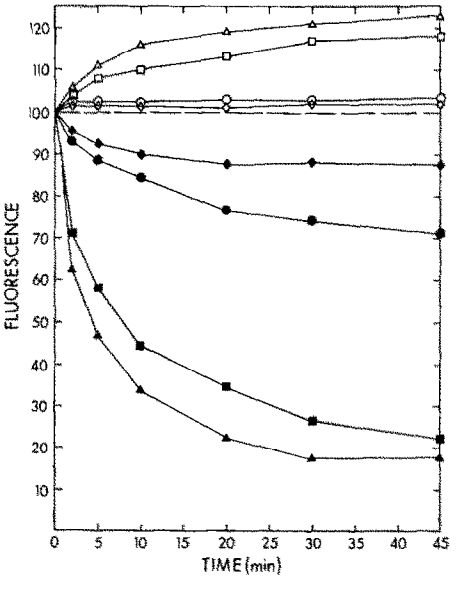

Fig. 4. Cleavage of PM2-CCC-DNA by TLM and its enhancement by reducing agents. Reactions were performed in deionized water at 37° in 50 mM potassium phosphate buffer, pH 9.0, and contained 1.50 A_{260} units/ml of PM2-CCC-DNA (87 per cent CCC). The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Additional components were: (○) 4.8×10^{-5} M TLM, (□) 4.8×10^{-5} M TLM and 1 mM NADPH, (△) 4.8×10^{-6} M TLM and 20 mM 2-ME, and (◇) 20 mM 2-ME, (----) control.

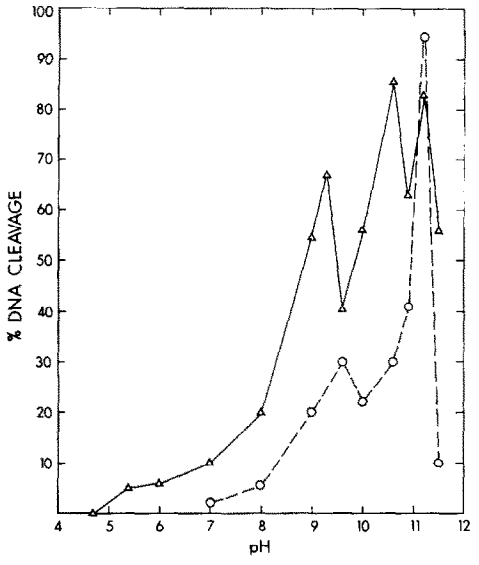

Fig. 5. Effects of pH on the cleavage of PM2-CCC-DNA by TLM and BLM-A_2. Reactions were performed in deionized water at 37° in 50 mM potassium phosphate buffer and contained 1.50 A_{260} units/ml of PM2-CCC-DNA (87 per cent CCC) and 4.8×10^{-5} M TLM or 6.66×10^{-5} BLM-A_2. Key: (○) 4.8×10^{-5} M TLM, and (△) 6.66×10^{-5} M BLM-A_2.

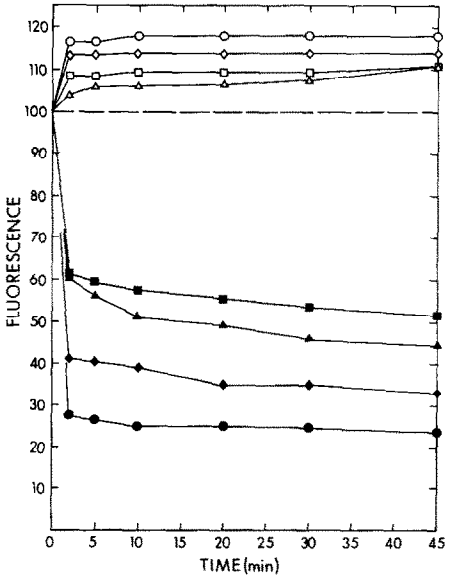

Fig. 6. Requirement of oxygen for the cleavage of PM2-CCC-DNA by TLM in the presence of Fe^{2+}. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Reactions were performed in deionized water at 37° in 50 mM potassium phosphate buffer, pH 7.0, and contained 1.50 A_{260} units/ml of PM2-CCC-DNA (87 per cent CCC) and with a constant concentration of 4×10^{-6} M $FeSO_4$ but with the following additional components: (□) 1.92×10^{-5} M TLM, (◇) 1.92×10^{-5} M TLM and oxygen, (○) 4.8×10^{-5} M TLM, and (△) 4.8×10^{-5} M TLM under argon, (----) control.

As with BLM, dissolved oxygen appears to be necessary for the cleavage of PM2-CCC-DNA by TLM-Fe^{2+}. In the presence of 4.8×10^{-5} M TLM, 4×10^{-6} M $FeSO_4$ nicked 77 per cent PM2-CCC-DNA in 45 min at 37° and pH 7.0. When degassed water was used and the reaction with DNA was performed in an atmosphere of argon, the cleavage was reduced to 55 per cent. On the other hand, saturating the reaction mixture with oxygen before the addition of $FeSO_4$ resulted in an increase in the extent of cleavage of PM2-CCC-DNA under similar reaction conditions. Thus, although 1.92×10^{-5} M TLM and 4×10^{-6} $FeSO_4$ were capable of nicking 49 per cent PM2-CCC-DNA at 37° and pH 7.0, saturation of the reaction mixture with oxygen increased the nicking to 67 per cent (Fig. 6).

There appears to be no difference in the reactivity of TLM-Fe^{2+} toward supercoiled and relaxed PM2-CCC-DNA. Thus, PM2-CCC-DNA, relaxed by treating with calf thymus topoisomerase (which acts by a nicking–closing mechanism), was cleaved to the same extent as native supercoiled PM2-CCC-DNA was by TLM-Fe^{2+} at TLM and $FeSO_4$ concentrations of 4.8×10^{-5} M and 4×10^{-6} M, respectively. The complex between supercoiled PM2-CCC-DNA and TLM or BLM was relaxed by topoisomerase to the same extent as uncomplexed supercoiled PM2-CCC-DNA.

In the above experiment with TLM, it was necessary to include an excess [20 mM] of EDTA to prevent DNA cleavage. When the EDTA concentration was reduced [1 mM] or completely omitted, the closing step (ligase action) of the topoisomerase was partially inhibited. Under the above reaction conditions (pH 7.0), TLM did not produce any appreciable cleavage of DNA. BLM, TLM and TLM-E_{1a} in independent assays at 0.64 μM all showed complete inhibition of T4-DNA ligase action. The inhibition of ligase action by BLM has been reported by Umezawa [12].

Both BLM and TLM are able to cleave depurinated PM2-CCC-DNA (prepared by incubating PM2-CCC-DNA at pH 3.05 [30]). With BLM, the process was very slow at a concentration of 6.66×10^{-5} M and took several hours for completion. With TLM (4.8×10^{-5} M), it was relatively faster. After 20 hr both produced the same extent of cleavage.

Under conditions where TLM and BLM induce strand scission (4.8 to 6.6×10^{-5} M), they did not excise detectable quantities of purine or pyrimidine bases, in contrast to the situation that obtains for nitrosoureas [30]. Depurination under these conditions would have been revealed by treatment of supercoiled or relaxed PM2-CCC-DNA treated with TLM or BLM with endonuclease VI. This enzyme specifically recognizes and cleaves apurinic PM2-CCC-DNA and thereby converts it into open circular DNA which results in a characteristic rise (100 per cent in the case of relaxed DNA) in ethidium fluorescence before heat denaturation when measured at pH 8.0. Thus, incubation of relaxed PM2-CCC-DNA with BLM and TLM (6.66×10^{-5} M and 4.8×10^{-5} M, respectively) for 3 hr at 37° and pH 7.0 and subsequent treatment with endonuclease VI did not cause an increase in fluorescence (assayed at pH 8.0) or any appreciable cleavage (assayed at

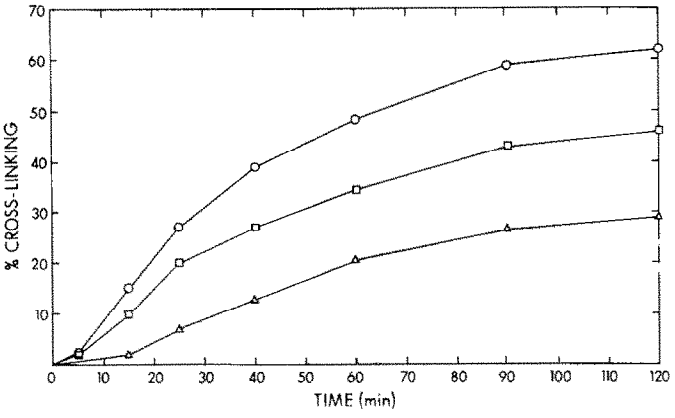

Fig. 7. Effects of TLM and BLM-A_2 on the photo-induced cross-linking of λ-DNA with psoralen. Reactions were performed in deionized water at 22° in 50 mM potassium phosphate buffer, pH 7.0, containing 1.50 A_{260} units/ml of λ-DNA and 2×10^{-4} M psoralen, and were irradiated at 360 nm at an intensity of 17.5 ergs/mm^2/sec. Fluorescence readings are those after the heat denaturation and cooling cycle. Additional components were: (○) none, (□) 4.8×10^{-5} M TLM, and (△) 6.66×10^{-5} M BLM-A_2.

pH 11.8. A sample of BLM which produced ~30 per cent nicking of PM2-CCC-DNA in 10 min at a concentration of 8.0×10^{-5} at 37° and pH 9.6 did not produce more than 35 per cent cleavage after incubation for 23 hr under identical conditions. Treatment with endonuclease VI after 10 min of incubation did not produce any increase in fluorescence. If there was depurination accompanying nicking, endonuclease treatment would have revealed it, since BLM is incapable of producing any appreciable cleavage of depurinated DNA after 10 min (see above). Similarly, a sample of TLM solution which produced *ca.* 10 per cent cleavage after 10 min at a concentration of 4.8×10^{-5} M at 37° and pH 9.6 did not produce more than 11 per cent cleavage after 16.5 hr under identical conditions. Treatment with endonuclease VI after 10 min of incubation did not produce any increase in fluorescence. TLM does not cleave depurinated DNA to any appreciable extent after 10 min. These series of experiments were repeated with TLM and BLM solutions of varying DNA-nicking capabilities and in no case was any depurination detected.

The effects of sequence specific reagents on the nicking of PM2-CCC-DNA by TLM-Fe^{2+} and BLM-Fe^{2+} were investigated. A slight enhancement of the rate of DNA cleavage was noticed in all cases. Olivomycin and chromomycin A_3, which in the presence of an equivalent of Mg^{2+} bind specifically to (G,C)-rich regions in the DNA, enhanced the nicking by 15 per cent and 16 per cent for TLM-Fe^{2+} at concentrations of 0.2 mg/ml. For BLM-Fe^{2+}, the corresponding figures are 5.5 per cent and 7 per cent, respectively. Netropsin and distamycin, which bind specifically to (A,T)-rich regions in the minor groove of DNA, enhanced the cleavage by 7 per cent and 9.5 per cent for TLM-Fe^{2+} and 3.5 per cent and 7 per cent for BLM-Fe^{2+} at concentrations of 0.1 mg/ml. All the above reactions were carried out at constant concentrations of TLM (4.8×10^{-5} M), BLM (2.66×10^{-5} M) and $FeSO_4$ (4×10^{-6} M).

Intercalated ethidium bromide has no influence on the nicking process produced by the antibiotics. Similarly, spermine (1 mM) and spermidine (1 mM) did not affect the extent of cleavage of PM2-CCC-DNA by either TLM-Fe^{2+} or BLM-Fe^{2+}.

The effects of TLM and BLM on the photo-induced cross-linking of λ-DNA by psoralen were

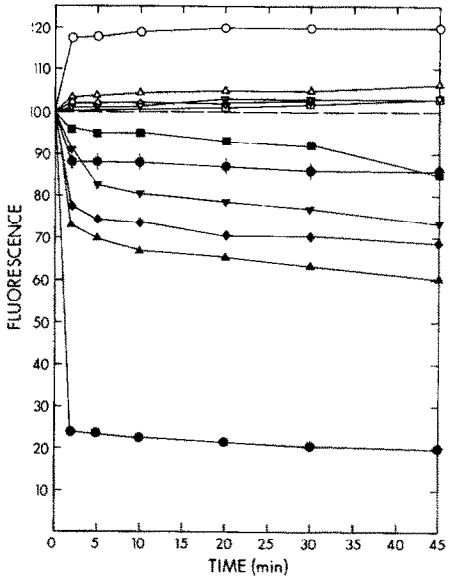

Fig. 8. Cleavage of PM2-CCC-DNA by TLM in the presence of Fe^{2+} and its selective inhibition. The before heat fluorescence readings are shown as open symbols and the closed symbols are fluorescence readings after the denaturation at 96° and rapid cooling. Reactions were performed using the conditions described in the legend for Fig. 2 and with a constant concentration of 4.8×10^{-5} M TLM and 4×10^{-6} M $FeSO_4$ but with the following additional components: (○) none, (□) 1 mM Cu^{2+}, (◆) 1 mM Ni^{2+} (the before heat denaturation readings are omitted for clarity), (▽) 1 mM Co^{2+}, (△) 1 mM Mg^{2+} and (◇) 1 mM EDTA, (----) control.

investigated. Thus, the presence of 4.8×10^{-5} M TLM and 6.66×10^{-5} M BLM inhibited the cross-linking by 24 per cent and 53 per cent, respectively, at a psoralen concentration of 2×10^{-4} M at 22° and pH 7.0 (Fig. 7).

The cleavage of DNA by TLM-Fe^{2+} is inhibited to a large extent by divalent metal ions and also by the metal ion complexing agent, EDTA. Thus, Cu^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+} inhibited the nicking by 81 per cent, 51 per cent, 67 per cent and 85 per cent, respectively, at a concentration of 1 mM. Similarly, EDTA at concentrations of 1 and 20 mM inhibited the cleavage by 49 per cent and 92.5 per cent, respectively. None of the above agents separately had any effect on DNA. All the above reactions were performed at a constant concentration of TLM (4.8×10^{-5} M) and $FeSO_4$ (4×10^{-6} M) at 37° and pH 7.0 (Fig. 8).

The cleavage of DNA by TLM (4.8×10^{-5} M) in conjunction with Fe^{2+} (4×10^{-6} M) at 37° and pH 7.0 was inhibited by catalase (0.08 mg/ml), superoxide dismutase (0.4 mg/ml) and the two enzymes in combination by 10 per cent, 17 per cent and 18 per cent, respectively. In addition, the hydroxyl radical scavenger sodium benzoate at a concentration of 20 mM inhibited the cleavage by 12 per cent and 21.5 per cent, respectively, at TLM concentrations of 4.8×10^{-5} M and 1.92×10^{-5} M at a constant Fe^{2+} concentration of 4×10^{-6} M at 37° and pH 7.0. However, in contrast to the situation that obtains for BLM, the cleavage of DNA by TLM-Fe^{2+} was not inhibited to any appreciable extent by the free radical scavenger isopropyl alcohol.

TLM-E_{1a}, in which the spermidine portion of the side chain of TLM-A has been degraded by spermidine oxidase to a 1,3-diaminopropane grouping [2], was isolated and purified from its copper complex. The copper complex and free TLM-E_{1a} showed less binding to DNA compared with TLM-A-Cu^{2+} and TLM-A, respectively, as evidenced by suppression of the ethidium fluorescence. Thus, TLM-A-Cu^{2+} and TLM-E_{1a}-Cu^{2+} suppressed the ethidium fluorescence of PM2-CCC-DNA by 18 per cent and 5.5 per cent at pH 4.7 and 37° at a concentration of 4.8×10^{-5} M. Similarly, TLM-A and TLM-E_{1a} suppressed the fluorescence of PM2-CCC-DNA by 19 per cent and 5.5 per cent under the above mentioned conditions.

TLM-E_{1+a} did not cleave DNA at pH 9.0 in contrast to TLM-A. This was due presumably to the absence of traces of Fe^{2+} in the antibiotic, since deionized water was used in the purification. TLM-E_{1a} (1.92×10^{-5} M) in the presence of 4×10^{-6} M $FeSO_4$ nicked 86 per cent PM2-CCC-DNA at 37° and pH 7.0 in 30 min. Under the above conditions, TLM-A (1.92×10^{-5} M) and $FeSO_4$ (4×10^{-6} M) cleaved 83 per cent PM2-CCC-DNA. As in the case of the parent antibiotic, the cleavage of DNA by TLM-E_{1a} in the presence of Fe^{2+} (1.92×10^{-5} M and 4×10^{-6} M, respectively) was inhibited by 0.08 mg/ml of catalase (13.5 per cent) and 0.4 mg/ml of superoxide dismutase (7 per cent) and by the two enzymes together (17 per cent).

Finally, attempts were made to trap the postulated hydroxyl radical with PBN. Thus, incubation of PBN with TLM, TLM-E_{1a} or BLM in the presence of NADPH for 16 hr produced the spin adduct, PBN·OH, identified by its characteristic e.s.r. spectrum [35]. The spectrum showed a triplet of doublets $a^N = 16.0$ G, $a_\beta^H = 3.4$ G and $g = 2.0061$ (Fig. 9). The e.s.r. spectrum of PBN·OH radical has been recorded after generation from a number of aminoquinone antibiotics [35] and also from the photoirradiation of H_2O_2 in aqueous solution [37, 38]. Inclusion of catalase and superoxide dismutase in the reaction mixture almost completely suppressed the formation of the $OH^{\cdot}$ and thereby the spin adduct. In control experiments, it was established that, in the presence of NADPH, CH_3OH and CH_3CN and buffer, the spin-trapping agent, PBN, did not give rise to radicals to an appreciable extent in the absence of the antibiotics.

DISCUSSION

TLM resembles BLM quite closely in its reactions with DNA. However, TLM binds much more strongly to DNA than does BLM [11] as evidenced in the present work by causing a greater suppression in the ethidium fluorescence of DNA.

As in the case of BLM [17, 18], the cleavage of DNA by TLM can be considerably enhanced by the addition of traces of Fe^{2+} and to a greater extent than a mere additive effect. This indicates that, like BLM, traces of Fe^{2+} are sequestered by the antibiotic which binds to the DNA and thus brings Fe^{2+} close to the target. It has been reported that TLM contains 12.4 μg iron per g as demonstrated by atomic absorption spectroscopy [11].

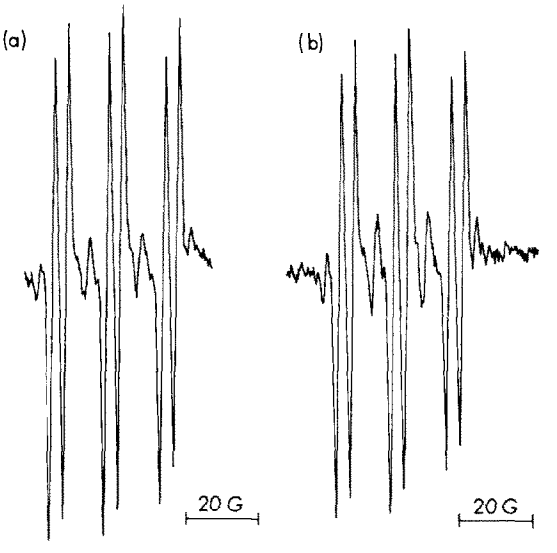

Fig. 9. Panel a: e.s.r. spectrum (microwave power 10 mW, modulation amplitude 2G, scan time 1000 sec) of PBN·OH radical obtained by incubating TLM-A and NADPH with 80 mM PBN at pH 8.0 in aqueous CH_3OH and CH_3CN. Hyperfine splitting constant (hfs) $a_\beta^N = 16.0$ G; $a_\beta^H = 3.4$ G; $g = 2.0061$. The additional signals are due to *tert*-butyl hydronitroxide formed by slow hydrolysis of the PBN [36]. Panel b: e.s.r. spectrum (microwave power 10 mW, modulation amplitude 2G, scan time 1000 sec) of PBN·OH radical obtained by incubating TLM and NADPH with 80 mM PBN at pH 8.0 in aqueous CH_3OH and CH_3CN. Hfs $a^N = 16.0$ G; $a_\beta^H = 3.4$ G; $g = 2.0061$.

Like BLM, TLM gradually loses its nicking potency on prolonged exposure to air [18]. This inactivation may be due to the oxidation of the traces of Fe^{2+} in the sample of the antibiotic. The nicking potency can be restored either by addition of traces of Fe^{2+} or by continuous re-reduction of the traces of Fe^{3+} in the antibiotics by a non-interfering reducing agent such as NADPH.

The dependence of the cleavage of DNA by TLM or TLM-Fe^{2+} on dissolved oxygen in the reaction mixture, the inhibition of the reaction by divalent metal ions such as Cu^{2+}, Mg^{2+}, Co^{2+} and Ni^{2+}, the inhibition by metal ion scavenging agents such as EDTA, the inhibition by radical scavengers such as sodium benzoate and also the fact that the reaction is inhibited by superoxide dismutase and catalase indicate the intermediacy of $O_2^{\cdot-}$, H_2O_2, and $OH^{\cdot}$ species, respectively, in accord with the following suggested mechanism [33]:

$$\text{TLM} + Fe^{2+} \rightleftarrows \text{TLM}\cdot Fe^{2+} \quad (1)$$

$$\text{TLM}\cdot Fe^{2+} + O_2 \rightarrow \text{TLM}\cdot Fe^{3+} + O_2^{\cdot-} \quad (2)$$

$$\text{TLM}\cdot Fe^{2+} + O_2^{\cdot-} \xrightarrow{2H^+} \text{TLM}\cdot Fe^{3+} + H_2O_2 \quad (3)$$

$$2O_2^{\cdot-} + 2H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} H_2O_2 + O_2 \quad (4)$$

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2 \quad (5)$$

$$\text{TLM}\cdot Fe^{2+} + H_2O_2 \rightarrow \text{TLM}\cdot Fe^{3+} + OH^{\cdot} + OH^- \quad (6)$$

The hydroxyl radicals thus generated by the TLM·Fe(II)–O_2 complex are the most reactive species in this system and can produce nicking in DNA in accord with the observed electrophoretic pattern [11]. The formation of malondialdehyde, a product of the BLM–DNA reactions [16] which requires a C–C bond cleavage in deoxyribose, may be produced by the attack of reactive OH radical at the 4′ position of the deoxyribose in DNA.

Additional proof for the above mechanism comes from spin-trapping studies on TLM and BLM. The observed e.s.r. spectrum of the PBN·OH nitroxide radical [37, 38] and the fact that its generation together with the concomitant DNA nicking are suppressed by catalase and by superoxide dismutase are clear indications that hydroxyl radicals are implicated in the degradation of DNA by TLM and by BLM. Strictly speaking, observation of the PBN·OH adduct is not absolute proof of the intermediacy of $OH^{\cdot}$ in these processes, since it primarily indicates that the PBN has been oxidized. Nevertheless the cumulative evidence for the $OH^{\cdot}$ radical is strong.

Both BLM and TLM are more effective in cleaving DNA at higher pH values than at pH 7.0 (range pH 7.0 to 11.2) (Fig. 5), although the reverse is true for Fe^{2+}. Thus, the previous interpretation for BLM [39] appears plausible for TLM also, that the higher pH optima reflect the existence of the unprotonated form of the α-amino group of the β-amino–alanine carboxamide residue of the antibiotics which is utilized in binding to the DNA and that, once bound, the sequestered Fe^{2+} generates radicals close to the DNA.

There have been a number of reports that BLM [11, 40–43] and TLM [11] at rather high concentrations of antibiotics and reducing agents, e.g. 100 μg/ml (6.7×10^{-5} M) BLM plus 50 mM dithiothreitol, cause the release of thymine bases [42] and at 12 mg/ml (8 mM) BLM plus 25 mM 2-ME cause the release of all four bases [40] from DNA and thereby renders it alkali-labile. However, under the conditions employed in the present study (i.e. antibiotic concentrations of 4.8×10^{-5} M and no added thiol), used to determine the characteristics of the DNA scission processes, there was no evidence for depurination either by TLM or BLM. The extremely sensitive assay employs endonuclease VI in conjunction with supercoiled and relaxed PM2-CCC-DNA which had been treated with the antibiotics at different pH values for different periods of time. This enzyme specifically recognizes and cleaves apurinic PM2-CCC-DNA and thereby converts it to open circular DNA which results in a characteristic rise in ethidium fluorescence (33 per cent for supercoiled PM2-CCC-DNA and 100 per cent for relaxed PM2-CCC-DNA), before heat denaturation when measured at pH 8.0 [30]. The present results point to the fact that release of free bases, rather than being an independent reaction, may be a secondary process caused by excessive degradation of DNA by very large concentrations of drug and reducing agents [40] (see Results).

It has been reported by Strong and Crooke [11] that the rate of strand scission by TLM and BLM was twice as great after alkaline denaturation as after neutral denaturation. However, it is to be noted that this may be due to a lower rate of denaturation under neutral conditions rather than to the difference in the rate of strand scission. For example, λ-DNA denatures at a slower rate under neutral conditions than under alkaline conditions.

From the viewpoint of any sequence specificity there are two factors to consider which may or may not be related: (1) base or sequence specificity of binding of the glycopeptide antibiotics, and (2) base specificity of DNA scission. In terms of the former, both TLM and BLM, like netropsin and distamycin, inhibited the 360 nm light photo-induced interstrand cross-linking of DNA by psoralen. Since psoralen has been shown to cross-link (A,T)-rich regions in the DNA [44–46], this may suggest a preference for binding to such regions by TLM and BLM which may then lead to the secondary and preferential release of thymine residues. It has been reported recently that, in the presence of ferrous ions, BLM promotes cleavage at G-T and G-C sequences [47] which suggests preferential binding in the major groove of DNA. As regards any base specificity for the DNA scission, however, both (A,T)-specific binders, netropsin and distamycin [48, 49], and (G,C)-specific binders, olivomycin and chromomycin A_3 [50, 51], alter the DNA scission to approximately the same extent in accord with a random attack by reactive hydroxyl radicals. The slight enhancement in the extent of DNA scission produced by these DNA binding agents, however, suggests a dependence on the topological form of the DNA of either the binding of the glycopeptide antibiotics or the susceptibility of the DNA to radical-induced scission.

A model for the complex of BLM, DNA, Fe^{2+} and O_2, which accounts for the observed facts of the mechanism, has been constructed [52]. The principal features of the model are a ferrous ion hexacoordinated to five nitrogens and one oxygen in one portion of the glycopeptide which are the five nitrogens indicated in Fig. 10, with the carbonyl group of the sugar carbamate group occupying the sixth coordination position. Evidence for this structure rests on p.m.r., c.m.r. and c.d. spectral evidence but more particularly on the close analogy with the corresponding hexacoordinate copper complex for which X-ray crystallographic analysis is available [53]. Binding of the carbamate carbonyl group is relatively weak, and it can be displaced by oxygen in the activated form of the antibiotic as shown in Fig. 10. The close similarity of the spectral information from this portion of the metal complexes for BLM and for TLM [10] leads one to expect a similar structure for the iron coordinated in TLM. Binding of the antibiotic to the DNA is due, in part, to intercalation of the bithiazole rings between base pairs in which one chain contains a G-T or G-C sequence. It has been shown by Povrik *et al.* [54] by fluorescence quenching that partial unwinding of DNA occurs during the binding process due to intercalation of the planar bithiazole moiety. Binding of the glycopeptide antibiotics to DNA may also be assisted through electrostatic interactions between the positively charged dimethylsulfonium moiety (in BLM-A_2) or the spermidinium grouping (in BLM-A_5 and TLM) and the negatively charged phosphate groups [52]. Another difference between TLM and BLM is that the former sequesters an additional metal ion by the four nitrogens of the β-lysine spermidine side chain together with the amino-group of the talose group [10]. These assignments were based on c.m.r. evidence, which also suggested that the amide nitrogen of the spermidine chain is deprotonated at pH 6–10 assisting in coordination to the metal [10].

It has been postulated that the electrostatic attraction of the protonated spermidine in BLM-A_5 or of the dimethyl sulfonium group in BLM-A_2, to the negatively charged phosphate groups on the DNA, assists in the binding of the glycopeptide antibiotics [52, 53]. Therefore, it seemed plausible that the protonated spermidine grouping in TLM might assist in binding to DNA either directly or by using the permanent positive charge of the second sequestered metal ion in which the spermidine chain is postulated to participate. To obtain some information on this, we examined the action on DNA of TLM-E_{1a} (i.e. the antibiotic modified by spermidine oxidase from *Serratia marcescens* in which the spermidine grouping has been replaced by 1,3-diaminopropane). TLM-E_{1a} and its copper complex bind to DNA with less efficiency than does TLM and the corresponding copper complex, as measured by ethidium fluorescence suppression. This indicates that the spermidine side chain contributes significantly to the DNA binding process of the metal-free antibiotic. However, in the presence of Fe^{2+}, the extent of DNA scission produced by TLM and the spermidine oxidase modified TLM-E_{1a} is comparable. This suggests that the spermidine side chain is not involved in binding Fe^{2+} at the 'active site' of the antibiotic. It is also likely that, at least in the case of the antibiotic–iron complex responsible for DNA degradation, of the two factors contributing to binding of the glycopeptide antibiotics to DNA, namely intercalation of the bithiazole moiety and electrostatic binding of the spermidinium chain, the former is more significant.

The model for the mechanism of action depicted in Fig. 10, in which binding of TLM to DNA brings the hexacoordinated iron close to the target where reaction with oxygen generates superoxide, hydrogen peroxide and hydroxyl radicals, is in accord with our observations that the cleavage of DNA by TLM is inhibited by superoxide dismutase, by catalase, and by free radical scavengers. While the present results indicate a close similarity between the molecular mechanisms of action of BLM and TLM on DNA, there are significant differences. For TLM there were definite but lower levels of inhibition of nicking by superoxide dismutase, catalase and sodium benzoate compared with BLM, but in contrast to the latter case no inhibition by other free radical scavengers including isopropyl alcohol and

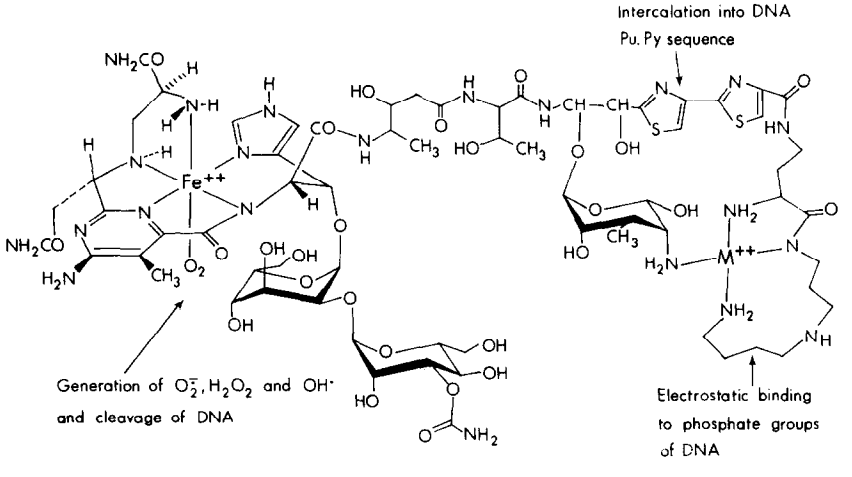

Fig. 10. Depiction of molecular mechanism of action of TLM-A in binding to and degrading DNA.

potassium iodide. In view of the evidence quoted above for the stronger binding of TLM than of BLM to DNA, this may account for the difficulty in complete suppression of cleavage by sodium benzoate. Our results confirm the findings of Strong and Crooke [11] that TLM is significantly less effective than BLM in producing strand breaks in DNA *in vitro*. The fact that TLM is a more effective antitumor agent *in vivo* may be due to its longer serum half-life compared with BLM [10].

In conclusion, steps 1–6 or their equivalent can account for the production, by DNA bound and activated TLM, of $OH^{\cdot}$ radicals which are known to degrade DNA [55]. Among the factors which may contribute to their selective action against neoplastic tissue are the more reducing environment of tumor tissue which activates the antibiotic-sequestered Fe^{3+} by reduction and the suppressed levels of superoxide dismutase and catalase in tumor cells [36, 56] which may account for preferential toxicity of the glycopeptide antitumor antibiotics.

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