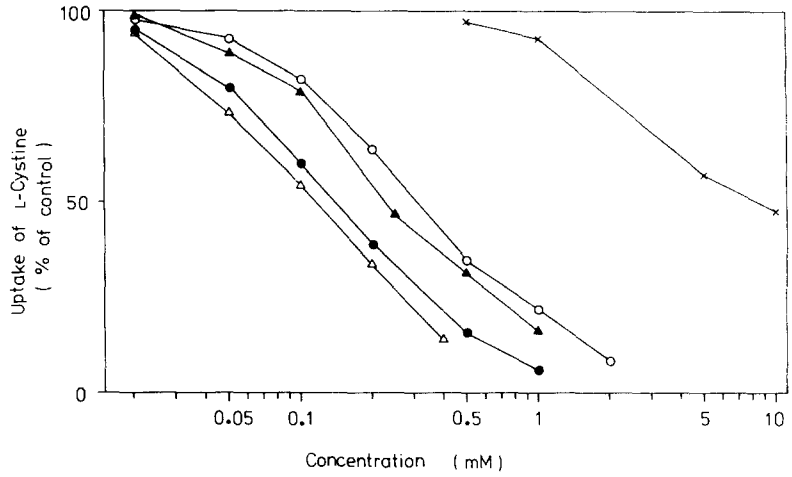

Fig. 3. Inhibition of L-[^{3}H]cystine uptake by some NSAID. NSAID added were indomethacin (●—●), mefenamic acid (△—△), piroxicam (▲—▲), phenylbutazone (○—○), and aspirin (×—×). The concentration of L-cystine in the uptake medium was 0.05 mM, and the rate of uptake was measured by taking the values for the 2-min uptake.

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Identification of a second binding isotherm for actinomycin D–deoxyribonucleic acid at low drug concentrations

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Actinomycin D is a heterocyclic compound consisting of a phenoxazinone ring linked to two cyclic pentapeptides. The interaction of the drug with DNA has been studied for many years because of its ability to inhibit RNA synthesis [1], its use as a chemotherapeutic agent in treatment of certain cancers [2], and as a system for studying protein-nucleic acid interactions [3–5]. The models proposed for the mechanism by which actinomycin D binds to DNA are based upon intercalation of the planar chromophore ring at GC base pairs in the double helix [4] or pseudointercalation between helical DNA chains at GC base pairs [6]. Wells and Larson [5], however, have reported that an analysis of actinomycin D binding to synthetic polydeoxyribonucleotides indicated that binding was not always dependent on the presence of GC base pairs. It is important to note, however, that few studies of actinomycin D–DNA interaction have been performed with concentrations of the drug which are relevant to biological (0.001 to 0.1 μg/ml or 0.8 to 80 nM) or therapeutic (0.5 to 2.0 mg/sq. m and <80 nM serum concentration) use. A study was undertaken to analyze the interaction of this drug with DNA at concentrations that fall within the range of biological or therapeutic use. A unique site (or sites) was found on rat liver DNA which could be observed when actinomycin D binding was assayed at concentrations of drug at <80 nM or 0.1 μg/ml. This site (or sites) appeared to be distinct from those previously observed in the presence of higher concentrations of actinomycin D [3–5, 7].

Methods and results

DNA was extracted as described by Maniatis *et al.* [8] from rat liver nuclei isolated by a modification [9] of the method of Hewish and Burgoyne [10] and subsequently digested with EcoRI restriction endonuclease [8]. The DNA was dialyzed under sterile conditions in 50 mM Tris–HCl, pH 7.6, 0.1 M NaCl. EcoRI cleaves DNA on the

average of one site for every 3400 base pairs based on the recognition sequence for the enzyme and the G+C content (44%) of rat DNA.

The assay for DNA bound actinomycin D was performed as described by Jones [7] with the following specifications. [^{3}H]Actinomycin D (14 Ci/mmole, Amersham), diluted in 95% ethanol, was added to DNA (0.5 μg/ml) in 50 mM Tris–HCl, pH 7.6, 0.1 M NaCl in triplicate 100-μl aliquots in 1.5 ml microfuge tubes treated with silicone (Sigmacote, Sigma). ^{32}P-Labelled rat liver DNA (10^7 dpm/μg), prepared by nick translation [8], was added to give 5–10 $\times$ 10^3 dpm/ml. After mixing and standing at room temperature for greater than 10 min, a 10-fold excess of cold (−20°) ethanol was added, and the samples were collected on glass filters (GF/C, Whatman), dried, and counted. Total free [^{3}H]-actinomycin D was determined by spotting 10-μl aliquots in triplicate onto 2.3 cm, 3 MM paper filter discs (Whatman), which were dried and counted. All dpm due to [^{3}H]actinomycin D was corrected for [^{32}P]DNA spillover into the ^{3}H-channel.

The data were converted to a form for plotting according to Scatchard [11] as described by Müller and Crothers [4]:

$$r/m = K_{app}(B_{app} - r)$$

where r is the ratio of bound molecules of actinomycin D to total DNA base pairs, m is the concentration in molarity of free actinomycin D, K_{app} is the apparent binding constant, and B_{app} is the apparent number of binding sites per base pair. K_{app} and B_{app} were calculated using a computer program (SCIFIT) developed by DeLean as described in Ref. 12 for simultaneous data analysis of saturation curves in systems involving binding of a ligand to multiple classes of binding sites according to the mass action law. Data were weighted by $1/x$. A theoretical curve was determined using the calculated values of K_{app} and B_{app}. The goodness of fit is based upon use of the critical F test [12].

The binding of actinomycin D to DNA was assayed not only at high concentrations of the drug as reported by others [3–5, 7] but at relatively low concentrations of the drug. Figure 1 shows the curves generated by non-linear least squares regression analysis of the data. The isotherm for binding of actinomycin D to rat DNA at low concentrations of the drug, defined by curve 1 of Fig. 1, yielded an apparent association constant (K_{app}) of 7.6×10^7 M^{-1} (K_D = 13 nM or 0.016 μg/ml). At higher concentrations of actinomycin D (curve 2, Fig. 1), binding was predominantly to sites having an apparent association constant at least 100-fold less than that determined from curve 1, Fig. 1 (K_{app} of 7.5×10^5 M^{-1}, K_D = 1.3 nM or 1.6 μg/ml), and compared favorably with the previously reported values obtained from data generated using high concentrations of actinomycin D by the method of equilibrium dialysis [4] as well as that reported by Jones [7]. The F value for comparison of one site versus two binding sites was calculated to be 47.2 or a $P < 0.05$ for a second binding isotherm at low concentrations of actinomycin D.

The intercept on the horizontal axis defines B_{app}, the apparent number of binding sites for actinomycin D per base pair of DNA. The B_{app} of curve 2, Fig. 1, at the high concentrations of actinomycin D is 0.066 (1 molecule bound per 15 bp) and is similar to those previously reported [3–5, 7]. Significantly fewer binding sites per unit base pairs of DNA were observed using the binding isotherm of curve 1, Fig. 1. The B_{app} was calculated to be 0.0030 or an average of 330 base pairs of DNA per actinomycin D bound.

The rate at which actinomycin D dissociates from DNA is another parameter for the definition of a binding site. [^{3}H]actinomycin D was combined with unlabeled EcoRI-cleaved DNA (0.5 μg/ml) at room temperature and trace amounts of [^{32}P]DNA to monitor DNA recovery in 7-ml aliquots. Triplicate 20-μl aliquots were spotted for determining the total concentration of actinomycin D, and 200-μl aliquots were removed and added to 1 ml of cold (−20°) ethanol, filtered, and counted for zero time. The remaining solution was made 50 mg/ml in Dextran gelatin-coated charcoal, and aliquots of 800 μl were taken at the indicated time points, centrifuged at 11,000 g, and processed essentially as described by Dokah *et al.* [13]. Time points were recorded when ethanol was added to 200-μl aliquots in triplicate, and subsequently filtered on glass fiber (GF/C, Whatman). The amount of DNA recovered on the filter was determined by the ^{32}P-specific radioactivity of the total DNA added, and ^{3}H-dpm on the filter was normalized for loss of DNA versus the amount of input DNA. The amount of charcoal added removed 99.9% of the free actinomycin D by the earliest time point examined (2 min). The loss of DNA by binding to charcoal was less than 55% of input DNA at the latest time point examined (130 min) and was independent of DNA concentration. Figure 2 shows the results. The relatively slow rate of dissociation ($T_{1/2}$ = 18 min) from rat liver DNA of actinomycin D bound at the low concentration of 0.045 μg/ml (36 nM) versus a $T_{1/2}$ = 9 min for that bound at 1 μg/ml (780 nM) illustrates the presence of a distinct high affinity binding site on DNA. When an intermediate concentration of 0.1 μg/ml of actinomycin D (78 mM) was employed, both rates were observed (Fig. 2, insert).

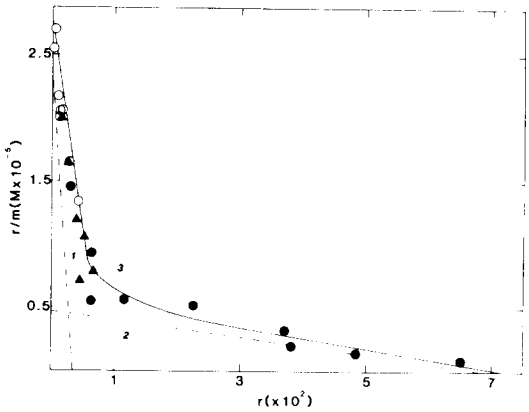

Fig. 1. Isotherms for the binding of actinomycin D to rat liver DNA. [^{3}H]Actinomycin D at various concentrations was combined with EcoRI-treated rat liver DNA, and the bound [^{3}H]actinomycin D was assayed and the results used to calculate the values for K_{app} and B_{app} as described in the text. A theoretical curve was generated to fit the data points (curve 3). The symbols represent the results from three separate experiments.

Discussion

The results presented here establish the presence of a site or sites on rat liver DNA that bind actinomycin D more tightly than previously observed. The sites were detected when binding was assayed at relatively low drug concentrations (Fig. 1).

The mechanism by which actinomycin D binds at a low concentration to DNA cannot be solely on the basis of actinomycin D intercalation at [4] or interaction with [3] the deoxyguanine residues of DNA. It is unlikely that the results presented here are due to overlapping dG sites or to positive ligand–ligand cooperative binding [14] since binding to overlapping dG sites would occur only at high concentrations of the drug, and there is no evidence of cooperative binding of actinomycin D to DNA [3–5, 7] with the exception of that observed by Winkle and Krugh [15]. However, an interpretation of their results should take into account the relatively high concentration of DNA employed in their experiments. These concentrations of DNA can significantly reduce the amount of unbound drug

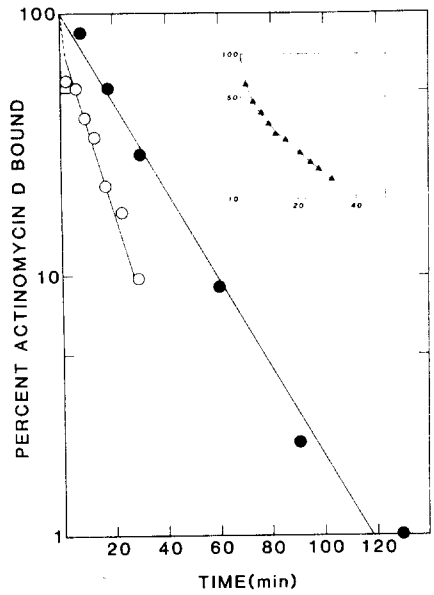

Fig. 2. Rate of dissociation of actinomycin D bound to DNA at either low or high concentrations. [^{3}H]Actinomycin D at 0.045 μg/ml (●) or at 1 μg/ml (○) was combined with EcoRI-treated rat liver DNA (0.5 μg/ml). The unbound drug was removed by charcoal, and the loss of bound [^{3}H]-actinomycin was assayed as described in the text. The curves were generated by linear regression analysis with regression coefficients of −0.991 and −0.971 for data using 0.045 and 1 μg/ml actinomycin D respectively. Insert: rate of dissociation of actinomycin D bound to DNA at 0.1 μg/ml.

to yield a Scatchard plot indicating cooperative binding (J. J. Duffy and T. J. Lindell, unpublished observations). It is possible, however that binding of actinomycin D at this site(s) may influence the binding of a second site several hundred bases removed by altering the DNA helix. But again, the binding cannot be based solely upon interaction with dG since no evidence of cooperative binding at high concentrations of the drug has been presented. The possibility that binding sites are created by EcoRI cleavage can be eliminated since this enzyme will cleave rat liver DNA on the average of once every 3400 base pairs, and the drug does not bind to single-stranded DNA.

Rates of dissociation (Fig. 2) also support the existence of at least two binding sites for actinomycin D. One would expect that an observed difference in K_{app} should be reflected in the rate of dissociation of the ligand. However, if the binding sites and mechanisms of binding are fundamentally different, this generally does not apply as shown by Müller and Crothers [4] in the case of DNA binding of actinomine and actinomycin C_3.

The molecular nature of the formation of this actinomycin D–DNA complex is not known, although investigations into the mechanism by which DNA interacts with the drug when present at high concentrations are well documented [3, 4, 6]. Wells and Larson [5] reported evidence that the presence of a dG residue alone may not dictate the binding of actinomycin D at high concentrations (>1 μg/ml) to DNA. They concluded that the nucleic acid structure and nucleotide sequence may also play a role in the specificity of actinomycin D binding. This evidence supports the existence of binding sites which are specified by parameters other than, but possibly including, those based on the specificity of actinomycin D for dG. Such sites may include those reported here.

The results presented here define an isotherm for the binding of actinomycin D at relatively low concentrations to rat liver DNA. The apparent binding constant ($7.6 \times 10^7\ M^{-1}$) defines a site on DNA which is clearly distinct from that observed at higher concentrations of the drug ($K_{app} = 7.5 \times 10^5\ M^{-1}$). The number of binding sites for actinomycin D per base pair of DNA was less numerous ($B_{app} = 0.0030$) at low concentrations of the drug when compared to the number of sites available at higher concentrations ($B_{app} = 0.066$). The rate at which actinomycin D dissociated from DNA when the drug was bound at 36 nM ($T_{1/2}$ = 18 min) was 2-fold slower than the rate of dissociation of actinomycin D bound at 780 nM ($T_{1/2}$ = 9 min). The results presented define a binding site for actinomycin D on DNA which may indicate a complexity of interaction greater than a G-C base pair requirement when the drug is present at low concentrations which are employed therapeutically and in the inhibition of RNA synthesis.

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