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TILORONE HYDROCHLORIDE—A SPECIFIC PROBE FOR A-T REGIONS OF DUPLEX DEOXYRIBONUCLEIC ACID

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Abstract—The interaction between tilorone hydrochloride and a variety of DNAs and synthetic polydeoxynucleotides was investigated in order to delineate the nucleic acid structural features necessary for binding. The binding parameters (B_{app} and K_{app}) derived from the Scatchard plots of $r/(u)$ vs r (determined by equilibrium dialysis experiments) show a very high specificity of tilorone binding to A-T rich DNAs; no specific binding Scatchard plot (cf. Fig. 1d) of tilorone to poly(dG-dC)·poly(dG-dC) was observed. The K_{app} value for poly(dG-dC)·poly(dG-dC) was less by a factor of 10^{-2}, compared with those for A-T containing DNAs. The number of binding sites (B_{app}) is correlated linearly to the A-T content of the DNAs. The closest distance between bound tilorone molecules is four base pairs.

The dihydro-chloride salt of 2,7-bis[2-(diethyl-amino) ethoxy]-fluoren-9-one, referred to as tilorone hydrochloride, or bis-DEAE-fluorenone (DEAE-F), is a broad-spectrum antiviral compound [1, 2] with antitumor activity [3–5]. Tilorone hydrochloride has been shown by us to form a molecular complex with DNA [6]. On the basis of our hydrodynamic studies with the tilorone–DNA complex we proposed an intercalative mode of binding of tilorone to DNA[7]. This interaction inhibits the DNA template functions in DNA- and RNA-polymerase reactions *in vitro* [6]. Tilorone was also reported to inhibit the DNA polymerase activity in RNA tumor viruses[8]. The latter inhibition was found to be selectively dependent on the chemical composition of the primer-template used in the viral enzyme reaction.

The present paper describes some of the binding parameters of tilorone to DNA and synthetic polydeoxynucleotides. The data obtained by equilibrium dialysis support our previous observations on thermal denaturation and enzymatic studies [7, 9] that tilorone specifically binds to dAT-sites of double-stranded DNA.

MATERIALS AND METHODS

Tilorone hydrochloride was a gift of Merrell National Laboratories, Cincinnati, Ohio, U.S.A. [9-^{14}C]Tilorone, sp. act. 5.5×10^{13} dis/min/mole was prepared by Dr. George Wright of the Merrell National Laboratories. ^{3}H-labelled ATP was obtained from NEN-Chemicals GmbH, Germany. Unlabelled ribonucleoside triphosphates were purchased from Boehringer Mannheim GmbH, Germany.

Polydeoxyribonucleotides were obtained from Collaborative Research, USA. Calf thymus DNA was isolated according to the method of Zamenhof [10]. Some samples were further purified by pronase digestion and phenol extraction. DNA from *Mic. lysodeikticus* was isolated by the procedure of Thomas *et al.* [11].

Equilibrium dialysis. Equilibrium dialysis was carried out by a procedure and an apparatus (Dianorm, supplied by Dr. Virus KG, Bonn, Germany) described by Weder *et al.* [12]. Dialysing membrane (0.025 mm thick) was sandwiched between two halves of a Teflon (round) macro-cell (dialysable volume = 1 ml). The DNA, or labelled tilorone solutions were introduced by separate micro syringes on either side of the membrane through the side valves. The valves were closed air tight and the macro-cells were fixed into a rotating machine. All equilibrium dialysis studies were carried out at 20°, and at 10 rotations/min. Under these conditions equilibrium was attained in 4–5 hr. After the equilibrium was reached, 0.8 ml of the solution from either side of the membrane was withdrawn by microsyringes and the radioactivity was determined using dioxan scintillation fluid. Protein was estimated by the method of Lowry *et al.* [13], and DNA was estimated by the method of Burton [14].

RESULTS AND DISCUSSION

Thermal denaturation studies performed on tilorone and DNA complexes with several DNAs and synthetic poly(dA-dT)·poly(dA-dT) have been reported earlier [7]. The ΔT_m of the complex was shown to be dependent on the AT content of DNA. Thus, at $r = 0.21$ (r designates the tilorone to DNA-P ratio), the complex between *Mic. lys.* DNA and tilorone has a ΔT_m of 11°, whereas, at the same drug ratio, the poly(dA-dT)·poly(dA-dT) complex shows a ΔT_m of 29.6° [7].

The AT-specificity in the binding of tilorone to DNA was also observed in the quantitative equilibrium binding measurements. The equilibrium binding data were plotted as $r/(u)$ vs r, where r is the moles of bound tilorone divided by the DNA concentration in base pairs [15], and (u) is the concentration of unbound tilorone. From this plot, binding parameters

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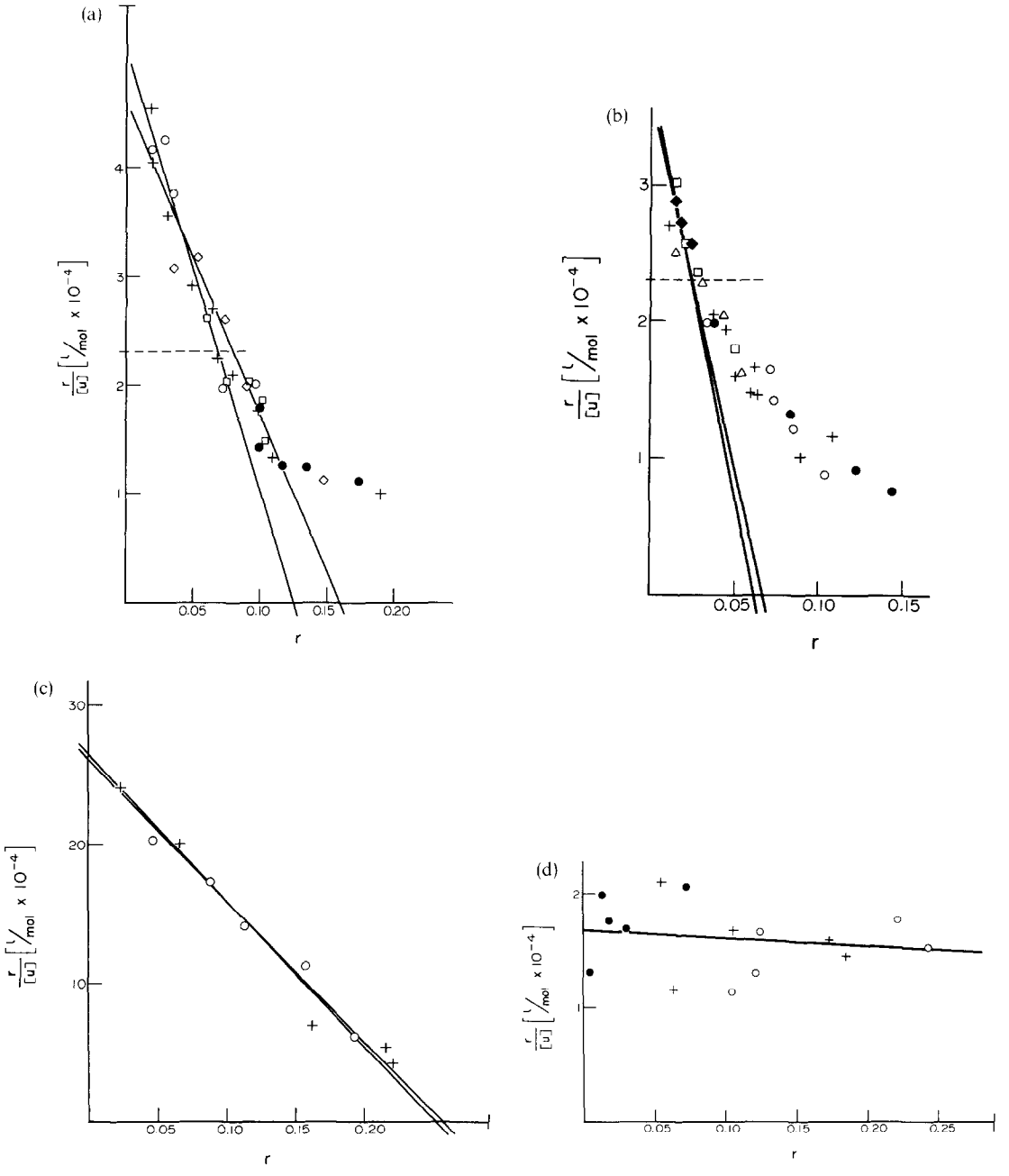

Fig. 1. Scatchard plots for the binding of tilorone hydrochloride to calf thymus DNA (1a); *Mic. lysodeikticus* DNA (1b); poly(dA-dT)·poly(dA-dT) (1c); and poly(dG-dC)·poly(dG-dC) (1d). Each different symbol corresponds to a separate experiment. Thus, each figure represents a set of 4 or 5 separate experiments. r is moles of bound tilorone/base pair concentration and (u) is the concentration of unbound tilorone. Further details are given in the text.

were obtained appropriate to a model in which all DNA binding sites are considered to be independent of each other [16]. The equation from this model is:

$$r/(u) = K_{app}\,(B_{app} - r),$$

where K_{app} and B_{app} are the apparent binding constant and number of binding sites per base pair, respectively; K_{app} is the negative of the slope, and B_{app} the $r/(u) = 0$ intercept of the linear region of the $r/(u)$ vs r plot.

The Scatchard plots for the binding of tilorone to calf thymus DNA (Fig. 1a), *Mic. lys.* DNA (Fig. 1b), poly(dA-dT)·poly(dA-dT) (Fig. 1c), and poly(dG-dC)·poly(dG-dC) (Fig. 1d) are shown in Fig. 1. As follows from the Scatchard plots for natural DNAs, (Figs. 1a and 1b), the independent site model does not fit for DNA–ligand interaction; however, we employed the binding parameters of this model to

Table 1. Binding constants for the interaction of tilorone hydrochloride with DNAs and synthetic polydeoxynucleotides

Source of DNA	B_{app}	K_{app} M^{-1}
A. Natural DNAs		
Calf thymus	0.16	2.9×10^5
	0.12	4.1×10^5
Mic. lysodeikticus	0.066	5.5×10^5
	0.062	6.0×10^5
B. Synthetic polymers		
Poly(dA-dT)·poly(dA-dT)	0.25	1.02×10^6
	0.25	1.04×10^6
Poly(dG-dC)·poly(dG-dC)	—	6.9×10^3

All experiments were carried out at 20° in 0.1 M Tris–HCl (pH 7.0). The binding parameters were derived from the Scatchard plots (Figs. 1a–1d); the experiments were carried out by equilibrium dialysis as described in Materials and Methods. K_{app} = apparent binding constant; B_{app} = number of binding sites per base pair.

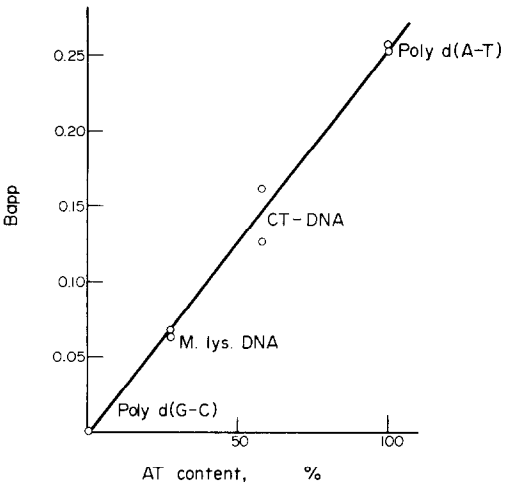

Fig. 2. A-T specificity of tilorone binding to DNAs. The B_{app} values were derived from the Scatchard plots of $r/(u)$ vs r, as shown in Table 1. Experimental details are described under Materials and Methods.

analyse the Scatchard plots. The binding parameters, K_{app} and B_{app} derived from the Scatchard plots (Fig. 1, a–d) are presented in Table 1. This table summarizes the results of several measurements.

As seen in Table 1 the apparent binding constants for both the natural DNAs are of the same magnitude, whereas the K_{app} for poly(dA-dT)·poly(dA-dT) is higher by a factor of two. The K_{app} value for poly(dG-dC)·poly(dG-dC) is less by a factor of 10^{-2}, compared with those of natural DNAs and the synthetic polydeoxynucleotide poly(dA-dT). poly-(dA-dT). The data on the number of binding sites per base pair (B_{app}) for various DNAs show a strict correlation with the AT-content of the biopolymer. On the basis of the B_{app} data, the closest distance between bound tilorone molecules is four base pairs. The values shown in Table 1 give almost a linear curve (Fig. 2), showing a linear dependency of B_{app} on the AT content of DNAs. The value for poly(dG-dC)·poly(dG-dC) has been extrapolated to zero (Fig. 2), since the Scatchard plot (cf. Fig. 1d) does not indicate any specific binding of tilorone to this polymer.

The AT-specificity of tilorone binding to DNA shown in the present experiments was also observed in the RNA-polymerase reaction catalyzed by calf thymus DNA, or poly(dA-dT)·poly(dA-dT) [9]. It was found that the template activity of poly(dA-dT)·poly(dA-dT) in the RNA-polymerase reaction is much more sensitive towards tilorone than that of calf thymus DNA. The tilorone concentration needed to inhibit the 50 per cent reaction were for the poly-(dA-dT)·poly(dA-dT)-dependent reaction 12 μM, and for the DNA-dependent reaction approx 50 μM [9].

Besides tilorone, some pyrrole–amidine antibiotics have recently been reported to exhibit A-T specificity in their interaction with DNA. Of these, distamycin [17, 18] and netropsin [19] have been studied in detail. The binding parameters of netropsin to DNAs of various composition using different methods have been reported by Wartell *et al.* [19]. They concluded that the inability of G-C paired regions of DNA to bind netropsin was a consequence of the 2-amino group of guanine. Using the linear density-gradient centrifugation method, we have been able to show that labelled tilorone binds quite efficiently to poly-(dI-dC)·poly(dI-dC). The quantitative evaluation of this binding by the equilibrium dialysis method is under investigation. If the K_{app} value of this binding turns out to be of the same magnitude as for AT-polymers, one might draw similar conclusions, that the inability of G-C pairs of DNA to bind tilorone is a consequence of the 2-amino group of guanine. Preliminary experiments using the density-gradient method show that tilorone binds poorly to polydA·polydT; however more experiments are needed to draw any conclusions about the role of conformation in tilorone binding.

In a previous publication [9] we reported the thermal transition temperatures of DNA complexed to tilorone, and a series of tilorone congeners. These studies indicated that tilorone interaction with DNA is dependent on some structural entities of tilorone. Of particular interest are the facts that side chains at both ends, i.e. C-2 and C-7 are necessary [9], and the substitution of the keto group by an alcoholic group, i.e. the fluorenol derivative, leads to an appreciable loss of its binding efficiency to DNA (manuscript in preparation). The tilorone congeners with side-chain modifications, or other substitutions such as dibenzofuran, fluorene, and dibenzothiophene [5, 9] exhibit an A-T specificity similar to tilorone, as observed in the RNA- and DNA-polymerase reactions catalyzed by DNA and synthetic polymers (unpublished results).

The capacity of a nucleic acid to bind actinomycin is a sensitive indicator of polynucleotide configuration. Earlier studies have shown [20–23] that actinomycin D binds specifically to helical DNA at G-C pairs, causing structural distortion. The availability of a probe with complimentary specificities, such as tilorone, may permit dual binding studies to investigate the transmission of conformational stability [24]. One such study has recently been reported by Burd

et al. [24] using the probes actinomycin D and netropsin, with complimentary specificities.

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