The use of radiolabelled triostin antibiotics to measure low levels of binding to deoxyribonucleic acid

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Triostin antibiotics, which contain a cyclic peptide with a disulphide bridge, have been prepared by growing *Streptomyces triostinicus* in the presence of inorganic [^{35}S]-sulphate. The labelled triostin A has been shown to behave in all respects similarly to the authentic natural product and to enable a much more sensitive radiochemical adaptation of the solvent-partition method for determining antibiotic binding to DNA. By this means, binding isotherms at low, biologically relevant levels (down to one antibiotic molecule per gene) have been measured. The results indicate the existence of some tight binding sites in natural DNA species that are preferentially occupied at low concentrations. No evidence has been found for any allosteric transitions provoked by interaction between these antibiotics and natural DNA species, though there is evidence for co-operativity in the binding of triostin A to poly(dA-dT). For the first time accurate isotherms have been determined for the binding of triostin C to DNA; its binding constants for a variety of polydeoxynucleotides are uniformly tighter than those of triostin A but fall into the same ranking order when different species of natural DNA are compared.

Quinoxaline antibiotics are secondary metabolites produced by several species of streptomycetes. Structural features common to all members of the group include a heterodetic cyclic octadepsipeptide bearing two symmetrically or quasi-symmetrically disposed quinoxaline chromophores (Katagiri *et al.*, 1975; Waring, 1979). They can be divided into two families, the quinomycins and the triostins, which differ in the nature of the bridge that crosses the peptide ring. The triostins are characterized by a simple disulphide cross-bridge. Triostin A is shown in Fig. 1; triostin C differs only in having L-N γ -dimethyl-*allo*-isoleucine residues in place of the L-N-methylvaline residues (Otsuka & Shoji, 1965). These antibiotics are powerful anti-microbial and anti-tumour agents whose toxic effects have been attributed to their binding to the DNA of susceptible cells (Ward *et al.*, 1965; Sato *et al.*, 1967; Waring & Makoff, 1974; Katagiri *et al.*, 1975; Waring, 1981).

Much of the interest in these compounds has centred around their unusual sequence preferences in binding to DNA. Echinomycin (quinomycin A) displays a broad preference for binding to DNA species rich in (G + C) residues (Wakelin & Waring,

1976). Triostin A retains this GC preference, but it is much less marked (Lee & Waring, 1978a). TANDEM, a synthetic des-N-tetramethyl analogue of triostin A, shows a pronounced selectivity for (A + T)-rich sequences in DNA (Lee & Waring, 1978b). The validity of these conclusions is limited to a rather gross description of the DNA-binding behaviour of the ligands, however, because the techniques employed to measure binding have generally lacked sufficient sensitivity to provide data below *r* values (antibiotic molecules bound per nucleotide) of about 0.02. On the other hand, experiments measuring the dissociation of quinoxaline antibiotics from DNA have also indicated the occurrence of different classes of intercalative binding sites (Fox *et al.*, 1981; Fox & Waring, 1981), reflecting the presumed existence of sequence selectivity.

The chief purpose of the present study was to develop a method for investigating the binding of these ligands to DNA at low ('biologically relevant') levels likely to be encountered *in vivo*. This we have accomplished by preparing radiolabelled triostin antibiotics. The first step was to investigate whether inorganic [^{35}S]sulphate could be incorporated into antibiotic molecules by the producing organism

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Fig. 1. Structure of Triostin A

Streptomyces triostinicus, and then to produce the ligand with sufficiently high specific radioactivity for use in solvent-partition experiments (Waring *et al.*, 1975). Next, the properties of the radiolabelled antibiotics were carefully examined to verify consistent behaviour compared with previous results. That done, we sought to extend the existing binding isotherms for triostin A to much lower levels, where deviations from the predicted curves due to sequence selectivity should be more apparent. A related objective was to investigate whether better evidence might be adduced as to the possible existence of co-operativity in the interaction between quinoxaline antibiotics and poly(dA-dT) (Lee & Waring, 1978a, b; Fox *et al.*, 1982). Finally, we took advantage of the increased sensitivity of the radiochemical assay to determine accurate DNA-binding curves for triostin C, which has not previously been amenable to detailed investigation because of its very low water solubility (Lee & Waring, 1978a).

Materials and methods

Calf thymus DNA (highly polymerized sodium salt, type I) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Bacterial DNA species were prepared by a modification of the method of Marmur (1961) as described in Lee & Waring (1978a). For the measurement of binding curves all natural DNA samples were sheared to a standard molecular weight by drawing a solution (2 mg/ml in 2.5 M-NaCl) twenty times through a no. 27 needle at 0°C (Pieritz *et al.*, 1972). Poly(dA-dT) was purchased from Boehringer Corp., London W.5, U.K. Nucleic acid concentrations are expressed in

terms of molarity with respect to nucleotides and are based on an assumed value for $\epsilon_{(P)}^{260}$ of 6600 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$, except for *Micrococcus lysodeikticus* DNA (6300 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$) (Tubbs *et al.*, 1964) and poly(dA-dT) (6700 litre $\cdot$ mol $^{-1} \cdot$ cm $^{-1}$) (Wells & Wartell, 1974).

All binding experiments were conducted at 20°C in a HEPES [4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid]/NaOH buffer at pH 7.0 and 10.01, designated '0.01 SHE buffer' (Wakelin & Waring, 1976). Solvent-partition analysis was conducted using either pure isopentyl acetate (IPA) or an isopentyl acetate/n-heptane mixture (1:1, v/v) designated '50/50 IPA/heptane' which was prepared as described by Lee & Waring (1978a).

Preparation of radiolabelled antibiotics

Streptomyces triostinicus (A.T.C.C. 21043) was provided by ICI Pharmaceuticals Division, Alderley Park, Macclesfield, Cheshire, U.K., and maintained on ISP7 slants (Shirling & Gottlieb, 1966). For the production of ^{35}S -labelled triostins the organism was grown in two 1-litre cultures of maltose minimal medium in 2-litre Erlenmeyer flasks by the method of Yoshida & Katagiri (1969). The inorganic-sulphate content of the growth medium was decreased to 0.29 mM (one-fifth of the normal value) by partially replacing the ZnSO_4 and MgSO_4 with ZnCl_2 and MgCl_2 . Inorganic [^{35}S]sulphate (carrier-free, purchased from The Radiochemical Centre, Amersham, Bucks., U.K.) was added to each culture at the time of inoculation (2 mCi/litre). Aeration and temperature control were provided in a New Brunswick G-25 orbital incubator operating at 240 rev./min and 28°C. Mycelia were collected after

8 days' incubation and extracted with acetone and ethyl acetate as described by Yoshida *et al.* (1968) to yield a white powder containing triostins A and C. It was found that 13.7% of the ^{35}S added to the medium was recovered in the antibiotics. They were separated by using the flash column-chromatography method of Still *et al.* (1978) using Merck Kieselgel 40 (40–63 μm particle size), with ethyl acetate as eluting solvent. The triostin C fraction was subjected to the same procedure a second time and twice recrystallized from acetone. Triostin A was further purified by preparative t.l.c. on Merck Kieselgel 60F₂₅₄ plates (0.75 mm thickness) developed in butan-1-ol/acetone/water (3:1:1, by vol.). The purity of the antibiotics was checked by analytical t.l.c. on Merck Kieselgel 60F₂₅₄ sheets (0.2 mm thickness) developed in butan-2-one. The R_F values of triostins A and C in this system are 0.27 and 0.35 respectively. The compounds were found to be homogeneous by direct autoradiography of t.l.c. sheets, and the upper limit for cross-contamination of the purified materials was estimated to be less than 1% by liquid-scintillation counting of t.l.c. sheets sliced into zones corresponding to the known R_F values of the two components. The purified antibiotics were stored as solutions in acetone at -20°C .

Measurement of radioactivity

Antibiotic concentrations were determined by scintillation counting in a Nuclear Enterprise model 8312 counter. Samples were prepared by drying portions (up to 500 μl of aqueous solutions or 200 μl of organic solutions) on to 2 cm-diameter Whatman GF/D filters. When dry the filters were placed in plastic vials and covered with 10 ml of scintillant [containing 5 g of PPO (2,5-diphenyloxazole) and 0.1 g of POPOP [1,4-bis-(5-phenyloxazol-2-yl)-benzene] per litre of toluene]. Duplicate samples were each counted for radioactivity for 5 min. Control experiments were conducted to check that the counting efficiency remained effectively constant at >97% under all the experimental conditions. By standardizing the procedure so that all samples were dried on to filters, it was possible to eliminate both the need for an emulsifier to count aqueous samples and any problems associated with counting different volumes in different experiments.

Determination of specific radioactivity

For each antibiotic, 100 μl of the stock solution in acetone was evaporated to dryness and dissolved in 1 ml of a solvent for which the molar absorption coefficient was known: isopentyl acetate for triostin A, and a dimethyl sulphoxide/buffer mixture (1:1, v/v) for triostin C. The concentration of this solution was determined by measuring its absorbance at 317–325 nm in a Pye-Unicam SP.8-200 spectro-

photometer, using the absorption coefficients of 12100 litre $\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (triostin A) and 13800 litre $\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ (triostin C) given by Lee & Waring (1978a). Duplicate portions of this antibiotic solution of known concentration were dried on to GF/D filters and counted for radioactivity as described above. The specific radioactivity of triostin A on the first day was found to be $21.8 \times 10^6 \text{ d.p.m.} \cdot \mu\text{mol}^{-1}$; that of triostin C was $21.7 \times 10^6 \text{ d.p.m.} \cdot \mu\text{mol}^{-1}$. The specific radioactivity on subsequent days was calculated from the known half-life of ^{35}S (87.4 days).

Partition coefficients

By way of a final check on the purity of the labelled material, and as an essential preliminary before investigating the consonance between results obtained by spectrophotometric and radiochemical methods, the partition coefficient of triostin A between isopentyl acetate and 0.01 SHE buffer was determined by using the labelled material. Samples were prepared and treated as described by Waring *et al.* (1975), but the concentration of antibiotic in each

Fig. 2. Partition of triostin A between isopentyl acetate and 0.01 SHE buffer

Samples were prepared and counted for radioactivity as described in the text. The line drawn is 'theoretical', i.e. that determined by Lee & Waring (1978a) from a least-squares fit to data obtained by spectrophotometric measurements. Its slope is 943 ± 23 [concentration in organic phase ($c_{\text{org.}}$)/concentration in aqueous phase ($c_{\text{aq.}}$)]. To ensure consistency with previously reported experiments this value for the partition coefficient was employed in all experiments with radiolabelled triostin A (Figs. 3 and 4).

phase was determined by removing duplicate portions, drying on to GF/D filters and counting for radioactivity as described above. The data obtained (Fig. 2) yielded a partition coefficient (concentration in organic phase/concentration in aqueous phase) of 957 ± 26 . This is in excellent agreement with the value of 943 ± 23 previously determined by Waring *et al.* (1975), providing further proof of the purity of the material.

For triostin C, preliminary experiments suggested that the partition coefficient between isopentyl acetate and 0.01 SHE buffer was greater than 5000. This is much too high for practical purposes, lying well outside the range recommended by Waring *et al.* (1975). However, addition of n-heptane reduced the partition coefficient, since the antibiotic is completely insoluble in it. The mixture '50/50 IPA/heptane' was chosen as the organic phase and the partition coefficient of triostin C between this solvent and 0.01 SHE buffer was determined to be 1210 ± 150 .

Solvent-partition analysis

The experimental procedure was as previously described (Waring *et al.*, 1975), except that the volume of the organic phase was decreased to 0.6 ml or 0.3 ml in order to conserve material and the aqueous, DNA-containing phase was 1.5 ml. Various DNA concentrations were employed in different experiments as described in the legends to Figs. 3–5 (below). Samples were shaken in silicone-treated glass-stoppered tubes for 1 h at 20°C to establish equilibrium, after which the phases were separated by centrifugation in an MSE Minor bench centrifuge at 3000 rev./min (r 15 cm) for 30 min. Values of r (antibiotic molecules bound per DNA nucleotide) and c (molar concentration of free antibiotic) obtained from the binding experiments were routinely analysed in terms of the non-interacting overlapping binding-site model defined by eqn. (10) of McGhee & Von Hippel (1974) as described by Lee & Waring (1978a):

$$\frac{r}{c} = K(0) (1 - nr) \left[\frac{1 - nr}{1 - (n-1)r} \right]^{n-1}$$

Here $K(0)$ represents the intrinsic association constant for binding to an isolated site on the polymer (given by the intercept on the r/c axis of a Scatchard plot) and n represents the number of nucleotides occluded by the binding of a single ligand molecule (given by the intercept on the axis of r). The experimentally determined values of r and c were fitted to this equation by using two computer programs. The first program, written by Dr. J. D. McGhee and installed in the Cambridge University computer by Dr. G. Ughetto, used an iterative

procedure to estimate $K(0)$ and n from an initial input guess of n ; these values were then recycled until they changed by less than 1%. Altering the stopping criterion to 0.01% caused less than a 1% change in the final values of $K(0)$ and n . An estimate of the success of this program at fitting the data could be obtained by using different initial guesses of n , both above and below the actual value, and comparing the different values of $K(0)$ and n obtained; generally these varied by less than 5%. The second program used a modified Marquardt method, as implemented in the Harwell library routine VBO1A in the Cambridge University computer. This program was able to provide estimates of $K(0)$ and n together with standard deviations, and has also been adapted to fit eqn. (15) of McGhee & Von Hippel (1974), which provides for a cooperativity parameter, ω :

$$\frac{r}{c} = K(0) \cdot (1 - nr) \cdot \left(\frac{(2\omega - 1)(1 - nr) + r - R}{2(\omega - 1)(1 - nr)} \right)^{n-1} \cdot \left(\frac{1 - (n+1)r + R}{2(1 - nr)} \right)^2$$

where $R = \sqrt{\{[1 - (n+1)r]^2 + 4\omega r(1 - nr)\}}$

Each experimental point was weighted in proportion to the inverse of the corresponding free antibiotic concentration. Repeated trials were made with different initial estimates of parameters, but these had no significant effect on the final values obtained. It should be noted that whereas binding data are presented in the form of Scatchard plots, having the dependent variable on both axes, the isotherms were calculated (by both programs) by using the experimentally determined values of r and c .

Results

Triostin A

Scatchard plots for the interaction between triostin A and calf thymus DNA determined by using the radioactive antibiotic are illustrated in Fig. 3. Fig. 3(a) shows a composite of data obtained by both the radiochemical and spectrophotometric methods; the curve drawn is that determined by Lee & Waring (1978a) based on the spectrophotometric data alone. It can be seen that in the range $0.02 < r < 0.05$, where the two sets of data overlap, there is excellent agreement; thus the two techniques for estimating antibiotic binding are clearly concordant. Fig. 3(b) displays the results determined by the radiochemical method alone. Despite the scatter in the large number of data points there is an upward deviation from the predicted curve, suggesting the occurrence of a small proportion of tighter binding sites. Fitting the spectrophotometric data alone

Fig. 3. Binding of triostin A to calf thymus DNA

The binding ratio r is expressed in terms of antibiotic molecules bound per nucleotide; c represents the free ligand concentration. (a) $\circ$, Data obtained by using radioactive material as described in the text; $\bullet$, data from Lee & Waring (1978a) measured spectrophotometrically using natural triostin A; Δ , data obtained using synthetic triostin A (a gift from Dr. R. K. Olsen). The curve drawn is taken from Lee & Waring (1978a). (b) Data obtained using only the radioactive antibiotic in separate experiments at the following DNA concentrations: $\circ$, $164.4\mu\text{M}$; Δ , $171.9\mu\text{M}$; $\bullet$, $127.3\mu\text{M}$; $\blacktriangle$, $151.5\mu\text{M}$; $\square$, $180.3\mu\text{M}$; $\blacksquare$, $207.6\mu\text{M}$. The line drawn was fitted to the data so as to satisfy eqn. (10) of McGhee & von Hippel (1974) and is characterized by the parameters $K(0) = 0.76 \pm 0.02\mu\text{M}^{-1}$ and $n = 7.8 \pm 0.2$.

yielded $K(0) = 0.67 \pm 0.03\mu\text{M}^{-1}$ and $n = 7.0 \pm 0.2$, whereas fitting the radiochemical data alone yielded $K(0) = 0.76 \pm 0.02\mu\text{M}^{-1}$ and $n = 7.8 \pm 0.2$. It is worth noting that the lowest level of binding measured corresponds to $r = 0.0008$, much lower than that previously achieved by spectrophotometric means.

Scatchard plots for the binding of triostin A to *Micrococcus lysodeikticus* DNA and to poly(dA-dT) are shown in Fig. 4. With *M. lysodeikticus* DNA the points obtained at intermediate values of r are again in satisfactory agreement with those determined by spectrophotometric methods. At low values of r , although the data are somewhat scattered, there again appears a substantial upward deviation from the curve determined by Lee & Waring (1978a), consistent with the view that the drug is binding selectively to a small subset of preferred sites on the DNA lattice. A best-fit curve to the spectropho-

metric data alone yielded $K(0) = 1.05 \pm 0.06\mu\text{M}^{-1}$ and $n = 7.4 \pm 0.2$, whereas applying the same procedure to the radiochemical data alone yielded $K(0) = 1.55 \pm 0.10\mu\text{M}^{-1}$ and $n = 10.2 \pm 0.4$, revealing a 48% increase in the apparent binding constant ($P < 0.01$). The experiments on binding to poly(dA-dT) were undertaken in order to permit an objective decision between the suitability of co-operative and non-co-operative binding isotherms (Fig. 4b). It is noticeable that nearly all the radiochemical data points lie well below the non-co-operative curve calculated by Lee & Waring (1978a), as would be expected for a co-operative mode of interaction at low binding levels. This conclusion seems more likely if we compare the results with those obtained for the two natural DNA species (Figs. 3 and 4a), where the deviation from the predicted curve clearly lies in the opposite, upwards, direction. Combining the spectropho-

Fig. 4. Binding of triostin A to *Micrococcus lysodeikticus* DNA (a) and poly(dA-dT) (b) (a) *M. lysodeikticus* DNA. Six series of experiments were conducted with radiolabelled antibiotic with the following DNA concentrations: ●, 215.9 μM ; △, 180.9 μM ; ▼, 180.1 μM ; ▲, 174.6 μM ; □, 175.4 μM ; ■, 146.8 μM . The curve shown is taken from Lee & Waring (1978a); it was fitted to their data points determined by spectrophotometric means (○) so as to conform with eqn. (10) of McGhee & von Hippel (1974), characterized by the parameters $K(0) = 1.1 \pm 0.1 \mu\text{M}^{-1}$ and $n = 7.4 \pm 0.2$. (b) Poly(dA-dT). Data obtained with radiolabelled triostin A in various experiments at the following poly(dA-dT) concentrations: ●, 44.0 μM ; △, 36.6 μM ; ▲, 45.5 μM ; □, 46.6 μM . The curves drawn are taken from Lee & Waring (1978a); they represent two possible extremes of interpretation of the original spectrophotometric data points (○): either a best fit to the non-co-operative model defined by eqn. (10) of McGhee & von Hippel (1974) characterized by the parameters $K(0) = 0.96 \pm 0.10 \mu\text{M}^{-1}$ and $n = 4.7 \pm 0.3$ (the concave-upwards curve); or alternatively a best fit to their model which allows for a degree of co-operativity defined by the parameter $\omega = 6.2$ with $K(0) = 0.8 \mu\text{M}^{-1}$ and $n = 7.5$ (the 'humped' curve).

metric and radiochemical data sets and subjecting them to the Marquardt-Harwell procedure for a best fit to eqn. (15) of McGhee & Von Hippel (1974) yielded the result $K(0) = 0.78 \pm 0.08 \mu\text{M}^{-1}$, $n = 7.1 \pm 0.5$ and $\omega = 6.1 \pm 2.0$, in excellent agreement with the predicted co-operative binding curve (Fig. 4b).

Triostin C

The results of five series of experiments on the interaction of triostin C with a variety of natural DNA species and poly(dA-dT), measured by using the radioactive antibiotic, are presented in Fig. 5. The curves drawn are isotherms calculated to provide a best fit to eqn. (10) of McGhee & Von

Hippel (1974), as usual. The binding parameters derived from these plots along with those determined for triostin A (Lee & Waring, 1978a) are listed in Table 1. It can be seen that the relative order of binding constants for the two antibiotics among the various DNA species is quite similar, with triostin C binding to each more tightly than triostin A. The only exception to this trend occurs with poly(dA-dT), which evidently binds triostin C less tightly than do any of the natural DNA species. There appears to be a very good correlation between the binding constant for triostin C and the gross base composition (G + C content) of the DNA species.

The estimates of n , the number of bases occluded by the binding of one ligand molecule, are all

Fig. 5. Scatchard plots for the binding of triostin C to various DNA species
○, *E. coli* DNA; ▲, calf thymus DNA; □, poly(dA-dT); △, *M. lysodeikticus* DNA; ●, *Cl. perfringens* DNA. Experiments with the natural DNA species were conducted at 160 μM polynucleotide concentration; with poly(dA-dT) the concentration was 41 μM.

Table 1. DNA-binding parameters for triostins A and C

$K(0)$, the intrinsic association constant, and n , the number of nucleotides occluded by the binding of one molecule, were calculated from an analysis of the data in terms of eqn. (10) of McGhee & Von Hippel (1974). The results shown for triostin A are taken from Lee & Waring (1978a), recalculated to indicate standard deviations, apart from the value determined for *E. coli* DNA (K. R. Fox & M. J. Waring, unpublished work) and the values recorded for calf thymus DNA and *M. lysodeikticus* DNA which were calculated from the combined spectrophotometric and radiochemical data sets presented in Figs. 3 and 4 respectively.

DNA	G + C (%)	Antibiotic ... Triostin A		Triostin C	
		$K(0) (\mu\text{M}^{-1})$	n	$K(0) (\mu\text{M}^{-1})$	n
<i>M. lysodeikticus</i>	72	1.19 ± 0.05	7.7 ± 0.1	8.1 ± 0.3	9.5 ± 0.2
<i>E. coli</i>	50	0.91 ± 0.03	7.5 ± 0.1	4.1 ± 0.2	9.4 ± 0.3
Calf thymus	42	0.70 ± 0.02	7.2 ± 0.1	3.2 ± 0.1	12.2 ± 0.3
<i>Cl. perfringens</i>	30	0.43 ± 0.02	7.7 ± 0.2	2.3 ± 0.2	17.1 ± 1.0
poly(dA-dT)	0	0.96 ± 0.10	4.7 ± 0.3	1.9 ± 0.2	8.4 ± 1.3

perceptibly higher for triostin C than for triostin A. Although the values of n determined for the interaction of various quinoxaline antibiotics with different DNA species do not fall in any logical order (Wakelin & Waring, 1976; Lee & Waring, 1978a, b) and great care must be exercised when

interpreting variations in this parameter, the differences are too large to be accounted for by experimental error. It is possible that this might reflect nothing more than the bulk of the peptide ring in the two antibiotics: no doubt the larger size of the *L*-*N* γ -dimethyl-*allo*-isoleucine side chains in triostin

C could influence the effective length of the DNA lattice occluded when the antibiotic is intercalated. On the other hand, it may be that the apparent difference in site size is not functionally significant but merely reflects the region over which the binding isotherms were determined. For triostin A, most of the data, having been determined by spectrophotometric assay, lay in the range $0.02 < r < 0.10$. For triostin C the binding data lie largely in the low- r region (Fig. 5) where any effects due to sequence selectivity should be exaggerated, resulting in a steeper Scatchard plot and an apparently large value of n .

Discussion

The present results establish the feasibility of producing ^{35}S -labelled quinoxaline antibiotics simply by growing the producing organism in the presence of inorganic ^{35}S sulphate under appropriately adjusted culture conditions. Purified radioactive triostin A can clearly be used to determine accurate binding isotherms which, at r values above about 0.02, agree well with those obtained by spectrophotometric techniques. Hitherto it has been impossible to investigate binding below r values of 0.02 because of the insensitivity of the detection method (Lee & Waring, 1978a). Now we can measure r values as low as 0.0008, in equilibrium with a free drug concentration less than 1 nM. This level of binding of one ligand per 1250 nucleotides corresponds, roughly speaking, to one antibiotic molecule per gene.

The increased sensitivity afforded by using radioactively labelled material has widened the range of antibiotics whose binding can be investigated by the solvent-partition method. Accurate binding studies with triostin C have not previously been possible because of its extremely low ($< 1 \mu\text{M}$) aqueous solubility (Lee & Waring, 1978a; Waring, 1979), and a variety of new semi-biosynthetic analogues of the natural quinoxaline antibiotics are available for study (Gauvreau & Waring, 1983; Cornish *et al.*, 1983). The binding constants determined for triostin C with various natural DNA species fall in the same ranking order as seen with triostin A, and are uniformly higher than those of triostin A as predicted by Fox & Waring (1981) on the basis of the slower dissociation time constants measured for triostin C.

Unfortunately much of the data collected at low binding levels is too scattered to yield a reliable estimate of either the frequency of high-affinity binding sites on the DNA or co-operative (allosteric) interactions at low occupancy. We are uncertain about the origin of this scatter, but are confident that it does not arise from sampling or counting errors, though admittedly there are fewer total radioactivity counts in the data obtained at low binding levels. A

possible source of scatter could lie in the purity of the DNA samples, since traces of impurities (RNA or protein) might affect the binding at low levels, whereas the resulting deviations would be insignificant at higher levels of binding. However, it is difficult to see why impurities should provoke an apparent *increase* in the strength of binding at low levels, and no systematic variations in values of r were noted when different preparations of DNA were employed.

At all events, it is clear that if any allosteric effects occur at low binding levels they are much less pronounced than those reported for the interactions of actinomycin D and netropsin with DNA (Winkle & Krugh, 1981; Dattagupta *et al.*, 1980). Both sets of binding data determined for triostin A with natural DNA species yield a steeper curve than predicted at low levels of binding, consistent with the view that there is heterogeneity in the binding sites available, such that at low occupancy the drug binds selectively to a few tight sites. The fact that this deviation from the predicted curve is not large, less than a factor of 2, may be accounted for when we consider that triostin A is known to be the least sequence-selective of the quinoxaline antibiotics (Lee & Waring, 1978a), displaying only a 2.5-fold variation in its binding constant for natural DNA species (Table 1). Moreover, Fox & Waring (1981) presented evidence from kinetic studies that tight binding sites are only present in very small quantities, and these may well be swamped by the large excess of sites having lower affinity, even at low binding levels.

The present results emphasize the importance of measuring accurate binding isotherms at low levels of occupancy, and establish that the preparation of radiolabelled antibiotics is an invaluable tool for this purpose. It will be interesting in future to apply these techniques to ligands such as echinomycin or TANDEM, both of which are believed to be endowed with considerable sequence selectivity (Wakelin & Waring, 1976; Lee & Waring, 1978b).

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