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THERMODYNAMICS OF THE INTERACTION OF DAUNOMYCIN WITH DNA

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The association constant for the interaction of daunomycin with DNA was determined as a function of temperature (using [^{3}H] daunomycin in conventional equilibrium dialysis cells) and ionic strength (using a spectrophotometric titration method). The association constant varied between 3.1×10^6 M^{-1} (4°C) and 3.9×10^5 M^{-1} (65°C). The free energy change was −8.2 to −8.8 kcal/mol, the enthalpy change −5.3 kcal/mol and the entropy change +10 to +11 eu, all values being consistent with that expected of an intercalation process. The apparent number of intercalation sites detected (0.15 to 0.16 per nucleotide) was independent of temperature. The large positive entropy change accompanying the interaction appears to be due to extensive release of water from the DNA and daunomycin. The apparent number of binding sites increased dramatically with decrease of ionic strength, although the apparent association constant remained largely unaffected by ionic strength.

1. Introduction

Daunomycin is now widely used as a chemotherapeutic agent for the treatment of leukaemia [1,2] and a variety of other tumours [3]. Its effectiveness is due to its ability to interact with DNA, thereby inhibiting the synthesis of DNA and RNA [4,5]. The DNA–daunomycin complex has now been extensively investigated [6–15], and several studies have indicated that daunomycin intercalates with native DNA [7,9, 11]. However, the only thermodynamic study of the interaction has been a calorimetric approach at low ionic strength, yielding ΔH values as a function of daunomycin:nucleotide ratio [15]. By using [^{3}H] daunomycin in conventional equilibrium dialysis cells, we have obtained direct binding data at several temperatures yielding ΔG^0, ΔH^0 and ΔS^0 values for the interaction of daunomycin with native DNA.

As all melting phenomena of DNA–daunomycin complexes must be studied at low ionic strength (because of the substantial thermal stabilisation of DNA when in the presence of daunomycin), and this data is often compared to other physico-chemical data obtained at higher ionic strengths, there is an obvious need to have a clear understanding of the effect of ionic strength on the DNA–daunomycin interaction. For this reason, binding studies have been carried out at several ionic strengths to document the correlation between ionic strength and the various binding parameters. Such information should allow meaningful correlations between the amount of daunomycin bound to DNA at different ionic strengths, and the various parameters of DNA–daunomycin interaction measured by different techniques at different ionic strengths.

2. Materials and methods

Calf thymus DNA (Worthington) was prepared in the appropriate concentration of NaCl. A buffer was not necessary as it has been shown (D.R. Phillips, unpublished) that there is no pH change accompanying the interaction of daunomycin with DNA. DNA concentrations were determined using the spectrophotometric analysis of Hirschman and Felsenfeld [16]. Daunomycin hydrochloride and [^{3}H] daunomycin hydrochloride (specific activity of 2×10^{13} dpm/mol) were obtained from Farmitalia. Solutions were prepared in the appropriate concentration of NaCl on the day required, and concentrations determined using $E_{234} = 35050$ M^{-1} cm^{-1} or $E_{480} = 11\,500$ M^{-1} cm^{-1}.

All variable temperature binding studies were carried out in the dark with a conventional equilibrium dialysis cell containing 1 ml of 5×10^{-5} M_p DNA, suspended in 1 ml of daunomycin solution (10^{-8}–10^{-4} M). The dialysis membranes (Visking, Union Carbide, USA) were pretreated by boiling in 5% bicarbonate for 30 min, repeatedly rinsed with distilled water, leached in distilled water for 2 days, then stored at 4°C in the presence of a trace of chloroform. Equilibration times required were 72 h (4°C), 48 h (25°C and 45°C) and 9 h (65°C). Approximately 0.75 g samples from both sides of the membrane (to avoid the problem of daunomycin binding to the membrane) were made up with 7.5 ml of a scintillation cocktail, dark adapted and cooled for at least four hours and then counted for 10 min. Descicote (Beckman Instrument Inc.) was used on all glassware to minimise radioactivity loss.

Separate calibration curves of cpm versus daunomycin concentration were constructed for each set of binding data. As the presence of DNA had no effect on these curves, daunomycin concentrations were determined from the same plot whether in the presence or absence of DNA.

The binding data has been represented in terms of Scatchard plots of r/C versus r, where r is the moles of daunomycin bound per mole of nucleotide and C is the free molar daunomycin concentration. As the binding parameters obtained from Scatchard plots are meaningful only in the limiting case of $r \to 0$ [17], they were determined from the relationships [18,19]:

$$\lim_{r\to 0} \partial(r/C)/\partial r = -K_{app},$$

$$\frac{\lim_{r\to 0} r/C}{\left|\lim_{r\to 0} \partial(r/C)/\partial r\right|} = n_{app},$$

where K_{app} and n_{app} are the apparent association constants and the apparent number of binding sites per nucleotide.

ΔG^0 has been calculated using the relation $\Delta G^0 = -RT \ln K_{app}$ while ΔH^0 has been evaluated from the van 't Hoff equation:

$$\Delta H^0/R = -\mathrm{d}\ln K_{app}/\mathrm{d}(1/T).$$

The spectral titration method of Blake and Peacocke [20] was used to obtain binding parameters at 20° at different ionic strengths. 100 μl additions of approximately 1×10^{-3} M_p DNA were made to 8–10 ml of 2×10^{-5} M daunomycin in 5 cm path length spectrophotometer cells, and the absorbance monitored at 480 nm.

3. Results and discussion

The [^{3}H]-daunomycin was required to enable concentrations of free daunomycin to be measured down to 10^{-7} M. This in turn enables the values of r obtained to be significantly lower than that obtained using the more conventional spectral titration method of Blake and Peacocke [20], thereby allowing more reliable extrapolations of the data as $r \to 0$.

Three separate experiments were carried out to obtain the binding data at each temperature. The highest temperature used was at least 20°C lower than

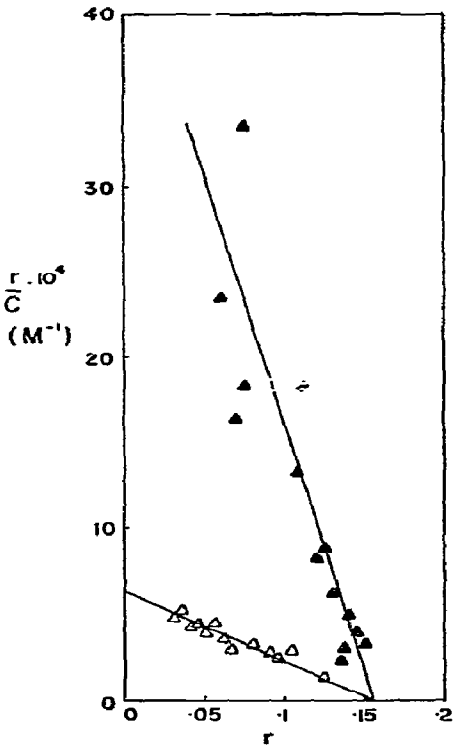

Fig. 1. Scatchard plots of the binding (dialysis method) of daunomycin to native DNA in 0.15 M NaCl at 4°C (▲) and 65°C (△).

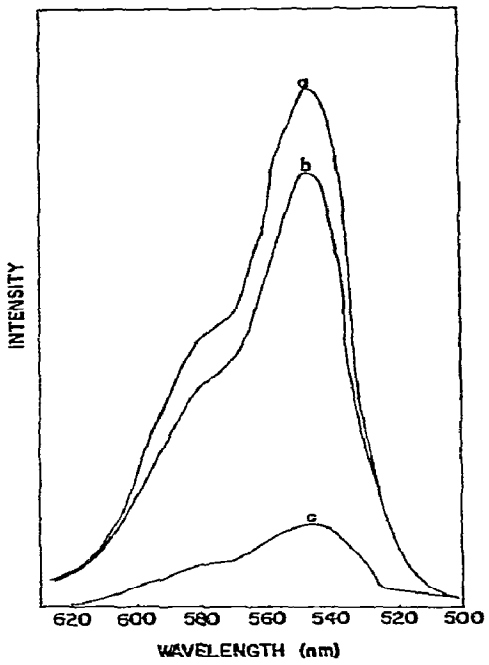

Fig. 2. Fluorescence spectra of daunomycin in 0.15 M NaCl. Excitation wavelength 436 nm. (a) Freshly prepared, (b) 65°C for 9 h, (c) acid hydrolysed [21].

the melting temperatures of the DNA or DNA–daunomycin mixtures. Fig. 1 shows two such plots of the data obtained at the temperature extremes used (4°C and 65°C). In all cases, almost all of the data could be fitted by a straight line up to r values of 0.14. This indicates the presence of only one type of binding at these daunomycin : nucleotide ratios, and is consistent with the fluorescence and calorimetric studies of Quadrifoglio et al. [15] which also showed only one type of interaction up to similar high ratios. In

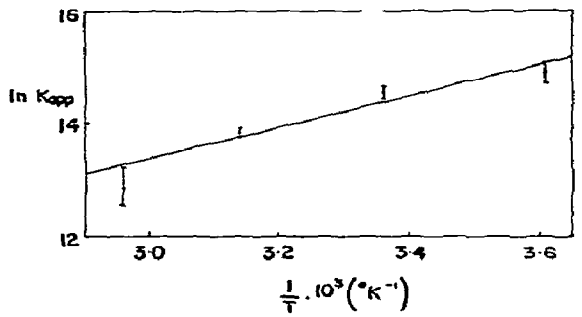

Fig. 3. Van 't Hoff plot of the apparent association constants determined using the dialysis method at 4, 25, 45 and 65°C in 0.15 M NaCl.

contrast, the DNA–cellulose chromatography results of Zunino [9] clearly indicate the presence of two distinct modes of interaction, and this is supported by the extreme curvature of Scatchard plots obtained from spectral titrations of DNA–daunomycin [11], which also indicates at least two modes of interaction. However, the present results do not preclude the presence of an additional binding site with a significantly lower association constant.

The binding data at 65°C has been corrected for partial hydrolysis of the daunomycin. Fig. 2 shows the 20% change of the fluorescence spectrum of daunomycin observed after 9 h in the dark at 65°C. No significant hydrolysis was detected for other daunomycin solutions subjected to the same conditions employed for equilibrium dialysis. As the binding of the aglycone to DNA is relatively weak [14], any contribution by the aglycone to the radioactivity on DNA has been ignored when correcting for this hydrolysis. The interaction of a ligand like daunomycin, with a heteropolymer such as DNA, has been described by eqs. (36)

Table 1
Binding parameters for the interaction of daunomycin with DNA at various temperatures, 0.15 M NaCl

Temp (°C)	n_{app}	K_{app} (M^{-1})	ΔG^0 (kcal/mol)	ΔS^0 (eu)
4	0.15_2	3.1×10^6	−8.2 ± 0.1	10 ± 2
25	0.15_8	2.1×10^6	−8.6 ± 0.1	11 ± 2
45	0.16_4	1.1×10^6	−8.8 ± 0.05	11 ± 2
65	0.15_8	0.39×10^6	−8.6 ± 0.02	10 ± 1

and (37) of Gurskii et al. [19]. However, as the apparent association constants for the binding of daunomycin to GC and AT pairs is known to be essentially identical (D.R. Phillips, A. DiMarco and F. Zunino, in preparation) these equations reduce to

$$1/2n_{app} = 2l - 1,$$

where l represents the number of base pairs (for each bound daunomycin) sterically excluded or altered in such a way that additional ligand is unable to bind. In this context, it is somewhat unexpected that the values of n_{app} are completely independent of temperature (table 1) and this indicates that there is no change in the apparent number of binding sites accompanying the increased flexibility of DNA at high temperatures. The exclusion distance, l, is approximately two base pairs for each daunomycin bound, and this is probably due almost entirely to steric exclusion of the daunomycin itself [10]. It would therefore seem probable that as the conformation of DNA changes at premelting temperatures [22,23], the value of l would increase (i.e. n_{app} decreases) as the conformational contribution to the exclusion distance would be expected to increase with temperature. As this is not detected, it must be concluded that there is little, if any, change of conformation of the DNA–daunomycin complexes in the temperature range studied.

The values for K_{app} are presented in fig. 3 in the form of a van 't Hoff plot, the slope of which yields an enthalpy change of -5.3 ± 0.6 kcal/mol for the interaction of daunomycin with DNA. This value is in substantial agreement with that of -6.5 ± 0.5 kcal/mol determined calorimetrically by Quadrifoglio et al. [15] at low daunomycin : DNA ratios. These values are also similar to those found for other intercalating agents (proflavine [15,24] and ethidium bromide [15]). The errors shown represent the contribution from the maximum error of each of the three determinations of K_{app}. There is a small but undefined additional error due to the contribution of the hydrolysis products of the non-specifically labelled daunomycin. This contribution increases with temperature and may account for the possible non-linearity evident in fig. 3.

The free energy change for the interaction (table 1) is essentially independent of temperature in the temperature range studied (−8.2 to −8.8 kcal/mol),

Table 2
Binding parameters, determined by spectrophotometric titration at 20°C, for the interaction of daunomycin with DNA at various ionic strengths

Ionic strength	K_{app} (M^{-1})	n_{app}	l
0.005	2.10^6	0.40	1.1
0.01	2.10^6	0.38	1.2
0.05	3.10^6	0.24	1.5
0.10	4.10^6	0.23	1.6
0.15	8.10^6	0.18	1.9

as is the entropy change detected (10 to 11 eu), consistent with the value of 7.7 eu obtained by others [15]. This value is in excellent agreement with that of 11 eu found for the interaction of proflavine with DNA [24]. As the experimentally determined value for the proflavine–DNA system has been shown to agree with a calculated value (assuming dehydration of both DNA and proflavine) [24], it is probable that dehydration is the major contribution to the positive entropy change of the DNA–proflavine intercalation system. This suggests that the large positive entropy change for the DNA–daunomycin system is also due to a similar extensive release of water from DNA and daunomycin.

The decrease of the apparent number of binding sites with increasing ionic strength is shown in table 2 and fig. 4. This fact, together with the observation that the apparent association constant is largely independent of ionic strength (see table 2) provides a clear picture

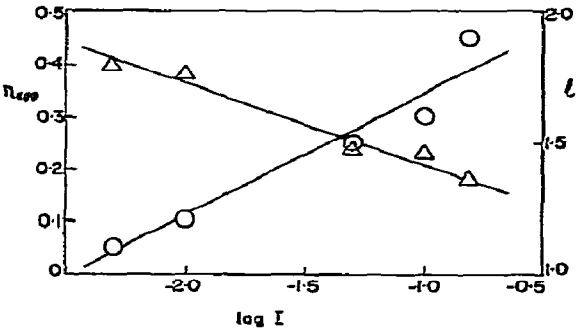

Fig. 4. Variation of n_{app} (△), the apparent number of binding sites per nulceotide and l (○), the exclusion length per base pair, with ionic strength (spectral titration method at 20°C).

of the two main contributions to the previously noted trend that more daunomycin is bound to DNA at lower ionic strengths than at high ionic strengths [6]. The alternative means of expressing this data in terms of the self exclusion length per base pair (l) is also shown in table 2 and fig. 4.

The values determined for the apparent association constant by the two different techniques differ somewhat (e.g. 8×10^6 M^{-1} as determined spectrophotometrically in 0.15 M NaCl, 20°C, compared with 2.1×10^6 M^{-1} obtained using equilibrium dialysis in 0.15 M NaCl, 25°C). The difference may reflect the limitations of the spectrophotometric method, and these have been discussed in detail by Blake and Peacocke [20]. Furthermore, the use of labelled daunomycin for the equilibrium dialysis studies enables much lower concentrations of free daunomycin to be determined, the implications of which have already been discussed.

The fact that the apparent association constant is relatively insensitive to changes of ionic strength contrasts with the marked decrease of K_{app} with increasing ionic strength detected for other charged intercalating agents [24]. As the charged amino sugar residue (and the stereochemistry of this group) of daunomycin is known to be involved in the stabilisation of the DNA–daunomycin complex [11,25], it is not clear why a thirty-fold increase of ionic strength has so little effect on K_{app}. However, it does suggest that the electrostatic contribution to the binding free energy of the complex must be relatively minor as polyelectrolyte models predict a dependence of the association constant on the logarithm of the ionic strength for electrostatic interactions [26].

As the ionic strength dependence of the binding of daunomycin to DNA is not identical to that observed for other known charged intercalating agents, it is clear that considerable caution is required when comparing the behaviour of one intercalating agent to another. The need for caution has also been shown by other ionic strength studies: the anthracyclines are not displaced by high concentrations of CsCl whereas the acridines are displaced [7].

4. Conclusions

The thermodynamic data obtained for the interaction of daunomycin with DNA are consistent with previous values obtained for other positively charged intercalating agents. The large positive entropy change appears to be due to extensive dehydration of the DNA (and daunomycin) accompanying the interaction.

There is a large decrease of the apparent number of binding sites as the ionic strength increases, although the apparent association constant is largely independent of ionic strength.

This data allows a more meaningful interpretation of much of the physico-chemical and biochemical studies of the DNA–daunomycin interaction as it is now possible to estimate the amount of daunomycin bound to DNA at a wide range of temperatures and ionic strengths.

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