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# THE INFLUENCE OF pH ON THE INTERACTION OF LIPOPHILIC ANTHRACYCLINES WITH BOVINE SERUM ALBUMIN

## QUANTITATIVE CHARACTERIZATION BY MEASUREMENT OF FLUORESCENCE QUENCHING

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**Abstract**—We have investigated the interaction of the lipophilic anthracyclines 4′-iodo-4′-deoxydoxorubicin (IDX) and 4-demethoxy-daunorubicin (DDN) with bovine serum albumin by the quantitation of fluorescence quenching. The protein binding of IDX was extremely sensitive to the pH of the solution in which the complex was formed and paralleled the effect of pH on dimerization of the drug. The effect of pH on the protein binding and self-association of DDN was less extensive. Both compounds exhibited curvilinear Scatchard plots indicating apparent cooperativity in the binding process. Because of the self-association of the drugs in aqueous solution, we attempted to resolve this cooperativity in terms of the preferential binding of the dimer to the acceptor. However, we found that similar Scatchard plots could be simulated by using slightly erroneous estimates of the fluorescence yield of the complex, rendering any such analysis inconclusive. Consequently, the relationship between acceptor concentration and the fraction of ligand bound was considered to be fitted adequately in terms of a single acceptor site per albumin molecule. The pH dependence of the association constants for bovine serum albumin was described best by the hydrophobic interaction of neutral drug monomer with a binding site with titratable affinity. We postulate that the pH-dependent binding of some anthracyclines with albumin may lead to their enhanced uptake, relative to that of non-target organs, into tumours with an acidotic extracellular milieu.

Anthracyclines are amongst the most widely used chemotherapeutic agents for the management of neoplastic disease. Although entry into the cell may not be essential for the cytotoxicity of doxorubicin [1], internalization is likely to be required for some of the proposed mechanisms of action of the anthracyclines, namely, DNA intercalation and scission, free radical injury, and inhibition of topoisomerase II activity [2]. The relative ineffectiveness of anthracyclines for the treatment of certain solid tumours [2] has been attributed to the poor penetration by these drugs into the tumour [3] and/or to the inherent or acquired resistance displayed by the target cells [4]. Assuming that entry of anthracyclines into cells occurs by the passive diffusion of the neutral (unionized) drug species [5, 6], the poor tumour penetration may be the result of limited cell permeability of this species and/or a predominance of the relatively impermeable ion [3] in the neutral or slightly acidic extracellular fluid of the tumour [7].

Recently, lipophilic anthracycline analogues have been synthesized which have an enhanced cytotoxic activity [8], presumably because of increased uptake into tumour cells [9, 10]. However, some of the chemotherapeutic advantage of this increased cellular penetration could be offset by a more extensive binding of the drugs to plasma proteins resulting in lower concentrations of unbound drug at the tumour cell surface. Such a correlation between lipophilicity and protein binding has been established with other homologous series of drugs [11].

Despite the importance of a quantitative assessment of such protein binding, very little information regarding this aspect of the anthracyclines is available. In rabbit and human plasma, approximately 70–75% of doxorubicin is bound [12, 13]. Similar values have been obtained for daunorubicin and daunorubicinol in the rabbit [14]. On the other hand, lower (43–66%) and species-dependent values have also been reported for doxorubicin [15]. Albumin appears to be a major binding protein of the anthracyclines, although there is some evidence of interaction with other proteins in rabbit plasma, notably with $\alpha_2$, $\beta$ and $\gamma$ globulins [12, 14]. This may explain why the degree of binding of doxorubicin, doxorubicinol and 4′epi-doxorubicin in human plasma is slightly greater than that attributable to albumin as the sole acceptor protein [13]. The extent of protein binding of daunorubicin in human plasma and that of doxorubicin in rat, rabbit and guinea-pig plasma has been reported to be independent of drug concentration over the therapeutic range [13, 15].

The pH of the solution is an important determinant

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of the extent of the interaction of anthracyclines with DNA and cell nuclei [16], and may also affect the well-documented self-association of these drugs in aqueous solutions [17]. Because of the clinical relevance of pH and protein binding, both of which could affect the concentration of neutral drug within the tumour, we investigated the pH dependence of the interaction between bovine serum albumin and the lipophilic anthracyclines, 4′-iodo-4′-deoxy-doxorubicin (IDX*) and 4-demethoxy-daunorubicin (DDN). As part of this work, we studied the pH dependence of the self-association of these drugs under similar conditions. Because preliminary studies revealed that both the binding and the self-association of IDX showed a greater dependence on pH than DDN, the former drug was investigated over a greater range of pH.

## MATERIALS AND METHODS

*Chemicals.* Crystalline bovine serum albumin (Fraction V) was used as supplied by the Sigma Chemical Co. (St Louis, MO, U.S.A.). The hydrochloride salts of the anthracyclines, IDX and DDN (purity > 98%) were donated by Farmitalia Carlo Erba (Melbourne, Australia).

Stock solutions (~5 mg/mL), prepared by dissolution of the anthracycline in 4.4% dextrose, were stored at −20° until required. Just prior to use, these solutions were diluted with 4.4% dextrose to yield the required final concentrations.

*Dimerization studies.* The self-association of IDX and DDN was quantified spectrophotometrically on the basis of the different molar extinction coefficients of the monomeric ($\varepsilon_m$) and dimeric ($\varepsilon_d$) forms of each drug [17]. Cuvettes with a 10-cm pathlength (Ultracell, Grayare Co., NY) were used for the absorbance measurements at 473 nm in a Cary 4E spectrophotometer (Varian Australia Pty Ltd, Brisbane, Australia), the cell compartment of which was maintained at 37°. Stepwise addition of aliquots of stock drug solutions (5–25 μL, total added < = 205 μL) to 30 mL of Dulbecco's phosphate-buffered saline [18] without calcium or magnesium (PBS) enabled examination of a range of concentrations of 0.11–36 and 0.14–42 μM for IDX and DDN, respectively. The absorbance of a solution containing both monomeric and dimeric drug can be described by [17]:

$$A = \varepsilon_d[D_t] + \frac{(\varepsilon_m - \varepsilon_d)[\sqrt{1 + 8Y[D_t]} - 1]}{4Y} \quad (1)$$

where $A$ is the absorbance per cm pathlength at the appropriate wavelength, $[D_t]$ is the total drug concentration and $Y$ is the molar self-association constant. Because the limited solubility of these lipophilic anthracycline derivatives precluded unequivocal estimation of $\varepsilon_d$, the results from experiments performed in triplicate in the pH range 6.86–8.25 were analysed by the simultaneous non-linear regression analysis [19] of all data on the basis of Eqn 1 to obtain pH-independent estimates of $\varepsilon_m$ and $\varepsilon_d$ and a value of $Y$ at each pH.

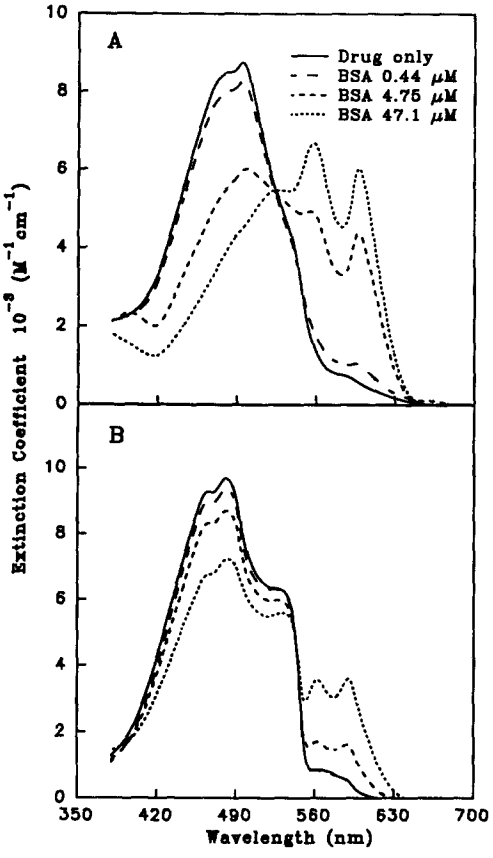


Fig. 1. The effect of bovine serum albumin (BSA) concentration on the extinction coefficients of (A) IDX (26.8 μM) and (B) DDN (28.8 μM) in PBS pH = 8.25 at 37°. The concentrations of albumin (indicated in figure) were achieved by the addition of aliquots (60 μL) of various albumin solutions to 3 mL of drug solution contained in disposable acrylic cuvettes (1 cm pathlength). The spectra were obtained with a Uvikon 810 spectrophotometer (Kontron, Switzerland).

*Spectrofluorimetric determination of protein binding.* The binding of the anthracyclines to bovine serum albumin was characterized by the quenching of drug fluorescence that accompanied formation of the complex. Binding studies in which a variety of ligand concentrations are studied at a fixed acceptor concentration have been argued to be more sound than those in which the acceptor (albumin) concentration is varied and that of the ligand fixed [20], provided that the acceptor is not contaminated by a competing ligand [21]. In our experiments, however, we wanted to limit the range of ligand concentrations to those which would yield reasonable fluorescence but be sufficiently low for the ligand to be in the monomeric form predominantly. This limited concentration range also prevented the spectrophotometric quantitation of the interaction of these compounds with bovine serum albumin, despite the presence of significant spectral shifts following complex formation (Fig. 1).

* Abbreviations: IDX, 4′-iodo-4′-deoxy-doxorubicin; DDN, 4-demethoxydaunorubicin; PBS, phosphate-buffered saline (Dulbecco's) without calcium or magnesium.

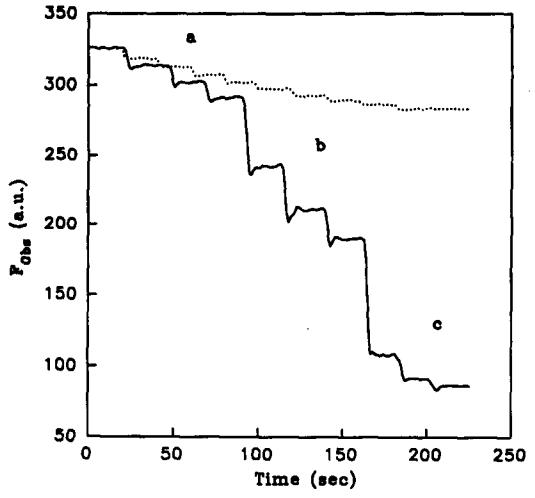


Fig. 2. Time trace of fluorescence emission ($F_{Obs}$, arbitrary units, 557 nm) in a typical experiment in which 3 mL of buffer (pH 7.45, 37°) containing 2.95 μM IDX is titrated with 3 stepwise additions of 60 μL aliquots of each of 1/100 (a), 1/10 (b) and stock (c) solutions of bovine serum albumin (pH 7.45). The dotted line represents the fluorescence intensity $F_f$ when the IDX solution is titrated with solutions of gelatin with the same absorbance as the bovine serum albumin solutions at the excitation wavelength (473 nm). The fluorescence decrements observed in the gelatin experiment corresponded closely to those calculated for the effect of dilution on drug concentration.

Stock solutions of albumin were prepared by dissolving approximately 2 g of protein in 10 mL of PBS, the pH of which had been adjusted to the desired value by the addition of NaOH or HCl and readjusted following dissolution of albumin. The albumin concentration of these solutions was determined spectrophotometrically at 280 nm on the basis of an absorption coefficient ($A^{1\%}_{cm}$) of 6.6 [22] and a relative molecular mass of albumin of 67,000. Parts of the stock solutions of albumin were diluted further (1/10 and 1/100) with PBS of the appropriate pH to provide several protein solutions with which to carry out the spectrofluorimetric titrations of the two anthracyclines.

Disposable acrylic cuvettes (Sarstedt, Germany) containing the drug solutions (IDX, 2.95 μM; DDN, 1.48 μM, in PBS, pH 6.86–8.25) were placed in a Perkin–Elmer LS-50 fluorimeter with the excitation and emission wavelengths set at 473 and 557 nm, respectively, the corresponding slit-widths being 10 and 20 nm. The higher fluorescent yield of DDN enabled the use of a lower concentration of this drug. The accumulation of data was controlled by FLDM software (Perkin–Elmer, Brisbane, Australia) operating in an Epson AX personal computer (Epson Australia Pty Ltd, Australia). The contents of the cuvettes were stirred magnetically and maintained at 37°. Each experiment, performed in triplicate, entailed the stepwise addition of aliquots (10–60 μL) of albumin to 3 mL of drug solution. When assessing the decreased fluorescence associated with each addition of albumin (Fig. 2), allowance was made for the effects of dilution and of protein absorbance on the fluorescence yield of unbound drug. This was done by measuring the change in the fluorescence of drug ($F_f$) when it was titrated with gelatin solutions which had the same absorbance as the equivalent albumin solution at the excitation wavelength (473 nm). Finally, the fluorescence of the drug mixtures that contained albumin ($F_{Obs}$) was corrected also for the slight fluorescence due to albumin alone.

The bound fraction of drug, $f_b$, was estimated from the expression [23]:

$$f_b = \frac{F_f - F_{Obs}}{F_f - F_b} \tag{2}$$

where $F_{Obs}$ is the observed fluorescence corrected for protein fluorescence, $F_f$ is the fluorescence of the equivalent drug solution with gelatin and $F_b$ is the fluorescence of the completely bound drug. The latter value, $F_b$, was estimated from solutions of IDX (2.95 μM) and DDN (1.48 μM) containing 0.82 and 2.45 mM albumin, respectively, and corrected for protein autofluorescence. These concentrations of albumin were chosen because they were greater than the inverse of the intrinsic association constant ($K^{-1}$) by the required factor of at least 10-fold [24] when $K$ was determined in preliminary experiments assuming $F_b = 0$.

*Evaluation of binding parameters.* In describing the usual relationship for the interaction of drug with $n$ equivalent and independent sites on albumin, the expression for the binding function, $r$, is:

$$r = \frac{f_b[D_t]}{[A_t]} = \frac{nK(1 - f_b)[D_t]}{1 + K(1 - f_b)[D_t]} \tag{3}$$

where $K$ is the intrinsic association constant for the binding interaction with albumin and $[A_t]$ and $[D_t]$ are the total albumin and drug concentrations, respectively. The slight reduction in $[D_t]$ resulting from the titration nature of the experiment was considered in all manipulations of the data. Non-linear regression analysis in terms of the relationship:

$$K[D_t]f_b^2 - [1 + K(n[A_t] + [D_t])f_b + K[A_t] = 0 \tag{4}$$

which follows directly from Eqn 3, can allow determination of both $K$ and $n$. However, the modest saturation of binding achieved even at low acceptor concentrations resulted in large dependencies (>99.9%) of these two variables during regression analysis. For this reason, the magnitude of $n$ was assessed independently by analysing the results in accordance with the double-reciprocal linear transform of Eqn 3 [25]. Once the stoichiometry of binding was defined, the intrinsic association constant was obtained by non-linear regression analysis [19] from Eqn 4. In the current study, the analysis was restricted to values of $f_b < 0.85$ (see Discussion).

*Binding function for a dimerizing ligand.* The binding function for a system which involves binding of both the dimeric and monomeric forms of a drug to $n$ independent equivalent acceptor sites is given by the expression [26]:

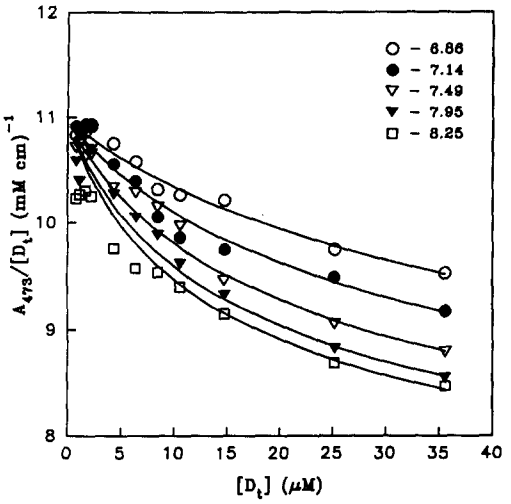


Fig. 3. Concentration dependence of the extinction coefficient of IDX in PBS (37°) at the pH indicated in the figure. The solid lines are theoretical relationships based on Eqn 1, the dimerization constants reported in Table 1 and the pH-independent extinction coefficient of 11,000 and 6200 $M^{-1}cm^{-1}$ for monomeric and dimeric forms, respectively.

$$r = \frac{n(4J[\sqrt{1+8[D_u]Y}-1] + 2L[\sqrt{1+8[D_u]Y}-1]^2)}{16Y + 4J[\sqrt{1+8[D_u]Y}-1] + L[\sqrt{1+8[D_u]Y}-1]^2} \quad (5)$$

where $J$ and $L$ are the binding constants for the interaction of albumin with monomeric and dimeric forms of the drug, respectively; $Y$ is the association equilibrium constant describing drug dimerization; and $[D_u] = (1-f_b)[D_t]$ is the concentration of combined monomeric and dimeric free drug. Comparisons of the predicted (Eqn 5) and observed binding functions were carried out following transformation of the data into Scatchard plots [27].

*Effect of uncertainty of* $F_b$ *on the binding function.* It is clear from Eqn 3 that the estimate of the binding function, $r$, of the interaction between acceptor and ligand is dependent on the accuracy of the estimate of the free fraction, $(1-f_b)$. From Eqn 1, it follows that:

$$1 - f_b = \frac{F_{Obs} - F_b}{F_f - F_b} \quad (6)$$

and that at high acceptor concentrations ($F_{Obs}$ approaches $F_b$), the value of $(1-f_b)$ is extremely sensitive to the estimate of the fluorescent yield of the fully bound ligand, $F_b$. To investigate the effect of small errors in $F_b$ on the shape of the Scatchard plots [27], we simulated values of the binding function using Eqns 2 and 4 with $K = 0.1\,\mu M^{-1}$, $[D_t] = 3\,\mu M^{-1}$ and $F_b/F_f = 0.200$. Scatchard plots were generated also for the binding function when incorrect values of $F_b/F_f$ (0.210, 0.195, 0.190) were substituted. The non-transformed simulated data were analysed also in terms of Eqn 4 to determine the effect of errors in $F_b$ on the magnitude of the intrinsic association constant, $K$.

Table 1. pH dependence of the dimerization of IDX and DDN at 37°

| | Dimerization constant, $Y$ ($mM^{-1}$)* | |
|---|---|---|
| pH | IDX | DDN |
| 6.86 | 8.8 (± 2.3)† | — |
| 7.14 | 13.7 (± 3.5) | 3.7 (± 0.7) |
| 7.49 | 21.2 (± 5.6) | 4.3 (± 0.8) |
| 7.95 | 28.4 (± 7.8) | 4.9 (± 0.8) |
| 8.25 | 33.3 (± 9.5) | — |

* Estimated spectrophotometrically [17] from the data of Fig. 3 on the basis of Eqn 1.

† Numbers in parentheses denote the uncertainty (± 2 SD) of the estimates.

## RESULTS

### *Dimerization of IDX and DDN*

The change in the absorbance of solutions of IDX and DDN as their concentration increased was compatible with a model of dimerization (Fig. 3). The dimerization constants of IDX, reported in Table 1, were found to be extremely dependent on the pH of the solution. In contrast, those of DDN, also listed in Table 1, were relatively pH independent. Molar extinction coefficients (± 2 SD) of 11,000 (± 210) and 6200 (± 170) $M^{-1}cm^{-1}$ were obtained for monomeric and dimeric IDX, respectively, based on the relative molecular mass of the monomer. For DDN, the corresponding coefficients were 10,000 (± 230) and 7500 (± 190) $M^{-1}cm^{-1}$. The pH dependence of the dimerization of IDX could be accounted for in terms of the predominant self-association of neutral drug with a constant of dimerization of 34.0 ± 3.1 ($mM^{-1}$, ± 2 SD) and a $pK_a$ (± 2 SD) of IDX of 6.88 ± 0.07 (dotted line, Fig. 4).

### *Fluorescence quenching*

The process of binding of anthracyclines to bovine serum albumin was similar to that reported for DNA [16, 23] inasmuch as binding of the drug was characterized by quenching of fluorescence (Fig. 2). The extent of this quenching, which occurred immediately upon the addition of albumin and indicated rapid equilibration of the unbound and bound drug, was dependent on the pH of the solution.

### *Analysis of fraction bound as a function of acceptor concentration*

The binding stoichiometry of the drug–albumin complex at the higher pH, at which the extent of binding was greatest, was investigated in double-reciprocal format [25]. Although these plots were non-linear at high values of $1/\{(1-f_b)[D_t]\}$, the probable number of binding sites appeared to be one per molecule of albumin when the analysis was

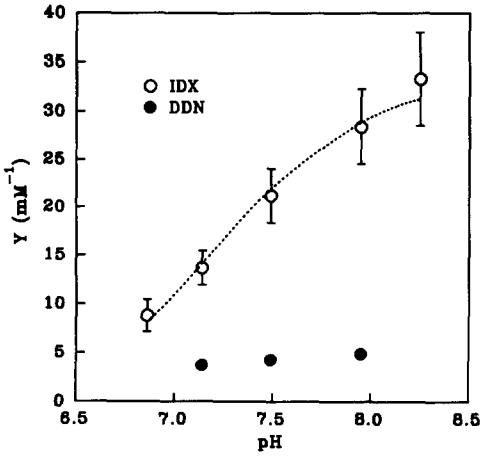


Fig. 4. Relationship between the constants of dimerization of IDX and DDN and pH. The dotted line is that described by a self-association constant of unionized IDX of 34 $mM^{-1}$ and a $pK_a$ of ionization of 6.88 (see text).

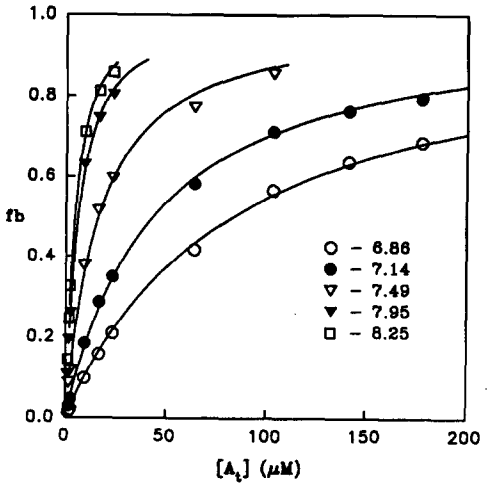


Fig. 6. Comparisons of the dependence of the bound fraction of IDX upon bovine serum albumin concentration observed experimentally with those predicted by Eqn 4 on the basis of 1:1 stoichiometry for the albumin–drug interaction and the binding constants reported in Table 2 at the indicated pH.

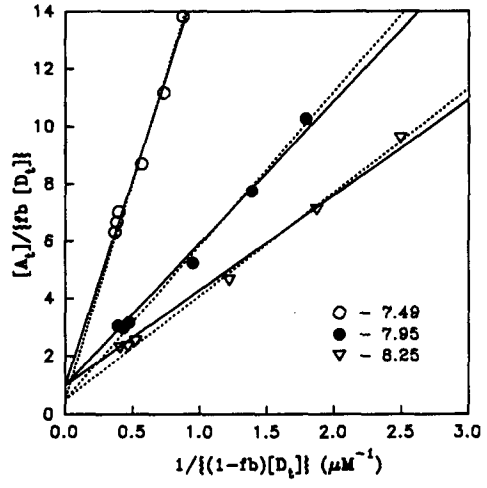


Fig. 5. Plot of binding data from the spectrophotometric titration of IDX with bovine serum albumin in double-reciprocal format to assess the reaction stoichiometry ($n$) at the indicated pH. Solid and broken lines denote the respective best-fit relationships for albumin–drug stoichiometries of 1:1 and 1:2.

Table 2. pH dependence of the binding interaction of IDX and DDN with bovine serum albumin at 37°

| | Association constant, $K$ ($mM^{-1}$)* | |
|---|---|---|
| pH | IDX | DDN |
| 6.86 | 12.2 (± 0.4)† | — |
| 6.87 | 14.6 (± 0.7) | 6.4 (± 0.1) |
| 7.14 | 23.8 (± 1.0) | — |
| 7.45 | 53.8 (± 1.5) | 10.6 (± 0.6) |
| 7.49 | 67.8 (± 5.4) | — |
| 7.87 | 164.6 (± 18.0) | 13.0 (± 0.8) |
| 7.95 | 214.0 (± 11.3) | — |
| 8.25 | 331.0 (± 19.7) | — |

* Obtained by non-linear regression analysis of the spectrofluorimetric titration data (Fig. 6) in terms of Eqn 4 with $n = 1$ (see text).

† Numbers in parentheses denote the uncertainty (± 2 SD) of the estimate.

restricted to the linear portion (Fig. 5). Although the theoretical plots for two drug binding sites also provided reasonable descriptions of the experimental results (broken lines in Fig. 5), comparison of the sums of squares of residuals favoured the value of unity for $n$. A similar conclusion followed from the non-linear regression analysis of the untransformed experimental data (Fig. 6) in terms of Eqn 4. The relative error in the evaluated binding constants was greater for $n = 2$. The values of $K$ (± 2 SD) obtained with $n = 1$ and using Eqn 4 are summarized in Table 2. These values demonstrated that the binding of IDX to bovine serum albumin was pH dependent and more avid than that of DDN.

The use of Eqn 4 in the analysis of the protein binding of a ligand which dimerizes in solution carries the inherent assumption that: (a) the dimer is present in negligible concentrations and does not bind preferentially, or (b) that the dimer has the same affinity for the acceptor as the monomer (i.e. $L = J = K$). The increase in binding affinity with increasing pH observed with IDX was modelled according to the titration of a single group on the acceptor molecule. The $pK_a$ and $K$ (± 2 SD) of this

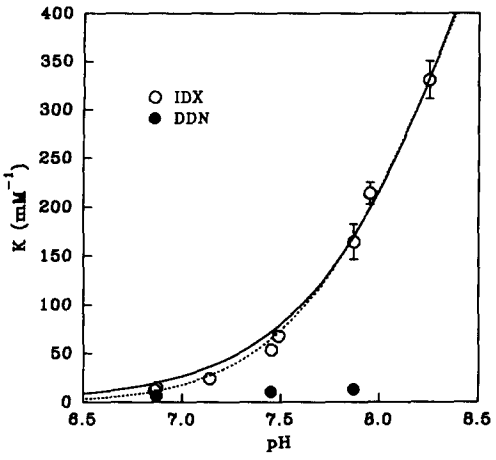


Fig. 7. The influence of pH on the intrinsic association constants of albumin for IDX and DDN. The solid line is that predicted by the interaction of IDX with an acceptor with a pH-dependent affinity determined by a p$K_a$ of 8.58. The dotted line describes the interaction of neutral IDX monomer only (p$K_a$ = 6.88) and a pH-dependent acceptor affinity determined by a p$K_a$ of 8.43.

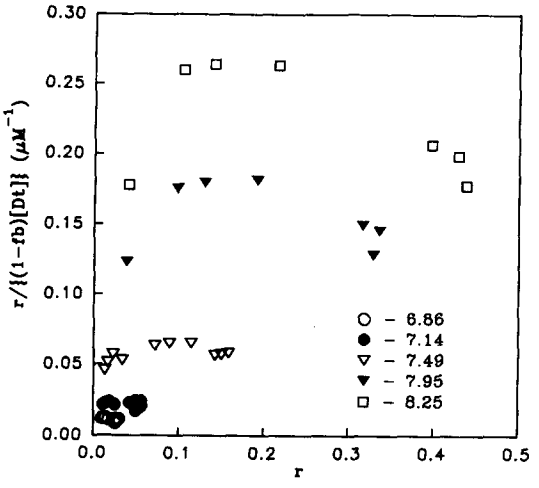


Fig. 8. Scatchard plots of binding data from the spectrophotometric titration of a single initial concentration of IDX (2.95 $\mu$M) at 37° with bovine serum albumin in PBS at the pH indicated in the figure.

process, consistent with the data of Fig. 7 and assumption (a) or (b), are 8.58 ± 0.08 and 1040 mM$^{-1}$ ± 135, respectively (solid line in Fig. 7). If the neutral form of the IDX monomer has a much greater affinity for the binding site and the dimer is present in negligible quantities [i.e. assumption (a) holds], the pH dependence of the affinity of the acceptor is consistent with a p$K_a$ and $K$ of 8.43 ± 0.06 and 873 mM$^{-1}$ ± 81, respectively (dotted line in Fig. 7), when the value of 6.88 is used for the p$K_a$ of IDX (as derived from the dimerization study).

*Scatchard analysis of binding*

Scatchard analysis of the binding function for both IDX (Fig. 8) and DDN (not shown) revealed curvilinear relationships suggestive of cooperativity in the binding process. The significant pH dependence of the binding function of IDX is also evident in this figure. True cooperativity occurs when the binding of one ligand molecule enables the binding of more ligand to the acceptor. Although several other mechanisms of cooperativity have been proposed (see Discussion), a probable cause in the present instance, given the apparent parallel in binding and self-association, was the preferential binding of dimer to albumin [26] (i.e. $L > J$ in Eqn 5).

The assumption that dimeric IDX was the sole form of the ligand that interacted with albumin ($J$ = 0) was untenable in that the value of $L$, calculated from each ($r$, [$D_u$]) point according to Eqn 5 and assuming $n = 1$, increased systematically with albumin concentration. Furthermore, consideration of the results in pairs to allow for binding of both monomer and dimer ($J \neq 0$) yielded the even more incongruous conclusion that the binding constant for dimeric ligand ($L$) was negative. Thus, consideration of preferential binding by dimeric ligand could not account for the curvilinear nature of the Scatchard plots.

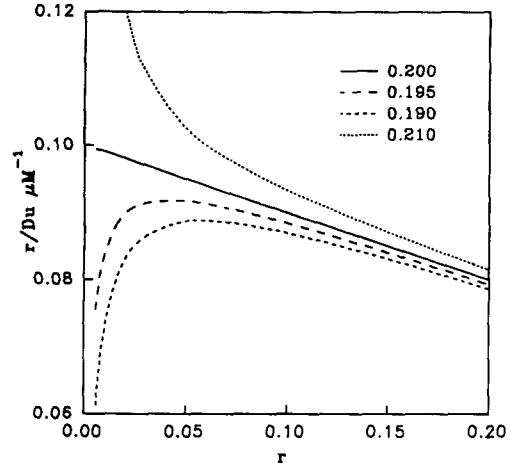


Fig. 9. Simulated Scatchard plot for the interaction of ligand and acceptor with association constant, $K = 0.1\ \mu M^{-1}$ and fractional fluorescent yield of the fully bound species, $F_b/F_f = 0.200$ (solid line) using Eqns 2 and 4. The dotted and dashed lines demonstrate the effect of analysing this simulated binding data with slightly incorrect estimates of $F_b/F_f$ (as indicated in figure). Note that the use of underestimates of $F_b/F_f$ creates apparent "cooperativity" at low values of $r$.

*Influence of potential errors on Scatchard plot*

The effect of analysing the simulated binding data with slightly different values of $F_b/F_f$ (0.19–0.21) is illustrated in Fig. 9. When the correct value (0.200) was used, the expected Scatchard plot was observed,

whereas underestimates of $F_b/F_f$ yielded curves suggestive of cooperativity. Overestimation of $F_b/F_f$ resulted in a Scatchard plot similar to that observed for multiple classes of binding sites. However, the values of $K$ recovered from the simulated data using Eqn 4 were all within 5% of the original value of 0.1 $\mu M^{-1}$ (data not shown). A further reduction in the error in $K$ was achieved by restricting the analyses of $f_b$ according to Eqn 4 to the values of $f_b < 0.85$. Therefore, this criterion was applied in the final analysis of the $f_b$ data of IDX and DDN shown in Table 2.

## DISCUSSION

In this study we have quantified the dependence of self-association and protein binding of two lipophilic anthracyclines on pH. Both processes were monitored under physiologically relevant conditions (37°, isotonicity).

Analysis of the dimerization of IDX and DDN showed marked differences in the self-association of these two drugs. Although the analysis of DDN was limited to three pH values, the dimerization of this drug appeared to be much less dependent on pH than IDX. At physiological pH, the major determinant of the ionization of the anthracyclines is the protonation of the heterocyclic nitrogen of the daunosamine sugar, which occurs to various extents depending on the drug. This factor could, at least in part, explain the differences in their tendencies to self-associate. In fact, the substantial pH dependence of the dimerization of IDX could be resolved in terms of the predominant dimerization of the neutral drug. The $pK_a$ of 6.88, obtained from this fitting, can be reconciled with the lower published value of 6.4, estimated at 23° and in water [28], when it is considered that the $pK_a$ of the parent anthracycline, doxorubicin, has been shown to increase significantly with ionic strength [5]. A predominant involvement of the unionized monomer in the dimerization process would explain the relative pH independence of the dimerization constant of DDN, which has a $pK_a$ of 8.5 [29], over the pH range studied.

The molar extinction coefficient of the IDX monomer observed in this study is between those reported for IDX in Ref. 28 (11,800 $M^{-1} cm^{-1}$, 480 nm) and in Ref. 30 (10,050 $M^{-1} cm^{-1}$, 485 nm). The dimerization constant of DDN has been reported previously to be 4.25 $mM^{-1}$ at 22° and pH 7.0 with an ionic strength of 0.196 M [17]. This is lower than that observed in this study, albeit under different conditions. However, the reported extinction coefficients of both DDN monomer and dimer [17] are in reasonable agreement with the current study.

The interaction between the lipophilic anthracyclines IDX and DDN and bovine serum albumin was quantified by measurements of fluorescence quenching. Although this technique has been applied to the study of interactions of anthracyclines with DNA (e.g. [16, 23]) and phospholipids [31], this is the first report of its use in the study of the interaction of anthracyclines with serum albumin. The use of fluorescence quenching to measure protein binding does have a number of limitations [24], but it is rapid and reproducible. The short periods of incubation at 37° reduce, in all likelihood, the degradation and non-specific adsorption of the anthracyclines which can occur during equilibrium dialysis [13]. IDX, in particular, is metabolized rapidly to non-fluorescent compounds in human plasma *in vitro* [32]. Thus, fluorescence quenching could be a practical technique to measure the binding of lipophilic anthracyclines under physiological conditions.

Both drugs revealed unusual forms of the binding curve when the results were transformed into Scatchard plots (Fig. 8). An unusual Scatchard plot has been reported also for the interaction of IDX with DNA [30]. Moreover, a "complex" curvilinear Scatchard plot was reported for an equilibrium dialysis study of doxorubicin binding to rabbit and human plasma [33]. Insufficient data were presented in that report for us to determine if the results represented cooperativity.

Cooperativity has been observed in studies with other ligands in which the concentration of ligand was constant but that of the protein varied [34–40]. Although factors such as the self-association [20] and contamination of the protein by a competing ligand [21] have the potential to account for positive cooperativity, the preferential binding of dimeric drug [26] was initially thought to be a more likely factor. However, despite the qualitative correspondence between the extents of ligand dimerization and apparent cooperativity of binding, the Scatchard analysis of binding was not compatible with this model. It is important to note that apparent cooperativity can occur also as a result of numerous experimental artefacts [41]. In our experiments, the similarity of the binding functions of Fig. 9 (with $F_b/F_f < 0.200$) with those of Fig. 8 suggests that the "cooperativity" observed experimentally is the result of a slight and systematic underestimation of $F_b$ for both IDX and DDN. We conclude that the more simple model of protein binding described by Eqn 4 is adequate.

The possible error in the fluorescent yield of the bound ligand was found not to influence greatly the value of $K$ determined from the simulated data and considerations of binding affinity in terms of the pH titration of drug and receptor site [16] were carried out. A model of the interaction of IDX with bovine albumin in which the ligand binds predominantly in the neutral form best accounted for the data. It is likely, therefore, that an electrostatic interaction of the cationic IDX with an anionic binding site, similar to that postulated for the binding of doxorubicin to DNA [16] and phospholipids [31], is not compatible with the observed pH dependence of the binding to albumin. These results, in conjunction with the observed parallel in the self-association and protein binding for IDX and DDN with changing pH, indicate that both processes are likely to be the result of hydrophobic interactions. Furthermore, the large red shift in the absorption spectra of both IDX and DDN in the presence of albumin (Fig. 1) suggests the presence of $\pi$-orbital interactions at the binding site. The pH dependence of the affinity of the acceptor does not necessarily imply that the titratable residue is part of the binding site. Rather, it may well reflect changes in the conformation of

bovine serum albumin associated with the neutral-to-base transition which occurs over a comparable pH range [42].

If applicable to human serum albumin, the binding affinity of both IDX and DDN to albumin is likely to be of clinical and pharmacokinetic significance [43]. On the basis of the intrinsic association constants estimated at pH ≈ 7.4 in this study, and the assumption that these are similar to those for human serum albumin *in vivo*, we predict unbound fractions of IDX and DDN of 4 at 15%, respectively, in a patient with a serum albumin level of 30 g/L [13]. pH dependence of the protein binding of IDX could be of clinical importance particularly for patients with solid tumours because the extracellular milieu of many of these tumours is considered to be acidotic [7]. The binding constants measured in this study suggest that the free fraction of IDX in a neutral environment (pH = 7.0) would be approximately three times greater than that in normal tissues (pH = 7.4). In terms of neutral IDX, the free concentration advantage would be somewhat lower but still greater than 2-fold. Thus, the reduced magnitude of the drug–albumin interaction of IDX at a lower pH could result in the greater uptake of the drug in the vicinity of the tumour than in non-target organs. Therefore, besides illustrating the importance of pH control in studies of drug binding, the dramatic influence of pH on the protein binding of IDX indicates a further factor to be considered in the design and development of new anthracycline analogues for the treatment of solid tumours. To take maximum advantage of the pH difference between tumour and healthy tissue, such a drug should undergo a maximal change in protein binding within the physiological range of pH.

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