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DISSOCIATION KINETICS OF DNA-ANTHRACYCLINE AND DNA-ANTHRAQUINONE COMPLEXES DETERMINED BY STOPPED-FLOW SPECTROPHOTOMETRY

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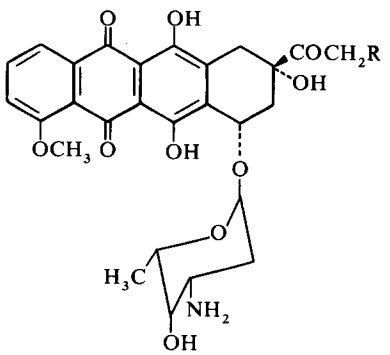
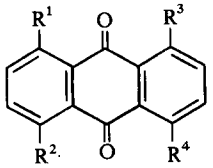
Abstract—The first order rate constants for the dissociation of daunorubicin, doxorubicin, and 1-; 1,4-; 1,5-; and 1,8-; N,N-diethylaminoethylamino-substituted anthraquinones from calf thymus DNA were determined using stopped-flow spectrophotometry. Sodium dodecyl sulphate was used to disrupt the equilibrium. In all cases there was an increase in the rate constant with temperature. The dissociation rate constants at 20°, 25° and 37°, were in the order 1-; >> 1,8-;> 1,4-;> daunorubicin and doxorubicin > 1,5-disubstituted anthraquinone. The 1,5-disubstituted anthraquinone (VII) thus shows the slowest rate of dissociation from DNA; the DNA complex dissociating more slowly than the DNA complexes of the anthracyclines, daunorubicin and doxorubicin. The result is consistent with the data from computer graphics modelling studies [39] which show that DNA-breathing (transient base pair unstacking) has to occur to allow the docking of the 1,5-disubstituted anthraquinone (VII) into the receptor site. Hence once the 1,5-disubstituted anthraquinone molecule has intercalated into DNA, DNA-breathing is required before dissociation can take place. This is not necessary with the other compounds (though the 1,4-disubstituted anthraquinone (V) can bind in this manner as well). So the very slow dissociation of the DNA/1,5-disubstituted anthraquinone complex relative to that of the DNA complexes of the other compounds examined here, supports the proposed mode of binding [39].

The anthracyclines daunorubicin I and doxorubicin II (particularly the latter) are widely used in cancer chemotherapy [1–3]. These drugs have a multiplicity of cellular effects, for example metabolic reduction in aerobic conditions leads to formation of reactive oxygen species (via redox cycling) hence to lipid peroxidation [4–7]. In anaerobic conditions, reductive deglycosylation occurs, again possibly leading to cellular damage [8–10]. It is becoming increasingly apparent that this *in vivo* reduction of the anthraquinone unit is linked to the cardiotoxic effects of the drug [11–13]. Other effects include direct interaction with phospholipids, for example in mitochondria [14–16], and a (possibly related) effect on calcium flux [17, 18]. Indeed it can be shown that doxorubicin can be cytotoxic without entering cells, presumably this is mediated via a membrane effect [19–22]. The most recognised cellular property of these drugs is their effect on the nucleus; they can intercalate into DNA [23–27], and cause sister chromatid exchange and chromosome aberrations [28]. The DNA shows protein associated single strand and double strand breaks possibly due to binding of topoisomerase which ineffectually attempts to relieve the strain in the helix induced by intercalative binding of the drug [29, 30]. Whatever the relevance of each of the reported cellular effects to the mechanism(s) of cytotoxic action, it is clear that DNA-intercalating drugs are potentially cytotoxic.

Scheme 1



I Daunorubicin R = H
II Doxorubicin R = OH



III $R^1 = R^2 = Y$; $R^3 = R^4 = OH$
IV $R^1 = R^2 = Y$; $R^3 = R^4 = H$
V $R^1 = X$; $R^2 = R^3 = R^4 = H$
VI $R^1 = R^2 = X$; $R^3 = R^4 = H$
VII $R^2 = R^3 = X$; $R^1 = R^4 = H$
VIII $R^1 = R^3 = X$; $R^2 = R^4 = H$

$X = NH(CH_2)_2\ N(C_2H_5)_2$
$Y = NH(CH_2)_2\ NHCH_2CH_2OH$

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In an attempt to reproduce the antitumour (but not the toxic) effects of these drugs in simplified analogues we prepared a series of DNA-intercalating substituted anthraquinones [31, 32]. Subsequent development of this type of agent elsewhere led to the introduction of mitoxantrone III and ametantrone IV [33, 34] which are now well into clinical trials. These compounds show similar cytotoxic effects to those of daunorubicin, for example chromosome aberrations and protein associated strand breaks [35]. These substituted anthraquinones intercalate into DNA but do not undergo redox cycling [36–45]: significantly they are less cardiotoxic. It is well established therefore that production of DNA-intercalating anthraquinones based on anthracyclines leads to cytotoxic compounds.

We are continuing our studies on such compounds with a detailed study of DNA-binding properties of anthraquinones with varying substituent patterns, comparing the compounds with daunorubicin and doxorubicin. Most studies on DNA-binding consider the affinity constant for the binding reaction, this being the ratio of the association and dissociation rate constants. However, it is not a fully adequate parameter: the dissociation rate constant itself is the crucial parameter. Here we report the dissociation rate constants for the complexes of anthraquinones V to VIII with DNA, compared with those of daunorubicin- and doxorubicin-DNA complexes, as determined by stopped-flow spectrophotometry.

MATERIALS AND METHODS

Solutions of daunorubicin (a gift from Farmitalia Carlo Erba, Milan, Italy), doxorubicin (Sigma Chemical Co., Poole, U.K.) and each of the anthraquinones V–VIII (the syntheses of these will be reported elsewhere) were prepared in Tris (0.015 M) NaCl (0.2 M) buffer pH 7.4, containing DNA (Type I calf-thymus DNA, Sigma Chemical Co. Poole, U.K.) at a drug to DNAp ratio of exactly 0.10 where DNAp is the concentration of DNA, calculated as DNA phosphate from the absorption of the solution at 260nm ($\varepsilon 260 = 6{,}600$).

The dissociation rate constant for the complex of each compound with DNA was determined at 20°, 25° and 37° as follows. The appropriate solution was placed in one reservoir of an SF3 series Stopped Flow spectrometer (Hi-Tech Scientific Ltd., Salisbury, U.K.). Sodium dodecyl sulphate (SDS), 0.4%, in the same buffer was placed in the other reservoir. The reservoirs were connected to two separate syringes and to the mixing chamber via a three way tap. Both syringes were driven at the same rate to deliver each solution to the mixing chamber; on stopping the flow, the absorbance, at the λ_{max} of the free drug, was monitored and the output recorded on a Tectronix storage oscilloscope.

The increase in extinction was monitored over a period of about 6 half lives. In each case at least eight data points were collected and fitted to a single exponential and to a biexponential equation using a microcomputer programme. In all cases the data could be fit to a single exponential with a correlation coefficient of 0.99 or better: the data did not fit a biexponential model (correlation coefficient less than 0.89).

Table 1. Dissociation rate constants for anthracycline- and anthraquinone-DNA complexes in (0.15 M) NaCl (0.2 M) buffer pH 7.4

Drug	Dissociation rate constant (sec^{-1}) 20°	25°	37°	Drug conc.* M × 10^5
I†	1.43 (0.17)‡	2.17 (0.20)	10.93 (0.76)	3.43
I	2.88§	—	—	0.60
I	3.3‖	—	27.0‖	4.00
II	1.84 (0.19)	2.07 (0.87)	9.87 (1.02)	3.45
V	> 150			3.42
VI	3.73 (0.14)	7.50 (0.38)	35.66 (3.30)	3.20
VII	0.91 (0.12)	1.80 (0.05)	7.50 (0.30)	3.05
VIII	6.99 (0.52)	17.26 (1.20)	47.97 (5.60)	3.74

* Final drug concn. after mixing with SDS = 0.5 × stated concn. Final SDS concn in all cases 0.2%.

† The values for daunorubicin (I) and doxorubicin (II) are not significantly different ($P > 0.95$).

‡ S.D. ($N = 5$).

§ Values for dissociation of daunorubicin-DNA (Salmon sperm) complexes in 1 mM PIPES buffer, 0.201 M NaCl, pH 7.8, at 15° [49].

‖ Values for dissociation of daunorubicin-poly (dG–dC). poly (dG–dC) complexes in 1 mM PIPES buffer, 0.15 M NaCl, pH 6.7 [46].

RESULTS AND DISCUSSION

The dissociation kinetics were found to be first order for more than four half-lives for all the DNA-anthracycline and DNA-anthraquinone complexes under these conditions. The first-order rate plots for the dissociation of DNA-daunorubicin, DNA-doxorubicin and DNA-anthraquinone (V–VIII) complexes at 20°, are shown in Fig. 1. The results at this and other temperatures (25 and 37°) have also been summarised in Table 1.

The anthracyclines and the substituted anthraquinones are ionised at physiological pH so as well as the intercalative mode of binding there will be a secondary mode of external binding to the helix, as occurs with other basic intercalating agents [23, 40]. The equilibrium can thus be envisaged as:

$$\text{DNA} + \text{Drug} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{DNA externally bound drug} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{DNA-intercalated drug.}$$

In support of this, previous T-jump and stopped flow studies with daunorubicin and polynucleotides or nucleic acids show a biexponential decay pattern [46, 47]. In this study all solutions were prepared in Tris–(0.015 M) NaCl (0.2M) buffer pH 7.4; at this ionic strength only the intercalation mode of binding should be present. Since the data obtained in this study could only be fit to a single exponential, this confirms the appropriateness of the ionic strength of the solution.

Sodium dodecyl sulphate has been used previously as an agent to disrupt intercalating systems [46, 48, 49] including daunorubicin/DNA complexes [46, 48]. In one study it was confirmed that alteration of the SDS concentration in the range 0.2–1.0% had minimal effect (±3%) on the dissociation rate [46].

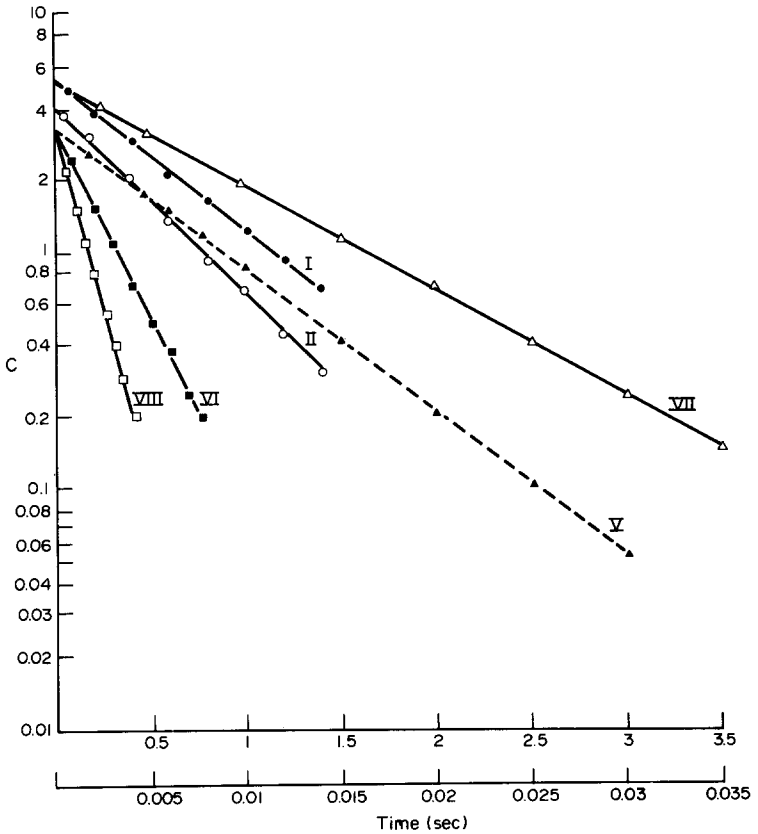

Fig. 1. First-order rate plots for the dissociation of DNA complexes of anthracyclines (I and II) and anthraquinones (V–VIII) in Tris (0.015M), NaCl (0.02M) buffer pH 7.4 at 20°, using sodium dodecyl sulphate (0.2%) to disrupt the equilibrium. The lower timescale is for compound V.

A concentration of 0.2% in the final solution was used in this study. The reaction can be represented as:

$$\text{DNA-drug} \xrightarrow{k_d} \text{DNA} + \text{drug}$$

$$\text{SDS} + \text{drug} \xrightarrow{k_s} \text{SDS-drug},$$

where k_d is the first order dissociation rate constant. The DNA-drug dissociation is the rate controlling step since the sequestering of drug with SDS is a diffusion controlled process [46, 48, 49]. It is not possible, by analogy with the rate constants for other intercalating systems, to unambiguously determine whether the measured rate constant, k_d, is identical to k_{-2} of the two-step mechanism. Therefore, we have confined our attention to overall dissociation rate constants.

In all cases there is an increase in the rate constant with temperature. The ranking order for the dissociation rate constants, of the substituted anthraquinone-DNA complexes, was the same at 20, 25, and 37°. Measurements were taken at 37° to ensure that the order is not changed at physiological temperature. All reactions were studied at 20° because this enabled a better stability and reproducibility of the data than at higher temperatures. Dissociation rate constants were also measured at 25° as this is standard temperature for physicochemical studies.

The dissociation rate constants for daunorubicin/DNA complexes (Table 1) are of the same order as those obtained by previous workers [46, 48] for polydeoxynucleotide-daunorubicin (3.3 sec^{-1} at 20 and 27.0 sec^{-1} at 37°) and for Salmon sperm DNA-daunorubicin complexes (2.88 sec^{-1} at 15°) in solutions of the same order of ionic strength (Table 1).

The dissociation rate constants for DNA-disubstituted anthraquinone complexes (Table 1) were of the same order as for DNA-anthracycline complexes. However, in the case of the monosubstituted anthraquinone (V) the dissociation rate constant was greater than 150 sec^{-1} at 20° and therefore could not be measured accurately at higher temperatures. The value for the 1,8-disubstituted compound (VIII) was significantly lower than that for the monosubstituted compound (V) but was greater than that for the 1,4-disubstituted compound (VI). The 1,5-disubstituted anthraquinone (VII) has the lowest dissociation rate constant, 0.91 sec^{-1} compared to 3.73 sec^{-1} for the 1,4-disubstituted anthraquinone (VI) even though the equilibrium constants for these compounds are similar [39]. It is not possible to calculate the association rate

constants (k_2) from these previously determined association constants [39] as the buffer used here was of a higher ionic strength (0.2 M NaCl compared to 0.05 M NaCl).

The slower dissociation of the DNA/1,5-disubstituted anthraquinone (VII) complex, as compared to the complexes of DNA with V, VI, VIII and the anthracyclines, is consistent with results from computer graphics modelling of the fit of the anthraquinones into an intercalation site [39] and with the X-ray crystallographic data on the complex of daunorubicin with the self-complementary hexanucleotide d(CpGpTpApCpGp) [26]. In order to accommodate the 1,5-disubstituted anthraquinone (VII), DNA-breathing (transient base pair unstacking) has to occur to allow the docking of drug molecule into the receptor site. Hence once the anthraquinone has intercalated into DNA, DNA-breathing is required before dissociation can take place. This is not necessary with the other compounds, though compound V can bind in this manner as well [39]. Thus analysis of the kinetics of DNA-drug binding is a vital adjunct to equilibrium studies as it can provide information of the ease of release of drug from DNA. This may be of biological relevance whether DNA is the site of action or whether it serves as an intracellular store of drug. The fact that the 1,5-disubstituted anthraquinone (VII) dissociates from its DNA intercalation complex less readily than does the 1,4-disubstituted compound (VI) correlates with the finding that it is as cytotoxic to HeLa cells as doxorubicin (II) whilst the 1,4-substituted anthraquinone (VI) is less cytotoxic [39].

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