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Infrared linear dichroism studies of DNA – drug complexes: quantitative determination of the drug-induced restriction of the B – A transition

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ABSTRACT

The B – A transition of films or fibers of NaDNA occurs at a relative humidity of 75 – 85%. The fraction of DNA that changed the conformation from B to A form can be determined quantitatively by infrared linear dichroism. DNA-binding drugs can 'freeze' a fraction of DNA in the B form. This fraction of DNA is in the B form and cannot be converted to A-DNA even at a reduced relative humidity of 54%. The 'freezing' potentiality of various drugs can be described by the 'freezing' index, *FI*, expressed in base pairs per added drug. Drugs with a high value of *FI* (more than eight base pairs per drug) were observed among both intercalating and groove-binding drugs. High values of *FI* imply restriction of the conformational flexibility of DNA significantly going beyond the binding site of the drug. This long-range effect of drugs on the conformational flexibility of DNA may be connected with the molecular mechanism of drug action. The freezing index *FI* is a new quantitative parameter of drug – DNA interaction that should be considered as a valuable tool for drug design.

INTRODUCTION

Double-helical DNA is polymorphic (1). The actual DNA conformation is dictated by several factors as base composition and sequence, salt content, amount and type of cation, supercoiling, and others. The most common conformations are B, A and Z. Under low salt conditions, natural NaDNA is usually in the B form at high water activity, and in the A form at reduced water activity. In fibers or films, the B–A transition can be observed at a relative humidity (RH) of the surrounding atmosphere of 75–80% (2–5). In solution, the B–A transition can be induced by progressive addition of ethanol or trifluorethanol which decreases the water activity. The transition occurs in the range of 65% to 80% (v/v) ethanol (6,7).

Until now, there are only few findings in favor of the occurrence of A-DNA *in vivo*. Some arguments, however, support the existence of special sequences in the A form at least during protein recognition (6). Recently, the change of the DNA conformation of B to A by binding of small acid-soluble spore proteins from *Bacillus subtilis* was reported (8). The conformational change to A-DNA protects the genetic material against UV irradiation damage. Notwithstanding, conformational flexibility of DNA is likely a pre-requisite for its proper biological function in the course of gene expression and gene regulation. Any restriction of the B–A transition may induce a malfunction of DNA.

Poly[(dA)·(dT)] does not go into the A form. On the other hand, poly[d(A-T)·d(A-T)] can adopt the A form (9). This behavior may be due to the special conformation of poly[(dA)·(dT)] which is significantly different from B-DNA and has a narrower minor groove (10,11). Films of natural DNA with a high GC content were shown to go easily and completely into the A form at reduced relative humidity (12,13).

Several studies have shown the incompleteness of the B–A transition in films of NaDNA (13–15). The reasons are due to milieu conditions and/or special physical forces that may shift the energetical preference towards either the B or the A form.

Independently of these findings, we recently reported the drug-induced restriction of the B–A transition of NaDNA films. We used the infrared linear dichroism for a quantitative description (16–20); the method was introduced in 1957 by Tsuboi and coworkers (21,22) and later supplemented (23,24). The potentiality of linear dichroism in DNA structure research was excellently reviewed very recently by B. Nordén *et al.* (25). Because of drug binding, a fraction of DNA is locked in the B form and is incapable to adopt the A form even under conditions of rigorous dehydration. The amount of the fraction of DNA 'frozen' in the B form is correlated with the drug concentration. The higher the amount of added drug per DNA base pair, the larger is the fraction of 'frozen' DNA. Corresponding to the size of the drug binding site on DNA, we would expect a maximum of 2–5 base pairs of DNA 'frozen' in the B form by one drug molecule. However, the 'freezing' potential of the investigated drugs varies considerably. The characteristic quantity of the 'freezing' potential is the 'freezing index', *FI*, specifying the number of DNA base pairs 'frozen' in the B form per added drug molecule. The 'freezing index' will be discussed as a new characteristic quantity to describe the action of drugs targeted to DNA and as a new tool for drug design. Of course, the results obtained on hydrated films cannot simply transferred to *in vivo*

conditions. However, one may argue that nuclear DNA in cells is rather compact and may be behave similar to DNA in hydrated films.

MATERIALS AND METHODS

DNA films

Oriented films of DNA were kindly supplied by Dr Allan Rupprecht, University of Stockholm, Sweden. The procedure was described elsewhere (26–28). In this study, wet-spun films of NaDNA from calf thymus (42% G+C) and from *Micrococcus lysodeikticus* (72% G+C) were used. The transfer of the wet-spun films from the Teflon deposit on infrared transparent windows was done according to (28).

DNA–drug complexes

The drugs were research samples and were used without further purification. The antibiotics daunomycin and distamycin A were kindly provided by Prof. Arcamone (Farmitalia Milano, Italy), and aclacinomycin A, mitoxanthrone, and anthrapyrazole by Prof. Umezawa (Institute of Microbial Chemistry, Tokyo, Japan). Amsacrine, pentamidine and the groove-binding bisquarternary ammonium heterocycles SN-6999, SN-18071 and NSC-101327 were gifts of Prof. Denny and Prof. Baguley, University of Auckland, New Zealand. All other drugs were from the former Institute of Microbiology and Experimental Therapy Jena.

The DNA–drug complexes were formed by addition of the appropriate amount of drug dissolved in 80% ethanol / 20% water (v/v) to the film.

Infrared linear dichroism

The spectra were recorded on a PERKIN ELMER model 325 and on a MATTSON GALAXY 4020 FT-IR spectrometer both equipped with a wire-grid polarizer. As described previously (29), the dichroic ratio R of the base stretching vibration at 1705 cm^{-1} (C2=O2 stretching of thymine) was used to determine the orientation parameter *f*, the fraction of perfectly ordered DNA according to Fraser's equation (30):

$$R = A_{\perp} / A_{\parallel} = [(1/2)f \sin^2\Theta + (1/3)(1-f)] / [f \cos^2\Theta + (1/3)(1-f)] \quad \text{(eq.1)}$$

$A_{\perp}$ and $A_{\parallel}$ are the absorbances of the investigated band for polarization perpendicular and parallel, respectively, of the infrared radiation with respect to the DNA helix axis of the oriented film. Θ is the angle between the DNA helix and the vibrational transition moment. In this special case, the vibrational transition moment can be assumed to be in the direction of the C2–O2 bond of thymine known from X-ray data. Here we used values of 90° and 80° for the angle Θ of B-DNA and A-DNA, respectively (29). Once the orientation parameter *f* of the oriented film is known, one can obtain the Θ values of any other vibration of DNA by equ. 1. Particularly, the dichroism of the symmetric and antisymmetric PO_2^- vibrations of DNA, absorbing at 1090 cm^{-1} and 1230 cm^{-1}, respectively, is related to transition moments aligned along the OPO bisector and the O...O connecting line, respectively. The corresponding inclination angles Θ_{OPO} and Θ_{OO} reflect the spatial situation of the PO_2^- subunit of DNA in relation to the axis of the double helix. The phosphate geometry is sensitive against conformational changes of DNA. The values of Θ_{OPO} and Θ_{OO} corresponding to B-, A- and Z-DNA, respectively, were compiled in (29,31). In this communication we focus on the B–A transition that occurs at 75–85% relative humidity (RH). B-DNA exists at high values of RH (>85 %), A-DNA at lower values of RH (<75%). The most significant change accompanying the B–A transition is the decrease of Θ_{OPO} from ~65° (Θ_B) down to ~45° (Θ_A). Values of Θ_{OPO} between 45° and 65° suggest a mixture of B-DNA and A-DNA. By linear interpolation we obtain the fraction of B-DNA from the experimental value Θ_{obs} according to:

$$\%B = [(\Theta_{obs} - \Theta_A)/(\Theta_B - \Theta_A)] * 100 \quad \text{(eq.2)}$$

Determination of the 'freezing' index *FI*

Drugs bound non-covalently to DNA are able to 'freeze' a fraction of DNA in the B form. This fraction does not adopt the A form even at low water activity (16). The 'freezing' potentiality of a drug can be described by the number of DNA base pairs which are 'frozen' by one added drug molecule. This quantity is the 'freezing' index, *FI*, that can be obtained from the experimental value Θ_{obs} of the symmetric PO_2^- vibration band at 1090 cm^{-1} determined at reduced RH (75% and 54%) by the simple equation:

$$FI = [(\Theta_{obs} - \Theta_A)/(\Theta_B - \Theta_A)] / r_t \quad \text{(eq.3)}$$

where r_t is the amount of added drug expressed in number of drug molecules per DNA base pair (drug/bp). The reported values of *FI* were obtained by extrapolation to infinite small amount of added drug, i.e. to values of $r_t = 0$.

As an example of the dichroic spectra, the polarized spectra of the DNA–amsacrine complex at 96% RH and 75% RH, respectively, and the determination of the 'freezing' index *FI* are given in Fig. 1. The extrapolation of the *FI* to $r_t = 0$ for the DNA-amsacrine complex is illustrated in Fig. 2.

RESULTS AND DISCUSSION

The infrared linear dichroism of DNA complexes with 19 non-covalently binding drugs was studied in dependence of the RH. Eleven of the drugs are known to intercalate between DNA base pairs (32,33). Among them are adriamycin (doxorubicin), daunomycin (daunorubicin) and aclacinomycin A, important anticancer drugs currently in widespread clinical use. The remaining eight drugs bind non-covalently in the minor groove of DNA (34,35). The values of *FI* were obtained by extrapolation to infinitely small drug input ratios. This excludes saturation effects which would result in lower values of *FI*.

Intercalating drugs

Anthracycline antibiotics and related anthraquinone analogues are intercalators. For comparison, the intercalators ethidium and amsacrine have been included in this study. The structures are compiled in Figs.3 and 4. The anthracycline chromophore with its three aromatic rings and one saturated ring are inserted between two consecutive base pairs of B-DNA gaining free energy by stacking interaction (36). All drugs cover 3–4 base pairs (bp) of DNA in the complex (32,33). A value of *FI* significantly higher than four bp/drug suggests a perturbation of the conformational flexibility of DNA beyond the binding site. Values of *FI* >8 represent a long-range perturbation of DNA.

Inspection of the *FI* values (Table I) shows long-range perturbations for the five anthracycline antibiotics adriamycin, daunomycin, violamycin BI, aclacinomycin A and β-rhodomycin II. The other investigated intercalators 'freeze' stretches of DNA equal to, or even smaller than, the size of the respective binding

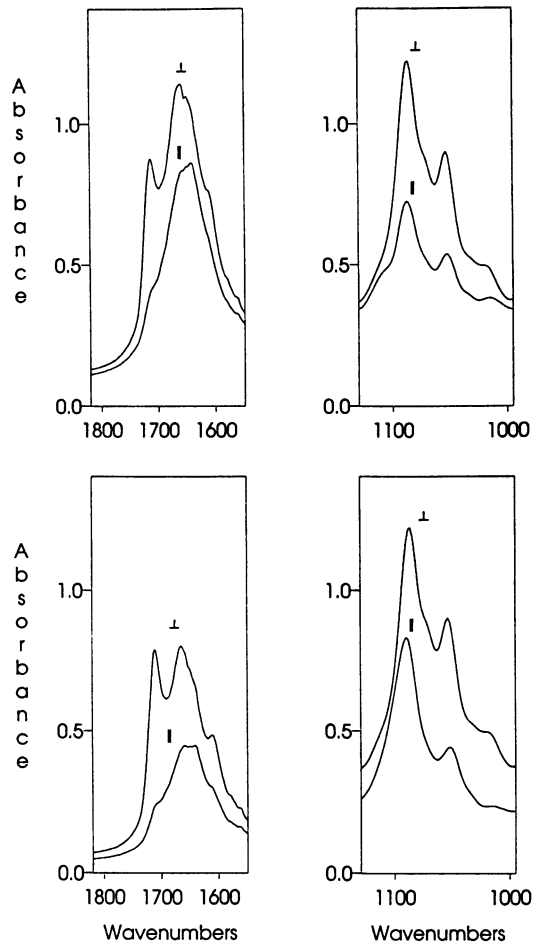


Figure 1. Polarized IR spectra of the amsacrin−DNA complex. The total drug input ratio amsacrin/DNA base pair is r_t = 0.2. The oriented DNA film is exposed to 96% RH (Fig. 1a) and 75% RH (Fig. 1b), respectively. The electric vector is perpendicularly (⊥) and parallely (∥) polarized with respect to the orientation direction of the sample which is the double helix axis. The orientation function f is calculated from the absorbance ratio of the 1710 cm^{-1} band and is f=0.896 (**a**) and f=0.995 (**b**), respectively. The values of Θ_{OPO} are obtained from the dichroic ratio of the band at 1090 cm^{-1} (R_{1090}= 2.099 [a]and R_{1090}= 1.327 [b]). Using the reported f values, we obtain by eq.1 Θ_{OPO}=65.2° (a) and Θ_{OPO}=58.4° (b). According to eq.2, the fraction of B-DNA in the sample at 75% RH is then 25.1%B, taking the values of Θ_{OPO}=65.2° and Θ_{OPO}=46.8° for B-DNA and A-DNA, respectively, and subtracting the B-DNA content of the drug-free DNA at 75% RH which is 37.9%. Finally, the 'freezing' index of this sample can be obtained from eq.3 and is then FI=2.51.

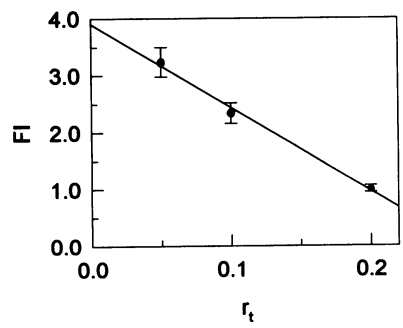
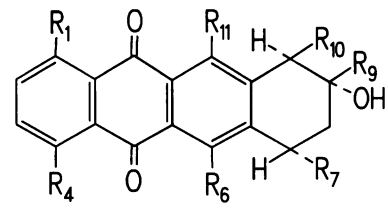


Figure 2. Plot of the 'freezing' index FI *versus* r_t, the drug/DNA input ratio. Extrapolation to r_t=0 gives the reported value FI~4 bp/drug (the exact value is FI=3.91 ± 0.19).



Anthracycline	R1	R4	R6	R11	R7	R10	R9
Adriamycin	H	OCH_3	OH	OH	das	H	$COCH_2OH$
Daunomycin	H	OCH_3	OH	OH	das	H	$COCH_3$
ß-Rhodomycin I	H	OH	OH	OH	rhs	OH	C_2H_5
ß-Rhodomycin II	H	OH	OH	OH	rhs	rhs	C_2H_5
Irmycin	H	OH	OH	OH	H	rhs	C_2H_5
Aclacinomycin A	H	OH	OH	H	tri	$COOCH_3$	C_2H_5
Violamycin:							
BI/1 = Rhm-II	H	OH	OH	OH	rhs	rhs	C_2H_5
BI/2	OH	OH	OH	OH	rhs	rhs	C_2H_5
BI/3	OH	OH	H	OH	rhs	rhs	C_2H_5

Figure 3. Structures of the anthracycline antibiotics. das: L-daunosamine; rhs: L-rhodosamine; tri: L-rhodosaminyl-L-2-deoxyfucosyl-L-cinerulose.

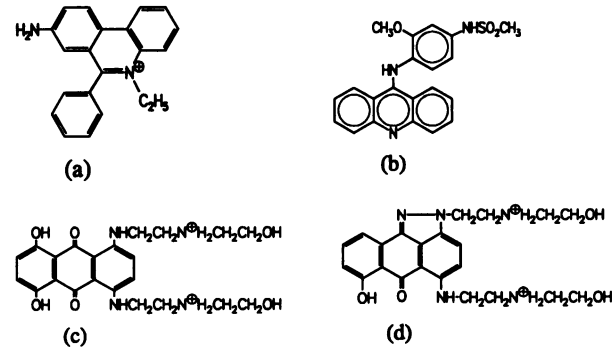


Figure 4. Structures of intercalating drugs: (**a**) ethidium, (**b**) amsacrine, (**c**) mitoxanthrone, (**d**) anthrapyrrazole.

Table 1. Freezing index FI of DNA complexes with intercalating drugs. The error of FI is approx. ±1 bp/drug

Drug	FI (bp/drug)
Adriamycin	28
Daunomycin	28
Violamycin BI	18
Aclacinomycin A	15
β-Rhodomycin II	15
Iremycin	9
β-Rhodomycin I	7
Mitoxanthrone	6
Anthrapyrazole	5
Amsacrine	4
Ethidium	4

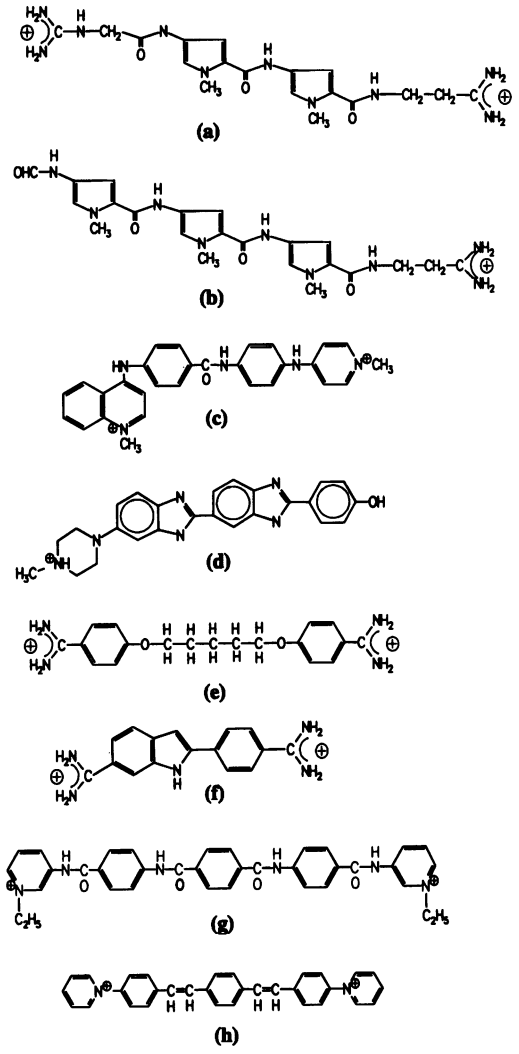


Figure 5. Structures of minor-groove binding drugs: (a) netropsin, (b) distamycin A, (c) SN 6999,(d) Hoechst 33258, (e) pentamidine, (f) DAPI, (g) NSC 101327, (h) SN 18071.

Table 2. Freezing index *FI* of DNA complexes with non-intercalating drugs

Drug	*FI* (bp/drug)
Netropsin	24
Hoechst 33258	24
Distamycin A	22
Pentamidine	12
SN 6999	9
DAPI	4
NSC 101327	2
SN 18071	2

sites. Merely iremycin with $FI=9$ is significantly beyond the level of 4–6 bp/drug, on the other hand, however, distinctly below the *FI* values of the five outstanding anthracycline antibiotics. Interestingly, all three clinically used anthracycline antibiotics, adriamycin, daunomycin and aclacinomycin A, are in the class with a very high value of *FI*. The other two are closely related: violamycin BI is a mixture mainly consisting of β-rhodomycin II and two closely related components differing in the substitution pattern of position 1 and 6 of the chromophore (33). Violamycin BI and β-rhodomycin II have some antitumor activity but have not been rigorously tested for clinical use. The fairly enhanced *FI* value of iremycin points to the irregular structure of this antibiotic. The aminosugar is in position 10 instead of the usual position 7 (all violamycin BI components have aminosugars both in position 7 and 10).

Non-intercalating minor-groove binding drugs

The structures of the eight minor-groove binding drugs are compiled in Fig. 5. They have in common a more or less pronounced preference of B-DNA and are situated in the small groove of DNA (34,35). They bind preferentially to AT-rich sequences of DNA. In general, a perfect fit of the drugs is disturbed by GC base pairs because of the amino group of guanine protruding in the minor groove. We selected two drugs of the netropsin type, a further imidazole derivative, an indole derivative, and four of the bis-quarternary heterocyclic ammonium compounds typically carrying two positive charges at their ends. The structure of SN 18071 and the main structural segment of pentamidine are lacking NH donor groups to form hydrogen bonds.

Among these eight DNA–drug complexes, we found four classes of *FI* values (Table II): netropsin, distamycin A and HOECHST 33258 with $FI > 20$; pentamidine and SN 6999 with $FI \sim 10$; DAPI with $FI \sim 10$; NSC 101327 and SN 18071 with $FI < 3$. Only the latter class 'freezes' less or equal numbers of base-pairs of DNA than are covered by the drug. The first class 'freezes' five to six times more base pairs, and the second class still two to three times more than the binding sites that span 4–6 base pairs (36,37).

The non-intercalating drugs show a large diversity of structures. A common feature, however, is the presence of two positive charges at the ends of the molecules (except distamycin A with only one positive charge). This favors the dominance of the electrostatic contributions to the binding energy and the localization in the minor groove of B-DNA at AT base pairs where the deepest electrostatic potential is localized (38). Two of the non-intercalating minor-groove binding drugs, NSC 101327 and SN 18071, have low values of *FI* not exceeding their sizes of binding sites on DNA. One may argue that the capability to form a maximum number of hydrogen bonds to DNA bases supports a high *FI*. This argument, however, fails since pentamidine is remarkably powerful to 'freeze' B-DNA despite its absence of hydrogen donor groups. On the other hand, the value of *FI* may be related with the flexibility of the drug. The high binding affinity of minor groove binding drugs like netropsin and distamycin A was correlated with their flexible adoption of an isohelical conformation (34). Both SN 1807 and NSC 101327 are very inflexible. This prevents an isohelical conformation with optimal contacts to the surface of the minor groove of B-DNA; the values of *FI* are extremely small. By contrast, the drugs pentamidine and SN 6999 are more flexible and fit more or less into the minor groove of B-DNA. The values of *FI* of these two drugs are significantly higher than their sizes of the binding site of DNA which is expected to be 4–6 base pairs. Summarizing, the flexibility of minor-groove binding drugs is the most important feature for the appearance of long-range 'freezing' effects. A similar conclusion was drawn for distamycin by Lu *et al.* (39) from Raman spectroscopic data.

Long-range 'freezing' effect

An unexpected result of this study is the long range 'freezing' effect of several drugs with respect to the suppression of the B−A transition of DNA and the wide variability of the values of *FI*. Ligand binding can change the conformational behavior at the binding site that is limited up to five base pairs for the investigated ligands. The B−A transition of DNA is known to be a cooperative process with a cooperative length of about 10−20 base pairs (6,7,40,41). As already pointed out by Ivanov *et al.* (41), this value of the cooperative length in the order of 10^1 base pairs contrasts sharply with the 10^3 base pairs of the helix-coil transition of DNA. It explains, however, the failure of a B−A transition when the drug 'freezes' B-DNA at the binding site and leaves stretches of DNA between the binding sites shorter than the cooperative length of at least 10 base pairs.

On the other hand, a very high value of *FI* in the DNA complexes with netropsin and distamycin A excludes the simultaneous binding of two drugs per binding site when the ratio drug/base pair is low. Under the conditions of NMR experiments has been found that DNA can accomodate two molecules of distamycin A simultaneously in the minor groove (42).

Intercalation as well as minor-groove binding of drugs is based upon B-DNA and may not function in A-DNA at all. Minor groove binding drugs like netropsin and distamycin A fit snugly into the minor groove of B-DNA but do not bind to A-DNA; they even revert A-DNA to B-DNA (43). A complete suppression of the B-A transition may occur when the drug occupies so much sites on the DNA that the 'free' spaces are shorter than the cooperative length of 10−20 bp. But this does not explain why various drugs with similar sizes of binding on DNA differ significantly in their values of *FI*. We have to assume a change of the conformational flexibility of DNA at the binding site of a drug which can extend up to ~ 12 bp on each side of the bound drug.

Recently was shown that binding of echinomycin to DNA causes hyperreactivity to DNase I up to 12 bp from the nearest binding site (44,45). Again, this puts evidence for a drug-induced long-range effect on the DNA conformation.

Some drugs are assumed to attack primarily of the DNA. For those drugs, *FI*—the measure of the 'freezing' potential of the drug—is a crucial quantity. The section of DNA 'frozen' in the B form by the bound drug may be unable to adopt other conformations or even to allow significant distortions of the structure necessary for the biological functioning of DNA. The strong inhibition of transcription by netropsin (34) can be regarded as an example of the consequences of the 'freezing' effect of drugs on DNA. The 'freezing' effect should be considered for the design of drugs with a long-range effect on the DNA conformation.

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