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ON THE INTERACTION OF CAFFEINE WITH NUCLEIC ACIDS. IV. STUDIES OF THE CAFFEINE-DNA INTERACTION BY INFRARED AND ULTRAVIOLET LINEAR DICHROISM, PROTON AND DEUTERON NUCLEAR MAGNETIC RESONANCE

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The DNA-caffeine interaction at high concentration of caffeine (Cf) leads to an arrangement of Cf molecules outside of the DNA double helix with an orientation parallel to the bases both in the A and B form as demonstrated by infrared linear dichroism. Addition of DNA broadens the ^{1}H NMR lines of Cf in aqueous solution indicating a specific binding. Intercalation is not the predominant mechanism, the deuterium relaxation time T_1 = (38 ± 7) ms and the estimated reorientation correlation time τ_{cb} = 0.17 ns of caffeine-8-[^{2}H] in the Cf-DNA complex suggest an outside stacking of the ligand. According to the results a model on the DNA-Cf complex at high Cf concentration (binding process II) is suggested including the caffeine-induced reorientation of the hydration shell of DNA.

1. Introduction

Previously, the existence of two mechanisms of caffeine-DNA interactions in dilute aqueous solution of DNA has been demonstrated [1–3]. The binding process I occurs at low concentration of caffeine (Cf) and stabilizes the DNA by Cf insertion. Contrary to this, binding process II is indicated by a destabilization of DNA. It predominates at high Cf concentration and is connected with an indirect interaction outside of the DNA chain.

In the preceding part of this series [4] we suggested a model of the partial insertion of Cf between adenine residues. By ^{1}H NMR investigations of Cf–5′-adenosine monophosphate and Cf–poly(riboadenylic) acid interaction we presented a refined model of the molecular arrangement of Cf between residues of the single-stranded stacked forms of poly(riboadenylic) acid.

To get more detailed information on the interaction processes and molecular arrangement of Cf molecules at high concentration in the presence of DNA (binding process II) we investigate the binding of Cf to DNA in high-concentrated solutions and in films, respectively. We used ^{1}H NMR spectroscopy for the detection of the specific binding of Cf to DNA. The NMR lines of Cf are broadened in the Cf-DNA complex. Additionally, the broadening of the Cf lines by paramagnetically labeled DNA is studied.

The deuterium relaxation time T_1 of deuterium-labeled Cf is measured in the presence of DNA in order to determine the mobility of the ligand in the complex.

The water proton relaxation times T_1 and T_2 of aqueous DNA solutions are measured in the absence and presence of Cf. This method is sensitive to changes in the hydration behaviour of DNA in the complex.

List of abbreviations:
NMR: nuclear magnetic resonance; Cf: caffeine; DNA: deoxyribonucleic acid (sodium salt); TSP: 3-(trimethylsilyl)-[$^2H_4$]-propionate; SSC buffer: standard sodium citrate buffer; EDTA: ethylenediamine tetraacetic acid; r.h.: relative humidity.

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The infrared linear dichroism of oriented films of the Cf–DNA complex informs us about (i) the ligand orientation in respect to the DNA helix axis [5] and (ii) the ligand-induced changes of the DNA conformation [6,5]. Cf-induced alterations of the DNA structure as a function of the humidity are indicated by the ultraviolet linear dichroism, too.

The biological activity of caffeine is discussed in terms of the results.

2. Experimental part

2.1. Materials

Calf thymus DNA has been isolated and deproteinized following standard procedures [7]. In the NMR experiments the DNA samples have been extremely deproteinized (protein content 0.18%). The aqueous DNA solutions (0.4%, SSC buffer + 10^{-5} M EDTA) have been sonicated under argon. Hypochromicity (32%) and molecular weight have been controlled (M = 450000 dalton).

Caffeine (pure, VEB Arzneimittelwerk Dresden) has been used without further purification. Deuterated caffeine-8-[^{2}H] has been prepared by a 120 h incubation of caffeine in D_2O solution (Isocommerz, GDR; isotopic purity: 99.7%) at 95°C. The isotopic purity of the reaction product caffeine-8-[^{2}H] has been better than 95% as checked by ^{1}H NMR.

2.2. Methods

^{1}H NMR. A 100 MHz instrument KRH 100 R (Zentrum für Wissenschaftlichen Gerätebau der Akademie der Wissenschaften der DDR, Berlin, GDR) working in the continuous-wave mode has been used. The measured chemical shifts are related to sodium 3-(trimethylsilyl)-[$^2H_4$]-propionate (TSP; Merck). A small amount of *tert*-butanol has been added as internal reference.

Some preliminary measurements have been run on a Varian HA-100 instrument (Karl-Marx-Universität Leipzig, Sektion Physik).

Relaxation time measurements. Both the ^{1}H and ^{2}H relaxation times have been obtained by the spin-echo technique using a pulse spectrometer B-KR 322s (Bruker, FRG) operating at 11 MHz (Karl-Marx-Universität Leipzig, Sektion Physik). The ^{1}H relaxation times have been measured by a $\pi-\pi/2-\pi$ (T_1) and a $\pi/2-\pi-\pi-$ ... pulse sequence (T_2), respectively. The pulse sequence $\pi-\pi/2-\pi$ has been used to discriminat small differences in the relaxation times T_1 of different samples from the time of the zero transition of th magnetization, despite of small systematic errors inherent to this method. Two parallel sets of samples have been used in the experiments. The sample tube diameter is 2 mm and the volume 100 µl. All reported T_1 and T_2 values are the average of 16 measurements, respectively. The longitudinal ^{2}H relaxation times T_1 of caffeine-8-[^{2}H] have been measured in tubes of 10 mm o.d. by accumulating 8192 scans.

Infrared linear dichroism measurements. The infrared spectra of oriented films of the DNA-caffeine complex (phosphate/drug mole ratio P/D = 2) have been recorded in the range of 4000–450 cm^{-1} by a Perkin-Elmer Model 325 spectralphotometer equippec with a wire-grid polarizer. Details of the procedure and calculation of the angles between the transition moments of the related vibrations and the helix axis of DNA are the same as described elsewhere [5,8].

Ultraviolet linear dichroism measurements. The UV linear dichroism of oriented films of the DNA-caffeine complex (P/D = 1.0) has been measured as described in [9].

3. Results

3.1. ^{1}H NMR

We have observed a fairly resolved splitting of the H-8 proton (quartet) and the N-7 methyl proton lines (doublet) in the high-resolution NMR spectra of Cf due to a long-range coupling of ^4J = (0.65 ± 0.05) Hz. This is a further evidence of the line assignment of the δ = 3.95 ppm line to the N-7 methyl protons of Cf [1(

Addition of DNA to an aqueous (^2H_2O) solution of Cf broadens the ^{1}H NMR lines of caffeine (table 1) As a control, the line widths of both the internal refer ence *tert*-butanol and of the $^1H^2HO$ line have been monitored. There is no indication of a general line broadening by addition of DNA by viscosity or other effects. The enhancement of line widths in the Cf NM spectrum is reduced by further Cf addition to the DNA-Cf mixture.

In a further experiment the DNA has been labeled

Table 1
^1H NMR line widths of caffeine in ^2H_2O solution (free and bound to DNA). Error of line width measurement: ± 0.1 Hz.

Sample	Line width [Hz]				
	H-8 a)	Me-7 b)	Me-3	Me-1	$^1H^2HO$
Cf, 0.05 M	1.8	1.4	0.7	0.8	0.5
Cf, 0.05 M, + DNA, 3.2×10^{-2} M phosphate	6.5	5.7	3.5	3.0	0.5
broadening difference	4.7	4.3	2.8	2.2	0

a) Unresolved quartet ($^4J_{H8-H7}$). b) Unresolved doublet ($^4J_{H8-H7}$).

by paramagnetic Mn^{2+} ions (phosphate/metal mole ratio = 10). This method has been introduced in the NMR studies of nucleic acids by Weiner et al. [11] (RNA) and Reuben et al. [12] (DNA). Again the line widths of the Cf ^1H NMR lines are considerably enhanced when the DNA–Mn^{2+} complex is added whereas in the control experiments the same Mn^{2+} concentrations in the absence of DNA are without influence on the line widths (table 2). A small overall effect of increase in the relaxation rate (line widths) is produced by the paramagnetic ions bound to DNA as demonstrated by the small increase of the reference line width. This effect is well known from the water proton relaxation rate enhancement studies introduced by Cohn and others as reviewed in [13,14,15].

3.2. Infrared linear dichroism

In a previous paper [5] we have demonstrated the determination of the orientation of a planar ligand relative to the helix axis of DNA by infrared linear dichroism. Assuming a transition moment of the out-of-plane deformation vibration of Cf at about 745 cm^{-1} passing perpendicular to the plane of the ring the strong parallel dichroism of this band in the DNA–Cf complex indicates a perpendicular arrangement of the ligand relative to the DNA helix axis or, in other words, a parallel arrangement of Cf in respect to the base pairs of DNA.

The characteristic parameters of films of the DNA–Cf complex as results of the infrared investigations covering the complete dehydration-rehydration cycle are presented in table 3. The most prominent result is the hysteresis of some characteristic parameters in the region of medium humidity (in this region pure DNA exists in the A form). The fraction f of perfectly oriented DNA molecules decreases in the course of the cycle, on the contrary the fraction of A form of DNA increases as indicated by the two conformation-sensitive phosphate angles $\theta_{O...O}$ (fig. 1)

Table 2
Influence of Mn(II)-labeled DNA on the ^1H NMR line widths of caffeine. Solvent: ^2H_2O; *tert*-butanol has been added as internal reference.

Sample	$[Mn^{2+}]$ M	Line width [Hz]				
		H-8 a)	Me-7 b)	Me-3	Me-1	*tert*-butanol
Cf, 0.048 M	0	1.8	1.3	0.6	0.7	0.4
Cf, 0.048 M	7×10^{-5}	2.0	1.4	0.7	0.7	0.7
Cf, 0.048 M, + DNA, 7.5×10^{-4} M phosphate	7×10^{-5}	2.6	2.5	2.6	2.4	0.6

a) Unresolved quartet. b) Unresolved doublet.

Table 3
Infrared spectroscopic data of an oriented film of the DNA-caffeine complex as a function of the relative humidity (phosphate/drug mole ratio P/D = 2). Orientation factor f, the phosphate angles $\theta_{O...O}$, θ_{OPO} and the ligand orientation angle θ_{Cf} are calculated from the infrared linear dichroism data and are defined as described in [5,8]. A'_{1185} is the absorbance of an A form specific absorption band of DNA at 1185 cm^{-1} [6] normalized to the absorbance of the 1230 cm^{-1} phosphate vibration band. The dehydration-rehydration cycle is divided into intervals of high, medium and low himidity, the specific parameters are mean values within one of these intervals.

Parameter	Relative humidity of the film				
	dehydration			rehydration	
	high (B type)	medium (A type)	low (part. disord.)	medium (A type)	high (B type)
water content A_{3400} in % of the starting value	100	ca. 75	ca. 52	ca. 75	ca. 135
orientation factor f (%)	61 ± 3	74 ± 4	35 ± 4	56 ± 3	52
DNA-conformation characterizing parameters:					
$\theta_{O...O}$ (degree)	55.1 ± 0.2	60.0 ± 0.6	63.8 ± 1.7	64.0 ± 0.3	55.0
θ_{OPO} (degree)	63.5 ± 1.0	49.1 ± 1.4	45.8 ± 1.0	46.9 ± 0.4	61.5 ± 0.5
A'_{1185} (A specific)	0	0.107 ± 0.016	0.077 ± 0.010	0.167 ± 0.016	0
ligand orientation rel. to helix axis					
θ_{Cf} (degree)	ca. 90	70 ± 4		71 ± 3	ca. 90
tilt ($90° - \theta_{Cf}$)	ca. 0	20 ± 4		19 ± 3	ca. 0

and θ_{OPO} [8] as well as the intensity of the band at 1185 cm^{-1} which is specific for the A form of DNA [6,8].

In respect to the initial state, the DNA–Cf complex in its final state is changed concerning two parameters: the water content of the film characterized by the absorbance at about 3400 cm^{-1} increases by approximately 35% whereas the angle θ_{OPO} in the region of high relative humidities (r.h.) decreases slightly but significantly by 2° down to (61.5 ± 0.5°) opposite to the values of (64.5 ± 1.0°) of pure DNA [6]. This is a change towards the so-called B* form introduced by Brahms and co-workers in their study of DNA–polypeptide complexes [16].

As demonstrated in table 4 the humidity region covering the A form of the DNA–Cf complex is extended compared with pure DNA towards both lower and higher r.h. The B–A transition of the complex is shifted to higher r.h. as well as the transition of the A form to the disordered form is shifted to lower r.h. in comparison to pure DNA.

Summarizing, the B–A conformational transition of DNA is mainly preserved in the complex but there are some peculiarities: (i) the conformational behaviour is significantly different in the course of dehydration and rehydration, respectively, and (ii) the humidity region of a well-defined A form is extended.

Parallel to the increase of the base tilt angle of DNA of 0° to 20° as a consequence of the B–A transition the orientation of the Cf molecules in the DNA–Cf complex is changed in an analogous manner characterized by θ_{Cf}, the angle between the Cf ring planes and the DNA helix (table 3). This is an evidence of a parallel mutual arrangement of both base pairs of DNA and bound caffeine in the full range of r.h.

Two bands at 1330 cm^{-1} and 1290 cm^{-1} which have been assigned to amino C–N stretching vibrations of adenine and guanine in DNA, respectively [17], show hypochromism as a consequence of caffeine binding by a factor of ca. 0.5.

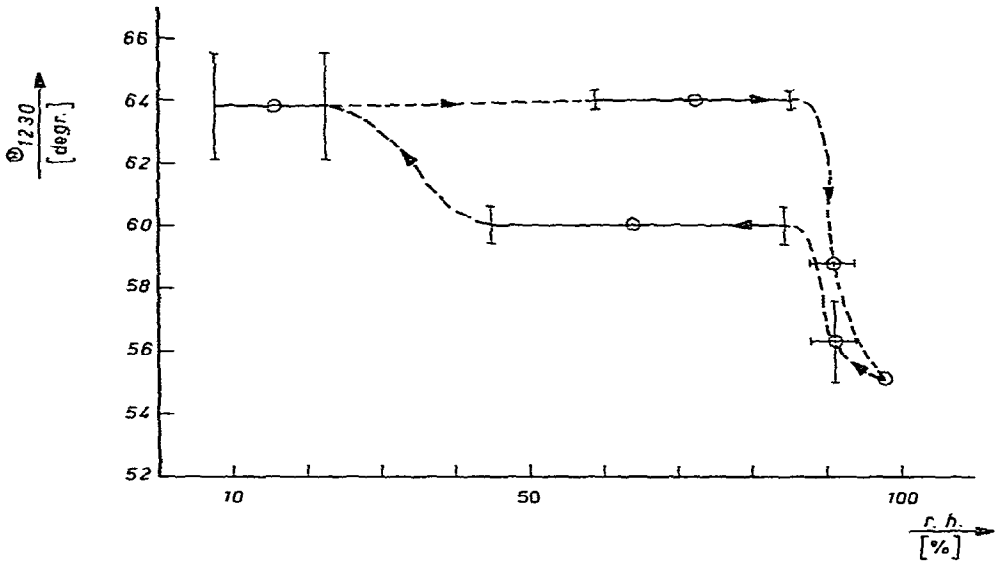

Fig. 1. Hysteresis of the phosphate group orientation, characterized by the angle $\theta_{O...O}$ (= θ_{1230}) of a DNA–caffeine film in the course of a complete dehydration-rehydration cycle. The humidity scale (r.h. = relative humidity in %) has been subdivided in regions of A and disordered forms, respectively. The mean values of θ_{1230} and their errors within each of these regions are presented.

Table 4
Fraction of A form of DNA films (free DNA and DNA–caffeine complex) in DNA conformation transition regions of B to A form (appr. 90% relative humidity) and of A form to the disordered state (appr. 20% relative humidity), respectively. The fraction of A conformation has been calculated both by the normalized absorbance A'_{1185} of the 1185 cm^{-1} band [6] and by the wavenumber of the 1230 cm^{-1} band [8].

Sample	relative humidity (%)	DNA conformations	fraction of A form (%)
DNA			22 ± 9
DNA–Cf	about 90	B + A	51 ± 3
DNA			33 ± 7
DNA–Cf	about 20	A + disordered	44 ± 1

3.3. Ultraviolet linear dichroism

The UV linear dichroism of oriented DNA films as a function of r.h. has been studied by several authors [18,9]. The transition moment of the π–π^* transition at about 260 nm has been expected in the plane of the bases. The transition of the ordered to the disordered form of DNA as a function of r.h. can be followed by the UV linear dichroism. Furthermore, a hysteresis effect in the complete dehydration-rehydration cycle of DNA has been demonstrated [9]. This effect has been discussed in terms of the DNA hydration as a prerequisite of both ordered conformations of DNA, B and A form.

The UV linear dichroism behaviour of the DNA–Cf complex is significantly shifted towards higher r.h. values both in the dehydration and in the rehydration phase of the cycle (fig. 2). The deorientation of bases connected to the dehydration starts in both free and complexed DNA at about 75–70% r.h. But the complex has a steeper increase of the normalized dichroic ratio D_N in the 50–30% r.h. region. The reorientation of bases begins at 50% r.h. in the complex compared to 40% r.h. in free DNA during rehydration.

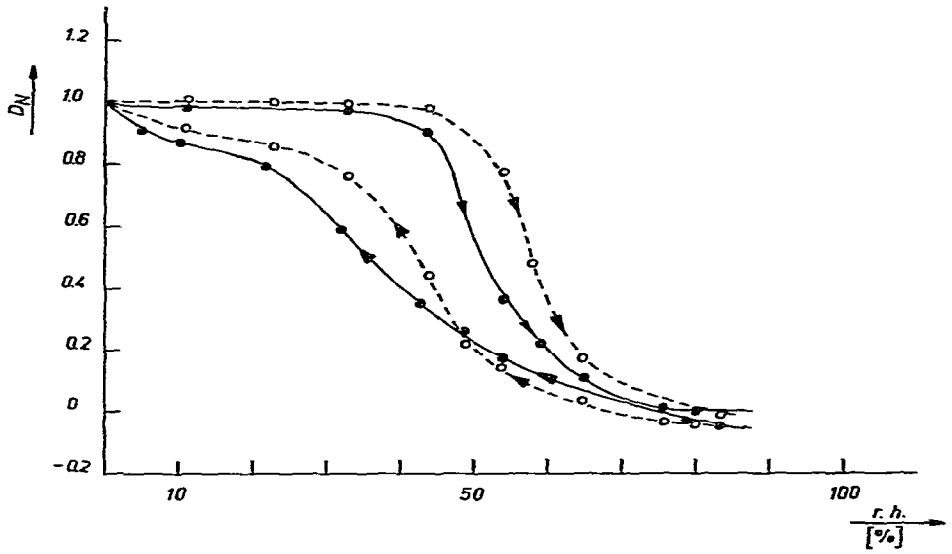

Fig. 2. Linear ultraviolet dichroism (wavelength 260 nm) of oriented DNA films without and with caffeine. The normalized dichroic ratio D_N is plotted versus the relative humidity. Molar ratio DNA phosphate/caffeine = 1. (Solid line: DNA; broken line: DNA–caffeine complex).

3.4. ^1H and ^2H magnetic relaxation time measurements

The results of the proton relaxation measurements are summarized in table 5. Obviously there exists no direct influence of Cf on the proton relaxation of water. However, both the relaxation times T_1 and T_2 aqueous DNA solutions are slightly shortened in the presence of Cf.

Using the deuterated caffeine-8-[^{2}H], a deuteron (^{2}H) relaxation time T_1 = (38 ± 7) ms has been measured in an aqueous solution containing DNA (5.5 × 10^{-2} M DNA phosphate) and caffeine-8-[^{2}H] (0.15 M). As a consequence of the low solubility of Cf in water, the ^{2}H magnetic relaxation time of Cf could not be measured in the absence of DNA.

It should be referred to the P/D ≈ 0.3 in these experiments which is lower as the P/D = 2 and P/D = 1 used in the infrared and ultraviolet dichroism studies of films, respectively. This difference originates from experimental demands: One needs an excess of Cf to produce a significant effect on the relaxation times of water protons as well as to measure a satisfactory signal-to-noise ratio in the ^{2}H NMR spin-echo experiment.

Table 5
Water proton relaxation times T_1 and T_2 of DNA and the DNA–caffeine complex in 0.15 M SSC buffer. Measurements at 11 MHz by the spin-echo technique using a $\pi-\pi/2-\pi$ pulse sequence for T_1 and a $\pi/2-\pi-\pi...$ pulse sequence for T_2.

Sample	Water proton relaxation times[s]	
	T_1	T_2
buffer (SCC, 0.15 M)	2.38	2.60
buffer + caffeine (0.055 M)	2.38	2.60
DNA (0.02 M phosphate)	1.87 ± 0.03	1.73 ± 0.06
DNA (0.02 M phosphate) + caffeine (0.055 M)	1.77 ± 0.03	1.57 ± 0.06

On the other hand, an excess of Cf is to avoid in the oriented films, otherwise the measured dichroism would be deteriorated by randomly distributed non-bound Cf molecules.

4. Discussion

4.1. Binding of Cf to DNA

The binding of caffeine to DNA has been investigated by Ts'o et al. [19,20] and recently by Lang [1–3]. Our results permit us to present a refined model of the Cf–DNA complex (binding process II).

The formation of a DNA–Cf complex has been established by the NMR line broadening of Cf in the presence of DNA both unlabeled and labeled by paramagnetic Mn^{2+} ions (tables 1 and 2). The H-8 and N-7 methyl protons have the most pronounced line width enhancement (4.7 and 4.3 Hz, resp.) compared to the N-3 and N-1 methyl protons (2.8 and 2.2 Hz, resp.). This may be an indication of preferential binding of the imidazol ring of Cf to DNA. But on account of the multiplet structure of both the H-8 and N-7 methyl proton NMR lines small differences in the DNA-induced broadening of the Cf NMR lines are not very strong indications of preferred binding sites of Cf to DNA. In fact the existence of preferential binding sites cannot be maintained if looking at the broadening of the Cf NMR lines by the Mn(II)-DNA (table 2). Addition of 7×10^{-5} M Mn^{2+} is without any influence on the line width of Cf, but the Cf lines broadens when the Mn(II)-DNA (metal/phosphate mole ratio $r = 0.1$) has been added to the Cf solution. It is well known that Mn^{2+} ions are bound to the phosphate groups of DNA [21].

In conclusion, the NMR line broadening of the Cf lines as well by DNA as by Mn(II)-DNA indicate a binding of Cf to DNA. The small effects demonstrate, however, that the exchange between free and bound Cf is very fast on the NMR time scale. The lack of striking differences in the line broadening values of the individual lines in the paramagnetic label experiment gives further support for a relative high mobility of the caffeine molecules in the complex and excludes a preference of individual ligand groups as binding sites. The decrease of the line broadening with increasing Cf concentration indicates a reversible binding of Cf to DNA and excludes a virtual effect produced by a breakdown of associated Cf species to monomers. The latter process would result in a downfield shift of the NMR lines of Cf [4] but not in a line broadening.

4.2. Parallel arrangement of outside-stacked Cf relative to the DNA bases

The Cf molecules in the Cf–DNA complex (P/D = 2) are arranged parallelly to the base pairs of the DNA double helix as indicated by the infrared linear dichroism of films (table 3). The caffeine orientation angle θ_{Cf} of 90° and 70° at high and medium humidity, resp., is adequate to the base tilt angle of approximately 0° and 20°, resp., of DNA in the B and A form. This fact excludes the mode of binding along the groove of the DNA double helix as suggested by Gursky et al. [22] for some DNA-drug complexes. We have to decide between two alternative arrangements of Cf in its DNA complex: (i) the intercalation or insertion of Cf between adjacent base pairs or (ii) the outside binding of stacked aggregates (dimers) in one or both grooves of DNA.

The mobility of the bound caffeine molecule has been estimated by the ^2H spin–lattice relaxation time (T_1) measurement of deuterated caffeine-8-[^2H] in its DNA complex in aqueous solution. For the ^2H relaxation time T_1 the quadrupole interaction mechanism dominates the other contributions. The relaxation process is determined by the mobility of the ligand in the complex [23,24]. Therefore, the following relation between the observed relaxation rate and the molecular correlation time τ_c holds under the assumption of an isotropic reorientation of the bound ligand [25] and a fast exchange between bound (b), self-associated (a) and free (f) Cf molecules:

$$\frac{1}{T_1} = \frac{12\pi^2}{80}\left(\frac{e^2qQ}{h}\right)^2\left[\frac{2\tau_{cb}}{1+(\omega\tau_{cb})^2} + \frac{8\tau_{cb}}{1+(2\omega\tau_{cb})^2}\right]p_b + \frac{p_a}{T_{1a}} + \frac{p_f}{T_{1f}}. \quad (1)$$

According to Egan [26] we use the quadrupole coupling constant $e^2qQ/h = 185$ kHz of benzene for a purine derivative deuterated in position 8. ω is the Larmor angular frequency in rad s^{-1}. The fractions of bound (p_b), self-associated (p_a), and free (p_f) Cf molecules are connected with the apparent association constant K (postulating one binding site for Cf per nucleotide) and the concentrations of Cf (c_c) and nucleotide (c_n):

$$K = \frac{p_b}{(c_n - p_b c_c)(p_f + p_a)}. \quad (2)$$

In a ^2H NMR study of the self-association of 5′-adenosine monophosphate a relaxation time $T_{1f(AMP)} = 35$ ms has been found for the monomer [26]. Using the Stokes-Einstein formula one can estimate the relaxation time for the Cf monomer:

$$1/T_{1f(Cf)} = (a_{Cf}/a_{AMP})^3 \cdot 1/T_{1f(AMP)}, \quad (3)$$

where a is the radius of the molecule ($a_{Cf} = 0.28$ nm). The resulting value $T_{1f(Cf)} = 103$ ms is considerably longer than the experimental relaxation time $T_1 = 38$ ms for deuterated Cf in DNA solution. This difference is a consequence of both the self-association of Cf and its interaction with DNA. The separation of these two contributions is difficult for experimental limitations. Neglecting the self-association of Cf ($p_a \approx 0$), the experimental value of T_1 results in an upper limit of 17 s^{-1} for the first term of eq. (1). Introduction of an evaluated value of $K \approx 10$ M^{-1} [20] results in a value of $p_b = 0.2$. Now we can calculate the reorientation time τ_{cb} of the bound Cf from eq. (1): $\tau_{cb} \leqslant 0.17$ ns or $\tau_{cb} \geqslant 0.5$ μs. A decision in favour of one of these two values succeeds on the basis of the measured line width of the H-8 line of Cf (table 1). The calculation of the contribution of the intramolecular dipole–dipole interaction [28] to the line width using the value of $\tau_{cb} = 0.5$ μs results in a too high value of the line width. Thus, the mobility of the bound Cf is characterized by a very short reorientation time $\tau_{cb} \leqslant 0.17$ ns. This is approximately the eightfold value of the reorientation time of the monomer. A similar ratio has been found for the self-association of 5′-adenosine monophosphate [26]. This value rules out any tight-binding process characterized by strongly hindered mobility of Cf. Especially, insertion or intercalation of Cf between adjacent DNA base pairs can be excluded, for this mechanism results in a reorientation time many orders of magnitude higher than our estimated upper limit [27]. This conclusion is confirmed by the narrow line width of the H-8 proton of Cf in a DNA solution (table 1).

Thus, the results confirm the earlier finding on the binding process II [2,3] characterized by outside-stacked Cf molecules in the Cf–DNA complex at high Cf concentration.

On the base of our data we suggest a model of the Cf–DNA complex (fig. 3) in which the Cf molecules lie parallel to the bases in the grooves of DNA forming closely packed aggregates. The proposed model includes a high exchange rate and high mobility of the individual Cf molecules in the complex. This would explain the lack of preferred binding sites found in the NMR experiment using DNA labeled by the paramagnetical Mn(II) ions.

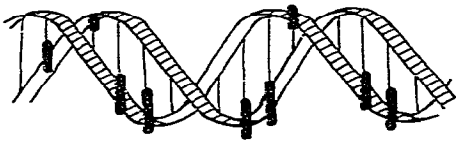

Fig. 3. Schematic drawing of the suggested model of the DNA-caffeine complex: external binding of stacked caffeine molecules in one of the grooves of DNA.

Furthermore, it should be emphasized that in the film of the Cf–DNA complex despite of the high Cf concentration (1 Cf per base pair!) all Cf molecules are arranged parallel to the DNA base pairs. The strong infrared hypochromism of the amino stretching vibrations of the DNA purine bases at 1330 cm^{-1} and 1290 cm^{-1} suggests a stabilization of the complex by hydrogen bonds between amino groups of the bases and carbonyl groups (or ring nitrogen) of Cf. An additional stabilization may be sponsored by the binding of stacked dimers of Cf to DNA bound by two hydrogen bonds.

4.3. Hydration shell of the DNA–Cf complex

The mode of interaction of the outside-stacked Cf molecules and DNA is accompanied by the disturbance of the hydration shell of the DNA chain. This is indicated by a significant change of the hydration behaviour of DNA induced by the binding of Cf as follows:

(i) changes in the ultraviolet linear dichroism as described in the preceding paragraph (fig. 2);

(ii) enhancement of the water content of the DNA–Cf film after running a complete dehydration-rehydration cycle as evidenced by the infrared absorbance at about 3400 cm^{-1} (table 3);

(iii) decrease of both water proton relaxation times T_1 and T_2 of the DNA–Cf complex in aqueous solution as compared to the free DNA (table 5).

The interpretation of the results of the proton relax-

ation measurements needs a classification of water referring to their relaxation behaviour into three different compartments: water bound on non-aggregated DNA molecules (b), water bound on aggregated DNA molecules (a), and bulk water (f). As evidenced in a previous paper [28] the hydration water included by DNA aggregates does not contribute to the effective relaxation rate under the experimental conditions of a spin-echo experiment because the exchange with bulk water is too slow on the NMR time scale.

For the compartment (b) the fast exchange limit can be postulated. Therefore, the total relaxation rate is the weighted average rate between bulk and bound water:

$$1/T_l = p_b/T_{lb} + p_f/T_{lf} \quad (4)$$

p_b and p_f are the fractions of bound water in the regions (b) and (f), respectively. T_{lb} and T_{lf} are the relaxation times ($l = 1$ and 2) in the hydration shell (b) and in the free water (f), respectively.

The decrease of the water relaxation times T_1 and T_2 after addition of Cf (table 5) may be interpreted alternatively by one of the following suggestions:

(1) The structure of DNA is changed in direction of a further restriction of mobility of the units.

(2) Caffeine evokes a dissociation of the DNA aggregates. The water fraction p_a now contributes to the total relaxation rate.

(3) Caffeine causes an increase of the hydration of DNA. Therefore the fraction p_b increases.

There are no arguments in favour of the first suggestion that the observed transition from the B to the B* conformation (cf. section 4.4) is connected with a stronger restriction of the mobility of the DNA units. Likewise, the second suggestion can be excluded: Taking into account the small protein content (0.18%) of the DNA the aggregation cannot dominate in aqueous solution as could be demonstrated in a previous paper [29].

Since the first and second suggestion are unlikely, it can be concluded that the hydration of DNA increases as a consequence of the formation of a DNA–Cf complex in aqueous solution. This interpretation is in agreement with our infrared studies on films of the DNA–Cf complex. The hydration capacity of DNA films increases dramatically after a run of a complete dehydration-rehydration cycle of the DNA–Cf complex (table 3).

All these data may be interpreted (a) by a displacement of water of the DNA hydration shell by Cf and (b) by an increase of the total number of water in the hydration shell of the complex caused by a stronger hydration around the stacked Cf molecules as compared with DNA. These phenomena are additional supports for an outside stacking of Cf according to the proposed model in fig. 3. All experimental findings complement each other in favour of this model independent on their origin from films or concentrated solutions.

4.4. Caffeine-induced changes of the conformational behaviour of DNA

We observe a variation of the Cf orientation with respect to the DNA helix axis as a function of the humidity according to the variation of the base-pair tilt angle of DNA due to the B–A transition in DNA films. The Cf molecules have always a parallel orientation to the DNA base pairs. This finding confirms the proposal of a hydrogen bond interaction between Cf and the DNA bases suggested above (cf. section 4.2) which is supported by the observed hypochromism of C–N stretching bands of adenine and guanine, respectively, with Cf.

The conformational behaviour of DNA in the complex is characterized by the following transitions in the course of a dehydration-rehydration cycle: B → A (partially formed) → disordered → A (completely formed) → B. The hysteresis of the fraction of A formed in the medium humidity region (fig. 1) may be interpreted by a partial inhibition of the B–A transition during dehydration whereas the regular A form is formed completely in the course of rehydration coming from the dry, disordered state. In the subsequent A–B transition the Cf induces a B* conformation of the DNA (cf. section 3.2) compared to the usual B conformation immediately after formation of the complex. Furthermore, the final DNA–Cf complex in its B* conformation is characterized by an increased hydration (table 3). All these phenomena may be explained by changes of the hydration behaviour. The originally formed DNA–Cf complex has some remaining water at the DNA bases which results in a non-uniform, less regular structure. This water is removed by dehydration going to the disordered state. The subsequent rehydration builds up a more regular

DNA–Cf complex displacing the water between bases and the outside-stacked caffeine. The more regular structure is characterized by tightly bound stacked aggregates (dimers) of Cf which displace water molecules formerly bound to the bases now attached by Cf. The A–B transition in the course of rehydration may be retarded by the shielding against water as a consequence of the tightly bound Cf dimers in the groove of DNA. The DNA in this regular structure now "sees" a lower actual water concentration inside the complex as in the more irregular structure originally formed in the course of dehydration. Therefore, the A–B transition is shifted towards higher humidities (fig. 1).

A new hydration sphere is formed around the DNA–Cf complex. The new situation of hydration of the DNA–Cf complex shifts both the B–A and the A-disorder transition on the humidity scale thus extending the region of A form (table 4). This is supported by the findings in diluted aqueous solution that the CD spectra of DNA in the presence of high Cf concentration are changed towards A-type spectra [3].

5. Conclusion

According to our data, a model on the outside binding of Cf molecules at high concentrations to DNA is suggested both for films and for concentrated aqueous solutions. This outside binding is characterized by a parallel arrangement of outside-stacked self-associated Cf molecules (preferential dimers) to the DNA bases. The parallel distribution of the Cf aggregates along the DNA chain is favoured by hydrogen bonds between adenine or guanine and Cf. Simultaneously the hydration shell of DNA is changed. The conformation of DNA at high humidities is slightly changed to the B* form by Cf. Furthermore, the humidity region in which the A form of DNA exists is extended by Cf in its DNA complex. The dehydration-rehydration cycle is characterized by a hysteresis of the conformational behaviour of DNA in the complex in respect to an increase of the fraction of A form at medium humidities in the course of rehydration.

In general we can conclude that the interaction of DNA with Cf at high concentrations lead to conformational changes by changing the hydration shell of DNA to outside-stacked Cf molecules [3]. The perturbation and redistribution of the first and second water shell around the DNA chain lead to the labilization of DNA and its partial structural collapse.

In some respects the biological role of Cf at high concentration in living cells may be explained. It is known that Cf at high concentration acts as a mutagenic agent in both procaryotic and eucaryotic cells and causes at certain conditions the phenomena of so-called "pulverization" of mitotic chromosomes [30]. It is imaginable that such biological events are caused by the second binding process of Cf to DNA. Due to tremendeous changes of the hydration shell of DNA in the presence of high Cf concentrations the DNA is destabilized and labilized against the action of endogenic or exogenic noxes, nucleases or other enzymes. Furthermore, the DNA–protein interaction in the interphase or mitotic chromatin can be influenced, too. Thus, some weak points, kinks, breaks etc. can be induced by Cf which disturb the intactness of DNA necessary for its biological activity. It is possible that Cf-induced events may be starting points for further biological failure up to mutagenic state.

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