Physicochemical studies on the interaction of Δ^5-dehydromalouetine with DNA. Further evidence for the partial insertion of a steroidal diamine into a DNA double helix

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The binding of Δ^5-dehydromalouetine (DHM), a bisquaternary steroidal diamine, to DNA induces several characteristic changes in the physicochemical properties of the nucleic acid. DHM produces a very effective protection of the duplex against thermal denaturation which decreases at higher input ratio. The interaction produces an enhancement of the u.v. absorbance of DNA suggesting a disorganization of the base stacking. Agarose gel electrophoresis of closed circular DNA treated with topoisomerase I in the presence of DHM demonstrates an unwinding of the double helix by the diamine. A 270 MHz proton n.m.r. study of the complexes of DHM with calf thymus DNA and with poly(dA–dT) reveals an unshielding of the DNA base protons and a shielding of several diamine protons. Our data and earlier observations on similar complexes provide evidence for a detailed discussion of different models of interaction. The experimental facts are best explained by the partial insertion of the steroid backbone between unstacked base pairs whereas the charged ends of the diamine interact electrostatically with the phosphate groups.

Keywords: DNA; steroidal diamine; Δ^5-dehydromalouetine; DNA; poly(dA–dT), u.v. spectroscopy; n.m.r.

Introduction

Conformational changes in the DNA double helix occur when varying the salt concentration and/or changing solvent conditions[1-3] in the molar range. Interaction of DNA with specific ligands or with macromolecules, in the millimolar range or lower, also leads to changes in the structure of DNA[4,5]. Steroidal diamines, which combine a bulky hydrophobic moiety with a di-cationic structure, can attach firmly to the poly-anionic double-stranded DNA by electrostatic interactions[6-8]. Several steroidal diamines (*Figure 1*) bind to DNA with very particular effects on its physicochemical properties. These effects differ deeply from those exerted by usual di-cationic ligands such as the natural polymethylene diamines (i.e. putrescine, cadaverine) in several respects: (a) malouetine and irehdiamine A (IDA) show the usual rise of melting temperature of DNA (T_m) at low input ratio observed for other diamines whereas they can actually decrease the T_m at higher input ratio[9-11]; (b) malouetine and IDA enhance the u.v. absorbance of DNA (hyperchromic effect) and alter its optical rotatory dispersion and circular dichroism spectra[6,10]; (c) IDA, malouetine and certain isomers of dipyrandium affect the hydrodynamic properties of circular supercoiled DNAs, as shown by ultracentrifugation or viscometry, in a way which is roughly similar to that observed with intercalating drugs[11-13].

All these effects indicate a deformation of the DNA duplex on binding of these ligands. The binding of these diamines involves the formation of an ionic pair with the DNA phosphates and also an important non-electrostatic contribution[7,8]. However the precise features of the complexes still remain obscure and up to now proton n.m.r. investigations on DNA–steroidal diamines complexes did not yield an unambiguous response to this question. An isothermal n.m.r. study of the DNA–malouetine complexes showed no sign of ring-current shifts in the steroid signals, suggesting an external binding of the diamine on the DNA duplex[11]. Conversely, large upfield chemical shifts were detected for several protons of the diamine during the thermal denaturation of the complexes of dipyrandium with poly(dA–dT), suggesting a penetration of the steroid into the core of the DNA duplex[14]. However, it must be pointed out that these two series of n.m.r. experiments have been carried out in very different experimental conditions and therefore it is difficult to draw a firm conclusion from them.

It appeared of interest to investigate in detail the complexes of DNA with a suitably chosen steroidal diamine using different physicochemical methods. The

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diamine we have selected is Δ^5-dehydromalouetine (*Figure 1*). This molecule combines the structural features of malouetine (quaternary amine functions) and of IDA (double bond in 5–6) which both bind to DNA with characteristic effects as outlined earlier. Moreover, Δ^5-dehydromalouetine has five well identified and well separated n.m.r. signals corresponding to protons or methyl groups located all around the steroid backbone which can be used to monitor the interaction with DNA[15].

Experimental

Materials

Δ^5-Dehydromalouetine, pregn-5-ene-3β, 20α-ylenebis (trimethyl chloride), (DHM, *Figure 1*), was prepared as already described[8]. Its purity was checked by elemental analysis and its structure was assessed by high field proton n.m.r. spectroscopy[15]. The concentration of the diamine solutions was established by exact dilution of carefully weighted dry samples of DHM.

Calf thymus DNA (CT–DNA), MW 3×10^5, was prepared and sonicated as already described[11]. Poly(dA–dT), $s_{20,w}=13$ was purchased from Collaborative Research. Plasmid pBR322 DNA was prepared from chloramphenicol-amplified *E.coli* cultures by the alkaline procedure of Birnboim and Doly[16] and purified in $CsCl_2$ gradients. The concentration of DNA (P_t, in mol/litre of phosphate or nucleotide) was determined spectrophotometrically. The absorption coefficients used were A_{258} = 6650 M cm^{-1} for CT–DNA and A_{262} = 6600 M^{-1} cm^{-1} for poly(dA–dT). Solutions of diamine and DNA were normally made up in a buffer containing 12.5 mM KH_2PO_4, 12.5 mM K_2HPO_4, 0.1 mM Na_2-EDTA at pH 6.9 and total ionic strength $I=0.05$. For n.m.r. studies, D_2O was used instead of H_2O.

Methods

Ultra-violet spectroscopy studies. The thermal denaturation profiles of poly(dA–dT) and CT–DNA in the presence of varying concentrations of DHM were recorded at 260 nm on a Unicam SP-8-100 spectrophotometer. The melting temperature T_m, mid-point of the thermal transition, was determined as previously described[11].

The increase of absorbance of a solution of CT–DNA produced by the addition of DHM was measured at 258 nm and at 25°C as described[8]. The increase Δh, (in %), was plotted as a function of m_t, total diamine concentration, or of $R=m_t/P_t$, the input ratio. The curve was analysed according to method 2 described by Gourévitch *et al.*[8] to get K_a, the overall binding constant of DHM for CT–DNA, and Δh_{max}, the maximal increase of the absorbance corresponding to the saturation of all the binding sites by the diamine. In this procedure, it is assumed that DHM binds to a unique class of sites which are independent and equivalent.

Topological analysis. Topoisomerase I assay of closed circular DNA in the presence of DHM was carried out essentially as described[17]. In this procedure the plasmid DNA is incubated with topoisomerase I from calf thymus (BRL) in 25 mM KCl, 1 mM $MgCl_2$, 0.5 mM DTT, 20 mM Tris-HCl, pH 7.5 at 37°C for 60 min in order to obtain relaxed DNA. After this time topoisomerase I is added to a final enzyme/DNA ratio of 20 units/μg DNA and aliquots are mixed with different quantities of DHM

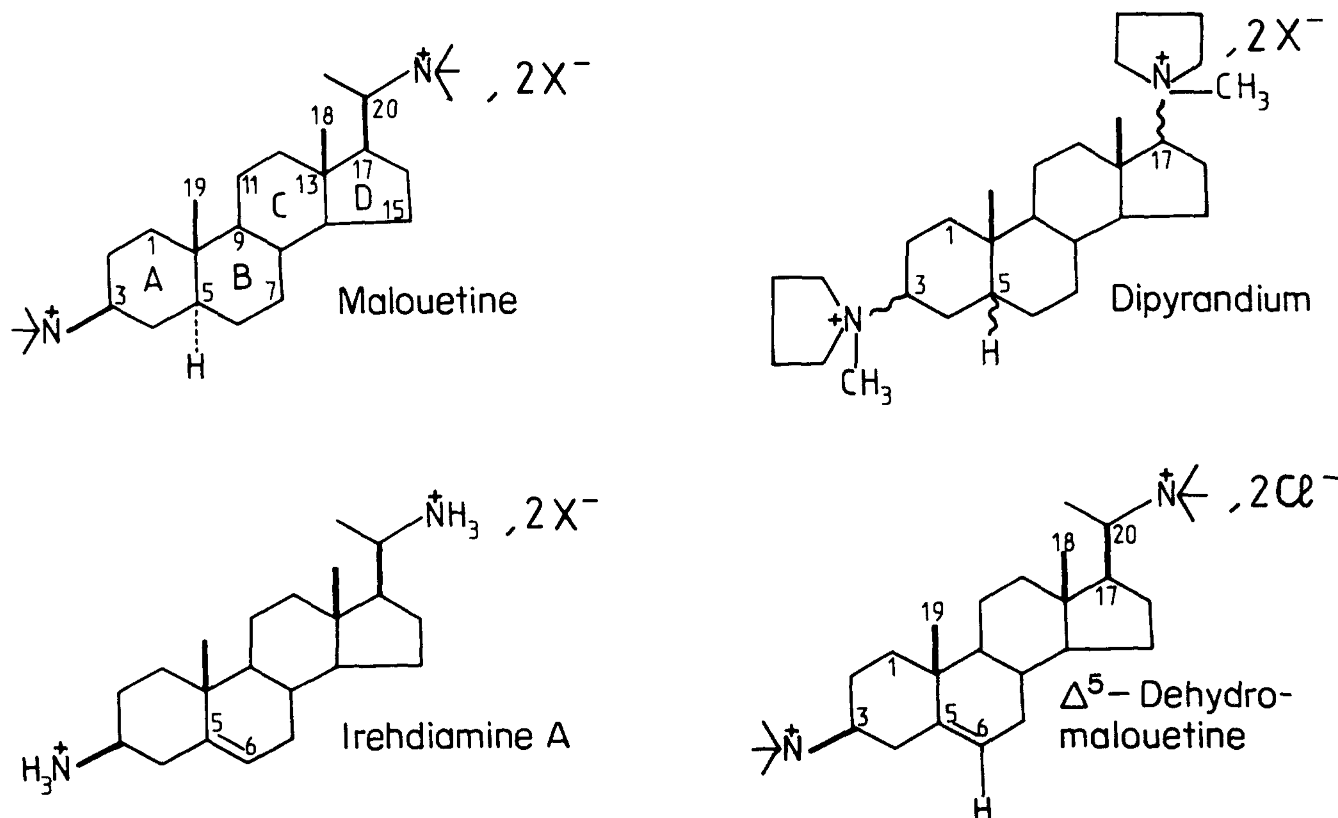

Figure 1 Structure of some steroidal diamines: malouetine, irehdiamine A (IDA) and Δ^5-dehydromalouetine (DHM) are based on the pregnane or pregn-5-ene ring system. Dipyrandium is a family of eight stereoisomers based on the androstane nucleus differing in the stereochemistry of the 3, 5 and 17 positions

dissolved in incubation buffer. After 60 min at 37°C the mixtures are made with 1.2% sodium dodecyl sulphate and charged on 1.6% agarose gels in Tris-phosphate buffer (35 mM Tris, 30 mM phosphate, 1 mM EDTA, pH 7.9). Gels in the presence of chloroquine were run in the same conditions with 6 μg/ml of chloroquine diphosphate (Sigma) added in the gel and in the sample buffer.

N.m.r. studies. ^{1}H-n.m.r. spectra were recorded at 270 MHz on a Bruker WH 270 spectrometer interfaced to an Aspect 2000 computer and operating in the fourier transform mode. The chemical shifts are referenced to an internal standard of DSS (2,2-dimethyl-2-silapentane-5-sulphonate) and are measured with a precision of ±0.005 ppm. The temperature of the sample was monitored by a B-ST-100-700 controller unit.

The proton n.m.r. spectrum of DHM shows sharp resonances which can be almost completely attributed to definite protons or methyl groups[15].

The chemical shift and the width D at half height of the signals of DHM were measured in mixtures of DHM with CT–DNA at different input ratio R and at different temperatures below the melting temperature of DNA in the complex. The resonances of the sonicated CT–DNA do not contribute to the spectrum since they are broadened out. Two kinds of experiments were carried out in order to determine different parameters of the interaction: (a) the total concentration P_t of DNA was kept constant and the input ratio was allowed to vary by changing the total concentration of diamine, m_t. (b) The total concentration, m_t, of diamine was kept constant and the input ratio was allowed to vary by changing the total concentration of nucleotide P_t. Because of line broadening at low R values, the parameters δ_b and D_b of the bound diamine could not be measured directly and had to be determined by extrapolation (the mathematical treatment of this aspect can be obtained from the authors upon request).

The chemical shifts of the non-exchangeable protons of the base pairs and sugar rings of the polynucleotide and those of the protons of the diamine were followed through the temperature-dependent melting transition in the complex of DHM with poly(dA–dT) as described[14,15]. The input ratio was low enough in these experiments (R=0.1–0.2) so that the nucleic acid was in large excess and all the diamine was bound to it. The temperature dependence of the chemical shifts of poly(dA–dT) alone was also recorded under the same conditions of ionic strength.

Results

Spectrophotometric methods

The variation in the melting transition mid-point (ΔT_m) of CT–DNA and of poly(dA–dT) on the addition of DHM is shown in *Figure 2*. The curves are similar with both the natural and synthetic DNA. At low input ratio (R) the increase in T_m is very sharp and reaches 28 and 24°C around $R=4$ for the complexes of CT–DNA and poly(dA–dT) respectively. Above this value of the input ratio ΔT_m decreases gradually indicating that the ability of the diamine to stabilize the DNA double helix at high input ratio decreases.

The addition of DHM to a CT–DNA solution in isothermal conditions enhances its ultraviolet absorbance. The hyperchromicity (Δh, in %) at 258 nm measured at 22°C is plotted as a function of R in *Figure 3*. The analysis of this curve gives $K_a = 9 \times 10^3\ \text{M}^{-1}$ for the overall binding constant and $\Delta h_{max} = 12.5\%$ for the maximal hyperchromicity corresponding to the saturation of the binding sites by the ligand. These values are comparable to those found with IDA–DNA or malouetine–DNA complexes under similar conditions of ionic strength[6,8].

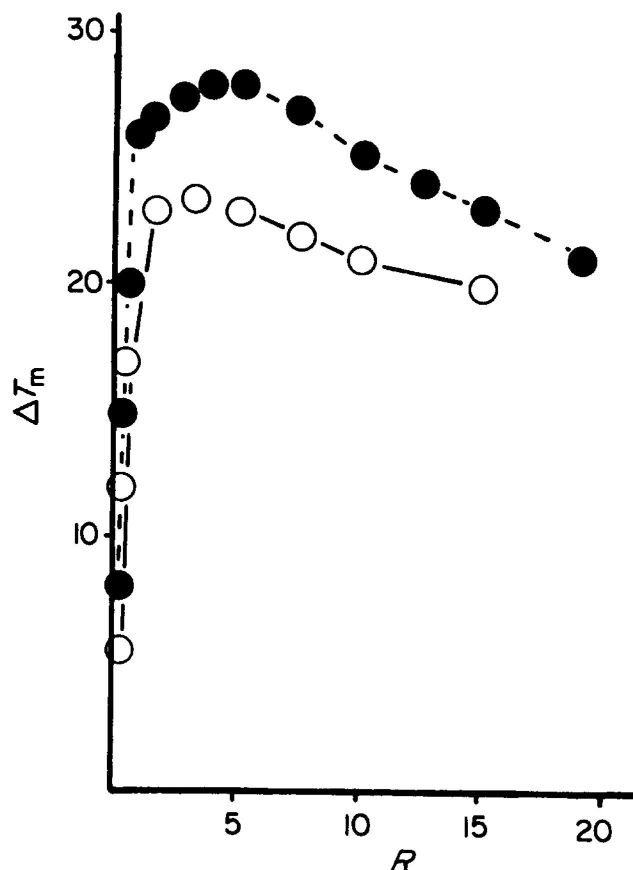

Figure 2 Variation of the melting temperature as a function of R, diamine to nucleotide ratio, for the complexes of DHM with calf thymus DNA (●) and with poly(dA–dT) (○). Nucleotide concentration P_t was kept constant at 0.075 mM

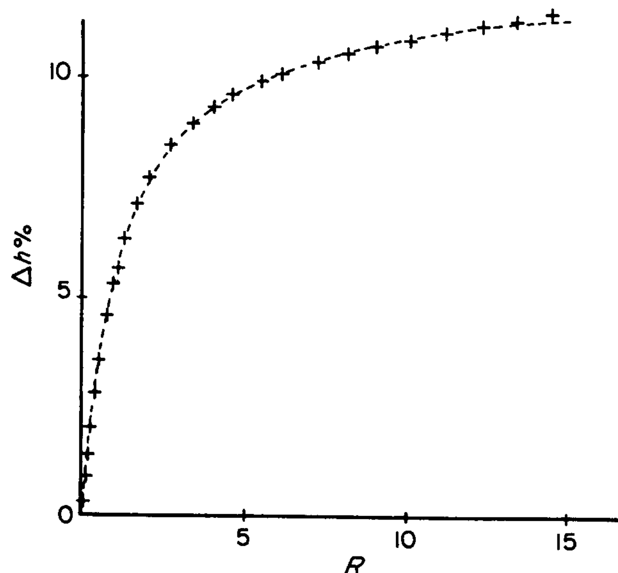

Figure 3 Relative increase of the absorbance of calf thymus DNA at 258 nm as a function of R, diamine to nucleotide ratio at 22°C. Nucleotide concentration P_t was kept constant at 0.1 mM

Binding experiments such as equilibrium dialysis that would allow the determination of the stoichiometric number n are not feasible in our case since they require very sensitive chemical or spectrophotometrical methods in order to measure the concentration of bis-quaternary diamines. Such a method is not available at present. Nevertheless an indirect estimation of the stoichiometric ratio of $n=0.18$ for the binding of malouetine to DNA has been obtained previously[8]. Also an estimation of $n=0.23$ to 0.28 has been obtained for the interaction of the di-primary diamine IDA with DNA[6]. Since the chemical structure of DHM is close to that of malouetine (quaternary amine functions) and to that of IDA (double bond in 5–6) it seems reasonable to admit that its binding to DNA is characterized by a similar stoichiometric ratio of 0.2.

The biphasic curves found in the melting experiments indicate that DHM loses its ability to stabilize the structure of CT–DNA and of poly(dA–dT) above a certain input ratio. The u.v. hyperchromic effect produced by the addition of DHM to DNA strongly suggests a disorganization in the stacking of base pairs. Such a local perturbation might affect the structure of DNA on a larger scale and indeed changes in the supercoiling of closed circular DNA produced by steroidal diamines have been detected by hydrodynamic methods[6,11–13]. In order to study the effects of DHM on the overall structure of DNA, we have followed, by gel electrophoresis, the changes in the topological properties of closed circular DNA incubated with topoisomerase I in the presence of varying concentrations of DHM.

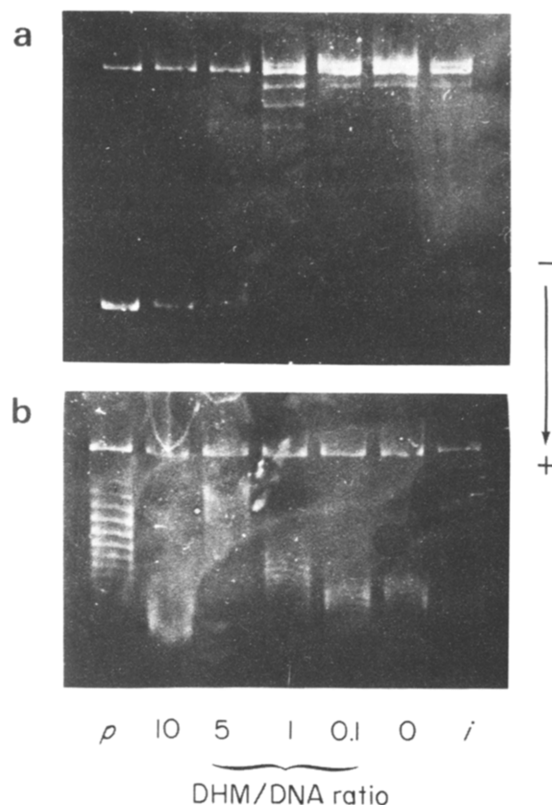

Figure 4 Agarose gel electrophoresis of closed circular DNA from plasmid pBR 322 incubated with topoisomerase I in the presence of different diamine/DNA ratios. Gel (a) was run in the absence and gel (b) in the presence of chloroquine disphosphate. Sample i is the initial DNA and p is the relaxed DNA used

Topological analysis

The effect of DHM on the topological properties of closed circular DNA was studied by treating with topoisomerase I the complexes of the diamine with plasmid pBR 322 DNA. The experiment is carried out by incubating with the enzyme previously relaxed DNA in the presence of increasing concentrations of DHM. In this way if the diamine produces a torsion in the DNA this will be reversed by the topoisomerase I action and the effect can be visualized in DNA gels. As could be expected from the previous results, the diamine acts upon the DNA superhelicity and this effect can be detected by this method. Increasing DHM/DNA ratios produces a larger proportion of supercoiled DNA following the action of the topoisomerase; this effect is complete at a proportion of 10 times the diamine excess calculated by weight. These results are shown in *Figure 4a*. By running agarose gels in the presence of chloroquine which acts as an unwinding agent on the DNA the sense of the topological variation of DNA can be observed (*Figure 4b*). It appears from this result that the sense of the topological variation produced by the diamine is negative. This effect can be correlated with an unwinding of the DNA double helix by DHM.

N.m.r. studies of calf thymus DNA–DHM complexes

Five resonances have been selected in the proton n.m.r. spectrum of DHM to monitor the interaction of the diamine with DNA because they can be followed even at low R values: the signals of the two N-methyl groups at positions 3 and 20 located at opposite ends of the molecule and which give nearly a single peak at 3.07 ppm; the signals of the two C-methyl groups at positions 18 and 19 which give two peaks at 0.83 and 1.02 ppm; the signal of the H6 proton giving a doublet at 5.58 ppm. The other resonances are less convenient for a quantitative analysis since they cannot be assigned at low R values: either they belong to a region of the spectrum where several signals overlap or they broaden out prematurely[15]. The chemical shifts of the resonances of DHM alone in solution are not affected by a variation in the temperature (from 10 to 100°C) and by a tenfold dilution (from 10^{-2} to 10^{-3} M). Therefore it can be concluded that there is no self-association of the free ligand in solution. The linewidth of the signals decreases regularly but weakly as the temperature is increased.

Measurements at constant nucleotide concentration

Solutions containing a fixed concentration of CT–DNA ($P_t = 10^{-3}$ M in nucleotide) and differing in their concentration (m_t) in DHM were examined at different temperatures. At a given input ratio R, turbidity appears in the solution above a certain temperature which decreases at high R values. This turbidity probably corresponds to the formation of micellar edifices. All the n.m.r. measurements we report hereafter were done under conditions of temperature and input ratio where no turbidity could be detected.

At a given R value the linewidth D of the resonances of DHM in the complex, especially those of the C-Me 18 and 19, are very sensitive to the variation of temperature. All the experiments reported below were carried out under conditions of temperature and input ratio where the diamine was exchanging rapidly between the bound and unbound states[11,18]. Therefore the mathematical formula

for the treatment of data mentioned in the experimental section can be applied.

The variation of the linewidths D and of the chemical shifts δ of the signals of mixtures of DHM with CT-DNA at 32°C as a function of the input ratio R is presented in *Figure 5*. The linewidths are very sensitive to the variation of R and they decrease sharply for R below 4 to 8. Limited but significant upfield shifts can be detected for R smaller than 6 at the resonance of H6 and the two C–Me resonances whereas a weak upfield shift is detected for R smaller than 2 at the N–Me resonance. Similar plots were established at 42 and 52°C.

The difference D–D_f between the linewidth D of a given resonance in the complex and the linewidth of the DHM alone was calculated at different values of R. The slope of a plot of R *versus* $(D-D_f)^{-1}$ is equal to $n \times \Delta D_{max}$ where n is the stoichiometry of the complex and ΔD_{max} the difference of linewidth D between the bound (D_b) and unbound (D_f) states. The value of the stoichiometry number n for the binding of DHM to DNA has not been calculated. However it is possible to calculate the relative ratios of ΔD_{max} for two protons of the same molecule. Such results are given in *Table 1*. If it is assumed that the value of n is equal to 0.2 for DHM as it has been measured for IDA or malouetine[6,8], then an estimation of D_b can be given (*Table 1*). It appears from these results that the broadening of the n.m.r. lines on complex formation is much larger for the two C–Me groups than for the two N–Me resonances. This indicates a more restricted motion of the C–Me than the N–Me groups when DHM is complexed to DNA.

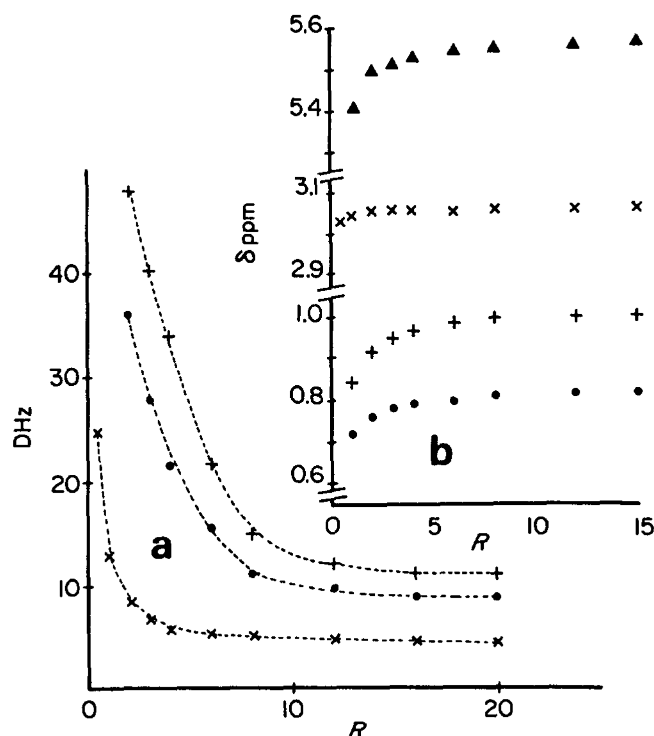

Figure 5 (a) Variation of the linewidths D and (b) variation of the chemical shifts δ of the main resonances of DHM as a function of R in the complexes with calf thymus DNA at 32°C. Nucleotide concentration was kept constant at 1 mM. (×) N–Me 3 and 20, (●) C–Me 18, (+) C–Me 19 and (▲) H6. Buffer conditions were 12.5 mM KH_2PO_4, 12.5 mM K_2HPO_4, 0.1 mM EDTA, pH 6.9 in D_2O

In principle, from the same experiment it is possible to calculate the value of the diassociation constant K_a. In practice this determination is rather unprecise and only a rough estimate of K_a can be obtained, being between 2.5×10^3 and 10^4 M^{-1} in the different experiments between 32 to 52°C. Despite the lack of precision the agreement with the value of 9×10^3 M^{-1} determined spectrophotometrically at 22°C for K_a is rather satisfactory.

Measurements at constant diamine concentration

The linewidth D and the chemical shift δ of the main resonances of DHM measured at 47°C, at a fixed concentration of DHM (10^{-2} M) and varying the DNA concentration are plotted in *Figure 6*. The linewidth of the C–Me and of the H6 signals decreases sharply when R increases up to 10–15 whereas the resonances shift downfield when R is smaller than 3–4. The parameters of the N–Me signal are much less affected than those of the other protons by the variation of R.

The data from *Figure 6* for R above 2 or 3, depending on the protons, can also be treated in order to calculate the ratio between ΔD_{max} for different protons. The values obtained at 47°C are presented in *Table 1*. A good agreement is found between these values and those obtained in the preceding section.

At low R values a linear relationship was found between $P_t/\Delta\delta$ and P_t where $\Delta\delta$ is the difference between the chemical shift of a given resonance in the presence of DNA and in the free state (δ_f). This relation allows to calculate the variation ($\Delta\delta_{max}$) of the chemical shift between the free

Table 1 Variations of the linewidth of the main n.m.r. resonances of DHM on complex formation with calf thymus DNA

Temperature of the experiment (°C)	Ratio of the linewidth variations $(\Delta D_{max})_A/(\Delta D_{max})_B$				Signal widths (Hz)					
	H6 / N–Me	C–Me 18 / N–Me	C–Me 19 / N–Me	C–Me 19 / C–Me 18	N–Me D_f	N–Me D_b	C–Me 18 D_f	C–Me 18 D_b	C–Me 19 D_f	C–Me 19 D_b
32[a]	–	9.7	15.4	1.6	3.8	48	2.4	430	2.8	630
42[a]	–	13.5	16.0	1.2	3.5	27	2.4	320	2.8	380
52[a]	–	9.1	11.4	1.25	3.3	22	2.2	175	2.6	220
47[b]	7.6	8.3	13.0	1.6						

The variation of the linewidth of a resonance is expressed by $\Delta D_{max} = D_b - D_f$ where D_f is the signal width in the free diamine and D_b the signal width in the bound diamine. The variations of the linewidth of the main resonances of DHM on complex formation are compared two by two by computing the ratio $(\Delta D_{max})_A/(\Delta D_{max})_B$ for the two resonances A and B. This ratio was derived from the data of n.m.r. experiments carried out at different temperatures either at constant nucleotide or at constant diamine concentration. The signal widths of the resonances of free DHM were measured directly. The signal widths D_b of DHM in the complex were calculated by assuming a stoichiometric ratio $n = 0.2$ diamine per nucleotide

[a] N.m.r. measurements made at constant nucleotide concentration

[b] N.m.r. measurements made at constant diamine concentration

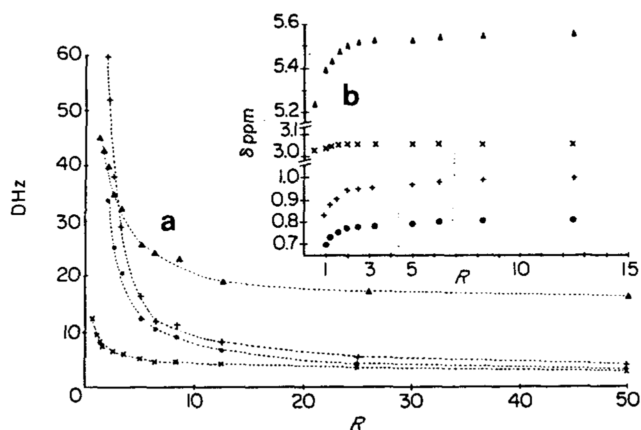

Figure 6 (a) Variation of the linewidth D and (b) variation of the chemical shifts δ of the main resonances of DHM as a function of R, diamine to nucleotide ratio, in the complexes with calf thymus DNA at 47°C. Diamine concentration was kept constant at 10 mM. Same buffer and symbols as in *Figure 5*

Table 2 Upfield shifts ($\Delta\delta_{max}$) in ppm of the resonances of DHM on complex formation with calf thymus (CT) DNA and with poly(dA–dT)

Nucleic acid	Resonances of the diamine			
	H6	N–Me	C–Me 18	C–Me 19
CT–DNA duplex[a]	1.1	0.05	0.6	1.0
poly(dA–dT) duplex[b]	0.8	0.09	0.55	0.75
poly(dA–dT) strand[c]	0.3	0.03	0.3	0.35

The values are derived from the data of *Figure 6* for the complex with CT–DNA and from Ref. 15 for the complex with poly(dA–dT)
[a] Extrapolated from measurements at 47 C
[b] Measured before the onset of denaturation
[c] Measured after the completion of denaturation

(δ_f) and bound (δ_b) states. These results are shown in *Table 2*. It is possible to observe that on complex formation large shielding effects occur for the two C–Me groups and proton H6 whereas a very limited upfield shift occurs for the N–Me groups. Upfield shifts upon complexation are not restricted to four of the five signals we have selected for the quantitative analysis. Upfield shifts comprising between 0.12 and 0.22 ppm were measured for several protons when comparing DHM alone to a complex with DNA at a ratio $R=1$. This is the case for the resonances at 3.56 ppm (proton H20), at 1.55 ppm (C–Me 21 and an unidentified signal Hz), at 2.55 ppm (proton H4) and at 2.09 ppm (H7 and an unidentified signal Hx).

N.m.r. studies of poly(dA–dT)–DHM complexes

The chemical shifts of the base and sugar protons of the polynucleotide and those of the main resonances of DHM were measured between 20 and 100 C in poly(dA–dT) alone, in DHM and in the complex poly(dA–dT)–DHM at $R=0.2$. In the absence of diamine the non-exchangeable protons of the bases (adenosine H2 and H8 protons, thymidine H6 proton and methyl 5 group) and of the sugar (H1′ and H3′) shift as average peaks during the duplex-to-strand transition with a mid-point at 55 C (*Figure 7*). The upfield shifts of the base protons on duplex formation reflect predominantly the effects of ring currents from nearest and next-nearest neighbour base pairs[14]. On addition of DHM to the poly(dA–dT) solution, the sugar H1′, adenosine and thymidine resonances of the duplex are subjected to unshielding effects: 0.1 ppm for the T(H1′), 0.2 ppm for the A(H1′) and the A(H8), 0.25 ppm for the T(CH_3–5), 0.3 ppm for the T(H6) and more than 0.6 ppm for the A(H2). Consequently the magnitude of the downfield shifts of the base and sugar protons during melting in the complex is severely reduced. The mid-point transition is at 80°C (*Figure 7*).

On complex formation, it was observed[15] that the H6 and the C–Me 18 and 19 protons (which are located at opposite edges of the steroidal backbone) experienced large upfield shifts. By contrast, a much smaller upfield shift was observed at the signal N–Me 3 and 20 located at the two ends of the steroid (see *Table 2*). On heating, the H6 and C–Me resonances of the diamine shift drastically downfield but, even after completion of the melting transition, they are still upfield from the position of the DHM alone[15].

Discussion

The interaction of a steroidal diamine, Δ⁵-dehydromalouetine (DHM), with DNA has been studied using different physicochemical techniques which give information about both the diamine and DNA in the complex. It appears that this compound has the typical behaviour of steroidal diamines which raise the melting temperature of DNA at low input ratio but lose this effect at high input ratio, produce a hyperchromic effect on DNA at constant temperature and act on the topological properties of DNA by inducing negative superturns in it.

The biphasic effect of DHM on DNA melting temperature confirms that this kind of profile is not

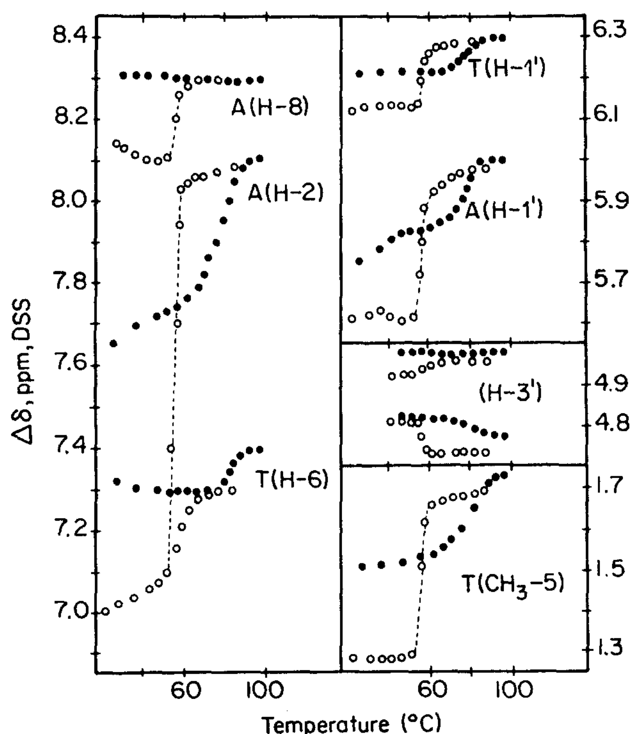

Figure 7 Temperature dependence of the chemical shifts of the non-exchangeable nucleic acid base and sugar protons in poly(dA–dT) (○) and in poly(dA–dT)/DHM complex at $R=0.2$ (●). Nucleotide concentration was 16 mM, same buffer as in *Figure 5*

restricted to only the di-primary and di-secondary steroidal diamines as originally proposed[9,10,19]. It appears, as it was observed with malouetine[11], that the degree of substitution of the amino groups does not play an essential role in producing a decreased stabilizing effect of diamines on DNA when added in sufficient amount.

The hyperchromicity of DNA observed upon addition of DHM reaches 12.5% at saturation. Values of 14 and 18% have been reported for malouetine[8] and IDA[6] respectively. This effect can be interpreted as a consequence of the disruption of the stacking of base pairs indicating that the diamine produces changes in the local structure of the nucleic acid. Such local changes may also have an effect on the superhelicity of DNA. Indeed, by incubating the complexes of DHM and closed circular DNA with topoisomerase I an unwinding effect of the diamine on DNA can be observed. Several lines of evidence indicate that the unwinding effects of the steroidal diamines are due to a non-intercalative mechanism[13]. The main reason is that the puckered steroid nucleus is much thicker than the aromatic planar ring system of typical intercalators such as ethidium bromide (6.5 Å as compared with 3.4 Å) and yet the unwinding angles reported for the steroidal diamines are two to four times smaller than those of ethidium[13]. Another reason is the absence of effect of steroidal diamines on the viscosity of sonicated DNA[6,18] contrary to the increase observed with intercalating agents[20] which is due to the lengthening of the double helix[21].

Several models for the interaction of steroidal diamines with DNA can be considered in the light of the results obtained. A possible mechanism is disruption of DNA base pairing at the site of interaction. In favour of this hypothesis is the increase of the rate of reaction of the bases by formaldehyde in mixtures of DNA and IDA[6]. However, the hyperchromicity expected from such an effect (3%) is much lower than that observed (12%)[6]. Furthermore, n.m.r. studies of poly(dA–dT) with diamines have established that base pairs are intact in the complex[14]. Finally, the opening of a base pair is equivalent to an unwinding of the double helix of 36°. The unwinding angle of IDA has been measured at 7.2°[6], and therefore five diamines would be required at the binding site. Such an arrangement appears difficult taking into account the bulky backbones of the steroid even if cooperativity can be invoked[6]. Other models such as local changes in the handedness of the DNA double helix can be ruled out using similar arguments.

It has been proposed[22] that a partial insertion of ligands such as steroidal diamines between the nucleic acid base pairs could explain the phenomenon observed with such compounds. A consequence of such a model is the formation of a kink in the DNA at the site of the interaction. The diamine would bind to the DNA through the minor groove fitting into the kink. The complex would be stabilized by electrostatic interactions between phosphate groups on both DNA strands and the amino groups of the steroid and by hydrophobic contacts between the steroid nucleus and the aromatic rings of the bases.

Precise structural features in the steroid backbone appear necessary to allow the insertion of a diamine at a kinked site. It is likely that the changes in the geometry of the steroid backbone modify the overall shape of the molecule and the orientation of the charged ends in such a way that the diamine can no longer bridge the phosphate groups of DNA, but at the same time the steroid nucleus can be inserted into the kink. Strong experimental support for this interpretation comes from n.m.r. studies on complexes of poly(dA–dT) with two different dipyrandium isomers[14,23], one of which is very effective in unwinding DNA while the other is not[13]. A limitation inherent in these studies lies in the fact that no individual dipyrandium protons were assigned to specific positions on the steroid ring. This problem can be overcome by the use of DHM, thus allowing a more precise analysis of the complex. We have established that the chemical shifts experienced by the steroid protons are similar in the complex with both the synthetic polynucleotide (poly(dA–dT)) and with natural DNA and this gives more significance to the results. We have observed large upfield shifts at a number of protons and methyl groups located at both edges of the steroidal backbone (C–Me 18, C–Me 19, H6, H20, H4 and probably H7 and C–Me 21). Large broadening of these resonances also occurs in complex formation. However the N–Me resonances are nearly not shifted and they broaden only to a limited extent. These observations are consistent with the proposed insertion of the steroid ring in the DNA groove whereas the amino groups remain outside in the vicinity of the phosphate groups and are almost free to rotate.

The concept of partial insertion of a steroid diamine between unstacked base pairs at a kinked site offers a general framework for understanding most, if not all, the available experimental facts. Within this framework, some adjustments of the originally proposed geometry will have to be made to account, for example, for the differences in the unwinding angle of the four isomers of dipyrandium which can effectively unwind the DNA[13]. The high values of the upfield shifts and the broadening of the C–Me 18 and 19 signals in DHM seem difficult to reconcile with some aspects of the model presented for the binding of IDA to DNA. A close inspection of the stereoscopic views of this model shows an insertion of the south edge of IDA between the base pairs whereas the north edge, where the C–Me groups are located, protrudes largely in the minor groove. This disposition puts the C–Me groups too distant from the plane of the neighbouring base pair to explain the large upfield shift and line broadening of the C–Me signals.

The study of the interaction of DHM with DNA confirms the partial insertion model in DNA of this type of ligand, producing a kink at the site of the interaction. Such an interaction may be relevant to understand the effects on nucleic acids of molecules which have a hydrophobic structure between charged groups. This is the case for aliphatic diamines H_3N–$(CH_2)_n$–NH_3 which for $n = 12$ and 14 have effects on DNA identical in many respects to steroidal diamines[11,24]. This may also be the case for peptides or proteins having clusters of hydrophobic residues surrounded by basic residues.

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