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Stereochemical aspects of the interaction between steroidal diamines and DNA

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ABSTRACT

A complete series of stereoisomeric quaternised diaminoandrostanes, differing in their stereochemistry at the 3,5 and 17 positions, has been examined for effects on the thermal denaturation of calf thymus DNA and for the ability to remove and reverse the supercoiling of closed circular duplex PM2 DNA. In both types of test the eight isomers rank in the same order of effectiveness. The preferred stereochemistry for the quaternary substituents at positions 3 and 17 is β; of these the orientation of the 17-substituent is the more critical. Folding of the steroid skeleton between the A and B rings, as in 5β-androstanes, diminishes effectiveness but does not necessarily abolish the effect on supercoiling. The over-riding importance of the C-D ring end of the steroid nucleus bearing a 17β-amino substituent is confirmed by a comparison of the effects of five mono-amino androstanes. Relative helix-unwinding angles per bound steroidal diamine molecule have been determined for four of the isomers; for three 17β compounds the estimated values are similar to that previously reported for irehdiamine A. For a fourth isomer the angle is 0.22 times that of ethidium, the lowest yet determined for any DNA-binding drug. The results lend further support to the argument that intercalation can be ruled out, and alternative models for the binding mechanism are discussed.

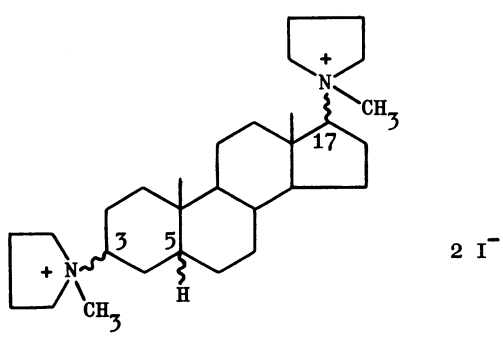
INTRODUCTION

Steroidal diamines have been found to exert a variety of effects in biological systems, ranging from antimicrobial activity and mutagenesis towards bacteriophages[1] to interference with membrane-mediated phenomena such as permeability barriers, transport processes[2,3], and the excitability of the neuromuscular junction in animals and man[4,5]. They include substances of natural origin, such as the plant alkaloids irehdiamine A and malouetine[3], as well as wholly synthetic compounds of potential interest as drugs and investigational tools[5-11]. Their chemical similarity to a number of hormonal steroids invites comparison with the properties of these agents too, particularly in respect of the known capacity of both groups of substances to interact with nucleic acids[7-16].

Our interest in steroidal diamines stems from the observation that they cause removal and reversal of the supercoiling in closed circular duplex

DNA[17,18]. This phenomenon, attributable to local unwinding of the DNA helix[17-22], is regarded as characteristic of intercalating drugs (though not necessarily exclusively so) and it is in this regard that steroidal diamines are particularly important. Several lines of evidence suggest that they constitute a well-defined class of substances whose binding to DNA is associated with unwinding of the helix by a uniquely non-intercalative mechanism. Firstly, they totally lack the aromatic character of acknowledged intercalating drugs. Secondly, they fail to elicit the increase in viscosity of DNA normally seen with intercalative binding of small molecules[10,11]. Thirdly, while nuclear magnetic resonance experiments provide evidence of restricted motion of protons in DNA-bound steroidal diamines[11,23] they yield little or no sign of ring-current shifts[23]. Fourthly, the steroid nucleus is substantially thicker than the aromatic chromophore of typical intercalating drugs[8-11] (5.9Å as opposed to 3.4Å), yet the binding to DNA of an irehdiamine A molecule results in less than half the unwinding caused by ethidium, the standard reference intercalator[18], and indeed the unwinding angle of irehdiamine A is the lowest heretofore reported for any DNA-binding ligand[22,24].

In an effort to elucidate the origins of this anomalous unwinding effect and to clarify the nature of the DNA-binding reaction we have sought to define the necessary stereochemical features of the steroid molecule. Results are presented for a complete series of eight isomeric quaternised diaminoandrostane compounds, together with five related (mono-)amino-androstanes. The diamines[6] are all bisquaternary 3,17-dipyrrolidin-1'-yl-androstane bismethiodides differing in their stereochemistry at the 3,5 and 17 positions:



Unlike the naturally-occurring steroidal diamines studied previously (irehdiamine A and malouetine, whose structures are based on the pregnane

ring system)[3,18] there is little or no conformational flexibility allowed in these synthetic molecules due to the direct substitution on the relatively rigid androstane nucleus. Consequently their molecular shape is more precisely defined, enabling firm correlations between molecular structure and biological response to be attempted — e.g. in respect of the charge separation or inter-onium distance[5] (see Table 1). The effect of the differing stereochemistry in these isomers is illustrated in Fig. 1. It can be seen that switching the 5-H from the α-configuration to the less common β-configuration, producing a cis junction between the A and B rings, yields a molecule in which the steroid nucleus is sharply kinked. By contrast, the trans A-B ring junction of the 5α-androstanes places the four rings in more nearly linear array. Steroids having the 5β configuration have not previously been examined for effects on DNA.

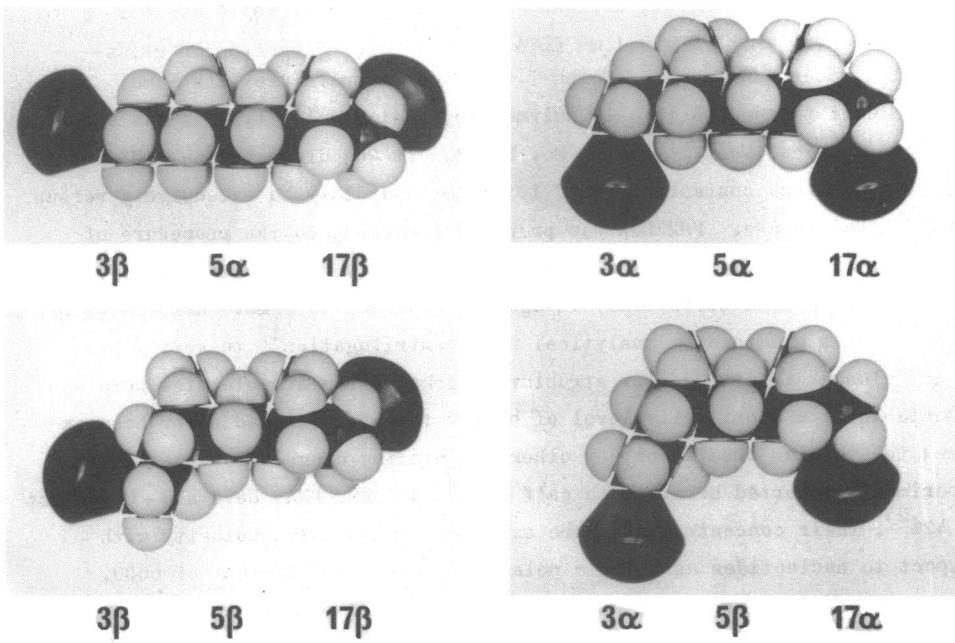


Fig. 1. Space-filling (CPK) molecular models of 3,17-substituted androstanes. The steroid nucleus is viewed from the side, with the β face bearing the two methyl groups uppermost and the A,B,C,D rings oriented left to right. In the interests of clarity the 3- (left) and 17- (right) substituents are represented by large halogen atoms. The A,B and C rings are assumed to retain the chair conformation throughout, while the D ring adopts the 'envelope' form[5]. Top left: a 3β,17β-substituted 5α-androstane. Top right: a 3α,17α-substituted 5α-androstane. Bottom left: a 3β,17β-substituted 5β-androstane. Bottom right: a 3α,17α-substituted 5β-androstane. Based on this direction of view, a pictograph has been devised and included in subsequent figures to facilitate assessment of the relation between stereochemistry and effectiveness of the compounds.

Of the eight isomeric diamines four were found to manifest a helix-unwinding effect qualitatively comparable with that previously detected for irehdiamine A and malouetine[17,18], but these four did not comprise all those having a common configuration at any one of the asymmetric centres. Based on quantitative comparisons between the various responses one end of the steroid nucleus can be identified as having primary importance for interaction with DNA, while the other end provides a secondary site.

MATERIALS AND METHODS

The steroidal diamines (mol. wt. 682.6), monoamines and ethidium bromide were products of May & Baker Ltd., Dagenham, England. Their constitution and homogeneity had been checked in the laboratories of May & Baker; they were used without further purification. Solutions were freshly prepared whenever possible and maintained in the dark at 0-4° or stored frozen at -22°. The buffer used throughout, designated 0.01 SHE, was of ionic strength 0.01; it contained 20 mM HEPES, 0.1 mM EDTA and 4 mM NaCl dissolved in glass-distilled water and adjusted to pH 7.0 at room temperature with NaOH.

Calf thymus DNA (highly polymerised sodium salt, type 1) was a product of Sigma Chemical Co., St Louis, Mo, U.S.A. It was dissolved in 0.01 SHE buffer at a stock concentration of 1.5 mg/ml and dialysed extensively versus 0.01 SHE before use. PM2 DNA was prepared according to the procedure of Espejo *et al.*[25] from virus and host bacteria kindly provided by Dr R.T. Espejo. After exhaustive dialysis against 0.01 SHE to remove all traces of phenol it was examined by analytical ultracentrifugation[17] to verify its intact closed circular duplex structure; nicked circular molecules were undetectable at a sensitivity level of better than 5%. It had never been exposed to ethidium bromide or any other DNA-binding drugs before use in the experiments reported here. Both calf thymus and PM2 DNAs have a G+C content of 42%[25]; their concentrations were expressed in terms of molarity with respect to nucleotides assuming a molar extinction coefficient of 6600.

Thermal denaturation profiles were recorded automatically in a Unicam SP 500 series II spectrophotometer with a programmed temperature rise of 0.6°C per min, using apparatus as previously described[26]. Each of the four cuvettes contained 100 μM calf thymus DNA; one served as the control and was equipped with a thermistor probe. The other three contained the test drug at different steroid/nucleotide ratios. The absorbance at 260 nm of each cuvette was recorded at intervals of 27 sec, providing an essentially continuous trace of absorbance versus temperature. No correction was applied for expansion of cuvette contents. 'Melting temperatures' (T_m, mid-point of the hyperchromic transition) were determined in the usual way[26]. Analytical ultracentrifuga-

tion and spectrophotometric measurements of ethidium binding were conducted by established procedures[17,24].

Viscosity measurements were performed essentially according to the method of Revet et al.[27] using a simple viscometer having a 10 cm capillary of 0.4 mm bore and a bulb of volume 0.7 ml, thermostatted in a 53 litre water bath at $20.0^{\circ} \pm 0.01^{\circ}$ by a Techne Accurostat heater/water pump. The flow time for water was 102 sec. For routine experiments the PM2 DNA concentration was 303 μM in nucleotides ($O.D._{260}$ 2.00; flow time 111.5 sec). Drugs were added in increments of 5 - 20 μl from a Burkard precision micrometer syringe of 1 ml volume via a fine plastic tube inserted down the ascending limb of the viscometer; after each addition complete mixing was effected by bubbling a gentle current of air down the descending limb. Solutions were freed from contamination with traces of particulate material by brief centrifugation in glass tubes in an MSE super minor bench centrifuge, to avoid possible losses of drug and/or DNA by adsorption to the matrix of filter materials. Flow times were measured in triplicate to an accuracy of 0.1 sec; the average deviation of a set of measurements was 0.1 - 0.2 sec, i.e. approximately 0.1%. No kinetic effects attributable to the rate of complex formation were detected: there was never any consistent trend towards longer or shorter flow times during a set of replicate measurements, even at drug/nucleotide ratios where the viscosity was changing rapidly with each increment in drug concentration. Thus equilibrium must have been attained within a substantially shorter time than the 2 - 3 min required to make a first measurement. Reduced viscosities η_{red} were calculated by established methods, taking into account the small dilution caused by addition of drug solutions[27].

RESULTS

Thermal denaturation. Because steroidal diamines having fully saturated ring systems generally lack absorption in the visible and near-ultraviolet regions of the spectrum their interaction with DNA cannot readily be assessed by traditional optical methods, so that effects on the T_m of DNA have become the method of choice[7-11]. In Fig. 2 the effects of the eight bisquaternary diaminoandrostanes on the thermal denaturation of calf thymus DNA are presented. It is evident that all the isomers are capable of binding to DNA, for all of them stabilise the helix to a far greater extent than can be accounted for simply by the increase in ionic strength resulting from their addition. Nevertheless they vary widely in effectiveness, in the order 3β,5α,17β > 3α,5α,17β > 3β,5β,17β > 3β,5α,17α > 3α,5α,17α ≃ 3β,5β,17α > 3α,5β,17β > 3α,5β,17α. This order is in agreement with that reported for isomeric bisprimary and bistertiary 5α-androstane diamines by Gabbay and

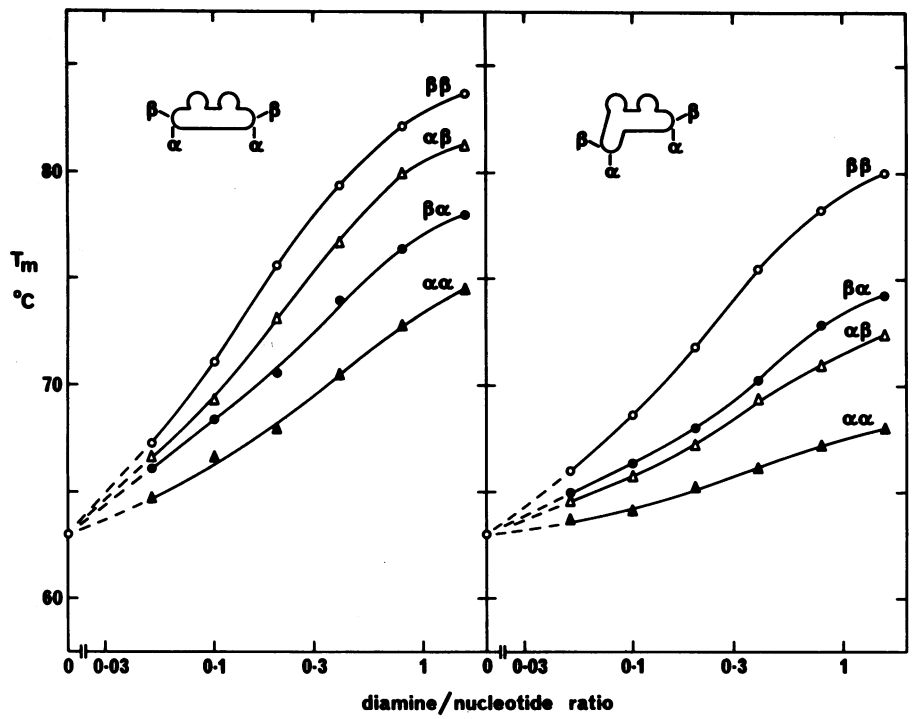


Fig. 2. Elevation of the melting temperature of calf thymus DNA by stereoisomeric 3,17-dipyrrolidin-1'-yl-androstane bismethiodides.
The left-hand panel shows the effects of 5α-androstanes, the right-hand panel those of the corresponding 5β-isomers. Each curve is labelled to indicate the disposition of the 3- and 17-substituents; thus 0 records the results for 3β, 17β compounds, Δ those for 3α, 17β compounds, and so on.

Glaser[11]; it establishes the preferred stereochemistry at the three asymmetric centres as β for both amino substituents and 5α for the steroid nucleus. While this order of effectiveness does not necessarily rank the steroids in the order of their intrinsic binding constants, it is likely that this is so unless there are large differences in the thermodynamics of their interaction with the helix and coil forms of DNA. Further evidence on this point will emerge in the next section.

Also in agreement with previous work[8,11] is the finding of a monotonic increase in T_m caused by all the isomers, at least up to the highest ratios tested (1.6 molecules per DNA nucleotide). This behaviour is reported to be characteristic of bistertiary and bisquaternary steroidal diamines (refs. 8,11), while the analogous primary and secondary diamino steroids produce a labilising effect at higher ratios which has been attributed to the formation of a secondary type of complex[8]. In actual fact a close inspection of the melting curves (Fig. 3) reveals that this distinction may belie the

facts to some extent, for at higher ratios a distinct tendency for the absorbance to creep upwards at temperatures well below the main hyperchromic transition was consistently observed for all the bisquaternary diamino-androstanes. Associated with this phenomenon was an increase in the total hyperchromic effect, following a decrease at ratios up to 0.4 (Fig. 3). Similar behaviour has recently been described for a number of other diamines[23], suggesting that the situation may be more complicated than has previously been supposed, and that features indicative of incipient destabilising and/or secondary interaction may be shared by more highly substituted

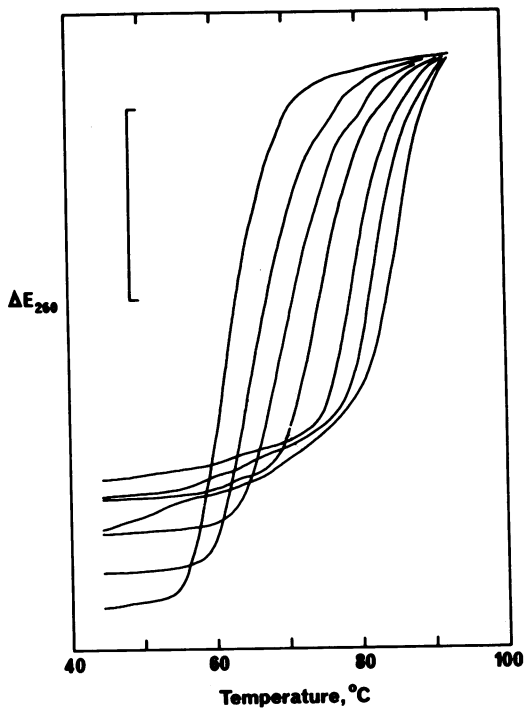


Fig. 3. Thermal denaturation profiles of calf thymus DNA in the presence of 3β,17β-dipyrrolidin-1'-yl-5α-androstane bismethiodide.
The curves correspond to the points plotted (0) in the left-hand panel of Fig. 2; in succession they show the melting of mixtures having steroid/DNA nucleotide ratios of 0 (control), 0.05, 0.1, 0.2, 0.4, 0.8 and 1.6. They have been normalised to the same absorbance at 93°, the practical upper limit of the recording equipment, and the bar represents an absorbance change of 0.1 unit.

diamines. Be that as it may, such secondary effects at high concentrations are unlikely to be relevant to the unwinding phenomena described in the next section.

Effects on the supercoiling of closed circular duplex DNA.

The characteristics of the viscometric system for measuring removal and reversal of the supercoiling of PM2 DNA are illustrated in Fig. 4. It can be seen that the equivalence point for binding of ethidium, defined as the binding ratio at the minimum and maximum of the sedimentation and viscosity plots respectively, is the same determined by either experimental method: 0.0516 ethidium molecules bound per nucleotide. This estimate is in good agreement with values determined previously by sedimentation analysis in buffers of similar ionic strength[18,24]. It serves to verify the reliability of the

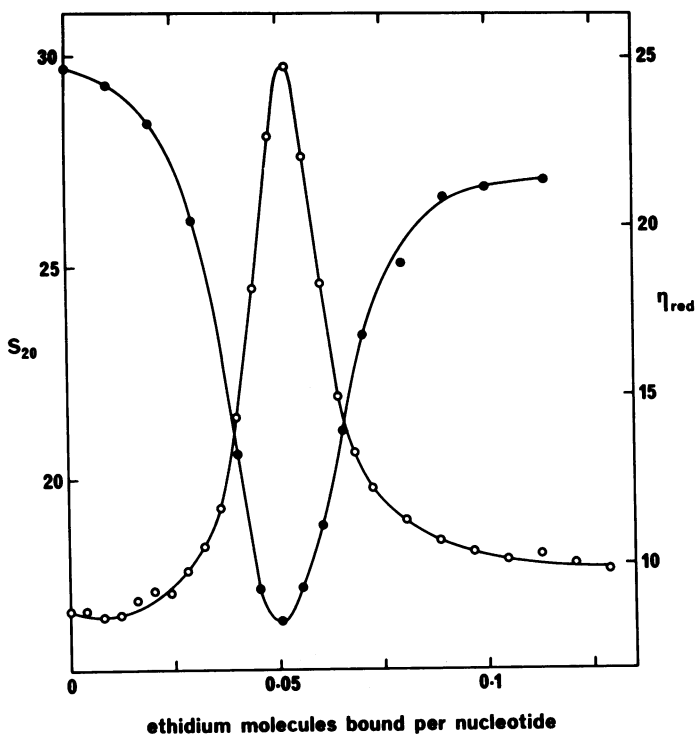


Fig. 4. Removal and reversal of the supercoiling of closed circular duplex PM2 DNA by ethidium bromide.
Closed symbols show the variation in sedimentation coefficient (left-hand ordinate); open symbols represent the changes in reduced viscosity (right-hand ordinate). For the viscosity measurements the DNA concentration was 303 μM in nucleotides; ethidium was added as a 220 μM solution in buffer.

viscometric technique as a means of assessing the actions of the experimental drugs and provides the essential control from which unwinding angles relative to that of ethidium may be calculated.

In Fig. 5 the effects of the 5α-androstane diamines are shown. Three out of the four steroids caused removal and reversal of the supercoiling. The fourth compound, the 3α,17α isomer, failed to remove the supercoils at ratios up to 1:1, the highest concentration tested. It did produce a steady increase in the viscosity however, and it is possible that this increase represents a genuine reduction in the extent of supercoiling such that complete relaxation of the closed circular DNA molecules might be observable at much higher concentrations. For the present its action can be recorded as tentatively negative; in any event its effect is very much

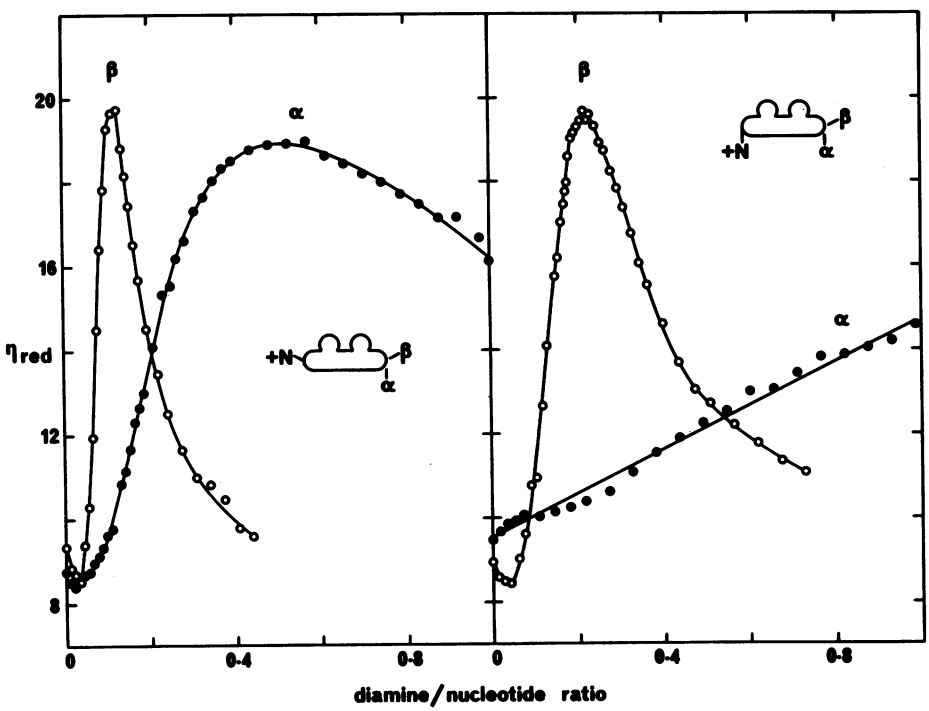


Fig. 5. Effects of stereoisomeric 5α-androstane-3,17-bisquaternary ammonium salts on the viscosity of PM2 DNA.
The initial DNA concentration was 303 μM in nucleotides; steroids were added as 1 mM solutions in buffer. The abscissae represent the molar ratio of added drug to DNA nucleotides. The left-hand panel shows the effect of 3β-pyrrolidin-1'-yl-5α-androstanes with the second quaternary amino substituent at position 17 oriented either β (open symbols) or α (filled symbols). The right-hand panel shows the effect of the corresponding 3α-pyrrolidin-1'-yl-5α-androstanes, again with the 17-substituent oriented either β (open symbols) or α (filled symbols).

weaker than those of the other three drugs.

The positive responses given by these three isomers show qualitative

similarities, though clear quantitative differences are apparent. Firstly, the reduced viscosities of the relaxed circular DNA complexes at the maxima of the curves are all much the same, 19 - 20 dl/g, and significantly lower than that of the relaxed ethidium complex (Fig. 4). Secondly, all three peaks display a considerable skew towards higher diamine/nucleotide ratios; a similar skew is apparent in the sedimentation coefficient profiles for ireh-diamine A and malouetine reported previously[17,18]. Thirdly, a small decrease in viscosity occurs before the onset of the principal rise and fall, an effect

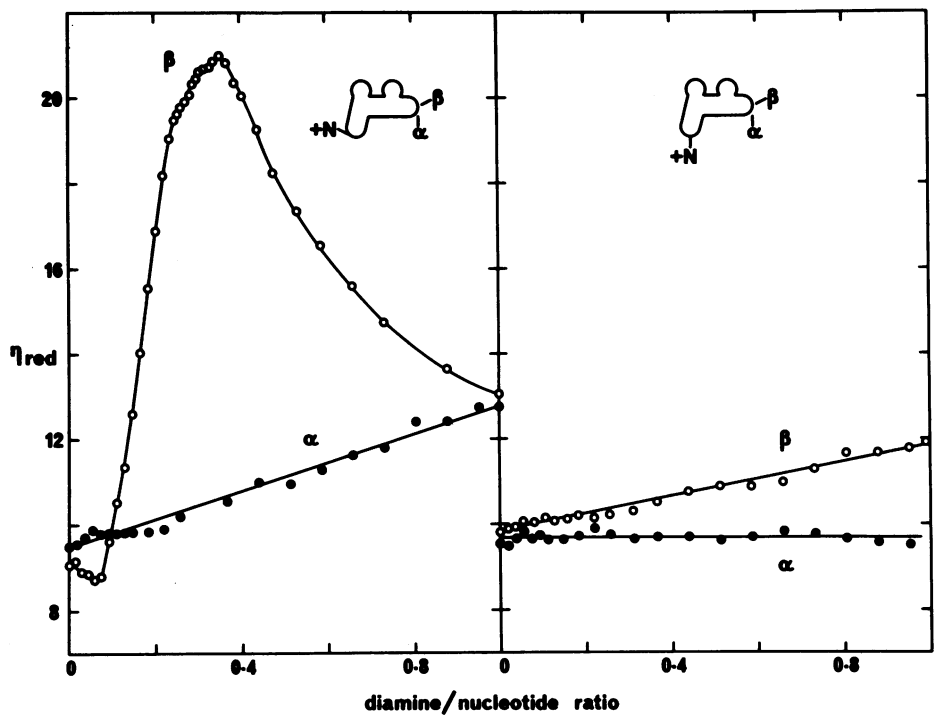


Fig. 6. Effects of stereoisomeric 5β-androstane-3,17-bisquaternary ammonium salts on the viscosity of PM2 DNA.
Details are as described in the legend to Fig. 5. The left-hand panel shows the effect of the two isomeric 3β-pyrrolidin-1'-yl-5β-androstanes, the right-hand panel that of the 3α-pyrrolidin-1'-yl-5β-androstanes.

not seen with any isomer which fails to remove the supercoiling (Figs. 5 and 6) and barely, if at all, with ethidium (Fig. 4).

The most potent response is clearly produced by the 3β,17β isomer. Taking this as a reference, it can be seen that switching either the 3- or the 17-amino substituent to the α configuration shifts the equivalence region to higher ratios. Moreover, epimerisation at the 17 position seems tohave

the more dramatic consequences. It can therefore be inferred that the optimal configuration at each of positions 3 and 17 is β, that the 17β orientation seems the more critical, and that with both 3- and 17-amino groups in the unfavourable α configuration activity is, for practical purposes, lost.

The results for the 5β-androstanes confirm these inferences and reveal in addition that the flatter 5α-steroid skeleton is much preferred over the more folded 5β-steroid nucleus (Fig. 6). In this series only the 3β,17β

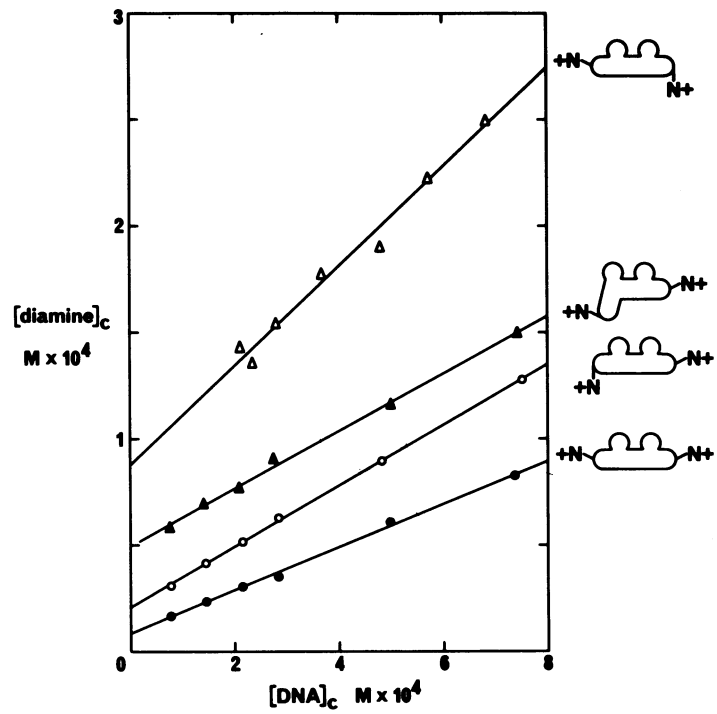


Fig. 7. Titration of the supercoiling of PM2 DNA by stereoisomeric androstane-3,17-bisquaternary ammonium salts.
The ordinate and abscissa show respectively the absolute concentrations of total steroid and DNA nucleotides in the viscometer at the maxima of reduced viscosity plots like those presented in Figs. 5 and 6. For the different experiments the starting DNA concentration was varied between 80 and 800 μM in nucleotides. The straight lines were fitted by computer using a least-squares programme. Identification of symbols:

●: 3β,17β-dipyrrolidin-1'-yl-5α-androstane bismethiodide
○: 3α,17β-dipyrrolidin-1'-yl-5α-androstane bismethiodide
▲: 3β,17β-dipyrrolidin-1'-yl-5β-androstane bismethiodide
△: 3β,17α-dipyrrolidin-1'-yl-5α-androstane bismethiodide

isomer yielded a positive response, the others producing even less effect on the viscosity than the 3α,5α,17α isomer — in the case of the 3α,5β,17α isomer no effect at all.

To determine whether the varied responses produced by the four positive isomers resulted from differences in their strength of interaction with DNA, different relative unwinding angles, or both, the position of the equivalence peak was determined for each drug at a range of DNA concentrations. In Fig. 7 the concentration of each steroid required to relax the supercoiling of the circles is plotted as a function of the DNA concentration. For a simple mass-action interaction such a plot should take the form of a straight line with slope equal to the equivalence binding ratio ν_c and intercept on the ordinate equal to the free drug concentration c' in equilibrium with the relaxed circular DNA complex[27]. The data in Fig. 7 reveal that this condition is satisfactorily obeyed, and the resulting parameters are collected in Table 1.

Table 1. SUMMARY OF RESULTS FOR STEREOISOMERIC ANDROSTANE-3,17-BISQUATERNARY AMMONIUM SALTS

The equivalence binding ratio ν_c, the free drug concentration c' in equilibrium with the relaxed circular DNA complex, and the unwinding angle per bound steroid molecule relative to that of ethidium ϕ_s/ϕ_e are calculated from the data presented in Fig. 7. Estimated ϕ_s values are related to an assumed value of 12° for ethidium. $[\eta]'$ is the apparent intrinsic viscosity of the relaxed circular DNA complex obtained by extrapolation to zero DNA concentration. ΔT_m represents the elevation of the T_m of calf thymus DNA at a steroid:nucleotide ratio of 1:1. The inter-onium distances ($\overset{+}{N}-\overset{+}{N}$) and estimates of relative neuromuscular blocking potency are taken from ref. 5.

Isomer	ν_c	c' (μM)	ϕ_s/ϕ_e	Estimated ϕ_s	$[\eta]'$ (dl/g)	ΔT_m (°C)	$\overset{+}{N}-\overset{+}{N}$ (Å)	Potency in vivo	
								Cat	Monkey
3β 5α 17β	0.102 ± 0.003	8 ± 2	0.51 ± 0.02	6.1° ± 0.2°	19.3 ± 0.1	20.0	11.0	—— 1.00 ——	
3α 5α 17β	0.143 ± 0.002	21 ± 1	0.36 ± 0.01	4.3° ± 0.1°	19.4 ± 0.4	17.5	10.4	0.56	0.86
3β 5β 17β	0.136 ± 0.005	49 ± 3	0.38 ± 0.02	4.6° ± 0.2°	20.2 ± 0.3	16.0	10.0	0.91	1.25
3β 5α 17α	0.233 ± 0.016	88 ± 10	0.22 ± 0.02	2.7° ± 0.2°	18.6 ± 0.4	14.0	10.6	1.00	0.92
3α 5α 17α	-	-	-	-	-	10.4	9.6	0.43	0.76
3β 5β 17α	-	-	-	-	-	10.4	9.3	0.67	0.79
3α 5β 17β	-	-	-	-	-	8.5	9.9	0.09	0.08
3α 5β 17α	-	-	-	-	-	4.5	8.7	0.09	0.07

Given the value of ν_c it is a simple matter to calculate the unwinding angle for each steroid relative to that of ethidium from the relation

$$\frac{\phi_s}{\phi_e} = \frac{\nu_c(\text{ethidium})}{\nu_c(\text{steroid})}$$

where ϕ_s and ϕ_e are the helix-unwinding angles per bound steroid or ethidium molecule respectively[17-22]. The quantity ν_c(ethidium) is taken from the data for ethidium bromide measured under identical conditions (Fig. 4). If ϕ_e is

known an absolute value for ϕ_s can be determined. In previous work[17-22] it has been customary to refer all unwinding angles to an assumed value of 12° for ϕ_e (ref. 28); if this is done the estimated values of ϕ_s presented in Table 1 result. Recent studies[29,30] suggest that this estimate of ϕ_e may be too low by a factor of approximately two. If so, the estimated values of ϕ_s must be increased by that factor. In any event the relative helix-unwinding angles ϕ_s / ϕ_e are not affected, and are correct as they stand in Table 1. It should be noted that the errors quoted in the Table are statistically real standard deviations since they are derived from least-squares fitted lines (Fig. 7), in contrast to the estimates of error made by comparing widths of equivalence regions of S_{20} plots in earlier work[17-22].

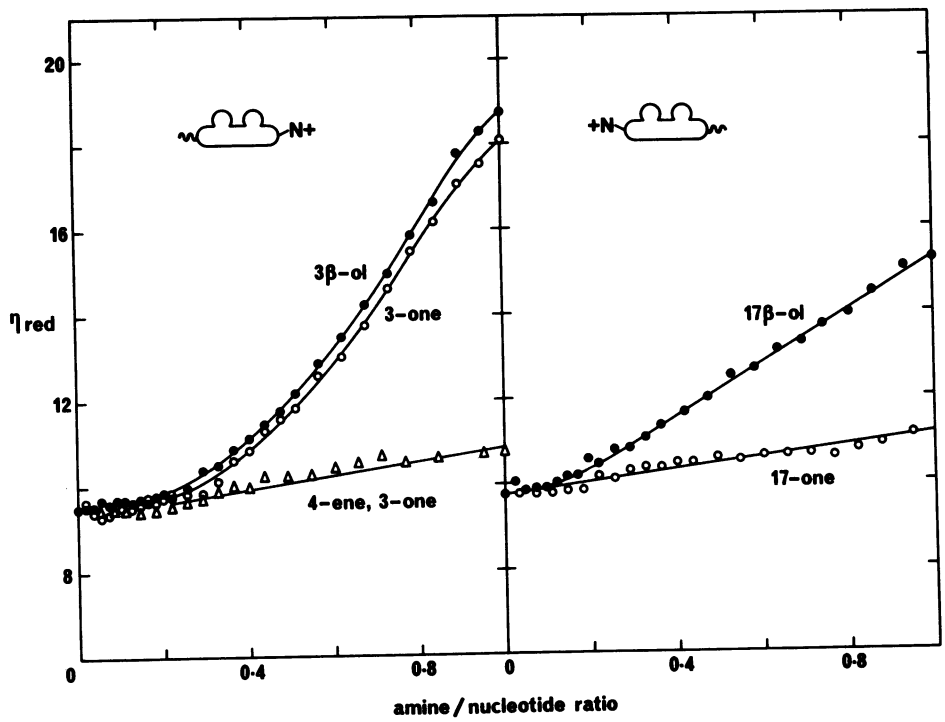


Fig. 8. Effects of monoquaternary 5α-androstane salts on the viscosity of PM2 DNA.
Experimental details are as described in the legend to Fig. 5. The abscissae show the molar ratio of added steroid to DNA nucleotides. Identification of symbols:
Left-hand panel: ●: 17β-pyrrolidin-1'-yl-5α-androstan-3β-ol methiodide
○: 17β-pyrrolidin-1'-yl-5α-androstan-3-one methiodide
△: 17β-pyrrolidin-1'-yl-androst-4-ene-3-one methiodide
Right-hand panel: ●: 3β-pyrrolidin-1'-yl-5α-androstan-17β-ol methiodide
○: 3β-pyrrolidin-1'-yl-5α-androstan-17-one methiodide

For the three 17β-isomers the relative helix-unwinding angles lie within the range 0.36 - 0.51, not very different from the values of 0.39 and 0.43 previously determined[18] for irehdiamine A. Among these three isomers the dominant factor responsible for the differences in their interaction with circular DNA lies in the binding parameters, for the value of c' varies over a six-fold range (Table 1). In contrast, the one isomer which seems to differ significantly from all the others is the only positive 17α-isomer which yields a substantially lower unwinding angle and evidently interacts relatively weakly with DNA as the high value of c' shows.

The simplest interpretation of these findings is that the preferred site for interaction with DNA leading to the observed unwinding of the helix lies towards the C - D ring end of the steroid nucleus. This is the only portion of the molecule common to the structures of the three most effective stereoisomers (cf. Fig. 7) and it seems to demand a 17β-oriented amino substituent. However, this is not the only structural constraint, witness the variation in c' among the 17β isomers and the anomalous response to binding of the 3β,5α,17α isomer. It seems that if the preferred site is rendered unavailable by α-orientation of the quaternary centre at position 17, a less favourable, perhaps qualitatively different, mode of interaction is revealed which may operate at the other end of the molecule since it demands a 3β quaternary centre as well as the trans A - B ring junction of the 5α-androstane nucleus.

Steroidal monoamines. These conclusions can in principle be checked by studying the effects of steroids bearing only a single β-amino substituent at either of the 3 or 17 positions. At the same time it might be possible to address the question whether unfavourably oriented (α-) substituents at these positions in steroidal diamines contribute anything to the stability of the complexes, or whether they exert a positively destabilising influence, e.g. by steric hindrance.

Five mono-amino androstanes stereochemically related to the diamines which had a positive action on supercoiling were tested for effects on circular DNA. The results are shown in Fig. 8. The two 17β-aminoandrostanes yielded closely similar curves which, while not actually reaching an equivalence point at the highest ratios tested, were clearly well on the way. All the other monoamino steroids, the 3β-aminoandrostanes as well as the 17β-aminoandrostene compound, were relatively ineffective. The much diminished effect of the latter steroid compared to the other 17β-amino steroids no doubt reflects the altered shape of the steroid skeleton due to the introduction of the 4 - 5 double bond, in agreement with the lesser effectiveness of 5β-steroids noted above. More importantly, the over-riding import-

ance of a 17β-substituent rather than a 3β-substituent is clearly confirmed. It is also evident that the second quaternary centre in a diamine, even if unfavourably oriented in the α configuration, nevertheless contributes positively to the helix-unwinding effect. Its influence is probably mediated by a contribution to the stabilisation of the complex, for the effects of the monoamines on the T_m of DNA are very much smaller than those of their diamine counterparts (Table 2).

Table 2. Elevation of the T_m of calf thymus DNA by steroidal monoamines. ΔT_m refers to a steroid:nucleotide ratio of 1:1

Compound	ΔT_m (°C)
17β-pyrrolidin-1'-yl-5α-androstan-3β-ol methiodide	2.5
17β-pyrrolidin-1'-yl-5α-androstan-3-one methiodide	2.5
17β-pyrrolidin-1'-yl-androst-4-ene-3-one methiodide	1.4
3β-pyrrolidin-1'-yl-5α-androstan-17β-ol methiodide	2.5
3β-pyrrolidin-1'-yl-5α-androstan-17-one methiodide	1.4

DISCUSSION

A striking feature of the present experiments is the variation in effectiveness between the different diamines and its relation to their precise stereochemistry. In respect of their capacity to unwind the DNA helix they vary from highly effective to apparently totally ineffective. Likewise their ability to stabilise the helix towards thermal denaturation spans a wide range. Perhaps more significantly the order of effectiveness deduced from the T_m experiments seems to apply equally well to their effect on the supercoiling of circular DNA. In Table 1 the eight isomers are listed in order of ΔT_m; for the first four compounds it can be seen that this also ranks them in order of c'. Although it remains impossible to state with certainty that this corresponds to the order of their intrinsic binding constants since the number of binding sites for each isomer remains unknown, c' rises sharply while the critical binding ratio changes much less, strongly suggesting progressively weaker binding. If the slopes of the viscosity plots for the remaining four isomers are compared (Figs. 5 and 6) it is clear that they too lie in order of diminishing effectiveness. Thus the correlation between responses in the two types of assay appears practically perfect.

On the other hand the correlation between DNA-binding properties and

distance between the charged centres is less good, though a certain trend is evident. The inter-onium distance is greatest for the most effective isomer and lowest for the least; for the four isomers which were capable of removing and reversing the supercoiling of PM2 DNA it is 10 Å or more; for those which did not it is less. Beyond that there is no strict relationship. When relative unwinding angles are compared they are seen to be quite uncorrelated with inter-charge separation. Inasmuch as ϕ_s / ϕ_e is the most sensitive indicator of the geometrical character of the interaction it is clear that the complex formed by the first three isomers, which seems to be of the same qualitative nature judged by this criterion, depends upon some feature(s) other than the $\overset{+}{N}-\overset{+}{N}$ distance, though it may demand a minimum of 10 Å. Conversely, the anomalously low unwinding angle of the fourth isomer is not attributable to its charge separation and must again be related to other molecular parameters. It is noteworthy that the relative unwinding angle for binding of this compound, the 3β,5α,17α isomer, represents a new low: 0.22 times the unwinding angle of ethidium. With the recent discovery of an antibiotic which unwinds the helix by an angle almost double that of ethidium[31] the range accessible to experimental study covers nearly a factor of ten.

What light do the present experiments shed upon the actual nature of the steroidal diamine-DNA complex? The possibility that it might involve some intercalative-type interaction is rendered still more remote by three findings: (1) the extraordinarily low unwinding angle of the 3β,5α,17α isomer referred to above; (2) the positive helix-unwinding effect of the 3β,5β,17β isomer, whose folded shape (Fig. 1) lacks even the near-planarity of 5α-steroids; and (3) the intrinsic viscosity of the relaxed circular PM2 DNA complexes (Table 1) which approximates to that of nicked circular molecules under these conditions, confirming the lack of any significant change in viscosity reported by other workers[10,11]. While the shear-dependence of apparent intrinsic viscosity determined under our conditions[27] results in values some 19% lower than the expected value as predicted by eq. (8) of Opschoor *et al.*[32] it is nevertheless certain that $[\eta]'$ for the steroidal diamines is much lower than the value for a typical intercalator such as ethidium (cf. Fig. 4). The only peculiarity in the viscosity measurements lies in the small decrease observed at very low diamine/nucleotide ratios: we have considered the possibility that this might be related to the existence of a small fraction of unpaired bases in superhelical DNA as suggested by Dean and Lebowitz[33] but have not pursued the issue further.

If intercalation is ruled out the mechanism of binding must presumably involve 'external' attachment to the helix, stabilised by electrostatic inter-

actions between the quaternary centres and negatively charged phosphates of the DNA together with hydrophobic and/or hydrogen-bonding interactions involving the remainder of the ligand molecule. Electrostatic interaction with adjacent or next-nearest-neighbour phosphates within the same strand of DNA may be considered unlikely because the separation between successive phosphates is approximately 7 Å, which does not compare favourably with the inter-onium distances of the effective isomers (Table 1). A more attractive idea is the possibility of bridging across one of the grooves of the helix, where minimal distances between phosphates are about 12 Å and 19 Å for the minor and major grooves respectively[23]. Such bridging might more readily explain the large increases in T_m caused by the diamines, and the local distortion of the helix which is manifestly associated with the binding might serve to optimise the inter-phosphate distance for effective interaction with the rigid diamine. The predominantly hydrophobic steroid nucleus would then be positioned suitably to interact with the base-pairs or their substituents in one or other of the grooves. One such form of interaction could involve local breakage of a base-pair to allow stacking between a 'flipped-out' base and the relatively flat α face of the steroid nucleus. Such a mode of interaction has been postulated in earlier work on the weaker interactions between hormonal steroids and nucleic acid components, where an apparent requirement for β orientation of 17-substituents leaving an unobstructed α face beneath the C and D rings was detected[13-15,34,35]. Our identification of the primary importance of the C - D ring end of the molecule bearing a 17β-amino substituent is entirely consistent with this view. The ineffectiveness of one out of the four 17β-isomers, the 3α,5β,17β compound, could easily be explained on steric grounds by the bulk of the methyl-pyrrolidinyl substituent at position 3 which, when α-oriented on the 5β skeleton might substantially hinder access to the space under the C and D rings, but leave it relatively free if oriented β (cf. Fig. 1).

One observation which might prove difficult to reconcile with this model is the finding of Gourevitch *et al.*[23] that relaxation of the 18- and 19-methyl group protons of malouetine differs by a factor of 3.7 from that of protons in its quaternising methyl groups when this steroidal diamine is bound to DNA. On this basis they postulated that the most bulky (β) face of the steroid ligand is in close hydrophobic contact with the DNA. This would effectively rotate the molecule about the axis connecting the quaternary centres compared to the model discussed above, leaving the α face exposed to the environment. There is no reason to believe that the malouetine-DNA complex differs fundamentally from complexes formed with other bisquaternary

steroid molecules — indeed there is every indication that they are essentially similar — thus this alternative model might equally well apply to the diamines studied here. In that case the stereochemical constraints which emerge from the present work will assist greatly in refining details of the model. Investigations along these lines are in progress.

Whatever the exact orientation of the steroid molecule in the complex, the origins of the helix-unwinding effect remain mysterious. Local distortion due to electrostatic forces acting to alter the distance between phosphates on the opposing strands might be responsible; alternatively the unwinding might be simply a consequence of some local denaturation caused by steroid binding. Both these phenomena are features of the first model discussed. The concept of local denaturation, perhaps of a quite non-specific kind, merits consideration if only because the local exclusion of water from the groove(s) of the helix occasioned by the proximity of a hydrophobic steroid nucleus immobilised at both ends would remove much of the driving force for the stability of base-pairing.

It remains to comment on the last two columns in Table 1. The diamines used in this work were originally synthesised as potential curare-like neuromuscular blocking agents and one, the 3β,5α,17β isomer (dipyrandium) proved to be a potent, non-depolarising curarising agent both in animals and man[4,5]. The relative effectiveness of the different isomers in respect of DNA-binding and neuromuscular blocking potency makes interesting comparison. In both cases the 3β,5α,17β isomer is the most potent, and the 3α,5β,17α isomer the least. 5β-compounds are generally less effective than 5α, and the preferred stereochemistry at each of positions 3 and 17 is β. However, with regard to neuromuscular blocking activity it appears that position 3 is the more important, for inversion at that centre produces a large decrease in potency whereas epimerisation at position 17 has little effect. We have found the converse to hold for effective interaction with DNA. There would seem little reason to expect stereochemical requirements to run in parallel for both systems since the two receptors have little in common, other than, perhaps, an array of negative charges on their surfaces.

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