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Topography of Nucleic Acid Helices in Solutions. Proton Magnetic Resonance Studies of the Interaction Specificities of Steroidal Amines with Nucleic Acid Systems*

Edmond J. Gabbay† and Robert Glaser‡

ABSTRACT: The interactions of steroidal amines with nucleic acid helices are reported. The studies were based on T_m of helix–coil transitions of nucleic acid–steroid complexes, as well as viscometric, circular dichroism, and temperature-dependent proton magnetic resonance techniques. The results indicate that the steroidal amines selectively stabilize the DNA helical structures, while causing the ribose-containing acids to unravel and denature.

The temperature-dependent proton magnetic resonance experiments show that single stranded random coils interact with the steroidal amines *via* hydrogen- and hydrophobic-type bonding. The capacity to form hydrogen bonding in the random coils is shown to be greater than that of the helical structure.

For the past 3 years, considerable work has been devoted in our laboratory to the elucidation of the interaction specificity of nucleic acid systems with mono- and polyammonium salts (Gabbay, 1967–1969; Glaser and Gabbay, 1968; Gabbay and Shimshak, 1968; Gabbay *et al.*, 1969a,b; Glaser and Gabbay, 1970; Passero *et al.*, 1970). The studies involved the use of diammonium salts of the general structure, I, $R_1R_2R_3N^+$-$(CH_2)_nN^+R_1R_2R_3 \cdot 2Br^-$. This paper reports the interactions of steroidal amines with nucleic acid helices. The effect of primary, tertiary, and quaternary steroidal ammonium salts as well as various epimers, *i.e.*, 3α-, 3β-, 17α-, and 17β-amino-5α-androstane, on the T_m of the helix–coil transition of deoxyhomopolymers, hybrid homopolymers, and ribohomopolymers are reported. Moreover, temperature-dependent studies of the proton magnetic resonance spectra of steroidal amines–nucleic acid complexes are utilized, together with ultraviolet absorption and circular dichroism studies, to elucidate the specificity of nucleic acid interactions.

Results and Discussion

T_m Studies with Steroidal Amines. Chart I lists the structure of the steroidal amines, II and III, used in this study. The synthesis and characterization of the salts II and III are reported

* From the School of Chemistry, Rutgers, The State University, New Brunswick, New Jersey 08903 (R. G.), and the Department of Chemistry, University of Florida, Gainesville, Florida 32601 (E. J. G.). *Received August 20, 1970.* This work was supported by Grants GM-15308 and GM17503 from the U. S. Public Health Service, and GB-16044 from the National Science Foundation.

† To whom correspondence should be addressed: Department of Chemistry, University of Florida. Recipient of a Career Development award from the U. S. Public Health Service (1970).

‡ National Defense Education Act Predoctoral fellow (1966–1969).

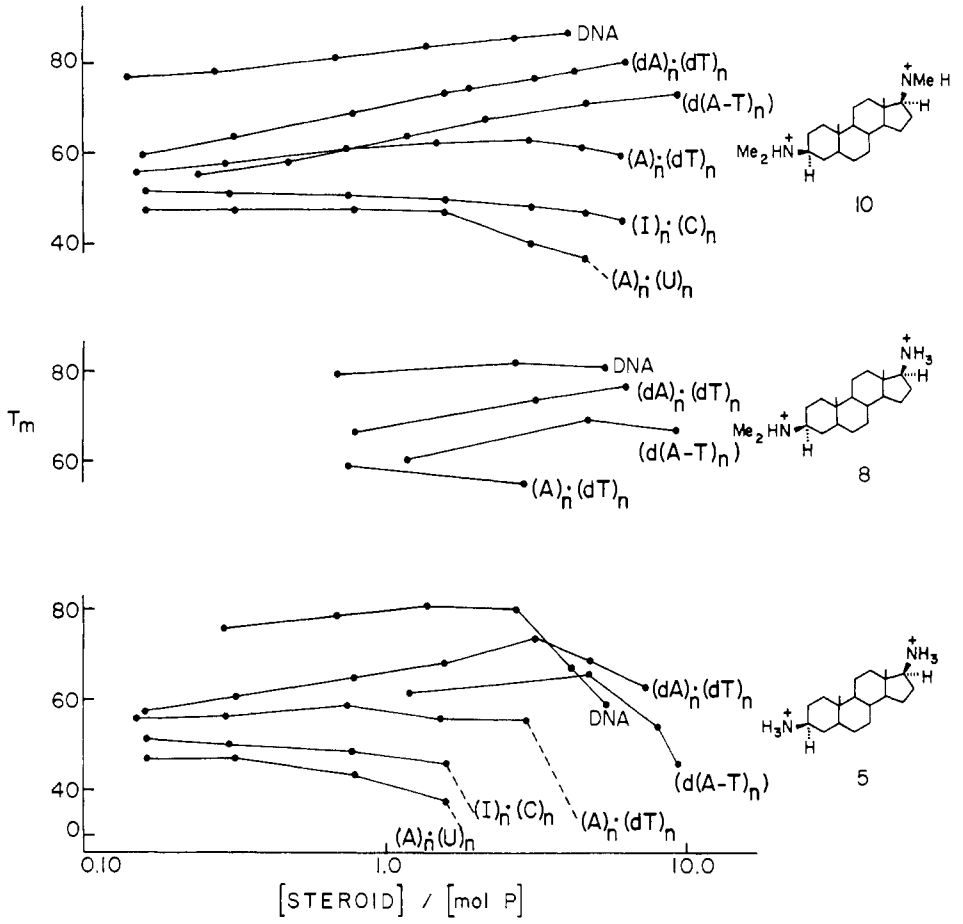


FIGURE 1: The effect of increasing concentration of steroidal amines **5**, **8**, and **10** on the T_m of nucleic acids.

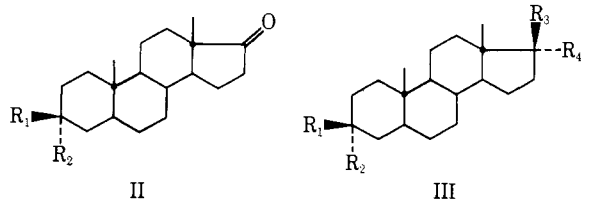
elsewhere (Glaser and Gabbay, 1970). The results of the interactions of the salts with $(dA)_n \cdot (dT)_n$, $d(A\text{-}T)_n$, salmon testes DNA, $(A)_n \cdot (dT)_n$, $(A)_n \cdot (U)_n$, and $(I)_n \cdot (C)_n$ helices have been obtained using 0.025 M sodium phosphate buffer (0.025 M in Na^+), pH 6.3, and are shown in Figure 1 and Table I.

In order to ascertain the number of positive charges per molecule at pH 6.30, the titration curves of the salts II and III were determined at 30, 50, and 75°. The results showed that at pH 6.30, the salts II and III are fully protonated at the above-mentioned temperatures. Therefore, differences in the interaction of the salts II and III with nucleic acids are assumed to reflect differences due to changes of the stereochemical configuration of the steroid, and are not simply due to differences in the number of positive charges/molecule.

Moreover, binary and ternary helical structures may be formed between polyriboadenylic acid, polyribouridylic acid, $(A)_n \cdot (U)_n$, and $(A)_n \cdot 2(U)_n$, as well as with $(dA)_n$ and $(dT)_n$, *i.e.*, $(dA)_n \cdot (dT)_n$ and $(dA)_n \cdot 2(dT)_n$, and $(A)_n$ and $(dT)_n$, *i.e.*, $(A)_n \cdot (dT)_n$ and $(A)_n \cdot 2(dT)_n$ (Stevens and Felsenfeld, 1964; Chamberlin and Patterson, 1965; Chamberlin, 1965; Riley *et al.*, 1966). In all these cases where a ternary complex may be formed, the nature of the helix–coil transition was examined at two wavelengths, *i.e.*, at 260 nm and at a second wavelength where the binary helix is isochromous with the random coils. In general, it is found that steroidal amines II and III do not induce the disproportionation reaction and formation of triple helices; however, the breadth of the transition is increased, and, in a few cases, complex melting curves are obtained.

The T_m of the helix–coil transition of $(dA)_n \cdot (dT)_n$, $(d(A\text{-}T))_n$, salmon sperm DNA, $(A)_n \cdot (dT)_n$, $(A)_n \cdot (U)_n$, and $(I)_n \cdot (C)_n$ at various steroidal amines concentrations are shown in Figure 1. Three types of effects are exhibited by the dicationic salts, **5**, **8**, and **10**, on the T_m of the helix–coil transition of nucleic acid systems, (a) stabilization effect, (b) biphasic effect (initial stabilization at low steroid concentrations followed by destabilization at higher concentrations), and (c) destabilization

CHART I



II III

Compound	Deriv.	R_1	R_2	R_3	R_4
1	II	$N^+H(CH_3)_2$	H		
2	II	H	$N^+H(CH_3)_2$		
3	II	$N^+(CH_3)_3$	H		
4	II	H	$N^+(CH_3)_3$		
5	III	N^+H_3	H	N^+H_3	H
6	III	N^+H_3	H	H	N^+H_3
7	III	H	N^+H_3	H	N^+H_3
8	III	$N^+H(CH_3)_2$	H	N^+H_3	H
9	III	$N^+H(CH_3)_2$	H	H	N^+H_3
10	III	$N^+H(CH_3)_2$	H	$N^+H(CH_3)_2$	H
11	III	$N^+H(CH_3)_2$	H	H	$N^+H(CH_3)_2$
12	III	H	$N^+H(CH_3)_2$	H	$N^+H(CH_3)_2$

effect. It is noted that the helical structure of deoxypolynucleotides, $(dA)_n \cdot (dT)_n$, $(d(A\text{-}T)_n)$, and salmon sperm DNA is stabilized by the bis(tertiary) ammonium steroid, **10**, whereas a biphasic effect is exhibited as the tertiary ammonium groups at positions 3β and 17β of the 5α-androstane are successively replaced by primary ammonium groups, *e.g.*, 3β,17β-bis-(tertiary) ammonium (**10**), 3β-tertiary ammonium, 17β-primary ammonium (**8**), and 3β,17β-bisprimary ammonium (**5**). The extent of the destabilization effect appears to depend on the nucleotide composition of the deoxypolymer (DNA > $(d(A\text{-}T)_n)$ > $(dA)_n \cdot (dT)_n$) as well as on the H-bonding abilities of the steroidal diamines, **5** > **8** > **10** (see Figure 1). The hybrid homopolymer helix, $(A)_n \cdot (dT)_n$, exhibits a biphasic response to the presence of the steroidal diamines. The destabilization effect of the $(A)_n \cdot (dT)_n$ helix is more pronounced than that exhibited by the deoxypolymer and again it is observed that this effect correlates with the ability of the steroidal amines to form H bonds. In fact, the $(A)_n \cdot (dT)_n$ helix is totally denatured by the bisprimary ammonium steroid, **5**, even at room temperature at a steroid per mole of P of 4.5. The helical structure of the ribohomopolymers, $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$, exhibits only a destabilization effect in the presence of the steroidal diamines, **5** and **10**. Moreover, the extent of destabilization is again found to depend on the nucleotide composition ($(A)_n \cdot (U)_n$ > $(I)_n \cdot (C)_n$) and the H-bonding capacity of the steroidal diamines, **5** > **10**. In order to understand and interpret these results it is instructive to inquire into the equilibrium process involved at the helix–coil transition. At the T_m of the helix–coil transition, the following equilibria must be considered

$$D + H \overset{K_H}{\rightleftharpoons} D \cdot H \overset{T_M}{\rightleftharpoons} D \cdot R \overset{K_R}{\rightleftharpoons} D + R$$

where D = diammonium salt, H = helix, R = random coils, and K_H and K_R are the binding constants of D to the helix and the random coils, respectively. Destabilization of the helical structure in the presence of the steroidal diamines can only be interpreted in terms of a greater affinity of the latter to the random coils, *i.e.*, $K_R > K_H$. This situation is obtained with the ribohomopolymers, $(A)_n \cdot (U)_n$ and $(I)_n \cdot (C)_n$. On the other hand, a net stabilization of the helical structure relative to the random coils as in the case for the deoxypolynucleotides is somewhat more difficult to interpret, *i.e.*, it may arise if $K_H > K_R$ and/or K_R is small or negligible. However, as will be shown below in the proton magnetic resonance studies, K_R is not negligible and in fact the steroidal amines **5**, **8**, and **10** have a very high affinity to single-stranded random coils of deoxypolynucleotides. Moreover, the proton magnetic resonance studies show that as the H-bonding capacity of the steroidal amines increcses the binding affinity to the deoxypolynucleotides also increases. The results are in line with the T_m studies and explain the destabilization effect of the bis primary ammonium steroid, **5**, upon deoxypolynucleotides at high steriod/mole of P.

Mahler and coworkers (1964, 1966, 1968) in an extensive study of the interaction of diamino steroids (derivatives of 5α-pregnane, pregn-5-ene, and androst-5-ene) with DNA, have also noted the biphasic effect at low and high steroid/mole of P. Their results indicate that destabilization of the DNA helices at high values of steroid/mole of P is found to be restricted to disecondary and diprimary amino steroids, and is more pronounced for the latter. Mahler *et al.* conclude that three types of DNA–steroid complexes are formed, *i.e.*, one in which a net stabilization of the helical structure is observed, a second in which the DNA helix is extensively destabilized by the presence of steroidal micelles on the surface of the helix, and a third in which the steroidal micelles are organized along the phosphate residue of the denatured form. Although the T_m result reported here does not rule out the above hypothesis, it does indicate that additional forces are also operating. For example, as can be seen from Figure 1, the destabilization effect appears to be due to relative stabilization of the random coils with respect to the helix by the diamino steroids as the H-bonding capacity of the latter is increased. This conclusion not only fits the T_m and proton magnetic resonance results, but is also reasonable since the H-bonding capacity of the random coils is greater than that of the helix.

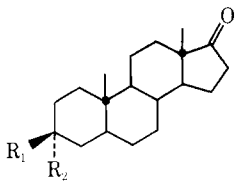
The stereochemical requirements of steroid–nucleic acid interactions were also studied for deoxy- and hybrid polynucleotides (Table I). In all cases, it was found that the ability to stabilize the helical structures relative to the random coils varied in the following order: (1) 3β- > 3α-dimethylamino-17-oxo-(5α)-androstane, (2) 3β- > 3α-trimethylamino-17-oxo-(5α)-androstane, (3) 3β,17β- > 3α,17β- > 3β,17α-bisdimethylamino-(5α)-androstanes, and (4) 3β,17β- > 3α,17β- > 3α,17α-diamino- (5α)-androstanes. Similar results are also obtained by Mahler's group using 3,20-bisamino-(5α)-pregnane systems. The significance of this order is still unclear at present.

Ultraviolet and Circular Dichroism Studies. Ultraviolet absorption and circular dichroism spectra of the complexes formed between 3β,17β-bisdimethylamino-(5α)-androstane (**10**) with deoxypolynucleotides, hybrid, and ribopolynucleotide at various steroid/mole of P were taken. The results are consistent with the T_m studies and are shown in Figure 2. The following interesting points may be noted: (1) increasing concentrations of **10** cause a hyperchromic effect for the entire absorption band in the region of 230–290 mμ, as well as a slight red shift in the absorption maxima of the polynucleotides; (2) the extent of hyperchromism and red shift is dependent on the polynucleotide and reaches a limiting value at high steroid/mole of P; (3) the $(dA)_n \cdot (dT)_n$, $(d(A\text{-}T)_n)$, and $(A)_n \cdot (dT)_n$ helices exhibit a slight hyperchromism and red shift in the maximum absorption as compared with the double-stranded polyribonucleotide, $(A)_n \cdot (U)_n$. The results are consistent with a slight configurational alteration of the $(dA)_n \cdot (dT)_n$, $(d(A\text{-}T)_n)$, and $(A)_n \cdot (dT)_n$ in the presence of excess concentration of **10**. The circular dichroism spectra are also in line with this interpretation and show the presence of new bands with characteristic molar ellipticities, [Θ]. It should be noted that the steroidal diamine, **10**, does not exhibit a circular dichroism spectra in this region of the spectra, *i.e.*, 220–300 nm.

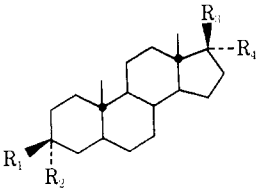
The rA-rU helix, on the other hand, is totally destabilized by **10** as evidenced by the large hyperchromism, red shift in λ_{max}, and diminished magnitude of the circular dichroism spectra characteristic of the random coils.

Proton Magnetic Resonance Studies. It is well known that if the rate of molecular tumbling of molecules in solutions is lower than the typical Larmor frequencies, W_0 (of the order of 10^8–10^9 rads sec^{-1} for protons in the conventional magnetic field), then T_2, the transverse relaxation time, is considerably diminished due to efficient intramolecular proton–proton dipolar coupling (Pople *et al.*, 1959; Jardetsky and Jardetsky, 1962). Since the line width is inversely proportional to T_2, considerable line broadening will be obtained due to slowing down of molecular reorientation. This situation is obtained if the proton is contained in a rigid macromolecule, *e.g.*, DNA (McTauge *et al.*, 1964; McDonald *et al.*, 1964, 1965, 1967),

TABLE I: Variation of T_m of $(dA)_n \cdot (dT)_n$, $(d(A\text{-}T)_n)$, DNA (Salmon Testes), and $(A)_n \cdot (dT)_n$ with 10^{-3} M of the Salts II and 5.0×10^{-5} M of the Salts III.



II



III

Derivative	Compd	R_1	R_2	R_3	R_4	T_m (°C)[a] $(dA)_n \cdot (dT)_n$	(Salmon Testes) $(d(A\text{-}T)_n)$	DNA	$(A)_n \cdot (dT)_n$
II	1	$N^+HMe_2Cl^-$	H			62.3	58.2	76.5	56.6
II	2	H	N^+HMeCl^-			58.1	55.3	74.4	53.5
II	3	$N^+Me_3Br^-$	H			61.5	58.3	76.6	56.3
II	4	H	$N^+Me_3Br^-$			60.2	58.0	76.0	55.4
III	5	$N^+H_3Cl^-$	H	$N^+H_3Cl^-$	H	64.3	61.5	78.5	58.8
III	6	H	$N^+H_3Cl^-$	$N^+H_3Cl^-$	H	62.7	58.9	77.8	56.8
III	7	H	$N^+H_3Cl^-$	H	$N^+H_3Cl^-$	59.2	56.7	77.3	56.5
III	8	$N^+MHe_2Cl^-$	H	$N^+H_2Cl^-$	H	66.5	60.3	79.4	59.4
III	9	$N^+HMe_2Cl^-$	H	H	$N^+H_3Cl^-$	61.5	56.8	77.5	56.0
III	10	$N^+HMe_2Cl^-$	H	$N^+HMe_2Cl^-$	H	68.3	63.5	80.5	60.3
III	11	$N^+HMe_2Cl^-$	H	H	$N^+HMe_2Cl^-$	60.4	56.5	76.8	56.7
III	12	H	$N^+HMe_2Cl^-$	$N^+HMe_2Cl^-$	H	64.2	59.7	78.3	57.7
None						55.2 ± 0.3	50.4 ± 0.3	74.2 ± 0.2	52.7 ± 0.3

[a] In 0.025 M sodium phosphate buffer (pH 6.3).

TABLE II: Chemical Shifts (Parts per Million) from DSS[a] (±0.01) and Line Width at Half-Height in Hertz ($\Delta\nu_{1/2}$) of Free and DNA-Bound Salts II and III at Various Temperatures.[b]

System	Temp, °C	ppm from DSS and Line Width ($\Delta\nu_{1/2}$) $C_{17}N^+CH_3$	$C_3N^+CH_3$	$C_{19}CH_3$	$C_{18}CH_3$	SP[c]
1[d]	32		2.85 (1.5)	0.88[e]	0.85[e]	0.50–2.50
1[f]	35		2.84 (1.8)	0.90 (2.0)	0.86 (2.3)	1.00–2.20
1[d]	90		2.84 (1.5)	0.87 (1.5)	0.83 (1.5)	0.50–2.50
1-DNA[f]	35		SUBLN[g]	SUBLN	SUBLN	SUBLN
1-DNA[f]	50		2.77 (7.0)	SNCLBN[h]	SNCBLN	SNCBLN
1-DNA[f]	88		2.81 (2.2)	0.73 (3.0)	0.63 (3.0)	0.80–2.00
2[d]	32		2.91 (1.5)	0.89 (1.8)[i]		0.50–2.50
2[f]	35		2.87 (1.7)	0.88 (4)		1.00–2.20
2[d]	90		2.90 (1.5)	0.87 (1.5)		0.50–2.50
2-DNA[f]	35		SUBLN	SUBLN	SUBLN	SUBLN
2-DNA[f]	50		2.85 (1.4)	SNCBLN	SNCBLN	SNCBLN
2-DNA[f]	88		2.89 (2.0)	0.77 (3)	0.73 (3)	0.80–2.00
3[d]	32		3.08 (1.8)	0.89[e]	0.86[e]	0.50–2.50
3[f]	35		3.03 (2.0)	0.88 (2.0)	0.85 (2.0)	1.00–2.20
3[d]	90		3.05 (1.5)	0.87[e]	0.84[e]	0.50–2.50
3-DNA[f]	35		3.00 (15)	SUBLN	SUBLN	SUBLN
3-DNA[f]	50		3.00 (6.0)	SNCBLN	SNCBLN	SNCBLN
3-DNA[f]	88		3.03 (2.4)	0.76 (3)	0.68 (3)	0.80–2.00
4[d]	32		3.08 (1.8)	0.89[e]	0.85[e]	0.50–2.50
4[f]	35		3.03 (2.0)	0.88 (2)	0.85 (2)	1.00–2.20
4[d]	90		3.07 (2.0)	0.87[e]	0.85[e]	0.50–2.50
4-DNA[f]	32		3.00 (12)	SUBLN	SUBLN	SUBLN
4-DNA[f]	50		3.01 (7)	SNCBLN	SNCBLN	SNCBLN
4-DNA[f]	88		3.02 (3.2)	0.77 (3)	0.71 (2)	0.80–2.00

TABLE II (*Continued*)

System	Temp, °C	ppm from DSS and Line Width ($\Delta\nu_{1/2}$)				
		$C_{17}N^+CH_3$	$C_3N^+CH_3$	$C_{19}CH_3$	$C_{18}CH_3$	SP[c]
8[d]	32		2.84 (1.5)	0.83[e]	0.80[e]	0.70–2.30
8[f]	39		2.84 (1.5)	0.83[e]	0.80[e]	0.70–2.20
8[d]	90		2.84 (1.4)	SNCBLN	SNCBLN	0.50–2.30
8–DNA[d]	32		SUBLN	SUBLN	SUBLN	SUBLN
8–DNA[f]	39		SUBLN	SUBLN	SUBLN	SUBLN
8–DNA[d]	58		SNCBLN	SUBLN	SUBLN	SUBLN
8–DNA[d]	90		2.80 (5.0)	SNCBLN	SNCBLN	SNCBLN
9[d]	32		2.85 (1.8)	0.84 (1.8)[i]		0.60–2.50
9[f]	39		2.84 (1.5)	0.83 (1.8)		0.60–2.50
9[d]	90		2.84 (1.3)	0.83 (2.3)		0.60–2.20
9–DNA[d]	32		SUBLN	SUBLN	SUBLN	SUBLN
9–DNA[f]	39		SUBLN	SUBLN	SUBLN	SUBLN
9–DNA[d]	58		SUBLN	SUBLN	SUBLN	SUBLN
9–DNA[d]	90		2.77 (8)	SNCBLN	SNCBLN	SNCBLN
10[d]	32	2.92 (2.0)	2.84 (1.7)	0.92 (.18)	0.83 (2.0)	0.60–2.40
10[f]	39	2.89 (1.5)	2.82 (1.5)	0.90 (1.8)	0.82 (1.8)	0.70–2.50
10[d]	90	2.91 (1.5)	2.83 (1.5)	0.91 (1.5)	0.82 (1.7)	0.70–2.40
10–RNA[f]	39	2.80 (13)[k]		SNCBLN	SNCBLN	SNCBLN
10–DNA[d]	32	SUBLN	SUBLN	SUBLN	SUBLN	SUBLN
10–DNA[f]	39	SUBLN	SUBLN	SUBLN	SUBLN	SUBLN
10–DNA[d]	58	2.79 (12)		SUBLN	SUBLN	SUBLN
10–DNA[d]	90	2.85[e]	2.80	0.69 (6.0)	0.48 (3.5)	0.35–2.10
11[f]	39	2.88 (2.3)[k]		0.89[e]	0.84[e]	0.60–2.50
11[d]	90	2.84 (1.8)		0.88 (2.0)	0.82 (2.2)	0.60–2.30
11–DNA[f]	39	2.84 (22)		SUBLN	SUBLN	SUBLN
11–DNA[d]	58	2.79 (7.5)		SUBLN	SUBLN	SUBLN
11–DNA[d]	90	2.80 (3.0)		0.68 (3.5)	0.53 (3.0)	0.40–2.00
12[f]	39	2.96 (2.6)[k]		0.92[e]	0.88[e]	0.70–2.50
12[d]	90	2.91 (2.2)		0.92 (2.0)	0.86 (2.2)	0.70–2.50
12–DNA[f]	39	SUBLN	SUBLN	SUBLN	SUBLN	SUBLN
12–DNA[d]	58	2.85 (11)		SUBLN	SUBLN	SUBLN
12–DNA[d]	90	2.88[e]	2.86[e]	0.72[e]	0.57[e]	0.40–2.00

[a] 2,2-Dimethyl-2-silapentanesulfonic acid. [b] In 10^{-4} M sodium phosphate buffer prepared in deuterium oxide, pD = 7.0 ± 0.2. [DNA] = [RNA] = 0.16 mole of P/l. and [steroid]/[mole of P] = 0.13. [c] Steroidal protons in A, B, C, and D rings. [d] Proton magnetic resonance spectra determined on Varian A-60A spectrometer. [e] $\Delta\nu_{1/2}$ not determined, since adjacent peaks overlap. [f] Spectra determined on Varian HA-100D spectrometer. [g] Proton signal is undistinguishable from base-line noise (SUBLN). [h] Proton signal is not clearly distinguishable from base-line noise (SNCBLN). [i] The chemical shift for $C_{19}CH_3$ and $C_{18}CH_3$ protons is similar, leading to a coalesced signal. [j] Spectra determined on Varian A-60 spectrometer. [k] The chemical shift for $C_{17}N^+CH_3$ and $C_3N^+CH_3$ protons is similar, leading to a coalesced signal.

or the proton is contained in a slowly tumbling small molecule bound to a macromolecule (Gabbay *et al.*, 1969a,b; Jardetsky *et al.*, 1963; Hollis, 1967; Fischer and Jardetsky, 1965; Jardetsky and Wade-Jardetsky, 1965; Metcalfe *et al.*, 1968; Gerig, 1968; Raftery *et al.*, 1968; Thomas, 1966; Spotswood *et al.*, 1967; Raftery *et al.*, 1969). It should be pointed out that the possible effect of chemical shift nonequivalence arising from varying binding sites within a system also plays a role in determining line widths of the proton signals. For example, a broadened line signal will be observed if the rate of exchange between various binding sites is approximately equal to the time scale of the proton magnetic resonance experiment. However, if the rate of exchange is either faster or slower than the time scale of the proton magnetic resonance experiment, sharp resonance lines are obtained. In the proton magnetic resonance experiments cited below (see Figure 3 and Table II) a total broadening phenomenon is being observed, *i.e.*, the proton magnetic resonance signals of the steroidal nucleus in the DNA complex were so broadened at 30–70° that the signals were indistinguishable from base-line noise of the instrument. These results can only be interpreted in terms of restricted molecular tumbling of the steroidal nucleus.

Proton magnetic resonance experiments on polynucleotide–amine complexes were performed in 10^{-4} M sodium phosphate buffer prepared in deuterium oxide, pD 7.0 ± 0.2. The polynucleotide concentration was 0.16 mole of P/l. and (steroid)/(mole of P) = 0.13. Under these conditions the steroidal amines II and III are fully bound as evidenced by the total broadening of the steroidal proton signals in the DNA complex at 32° (Table II and Figure 3). Proton magnetic resonance experiments were also performed using aliphatic polyammo-

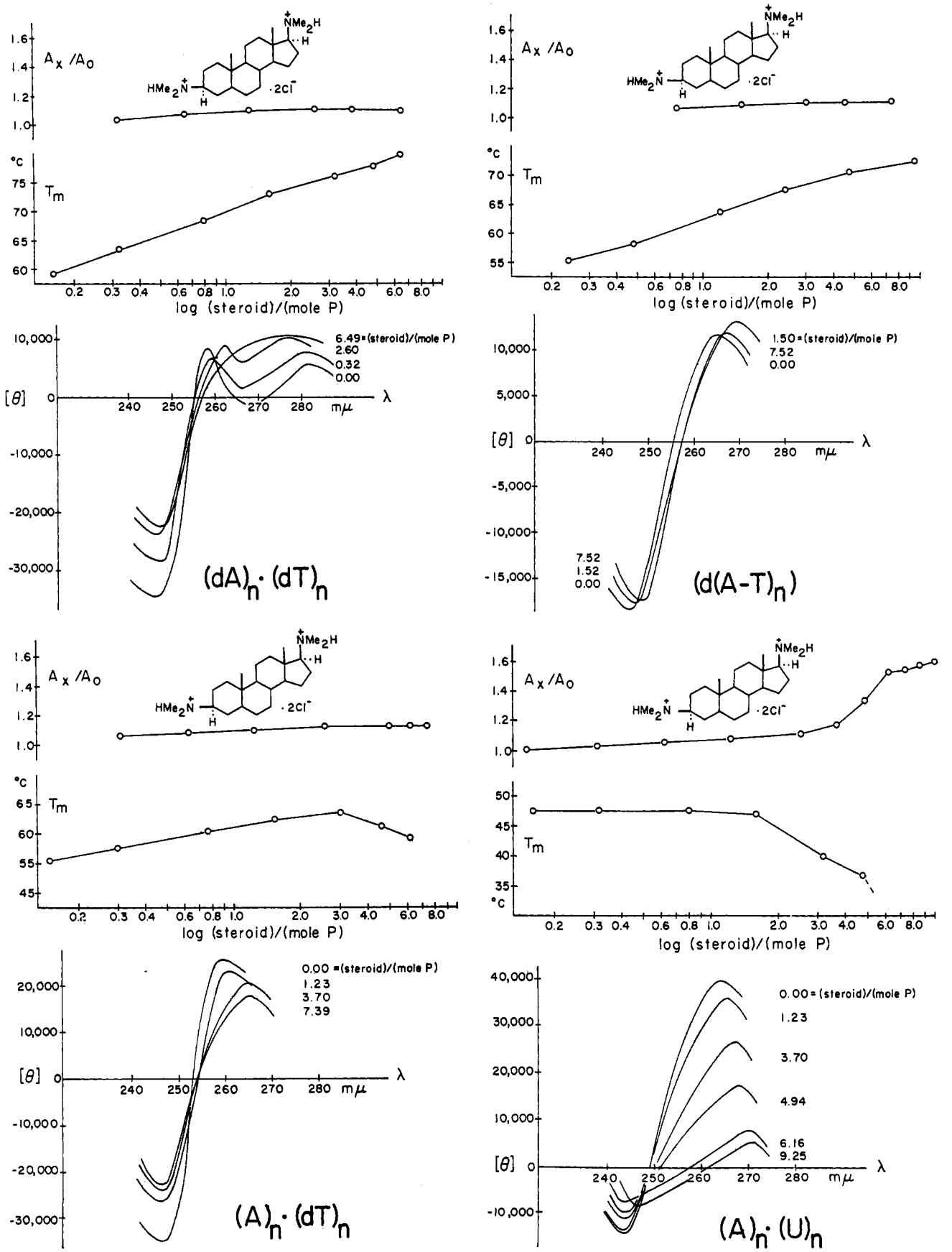


FIGURE 2: The effect of increasing concentration of steroidal amine, **10**, on the absorption of nucleic acids at 260 mμ (A_x) relative to the absence of **10** (A_0), the T_m of the nucleic acids, and the circular dichroism of the nucleic acids. (a) $(dA)_n \cdot (dT)_n$, (b) $(d(A\text{-}T)_n)$, (c) $(A)_n \cdot (dT)_n$, and (d) $(A)_n \cdot (U)_n$.

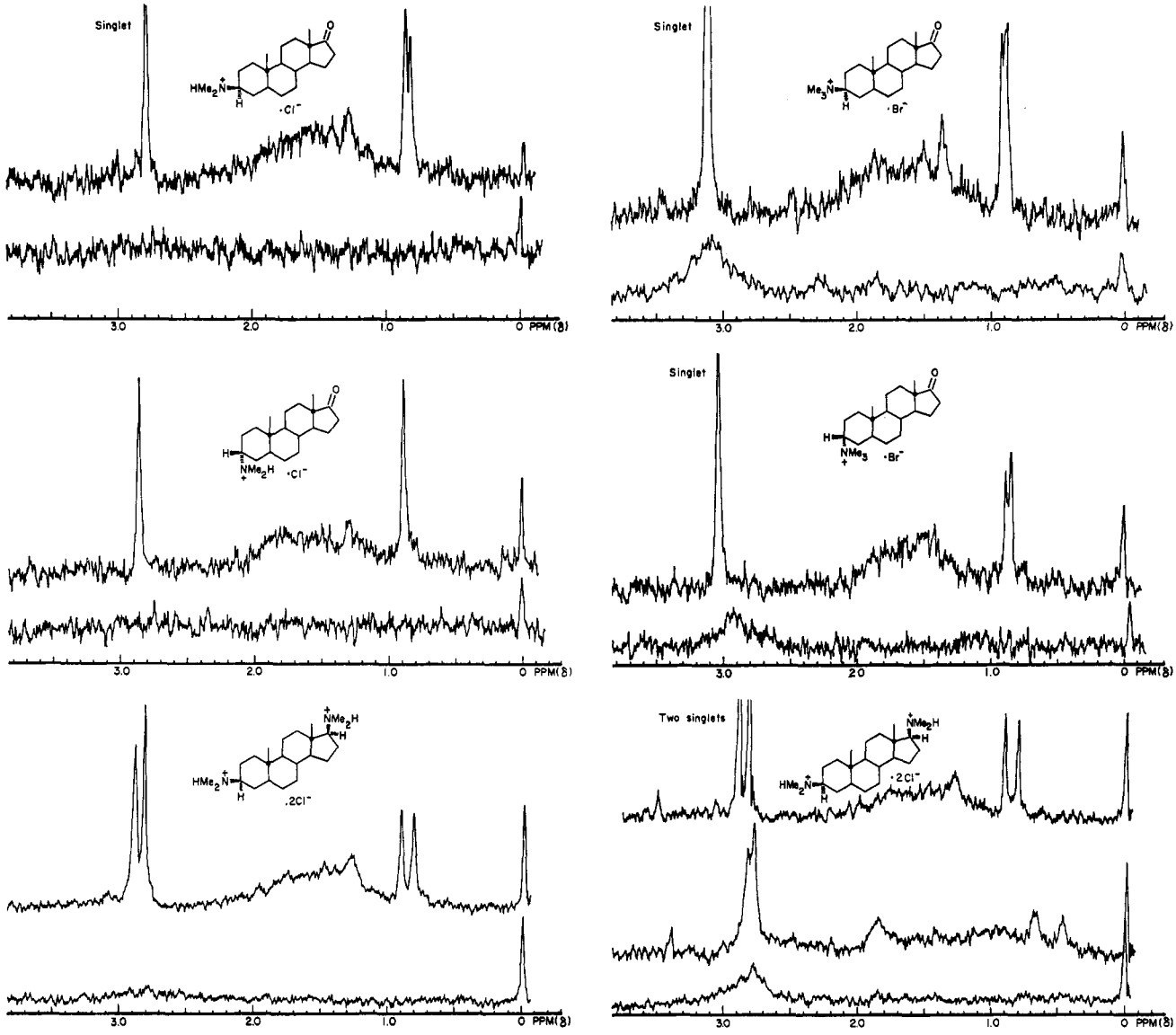
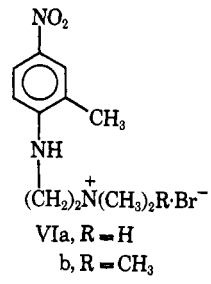


FIGURE 3: The partial 60-MHz proton magnetic resonance spectra of steroidal amines in the presence (bottom) and absence (top) of DNA at 39°; (a) steroidal amine **1**; (b) steroidal amine **3**; (c) steroidal amine **2**; (d) steroidal amine **4**; (e) steroidal amine **10**; (f) steroidal amine **10**; at 90° (top); in the presence of DNA at 90° (middle) and 58° (bottom).

nium salts, *i.e.*, I, $R_1R_2R_3N^+(CH_2)_nN^+R_1R_2R_3 \cdot 2Br^-$, where $R_1 = R_2 = R_3 =$ H and CH_3, and $n = 2$–6; IV, spermidine derivatives, $RRHN^+(CH_2)_3N^+HR(CH_2)_4N^+HRR \cdot 3Cl^-$, where R = H and CH_3; and V, spermine derivatives, $RRHN^+(CH_2)_3N^+HRCH_2(CH_2)_2 \cdot 4Cl^-$, where R = H and CH_3. Under the conditions of the proton magnetic resonance experiments, *i.e.*, 0.16 mole of P/l. of DNA and (amine/mole of P) = 0.13, the alkylpolyammonium salts are assumed to be fully bound to DNA. The assumption is reasonable since the diammonium salts, I, are known to bind to DNA about −200 fold more strongly than Na^+ (Passero *et al.*, 1970). Moreover, at lower DNA concentrations, *i.e.*, 0.02–0.08 mole of P/l., where (amine/mole of P) ≈1.0, precipitation of the DNA complex occurs—presumably due to the formation of a neutral 1:1, ammonium–phosphate, complex. It is found that the pmr spectra of the free and the DNA bound alkylpolyammonium salts I, IV, and V are identical. The significance of this is that the rate of tumbling of amine molecules in DNA–aliphatic diamine or polyamine complexes is considerably greater than in DNA–steroidal amine complexes.

Comparing the DNA complexes to steroidal tertiary ammonium salts **1** and **2** and with the corresponding quaternary ammonium salts **3** and **4** as reported in Table II and Figure 3, a number of observations can be made. First, at 35°, the proton magnetic resonance signals of the N^+-methyl protons of tertiary ammonium salts **1** and **2** in the DNA complex were so broadened that the signals were undistinguishable from base-line noise. However, in the case of DNA complexes with the corresponding quaternary ammonium salts **3** and **4**, the resonance signals of the latter were clearly distinguishable from base-line noise at the same temperature and exhibited a signal width at half-height ($\Delta\nu_{1/2}$) of 15 and 12 cps, respectively. These results may be explained in terms of restricted rotation of the tertiary ammonium group as compared to the quaternary ammonium group. This suggests but does not unequivocally prove that there are hydrogen-bonding interactions in the DNA complexes with the epimeric tertiary ammonium salts **1** and **2**. Another explanation for this behavior is that the steric requirements of the quaternary ammonium group cause the steroid to assume a different type

of orientation than that when the tertiary ammonium group is present. Evidence against this explanation is that similar behavior has been noted in a completely different system, VIa and -b (Gabbay *et al.*, 1969b). The differences in stereochemical configuration of VI *vs.* the steroids II are quite considerable, and hence it is unlikely that VI and II must both assume an analogous change in orientation in the DNA complex upon going from tertiary ammonium to quaternary ammonium moieties.



VIa, R = H
b, R = CH_3

Secondly, the proton signals of the steroidal nucleus (including the C-18 and C-19 methyl groups) in DNA complexes of the salts **1–4** are undistinguishable from base-line noise at 35°. These results suggest that the rate of tumbling about the steroidal nucleus is considerably slowed in the DNA complex due to steric hindrance. In addition, increasing temperature causes a shielding and sharpening of the resonance lines for the protons of the steroidal nucleus. This is indicative of more rapid tumbling and efficient averaging of the chemical shift environment of the steroidal protons at the higher temperature. Furthermore, the pmr spectra at 88° showed that melting of the DNA helix also occurred, as evidenced by the appearance of the thymine methyl peaks at 1.88 and 1.76 ppm (McDonald *et al.*, 1964, 1967). Moreover, it can be seen that denatured DNA at 88° still binds to the steroidal molecules, as indicated by the different chemical shifts of the steroidal protons, including the C-18 and C-19 methyl groups, when compared to the free molecule.

DNA complexes with the epimeric primary–tertiary diamines **8** and **9** also exhibit broadened N^+-methyl and steroidal proton signals that are undistinguishable from base-line noise at 32°. The steroidal proton signals in the DNA complex, including the C-18 and C-19 methyl protons, are not clearly distinguishable from the base-line noise at 90°. This behavior at 90° is different from that observed with the amino ketones **1–4** discussed earlier, and the bistertiary amino steroids **10–12**. In the case of DNA complexes with the salts **1–4** and **10–12** at 90°, the C-18 and C-19 methyl proton signals are shifted upfield, and together with the steroidal protons are clearly distinguishable from the base-line noise. This suggests that the primary–tertiary diamino steroids **8** and **9** undergo a slower rate of tumbling about the steroidal nucleus in the random coil DNA complex at 90° than the salts **1–4** and **10–12**. This may be interpreted in terms of the salts **8** and **9** as having a stronger affinity for the random coil form of DNA than the salts **1–4** and **10–12**. The interpretation is also consistent with the T_m experiments reported in Table I and Figure 1. In the case of salmon testes DNA, increasing concentration of the salts **1** and **10** exhibits increasing T_m. However, with the salt **8** a biphasic response is exhibited, *i.e.*, the T_m increases to a maximum value and then starts to decrease as steroid concentration is further increased. The helix–coil transition in the presence of high concentrations of the salt **8** exhibits stabilization of the random coils relative to the helical structures of the deoxypolynucleotide. However, in the presence of high concentrations of the salts **1** and **10** the opposite effect on the transition is observed, *i.e.*, stabilization of the helical structures relative to the random coils. The pmr results at 90° indicate that the salt **8** has a higher affinity for the random coil form of DNA than **1** or **10**. These results together with the T_m studies are interpreted in terms of a greater H-bonding interaction between the random coil form of DNA and 3β-(tertiary)amino-17β-amino-(5α)-androstane (**8**) as compared to 3β,17β-bis(tertiary)amino(5α)-androstane (**10**).

Nuclear magnetic resonance experiments of DNA complexes with the bis(primary amino) steroids **5–7** were unsuccessful when performed under the same conditions as the salts **1–4** and **8–12**, since the polynucleotides precipitated out of solution upon addition of the steroidal diamine. This may be explained in terms of a highly cooperative binding of the salts **5–7**, since the (steroid)/(mole of P) ratio is less than 0.2.

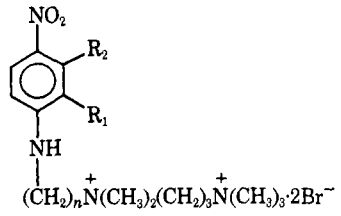
Differences in the stereochemical configurations of the diamines were also apparent from the proton magnetic resonance study. At 39°, the N^+-methyl proton signals of the 3β,-17α-bis(tertiary amino) derivative **11** in the DNA complex are clearly distinguishable from base-line noise but quite broadened ($\Delta\nu_{1/2}$ = 22 cps) (Table II). However, this is definitely not the case for either of the diastereomeric salts **10** or **12** (3β,17β and 3α,17β, respectively). DNA complexes with **10** or **12** at 39° both show that the signals of the N^+-methyl protons are undistinguishable from base-line noise. Furthermore, the resonance signals of the steroidal protons in the nucleus are definitely present in the salt **11**, although the signals are not clearly distinguishable from the base-line noise, whereas the corresponding signals in the salts **10** and **12** are undistinguishable from base-line noise. These results suggest that the rate of tumbling about the steroidal nucleus in the DNA–steroid complex is greater for **11** than for either **10** or **12**. This is in agreement with the T_m experiments reported in Table I which show that the salts **10** and **12** gave greater stabilization than the salt **11** of the helical structures relative to the random coils for the deoxy and hybrid polynucleotides.

Additional evidence for the interpretation that the steroidal amine derivatives II and III selectively interact with a hydrophobic site on deoxypolynucleotides relative to ribopolynucleotides is reported in Table II. The bis(tertiary)amino steroid **10** was studied with torula RNA at 39°. The N^+-methyl protons in the RNA complex are definitely distinguishable from the base-line noise although somewhat broadened ($\Delta\nu_{1/2}$ = 13 cps), as opposed to the DNA complex, where the signals are broadened to such an extent that they are undistinguishable from the base-line noise. The resonance signals of the steroidal protons, including the C-18 and C-19 methyl groups, also differ in the RNA complex compared to the DNA complex. In the RNA complex they are definitely present but are not clearly distinguishable from the base-line noise, whereas they are undistinguishable in the DNA complex. The behavior of the resonance signals in the RNA complex relative to the DNA complex suggests that the rate of tumbling of the steroidal nucleus is greater in the RNA complex than it is in the DNA complex. This is consistent with the T_m, circular dichroism, optical rotatory dispersion, and proton magnetic resonance experiments reported earlier which indicate the presence of a hydrophobic site in deoxypolynucleotides that is larger than that in ribopolynucleotides (Gabbay, 1969; Gabbay *et al.*, 1969a,b).

Steroidal Amine–Nucleic Acid Complex. What is the nature of the steroidal amine interactions with deoxypolynucleotides? Evidence for hydrophobic, H-bonding, and electrostatic inter-

action has already been presented. From molecular framework models, the distance between positively charged diammonium centers in the salts III is approximately 10 Å. While this distance may permit the steroidal diamines III to bridge the 11-Å distance between adjacent phosphate anions on different chains as has been suggested by Mahler for the diaminopregnane systems (Mahler and Dutton, 1964; Mahler *et al.*, 1966, 1968), the exact nature of the complex is still unclear.

For example, where is the hydrophobic site to which the steroidal amines II and III bind? Gabbay (1969), utilizing nitroaniline reporter-labeled diamines, VII, have presented evidence implying that the reporter diamines interact with a hydrophobic site located in the minor groove. Examination



VII

of molecular framework models of a Watson–Crick–Wilkins double helix shows a number of interesting observations. First, the major and minor grooves of the Watson–Crick–Wilkins double helix arise as a result of the position of substitution of the sugars. Inspection of molecular framework models shows that the sugar–phosphate backbone connected to the individual bases is closer on one side of the plane of the base pairs than the other. Therefore, substitution at the 5 position of pyrimidines will place the group in the major groove. Furthermore, the additional hydrogen bond in the G:C base pair lies in the minor groove. It is tempting to state that the site of hydrophobic interactions in deoxypolynucleotide–steroid complexes is located in the minor groove of the double helix. The lower degree of stabilization of salmon sperm deoxyribonucleic acid relative to $(dA)_n \cdot (dT)_n$ and $(d(A\text{-}T)_n)$ may perhaps be explained in terms of a more sterically crowded minor groove in DNA caused by the additional hydrogen bond in G:C base pairs. However, the validity of these interpretations still awaits further clarification and proof.

The possibility of intercalation of the steroidal nucleus between the bases of the double helix was investigated. Perhaps the restricted rotation of the steroidal nucleus in the DNA complex, as shown by proton magnetic resonance, was due to this phenomenon. Molecular framework models of the steroidal nucleus were constructed (Fieser and Fieser, 1959), and the distance between the bottom side and top side of the nucleus (including the van der Waals radii) was found to be approximately 5.9 Å. It was felt that the relatively thick steroidal nucleus would be unlikely to undergo intercalation. However, it was decided to check this hypothesis experimentally. It is known that the viscosity of a DNA solution increases when a planar dye molecule such as proflavin or acridine orange intercalates between base pairs of a double-stranded DNA helix (Lerman, 1961; Drummond *et al.*, 1966; Cohen and Eisenberg, 1969; Armstrong *et al.*, 1970). This increase in viscosity is presumed to be related to the lengthening of the DNA helix upon intercalation of the planar dye molecule. The solution viscosity of salmon testes DNA (3.0×10^{-3} mole of P/l.) in the absence and presence of the bis(tertiary)-amino steroid compound **10** ((steroid)/(mole of P) = 0.69) in 0.025 M sodium phosphate buffer (pH 6.3) was determined. A slight decrease in viscosity was noted in the DNA–steroid complex relative to the same quantity of DNA alone as the control. These results indicate that the steroidal diamine **10** is not undergoing intercalation into the DNA double helix.

In conclusion, it has been shown that there are differences between the interactions of aliphatic and steroidal amines with deoxyribonucleic, hybrid, and ribonucleic acids in solutions. At room temperature, the steroidal amines II and III can selectively stabilize the DNA helical structures, while causing the ribose-containing nucleic acids to unravel and denature. It is shown that the capacity for H bonding in the single-stranded random coils is greater than that of the bihelical structure. Thus, as the H-bonding capacity of the steroidal diamines increases, the interaction with the random coils is enhanced. The results obtained in these studies, together with previous work, strongly suggest the presence of a larger hydrophobic site in deoxyribose- than in ribose-containing helices. Moreover, it is indicated that there are at least two possible modes of binding in diamine–nucleic acid complexes. One mode seems to involve electrostatic interactions, as evidenced by the T_m experiments, and exhibits a relatively greater degree of flexibility, as shown by the proton magnetic resonance results. This mode is exemplified by DNA complexes with aliphatic diamines, I, and polyamines, IV and V. The second mode exhibits greater rigidity, and involves electrostatic and H-bonding interactions as well as a larger degree of hydrophobic bonding. This mode is exemplified by DNA complexes with steroidal amines.

Experimental Section

Materials and Methods. Polyriboadenylic acid (lot no. 55451), polyribouridylic acid (lot no. 63671), polyribocytidylic acid (lot no. 63217), and polyriboinosinic acid (lot no. 46114) were purchased from Calbiochem and were utilized in the polyamine experiments. Polyriboadenylic acid (lot no. 12850), polyribouridylic acid (lot no. 46859), polyribocytidylic acid (lot no. 212726), polyriboinoisinic acid (lot no. 35829), and polydeoxyadenylylthymidylic acid (lot no. 11-12-317) were purchased from Miles Laboratories and were utilized in the diamine experiments. Salmon testes deoxyribonucleic acid (DNA 8BA, ϵ_p^{260} 6500) was purchased from Worthington Biochemical Corp. Stock solutions of the ribohomopolymers were made in 0.025 M sodium phosphate buffer (0.025 M in Na^+) pH 6.3, at $1.0\text{–}1.4 \times 10^{-3}$ mole of P/l. The stock solution of the copolymer deoxyadenylylthymidylic acid was made in 0.025 M sodium phosphate buffer (pH 6.3) at 3.5×10^{-4} mole of P/l. The stock solution of the salmon testes DNA solution was removed and diluted with sodium phosphate buffer to a final concentration of 6.0×10^{-4} mole of P/l., and 0.013 M sodium phosphate buffer (pH 6.3). Aliquots were removed from the stock solutions to determine the polynucleotide concentration, using a Cary Model 14 spectrophotometer.

Polydeoxyadenylic and polydeoxythymidylic acids were lyophilized out of 10^{-3} M Tris-HCl buffer (pH 8.0) and were a gift of Dr. F. J. Bollum. Solutions of the deoxyhomopolymers were made in 0.025 M sodium phosphate buffer (pH 6.3) passed through a column of Sephadex G-25 fine (23 cm long, 1.5-cm diameter, and 75 ml/hr flow rate), and eluted with 0.025 M sodium phosphate buffer (pH 6.3) to remove the Tris-HCl salt. Fractions of the eluent were collected and aliquots were removed to determine the polynucleotide concentration, using a Cary Model 14 spectrophotometer.

T_m determinations were run in 1-ml quartz cuvets thermo-

stated with a Haake constant-temperature water circulator equipped with a Neslab temperature programmer. A Gilford Model 20 spectrophotometer equipped with automatic recording accessories was used, and the temperature of the cell compartment was measured directly with an iron-constantan thermocouple connected to a Leeds-Northrup Model 8680 potentiometer.

Circular dichroism curves were run in the region 200–300 mμ on a Cary Model 60 recording spectropolarimeter equipped with a Model 6001 CD accessory. A 3-ml quartz cuvet thermostated at 25.0° with a Tameson Model T9 constant-temperature water circulator was used.

Proton magnetic resonance spectra were determined on a Varian Model A-60 spectrometer at ambient temperature. Proton magnetic resonance spectra were also determined on Varian Model A-60A and HA-100D spectrometers equipped with Varian variable-temperature controllers, Models V-6040 and V-4343, respectively. Sonicated salmon testes DNA (mol wt <500,000) was used. Torula rRNA (lot no. 44585) was purchased from Calbiochem, and was used without further purification. Sodium 2,2-dimethyl-1,2-silapentanesulfonate was purchased from Nuclear Magnetic Resonance Specialties. The sulfonate was dissolved in deuterium oxide (13 mg/10 ml), and was utilized as the standard. Stock solutions of the polynucleotides (75 mg/ml) were made in deuterium oxide–sodium dimethylsilapentanesulfonate in 10^{-4} M sodium phosphate buffer (pH 7.0 ± 0.2).

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