Nucleic Acid Interactions. VI. Effects of Steroidal Diamines*

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ABSTRACT: The interactions between deoxyribonucleic acid (DNA) and a number of simple steroidal diamines have been investigated and compared, using Mg^{2+} and cadaverine^{2+} as reference compounds, largely by means of thermal transition profiles at very low ionic strength. Under these conditions definite end points are observed that are shown to occur at stoichiometric ratios r = [ligand]/[DNA-P] of 0.5, 0.35, and 0.2 for Mg, cadaverine, and malouetine [5α-pregnan-3β,20α-ylenebis (trimethyl iodide)], respectively. The maximum stabilization obtainable at the end point is about equal for different steroidal ligands and varies only moderately with the (G-C) content of the DNA investigated. The slopes of a plot of T_m *vs.* r, or of r at the end point, therefore, provide one with adequate "Figures of Merit" for a comparison of the stabilizing efficacy of different compounds.

They are strongly dependent on the nature and stereochemistry of the basic substituents in the steroid nucleus, and depend to a significant extent on subtle structural changes, such as the hydrogenation of a single double bond. Diquaternary amines are most effective as stabilizing agents > di 3° ≥ di 2° ≥ di 1° of the same group. Monoamines are ineffective. Diprimary and disecondary, but not ditertiary or diquaternary amines, in addition to stabilization at low r, produce a destabilizing effect at somewhat higher values of r. When irehdiamine (pregn-5-ene-3β,20α-diamine) is added to DNA, T_m is depressed, at values of $r \geq 0.3$, below that characteristic of Na-DNA, while at somewhat higher concentrations its addition leads to the precipitation of denatured DNA. Examination of the transition profiles discloses a decrease in the total hyperchromicity throughout both the stabilizing and the labilizing range, a greatly increased transition width in the central portion of the stabilizing region, followed by a reduction at the equivalence point, followed by a second increase and subsequent decrease in the labilizing region. All these results as well as those on other physical and biochemical properties presented can be accommodated by a model which postulates four possible and successive modes of interaction beween originally helical polynucleotides and steroidal diamines as the concentration of the latter is increased: (a) a phosphate bridging, charge-neutralizing, and hence stabilizing interaction producing a modified helix, (b) a nonbridging, charge-reversing, and hence labilizing interaction with a partially disoriented polymer, (c) formation of induced micelles by stacking of ligand molecules organized along the phosphate residue of the denatured form, and (d) phase separation and precipitation.

In a recent publication we described some of the effects of the alkaloid cyclobuxine (a steroidal diamine) on the thermal, reversible helix → coil transition of highly ordered polynucleotides such as deoxyribonucleic acid (DNA) and soluble ribonucleic acid (s-RNA) (Mahler and Dutton, 1964). These interactions were peculiar in the sense that one and the same agent appeared to stabilize the helical conformation when added in low, and the coil when present at slightly higher concentrations. Certain important questions were, however, left unanswered. Among them were the stoichiometry of the reactions, the possible implications of such reactions on chemical and biological activity of the reactants, and finally the nature and the mechanism of the actual chemical reaction involved. In the present report, we shall attempt to address ourselves to all these problems by somewhat more refined studies on the interaction of DNA (and certain other helical polynucleotides) with irehdiamine A, a simple steroidal diamine (pregn-5-ene-3β,20α-diamine),[1] and then extend them to a large number of other steroidal amines of known structure.

Materials and Methods

DNA Samples. The calf thymus DNA used was a commercial sample (Worthington Biochemicals). Its physical properties have been described (Mahler *et al.*, 1964) as have those of bacteriophage T2 (R. May) and

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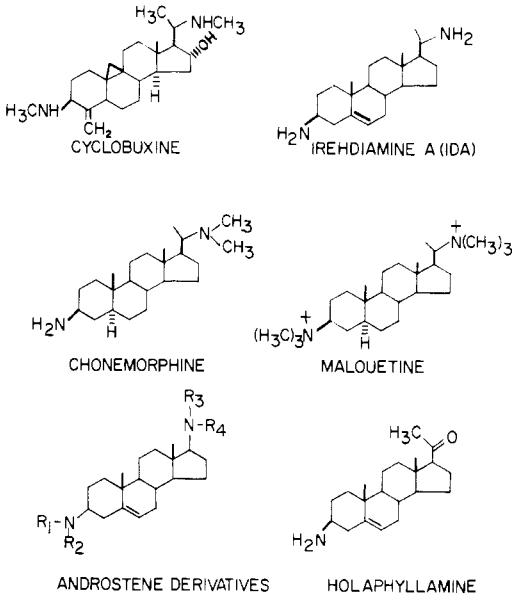
[1] Abbreviations used: CB, cyclobuxine D; IDA, irehdiamine A; T_m, midpoint of thermal transition; σ^2/s, dispersion of the transition; h_{max}, maximal hyperchromicity = A_{260} (maximal)/A_{260} (15°); STE, sodium chloride–Tris–disodium EDTA; ATP, GTP, UTP, and CTP, adenosine, guanosine, uridine, and cytidine triphosphates; EP, the end point for stoichiometric binding.



SCHEME 1: structures of steroids used in this investigation.

Pseudomonas aeruginosa and *Bacillus subtilis*. The latter two were furnished us by Professor M. Mandel. The sample of *Micrococcus lysodeikticus* DNA was prepared by the method of Marmur (1961). The DNA's from *Escherichia coli* K 12 and *Clostridium perfringens* as well as the s-RNA from *E. coli* (stripped) were commercial samples purchased from Worthington Biochemicals, Freehold, N. J., and the General Biochemicals Co., Chagrin Falls, Ohio. Their $\epsilon_{mM}(P)$ values were determined to be, respectively, 6.50, 6.40, and 7.80, all in 0.15 M NaCl–0.015 M Na citrate. Their maximal hyperchromicities h_{max} at 260 mμ in the same buffer were 1.36, 1.30 and 1.25, respectively. The subunit DNA and the corresponding nucleohistone have been described by Welsh (1962). The DNA was fractionated on methylated albumin Kieselguhr columns (Mandell and Hershey, 1960) and three main fractions, eluted by increasing the ionic strength, were pooled and used here (Dutton, 1965).

Steroidal Amines. Cyclobuxine (Brown and Kupchan, 1962) was a gift of Dr. Marvin Carmack. The isolation and properties of all other steroidal di- and monoamines used have been described by one of us (R. G.) and his collaborators (Janot *et al.*, 1959, 1960, 1962; Truong-Ho *et al.* 1963; Khuong-Huu-Lainé *et al.*, 1965; Leboeuf *et al.*, 1965). All amines not already furnished as salts were made as 0.0100 M stock solutions of their hydrochlorides by dissolving a carefully weighed sample of the free amine in 1 ml of spectral grade methanol, adding the calculated quantity of standardized HCl sufficient for exact neutralization, and making up the solution in a volumetric flask to 10.00 ml with triple glass-distilled H_2O. Hydrochlorides were made up directly in H_2O. Malouetine was the diiodide.

Buffers. In order to hold metal contamination to a minimum the polynucleotide samples used were dissolved in and then dialyzed for 24 hr *vs.* STE buffer (4.0×10^{-3} M NaCl–1.0×10^{-3} M Tris–1.0×10^{-3} M disodium EDTA, pH 7.6). For work at low ionic strength (stoichiometric experiments), the optical density at 260 mμ (A_{260}) of the sample was adjusted to 15.0, and 0.100 ml was added with a calibrated micropipet to 2.90 ml of triple glass-distilled water (final $A_{260} = 0.50$).

Thermal Transition Profiles. Most of the early work was carried out with a manual Beckman spectrophotometer adapted for studies of this kind as previously described (Mahler and Dutton, 1964; Mahler and Mehrotra, 1963). More recently we have used a recording "thermospectrophotometer" (Szybalski and Mennigman, 1961) manufactured by the Gilford Instrument Co., Yellow Springs, Ohio. Typical recordings produced by this instrument which monitors and records the absorbancy in three experimental and one blank cuvet equipped with a thermosensor, as well as the temperature in the latter by means of an auxilliary channel, are shown as Figure 8. Heating rates were generally of the order of 1°/min. All data were analyzed and the parameters extracted as described in our previous publication (Mahler and Dutton, 1964).

Other Physical Measurements. Analytical ultracentrifugations were performed at 20° in a Spinco Model E ultracentrifuge equipped with ultraviolet optics. The speed used generally was 29,500 rpm. Photographs were scanned and analyzed by a Spinco Analytrol densitometer. Viscosities were determined in Cannon-Fenske viscometers of the bulb type.

DNA-Dependent RNA Polymerase. The enzyme was purified and the activity assayed as described by Chamberlin and Berg (1962). The concentrations of reactants, all per 1.0 ml, were: DNA 30–40 μg, 20 mμmoles of ATP, GTP, UTP, and CTP of which one (usually C or G) was labeled with ^{14}C, 1 μmole of $MnCl_2$, 4 μmoles of $MgCl_2$, and 50 μmoles of Tris, pH 7.9.

Results

Comparison of IDA with CB and Other Counterions. The presence of a number of additional functional groups in CB made interpretations of results with this agent tenuous since the observed interactions might have been due, at least in part, to these functions rather than the two, appropriately spaced, methylamino groups. It therefore appeared imperative to extend our studies to the simplest steroidal diamine possessing the desired structural features. This desideratum was admirably satisfied by IDA (Scheme I) and we therefore first compared the two steroids in our standard saline–Tris–EDTA (STE) buffer. As seen in Figure 1, IDA added to calf thymus DNA does indeed elicit the characteristic biphasic response first observed with CB. As a matter of fact IDA, lacking all elements of the additional functionality present in CB, is more effective than the latter by about one order of magnitude, when the two agents are compared on the basis

TABLE I: Relative Effectiveness of Cations in Stabilizing Calf Thymus DNA in STE Buffer.

DNA Prepn	Nature	Added Cation Concn ($\times 10^5$)	T_m (°C)	ΔT_m (°C)	$\sigma_{2/3}$
1[a]	CB^{2+}	2.0	71.7	10.2	10.5
		10.0	75.7	14.2	10.0
		42	59.9	−1.6	10.0
		100	49.4	−12.1	7.0
	IDA^{2+}	0.25	64.0	2.7	11.0
		0.50	65.3	4.0	15.7
		1.0	71.5	10.0	14.0
		4.0	70.2	8.7	17.3
		10.0	41.6	−19.7	7.5
	Mg^{2+}	100	71.8	10.5	6.0
	$Cadaverine^{2+}$	10	74.8	13.5	6.8
	$Spermine^{2+}$	0.5	79.8	18.5	9.9
	Na^+	1000	69.1	7.8	9.8
		5000	75.5	14.2	8.0
2[b]	CB^{2+}	10	76.1	17.1	13.3
		100	49.2	−10.0	4.8
	IDA^{2+}	1	72.0	12.8	16.6
		10	41.2	18.0	10.5
	Mg^{2+}	100	68.3	9.1	20.2
	$Cadaverine^{2+}$	100	84.6	25.4	7.9
	$Spermine^{2+}$	0.5	78.9	19.7	14.7

[a] DNA preparation 1 was our standard commercial preparation — $T_m = 61.3$ ($\sigma_{2/3} = 10.5$). [b] DNA preparation 2 was the unfractionated subunit preparation described in the section on Materials and Methods. Its T_m and $\sigma_{2/3}$ were 59.2 and 11.1°, respectively.

of the molar concentration required to produce equivalent stabilizing or destabilizing effects (Table I). We therefore conclude that the primary phenomena of interest have at least not been *enhanced* by the introduction of additional functional groups into the steroid nucleus. In Table I we also present a qualitative comparison of the relative effectiveness of a number of other countercations as measured by the concentration required to produce roughly equivalent increases in T_m for a number of different preparations of calf thymus DNA. Among those used were our standard commercial preparation, a subunit preparation furnished us by Dr. Welsh, and three fractions prepared from the latter by chromatography on methylated serum albumin. With all these preparations the results are similar, and the order of effectiveness is for stabilization: spermine ≈ IDA > cadaverine ≈ CB > Mg ≫ Na; for labilization: IDA > CB. It also appeared from these studies that the *maximal* stabilization obtainable with all the different oligocations is roughly equal as is, for the two steroids, the extent of labilization. The latter (with 2×10^{-3} M CB or 2×10^{-4} M IDA) leads to complete denaturation of the sample at a temperature of <20° accompanied by precipitation of the denatured material.[2] Another characteristic feature for the transition in the presence of IDA is its extreme broadness (*i.e.*, a greater than usual increase in $\sigma_{2/3}$) in conjunction with a decrease in height (a decrease in h_{max}). More explicit and quantitative measurements bearing on all these phenomena could be obtained in a medium of low ionic strength and are therefore deferred to the next section.

Nature of the Transition at Low Ionic Strength. Dove and Davidson (1962) established that strong interactions between DNA and certain inorganic cations could be rendered stoichiometric at an ionic strength just sufficient to maintain the polymer in its native, helical conformation at room temperature. The bulk of our measurements described here were performed under these conditions and some typical experiments with IDA, cadaverine, and Mg are shown in Figure 2. It will be observed that all the features established earlier are retained, especially the great increase in $\sigma_{2/3}$ for IDA. Dove and Davidson also observed that $\sigma_{2/3}$ first increased, reached a maximum at a stoichiometric ratio one-half that of the end point, and then decreased again as the end point was approached. They explained

[2] The fact that the precipated DNA is denatured can be established easily: on redissolving the sample in a more dilute solution, and reheating, there is a broad diffuse increase in absorbancy over a broad temperature range with a maximal hyperchromicity amounting to only 5–10%, characteristic of the thermal profile for denatured DNA.

this phenomenon in terms of preferential ligand binding to the helical form of the DNA. A similar pattern is observed with IDA: $\sigma_{2/3}$ first increases, then decreases as the point of maximal stabilization is reached; it then increases and decreases again in the labilizing region and eventually reaches a minimum value at the point of maximal labilization. Throughout the stabilizing range, however, the value of $\sigma_{2/3}$ is greater than that observed with a nonsteroidal stabilizing agent such as the aliphatic diamine cadaverine used throughout these studies for purposes of comparison (Table II). These observations are equally valid, not only for calf thymus DNA where they might have been related to its highly heterogenous nature, but also for all other DNA's investigated. Results for the most homogenous DNA studied, that of bacteriophage T2, are also shown in Table II and Figure 2B.

TABLE II: Effects of IDA and Cadaverine on ΔT_m and $\sigma_{2/3}$.[a]

DNA	Agent	r	ΔT_m (°C)	$\sigma_{2/3}$
Calf thymus			(T_m = 32.5)	15
	Cadaverine	0.11	11.0	19.8
		0.22	20.5	11.6
		0.33	31.7	11.1
		0.44	33.7	8.2
		0.55	40.8	7.7
		0.66	42.6	4.6
	IDA	0.035	5.0	21.8
		0.070	10.0	24.0
		0.11	18.0	26.5
		0.14	31.0	19.7
		0.18	34.2	19.0
		0.22	41.6	8.3
		0.28	42.0	13.2
		0.33	34.6	28.5
		0.36	12.1	23.2
		0.44	11.0	15.6
		0.55	4.6	13.3
		0.66	0.5	12.5
T2			(T_m = 33.5)	10.9
	Cadaverine	0.050	3.2	12.1
		0.10	10.0	11.0
		0.20	15.7	9.4
		0.40	19.0	9.0
		0.60	21.0	8.3
		0.80	28.7	7.5
	IDA	0.025	2.6	14.3
		0.050	3.8	13.3
		0.10	12.0	12.9
		0.20	29.3	8.0
		0.30	32.7	11.5
		0.40	10.0	12.4
		0.50	4.8	19.1
		0.60	1.2	14.1
		0.70	−4.2	7.4
		0.80	−3.4	7.6
		0.90	−5.8	9.7

[a] All results obtained from recorded absorbance temperature profiles as described in the "Methods" section; CT [DNA-P] = 8.0×10^{-5} M in the experiments with cadaverine, 9.0×10^{-5} M in those with IDA; T2 [DNA-P] = 3.5×10^{-5} M in all experiments.

Stoichiometry of the Interaction with Cadaverine and IDA. Preliminary experiments with both T2 and calf thymus DNA completely confirmed Dove and Davidson's contention that for Mg^{2+}, EP, the end point for stoichiometric binding, as measured by the increase in the transition midpoint (ΔT_m) for several different DNA's, occurred at $r = 0.5$, where r = [ligand]/[DNA-P]. It therefore, appeared of interest to extend these studies first to cadaverine and then to IDA. As shown in Figure 3 the end point for cadaverine is observed at $r = 0.35$, with DNA's varying widely in base ratios, and differs from that for the inorganic divalent cation in this and other respects. The slope $\Delta T_m/\Delta r$ exhibits only a weak negative correlation with X_{GC}, the mole per cent (G + C) in the DNA, while the T_m at the end point proves to be a linear function of X_{GC} but with a slope of $^{14}/_{24} = 0.58$ that of T_m *vs.* X_{GC}. Thus, under these conditions of stoichiometric interaction ΔT_m [*i.e.*, T_m(EP) − T_m] is an inverse function of X_{GC}, in complete analogy to the situation in the presence of higher concentrations of monovalent counterions discussed previously (Mahler and Mehrotra, 1962, 1963).

Similar stoichiometric experiments with IDA are shown in Figure 4. Here the position of the end point cannot be established with certainty since the characteristic labilizing interaction supervenes at values of $r > 0.25$. Instead of an end point then, T_m reaches a maximum value in this region and then declines. Although the exact stoichiometry cannot be established

TABLE III: Dependence of Stabilization or Labilization by IDA on Stoichiometric Ratio.

Concn (× 10^5)			
DNA	IDA	r	T_m (°C)
2.25	0.60	0.266	69.5
4.5	1.2	0.266	71.5
7.5	2.0	0.266	72.6
9.0	2.4	0.266	71.5
			Av 71.0 ± 1.0
2.25	1.0	0.444	45.6
4.5	2.0	0.444	43.5
7.5	3.3	0.444	44.5
9.0	4.0	0.444	44.6
			Av 44.5 ± 0.9

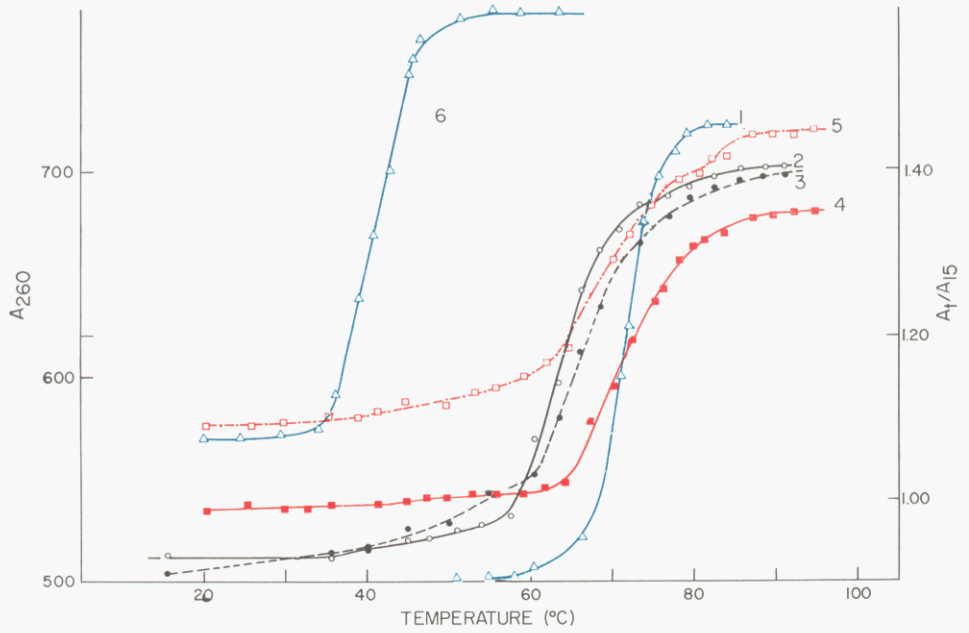


FIGURE 1: Thermal transitions of calf thymus DNA in STE buffer. Curves: 1, 10^{-3} M Mg; 2, 2.5×10^{-6} M IDA; 3, 5.0×10^{-6} M IDA; 4, 1.0×10^{-5} M IDA; 5, 2.0×10^{-5} M IDA; 6, 1.0×10^{-4} M IDA.

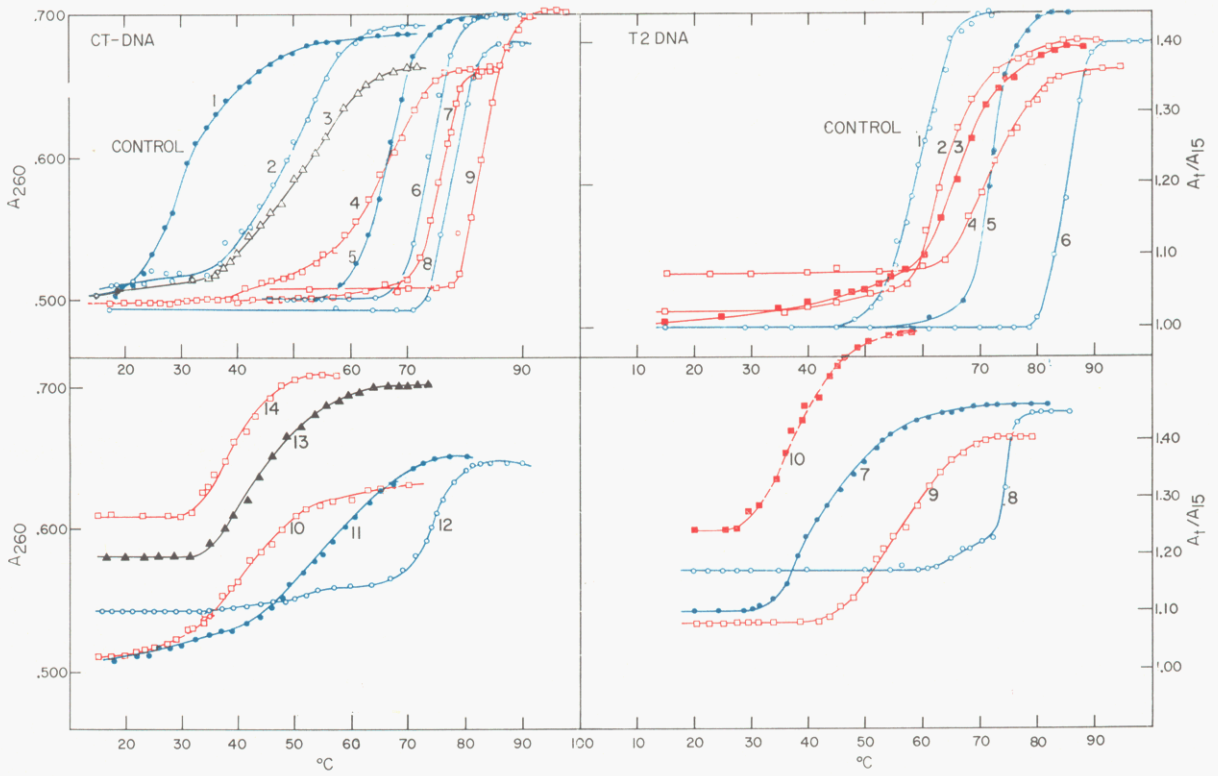


FIGURE 2: Thermal transitions of DNA in presence of various cations. Calf thymus DNA in dilute STE buffer (2A). Curves: 1, control; 2, 2×10^{-5} M Mg; 3, 1×10^{-5} M cadaverine; 4, 2×10^{-5} M cadaverine; 5, 4×10^{-5} M Mg; 6, 6×10^{-5} M Mg; 7, 4×10^{-5} M cadaverine; 8, 1.0×10^{-4} M Mg; 9, 1.0×10^{-4} M cadaverine; 10, 5×10^{-6} M IDA; 11, 1.0×10^{-5} M IDA; 12, 1.95×10^{-5} M IDA; 13, 3.9×10^{-5} M IDA; 14, 4.2×10^{-5} M IDA. Bacteriophage T2 DNA in STE buffer (2B). Curves: 1, control; 2, 2.5×10^{-6} M cadaverine; 3, 5.0×10^{-6} M cadaverine; 4, 1.0×10^{-5} M cadaverine; 5, 10^{-3} M Mg; 6, 10^{-3} M cadaverine; 7, 4.5×10^{-6} M IDA; 8, 9.0×10^{-6} M IDA; 9, 1.8×10^{-5} M IDA; 10, 3.6×10^{-5} M IDA.

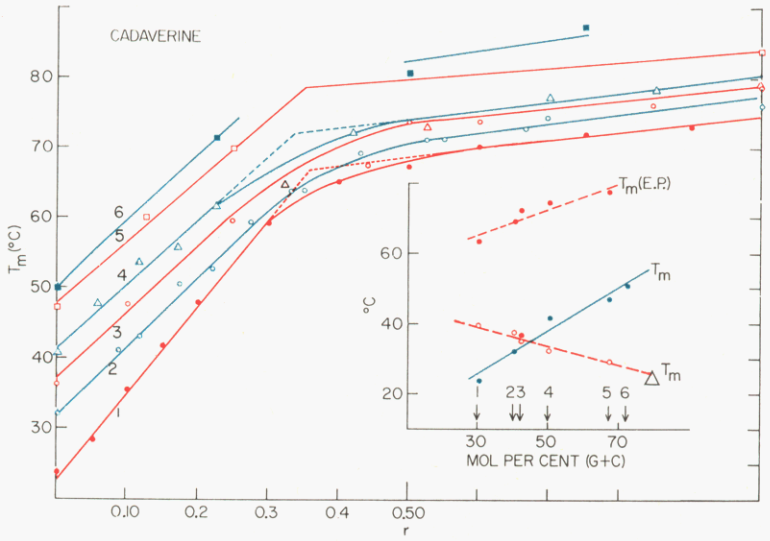


FIGURE 3: T_m as a function of cadaverine concentration. T_m in dilute STE buffer is plotted *vs.* $r \equiv$ [ligand]/[DNA-P]. The DNA's used were obtained from the following organisms: 1, *Cl. perfringens;* 2, calf thymus (results of two separate experiments); 3, *B. subtilis;* 4, *E. coli B;* 5, *Ps. aeruginosa;* 6, *M. lysodeikticus.* In the insert are plotted T_m, the transition midpoint in the absence of ligand; T_m(EP), the transition midpoint at the equivalence point, *i.e.*, the point of intersection in the main figure; and $\Delta T_m = T_m(EP) - T_m$ *vs.* base composition of the DNA used.

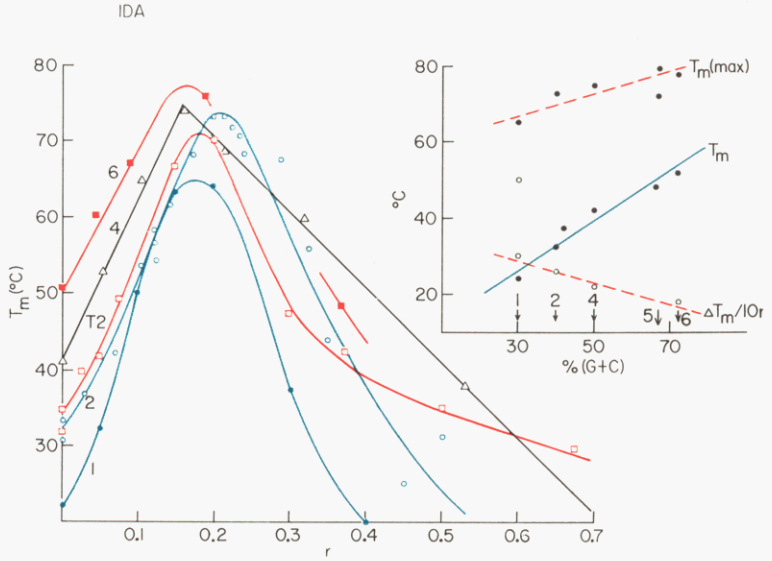


FIGURE 4: T_m as a function of IDA concentration. For details see legend to Figure 3. In the insert we plot T_m, T_m(max), the transition midpoint at that IDA concentration which gives maximum stabilization, and $\Delta T_m = T_m(max) - T_m$ *vs.* base composition.

on the basis of these experiments, several points are of interest: (1) the values of T_m(max) for IDA are very close to those of T_m(EP) for cadaverine; (2) the slopes $\Delta T_m/\Delta r$ show the customary negative correlation with X_{GC} but are considerably greater than those with cadaverine, which therefore is a less effective stabilizing agent mole for mole than is IDA; (3) there is some indication of nonlinearity in both the rising and the descending branch of the experimental curves which therefore assume a bell shape. This might be interpreted in terms of either nonequivalence of binding sites or cooperativeness in the binding steps, or both.

If the interaction between ligand and DNA is indeed stoichiometric, and the dissociation of the resulting complex virtually zero, one would expect that the extent of stabilization (or labilization) depends solely on r and not at all on the actual concentrations of the reactants involved. An experiment designed to test this

prediction is shown in Table III. The two mole ratios chosen were selected from the data of Figure 4 to afford close to maximal stabilization and labilization respectively. The hypothesis is borne out in a satisfactory manner.

Another, more direct, demonstration of complex formation is provided by dialysis experiments. When IDA is added to DNA in stabilizing concentrations, and the resultant solution dialysed extensively against our standard dilute STE buffer (STE diluted 1:30), the thermal profiles of the solution before and after dialysis are identical.

Comparison of Different Steroidal Amines. The availability of a large number of different steroidal mono- and diamines of known and defined structure made it possible to assess and compare the relative importance of various parameters in these molecules in their interaction with DNA. For this purpose we selected calf thymus DNA as our standard and conducted our comparisons by testing the various compounds selected at five or six different concentrations under conditions analogous to those just described for IDA. All absorbance–temperature profiles were recorded automatically and the data evaluated on the basis of a number of "Figures of Merit" extracted either directly from the profiles or from plots of T_m *vs.* r (Figures 5–7). Three different series of amines were studied: the simplest set of diamines constituted by the androst-5-ene-3,17 series (Figure 5), those of the pregn-5-ene series to which IDA itself belongs (Figures 6A and B), with the amino group at positions 3 for monoamines, and at both 3 and 20 for diamines, and the related dihydro series, *i.e.*, those belonging to the 5α-pregnane series (Figure 7). The Figures of Merit (Table IV) include the slopes for the stabilizing ($\Delta T_m/r$) and labilizing ($-\Delta T_m/r$) interactions; the T_m shift at the end point or point of maximum stabilization (ΔT_m(EP)); the mole ratio r at this point (r_{EP}); the hyperchromicity at this point (h_{EP}); and the dispersions at this point, at $r = r_{EP}/2$ and at $r = 2r_{EP}$. The following conclusions can readily be drawn from the data presented: (1) Strong interaction requires the presence of two amino groups on the steroid nucleus. A single amino group in either ring A or D is completely ineffective; an amino function in one position in conjunction with a carbonyl or hydroxyl function at the other produces some stabilization, but with properties quite unlike those exhibited by steroidal diamines. (2) Within any one group of related diamines with identical configurations, the stabilizing effectiveness increases as the number of methyl groups on either of the amino functions is increased (*e.g.*, the series A-2–4; A-4–5; C-1, -2, -6). (3) Stabilization is highly structure and stereoselective. The order of effectiveness between groups appears to be pregnene > pregnane $\geq$ androstene. For the ditertiary amines in the pregnane series at least the order is $\alpha,\alpha \geq \beta,\beta > \alpha,\beta$ (compounds C-3–5); the order $\beta,\beta > \alpha,\beta$ also holds in the androstene series.[3] Introduction of additional

[3] The configuration 17β is the naturally occurring one in this series, while in the pregnane and pregnene series it is 20α.

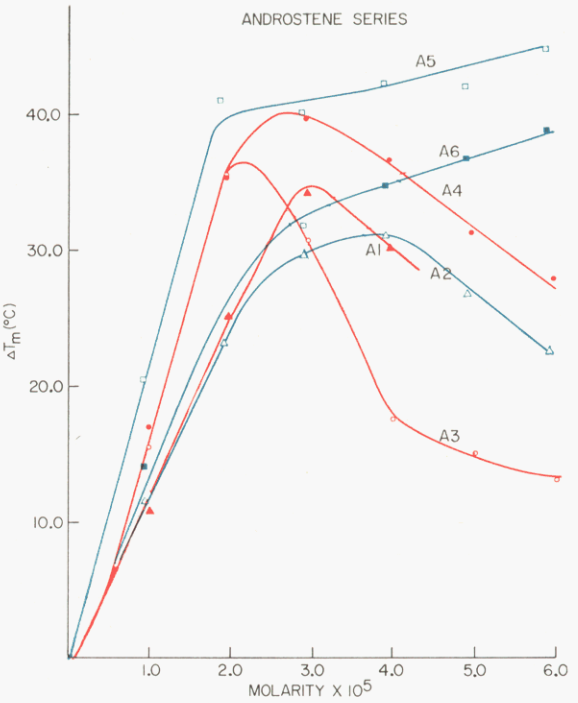


FIGURE 5: T_m of calf thymus DNA as a function of concentration for various androst-5-ene-3,17-diamines. Numbers for the various compounds are those of Table IV. A_{260} (control) = 0.500.

functional groups such as OH groups in the 18 position of the pregnane skeleton (B-2 *vs.* B-1, B-4 *vs.* B-3) affects stabilizing ability as does hydrogenation of the ring (IDA $\equiv$ B-1 *vs.* dihydro-IDA $\equiv$ C-1). (4) Labilization is observed with diprimary (compounds A-1, -2; B-1, -2), primary-secondary (A-3), primary-tertiary (A-4, C-2), disecondary (cyclobuxine), but never with ditertiary (A-5, -6, B-3, -4, C-3–5, conessine), or diquaternary amines (C-6). The order of labilizing effectiveness for diprimary amines appears identical with that mentioned under (3), *i.e.*, pregnene > pregnane > androstene. The introduction of additional methyl groups in either ring generally *decreases* labilizing ability.

The Reaction with Malouetine (Compound C-6). Malouetine, the diquaternary 3β-20α-pregnane-5 derivative, is the most effective stabilizing diamine found so far. For this reason, and since its interaction parameters closely resemble those previously established for our standard compound IDA, without, however the latter's ability to destabilize at $r >$ EP, we have investigated some of its reactions in greater detail. For instance, we were interested whether the great increase in transition width and decrease in hyperchromicity observed with IDA in the stoichiometric range was inherent in interactions with steroidal diamines or brought about by the ability of IDA to engage in a secondary labilizing interaction simultaneously with the primary, stabilizing one, even at low concentrations. Examination of the entries in Table IV, as well as of

TABLE IV: Figures of Merit.[a]

No.	Compound			$\Delta T_m/r$ (°C)	ΔT_m (EP)	$r_{EP} \times 10$	h_{EP}	$\sigma_{2/3}$ (EP/2)	$\sigma_{2/3}$ (EP)	$\sigma_{2/3}$ (2EP)	$-\Delta T_m/r$ (°C)
	Δ⁵-Androstene										
	3	17									
A-1	α-NH_2	β-NH_2		92	36	3.9	1.23	16.2	8.8		35
A-2	β-NH_2	β-NH_2		90	32	4.0	1.31	18.5	10.6	10.0	34
A-3	β-$NHCH_3$	β-NH_2		121	37	2.8	1.29	21.5	9.5	10.5	100
A-4	β-$N(CH_3)_2$	β-NH_2		121	40.5	3.0	1.29	20.0	10	9.3	40
A-5	β-$N(CH_3)_2$	β-$N(CH_3)_2$		151	40.5	2.64	1.26	21.3	11.5	11.0	...
A-6	α-$N(CH_3)_2$	β-$N(CH_3)_2$		104	32	2.64	1.29	16.0	10	9	...
	Δ⁵-Pregnene										
	3	20	18								
B-1	β-NH_2	α-NH_2		250	44	2.20	1.20	25.0	8.3	23.0	360
B-2	β-NH_2	α-NH_2	OH	129	33	2.6	1.24	18.9	9.5	6.0	129
B-3	β-$N(CH_3)_2$	α-$N(CH_3)_2$	..	122	46	3.9	1.16	20	8.0	13	...
B-4	β-$N(CH_3)_2$	α-$N(CH_3)_2$	OH	200	34	2.8	1.25	38	11	13	...
B-5,6	β-NH_2 or α-NH_2	...		0	...	>8	...	...	...	...	...
B-7[b]	β-NH_2	C=O	..	49	...	>6	1.27	14	...	26	...
	5α-Pregnane										
	3	20									
C-1	β-NH_2	α-NH_2		100	41	4.5	1.17	21	8.1	21	300
C-2	β-NH_2	α-$N(CH_3)_2$		140	50	4.0	1.21	24	12.3	14	100
C-3	α-$N(CH_3)_2$	α-$N(CH_3)_2$		222	36	1.8	1.25	16	10.6	23	...
C-4	α-$N(CH_3)_2$	β-$N(CH_3)_2$		76	28	4.5	1.26	21.5	14	4.0	...
C-5	β-$N(CH_3)_2$	β-$N(CH_3)_2$		100	35	4.5	1.25	35	11	20	...
C-6	β-$N(CH_3)_3$	α-$N(CH_3)_3$		250	42	1.8	1.25	17	14	22	...
C-7,8	α- or β-NH_2	α-H		9	...	>8	...	...	...	...	...
C-9	C=O	α-$N(CH_3)_2$		30	12	4	1.23	16	12	11	...

[a] All for calf thymus DNA ($\epsilon(P) = 6600$; $A_{260} = 0.500$; $h = 1.36$; $\sigma_{2/3} = 12.0$). [b] Compounds B-8–11 (Figure 6A) have the following substituents: B-8, 3β-$(CH_3)_2NH$, 20-CO; B-9, 3β-OH, 20-α-NH_2; B-10, 3α-NH_2; B-11, 3β-NH_2.

Figure 8, which reproduces the tracings of the automatically recorded profiles of *E. coli* DNA with malouetine, indicates that these peculiarities are inherent in the stabilizing interactions of steroidal diamines with DNA *per se*.

Stoichiometry of Interactions. In a manner completely analogous to the more extensive studies on cadaverine and IDA with DNA's of different base compositions we have also studied the interactions of three DNA's (those of *Cl. perfringens* with $X_{GC} = 33$, calf thymus with $X_{GC} = 42$, and *E. coli* with $X_{GC} = 50$) with malouetine and with chonemorphine (compound C-2, N^{20},N^{20}-dimethyl-5α-pregnane-3β,20α-diamine). The latter was chosen as a sample of a primary-tertiary amine, to be contrasted to the two primary amines (cadaverine and IDA), the bisecondary amine CB, and the biquaternary amine malouetine. The pertinent data are presented in Figures 9 and 10. A comparison of the corresponding Figures of Merit for the various diamines as well as for the stabilizing aminoketone holaphyllamine (compound B-7) 3β-amino-pregn-5-en-20-one is presented in Table V.

Competitive Interactions. One of the interesting features in the interaction between DNA and CB concerned the ability of various other cations to displace the ligand from the polymer and *vice versa:* while high ionic strength, such as elevated concentrations of Na^+, appeared to screen the polymer from interaction with CB and thus eliminated both the stabilizing and the labilizing effects of the ligand, polyvalent cations added in stoichiometric amounts appeared to be displaceable by CB. It was, therefore, pertinent to investigate the effect of IDA on the thermal transition of DNA in the presence of other stabilizing countercations and to extend the investigations with CB to nucleoproteins. Table VI presents a summary of some of the data obtained. As with CB the stabilizations afforded by IDA and other cations appear independent of one another provided both ions are added at stoichiometric ratios $<r$. Both CB and IDA at higher concentrations are able to displace stabilizing cations, including histones, from the polymer and thus lead to labilization of the latter.

Interactions with s-RNA. As an example of a ribonucleic acid with high helical content s-RNA was in-

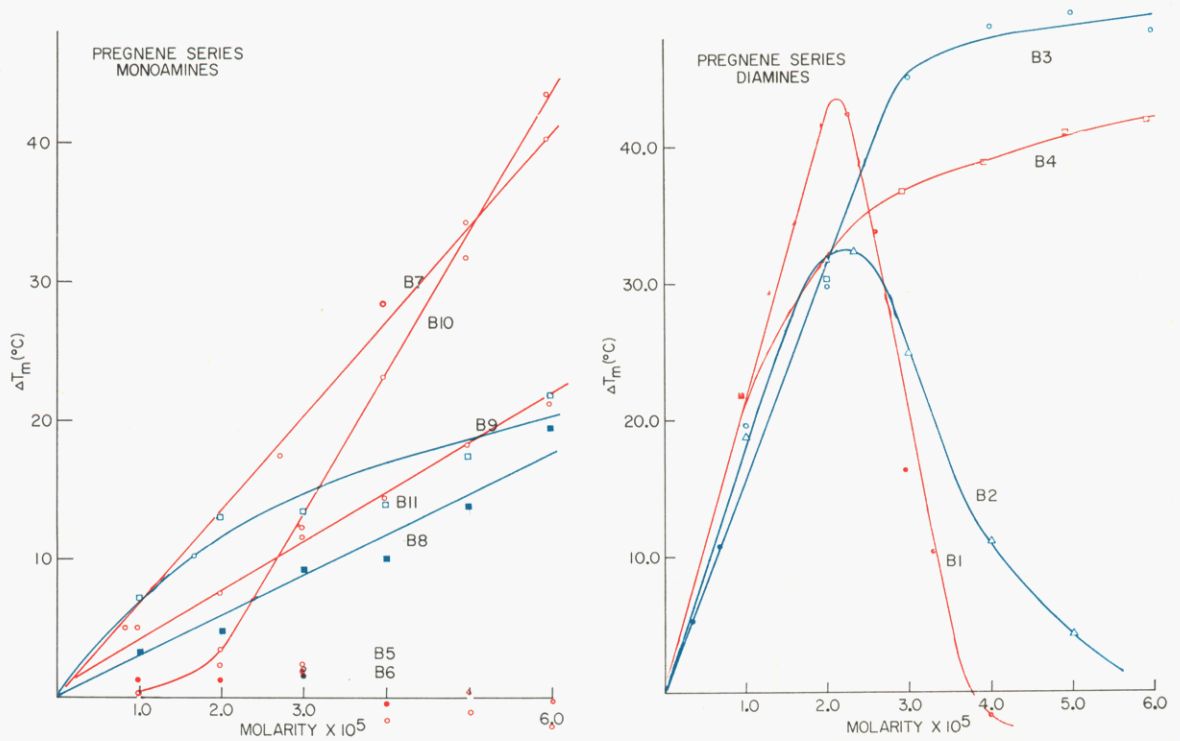


FIGURE 6: T_m of calf thymus DNA as a function of concentration for various pregn-5-ene derivatives. (A) 3-monoamines; (B) 3,20-diamines. Numbers of the various compounds are those of Table IV; A_{260} (control) = 0.500.

vestigated with regard to its interaction with our standard divalent cations at low ionic strength. Some of the results of these studies are shown in Figure 11. The difference between the effects observed is striking: Mg^{2+} not only affords stabilization but greatly increases the cooperativeness of the transition, so much so that the polyphasic and complex profile is converted into a simple sigmoid with a $\sigma_{2/3}$ of 15° when $r_{Mg} \geq 0.5$. On the other hand, although cadaverine is able to provide considerable stabilization (T_m for 2.1×10^5 M cadaverine = 53.5, compared to a T_m of 46.5 for an equal concentration of Mg^{2+}; for concentrations of 4.2×10^5 M the T_m values are 58.0 with either Mg^{2+} or cadaverine), there is no significant alteration in transition width: $\sigma_{2/3}$ remains constant at 25° throughout the range investigated. Finally IDA stabilizes slightly at the lowest concentrations tested, without alteration in transition width, but produces increasingly severe denaturation as r increases to and beyond 0.25.

Other Physical Properties. Almost all the data so far presented have described the system helical polynucleotide–steroidal diamine in terms of thermal denaturation profiles; *i.e.*, the existence of strong interactions between the two partners was only *inferred* on the basis of their behavior once a perturbation, here the exposure to heat, had been introduced. Some preliminary measurements of the hydrodynamic properties of T2 DNA indicate that the polymer is profoundly affected even at 20° in the absence of any such perturbation. They are presented in Table VII. Evidently there exists a

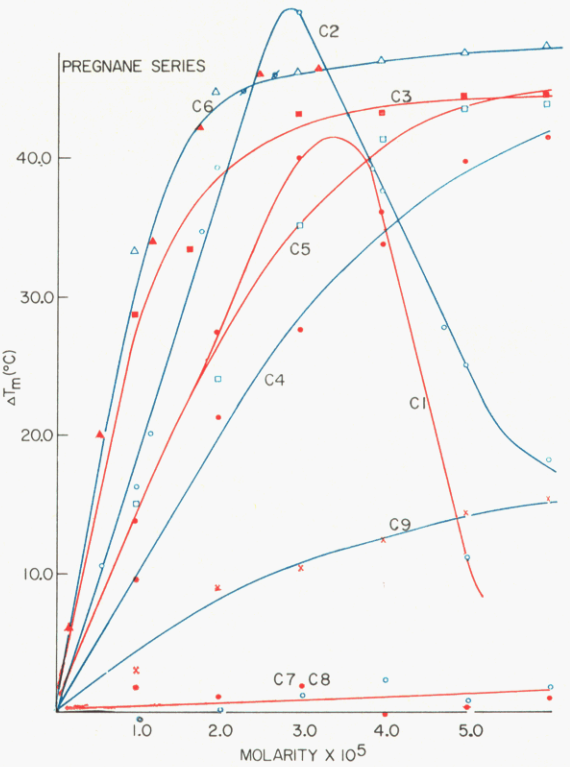


FIGURE 7: T_m of calf thymus DNA as a function of concentration for various 5-α-pregnane derivatives. Numbers of the various compounds are those of Table IV; A_{260} (control) = 0.500.

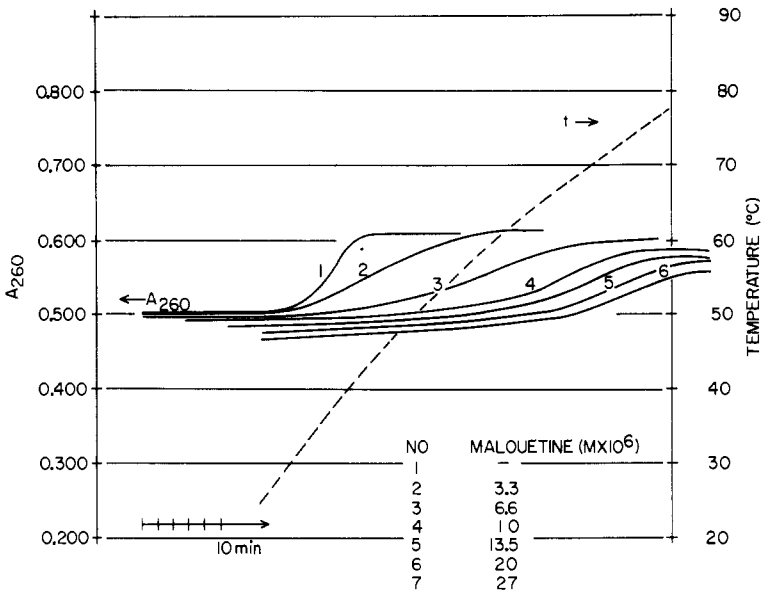


FIGURE 8: Effects of malouetine on the thermal transition of *E. coli* DNA. These are tracings of the actual records obtained with the thermospectrophotometer. Solid lines = absorbance; dashed line = temperature.

TABLE V: Figures of Merit.

DNA	Compd	T_m(EP) (°C)	$\Delta T_m/r$ (°C)	r_{EP} × 10	h_{EP}	σ^2/s (EP/2)	$-\Delta T_m/r$	σ^2/s (2EP)
C. perfrigens	Cad[a]	67.0	130	3.9	1.26	13.0	...	3.0
(30% A + T)	IDA	65.0	285	1.7	1.16	18.0	233	22.0
	Chon	72.5	152	3.2	1.15	20.3	100	14.5
	Mal	75.0	255	2.0	1.18	34.0	...	22.8
	Hol	*b*	68	*b*	1.22	*b*	...	...
Calf thymus	Cad	71.0	95	3.6	1.39	18.1	...	7.0
(59% A + T)	IDA	63.0	250	2.1	1.20	25.0	360	23.0
	Chon	72.0	129	3.9	1.28	16.0	129	20.0
	Mal	79.0	250	2.0	1.22	27.0	...	22.0
	Hol	*b*	43	*b*	1.34	...	...	...
E. coli	Cad	62.5	92	3.4	1.30	12.5	...	5.5
(50% A + T)	IDA	75.0	205	1.6	1.26	19.0	100	21.0
	Chon	84.5	155	2.7	1.15	25.5	100	15.6
	Mal	81.5	215	1.9	1.18	19.5	...	37.7
	Hol	*b*	45	*b*	1.16	*b*	...	...

[a] Cad, cadaverine; IDA, irehdiamine (B-1); Chon, chonemorphine (C-2); Mal, malouetine (C-6); Hol, holaphyllamine (B-7). [b] Equivalence point not yet reached at $r = 0.75$.

correlation between the effects of IDA at various concentrations on both the hydrodynamic properties and the thermal stability of the polymer. Especially noteworthy are the indications of strong interactions between the ligand and the denatured, coil form of DNA as indicated by the formation of complexes or aggregates with very high s values. These conclusions are only preliminary and must be regarded with some caution, however, since the sedimentation coefficients have not been extrapolated to zero concentrations.

Effects on RNA Polymerase. IDA was added to the standard *E. coli* polymerase assay system at concentrations of 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, and 10^{-3} M. No significant effect was observed at the two lower concentrations. At 10^{-4} M there was a stimulation of $20 \pm 5\%$, at 5×10^{-4} M there was an inhibition of 40,

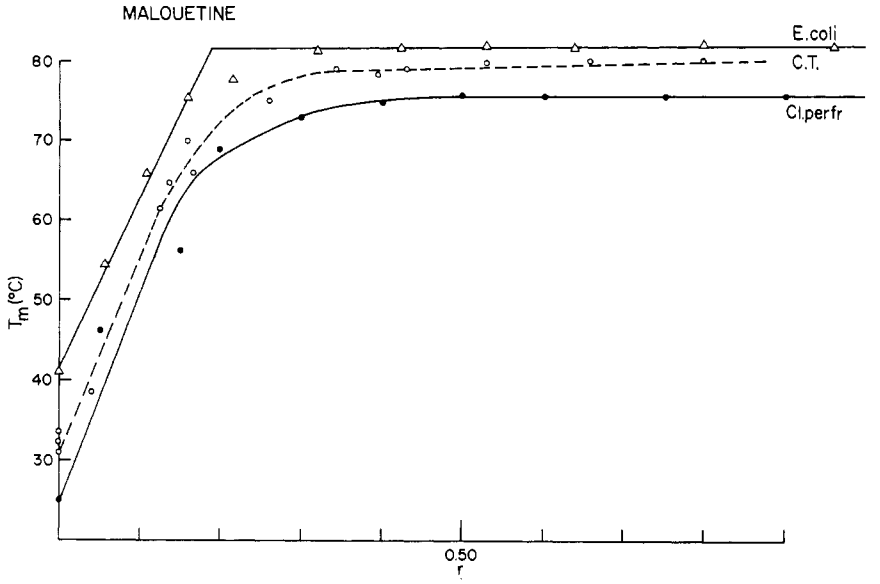


FIGURE 9: T_m as a function of malouetine concentration. For details see legend to Figure 3.

TABLE VI: Effects of IDA on DNA–Cation Complexes at Low Ionic Strength.

Expt[a]	Agent Added	Concn (M)	Concn of IDA (M)	T_m (°C)
A	...	...	...	31.5
	...	...	1.0×10^{-5}	54.0
	...	...	4.0×10^{-5}	39.0
	Mg^{2+}	4.0×10^{-5}	...	65.8
	Mg^{2+}	4.0×10^{-5}	4.0×10^{-5}	42.3
	Cadaverine^{2+}	2.0×10^{-5}	...	64.0
	Cadaverine^{2+}	2.0×10^{-5}	1.0×10^{-5}	74.1
	Cadaverine^{2+}	2.0×10^{-5}	4.0×10^{-5}	46.3
B	Thymus nucleoprotein[b]		...	70.7
	dto		10^{-4} CB	71.5
	dto		10^{-3} CB	50.1
	DNA[b]		...	59.2
	dto		10^{-4} CB	76.1
	dto		10^{-3} CB	45.4

[a] In expt A the total concentration of added monovalent cation was 2.0×10^{-4} M and commercial calf thymus DNA was used; in expt B the concentration of added monovalent cation was 6.0×10^{-3} M and the polymers used were those indicated (see also Materials and Methods). [b] Welsh, 1962.

and at 10^{-3} M, an inhibition of 47 ± 5%. The T_m values for the control, containing all the components of the assay mixture except β-mercaptoethanol and the nucleoside triphosphates, was 80.0° and for the experimentals in ascending order of concentrations 79.5, 80.5, 78.7, 73.5, 58.0, and 45.0°. Thus at ligand concentrations sufficient to destabilize the structure of DNA, *i.e.*, under conditions of partial strand disorientation and, perhaps, separation, the ability of the system to carry out RNA synthesis was impaired.

Discussion

The phase of this investigation described in the present report was concerned with four aspects of the problem: (1) to establish the stoichiometry of the interaction between DNA and selected steroidal diamines, most notably IDA, and to compare it to that between DNA and other cations; (2) to compare IDA with steroids containing other functional groups and establish those groups and their steric relationships re-

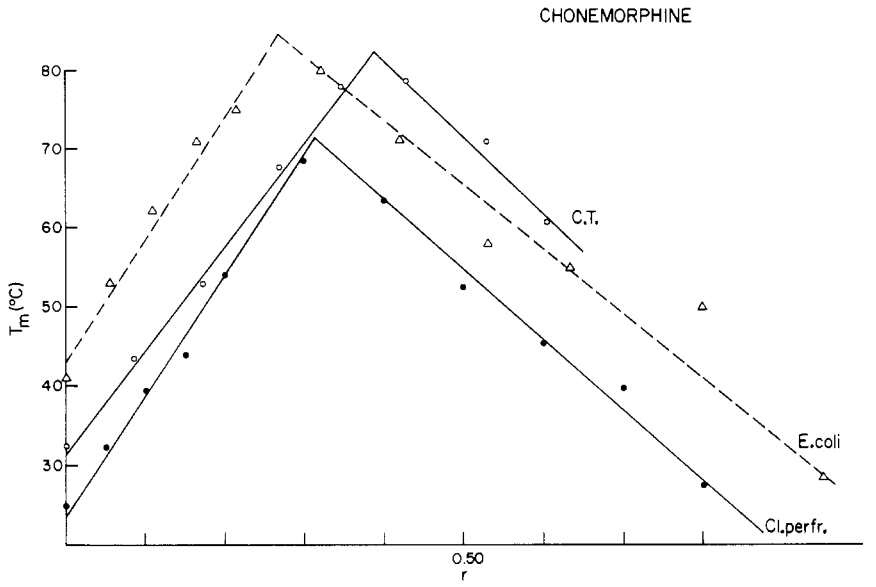


FIGURE 10: T_m as a function of chonemorphine concentration. For details see legend to Figure 3.

TABLE VII: Some Physical Properties of T2 DNA in the Presence of IDA. Series I.[a]

Medium (M)	DNA Added: $s_{20,w}$ (Svedbergs) Native	DNA Added: $s_{20,w}$ (Svedbergs) Denatured	T_m (°C) Native
NaCl (1)	39.5		82
NaCl (0.01)	31.5	22.5	50.0
NaCl (0.01) plus $MgCl_2$ (1.0×10^{-4})	29.0		75.0
NaCl (0.01) plus IDA (1.0×10^{-4})	36.0	>500	57.5
NaCl (0.01) plus IDA (4.0×10^{-5})	34.4	>500	52.5

[a] The DNA in series I showed a $s^0_{20,w}$ of 42.8 on extrapolation of three different samples to zero concentration. Substituting in the empirical equation relating $s_{20,w}$ to molecular weight (Studier, 1965; Eigner and Doty, 1965), this indicates that we were dealing largely with half molecules of M $\simeq 60 \times 10^6$. All DNA-P was present at a concentration of 8.0×10^{-5} M.

quired for stabilization and labilization of the helical conformation of DNA; (3) to investigate the dependence of the parameters defined under (1) and (2) on the base composition of the DNA; (4) to inquire into the mechanism of the interactions between DNA and steroidal ligands, most notably the question whether they are due to the formation of actual complexes rather than an alteration of the ionic "atmosphere" surrounding the polymer, and the nature of the groups on steroid and polymer responsible.

As far as the first point is concerned we have shown that maximum stabilization for IDA occurs at a value of $r = 0.2$. In this it resembles other strongly stabilizing steroidal diamines such as malouetine, but differs from aliphatic diamines such as cadaverine ($r_{EP} \approx 0.35$) or divalent inorganic cations such as Mg ($r_{EP} = 0.5$). Thus the number of apparent binding sites which have to be occupied to obtain maximal stabilization for any one DNA varies in the order steroidal diamines < aliphatic diamines < alkaline earths cations. The maximal stabilization obtainable by such stoichiometric interactions appears to be a constant for any DNA, and effects of different ligands are additive until this maximal value is attained. The steric prerequisites for the achievement of stabilization, or destabilization for that matter, are rather stringent. Even quite subtle changes, such as the one from the pregnene to the androstene series or, even more strikingly, the hydrogenation of the single double bond in the former, or a change in the relative configuration at C-3 and -20 (or C-3 and -17 in the androstene series), all produce alterations in the effectiveness of the interactions. Frequently the actual N/N distance is altered by as little as 1 A; it is, *e.g.*, 11.6 A in IDA or CB, 10.5–10.8 in various other configurations of the pregnene, pregnane, and androstene series. These values have been obtained from measurements on Dreiding or space-filling stereomodels. Stabilization is relatively general since it is produced by biprimary, secondary, tertiary, or quaternary amines, although the latter appear to be the most effective. Bifunctionality in appropriate positions appears absolutely essential, with an amino group in the second position greatly more effective than a hydroxyl or carbonyl function. Bifunctionality restricted to primary and secondary amines also is the essential prerequisite for destabilization.

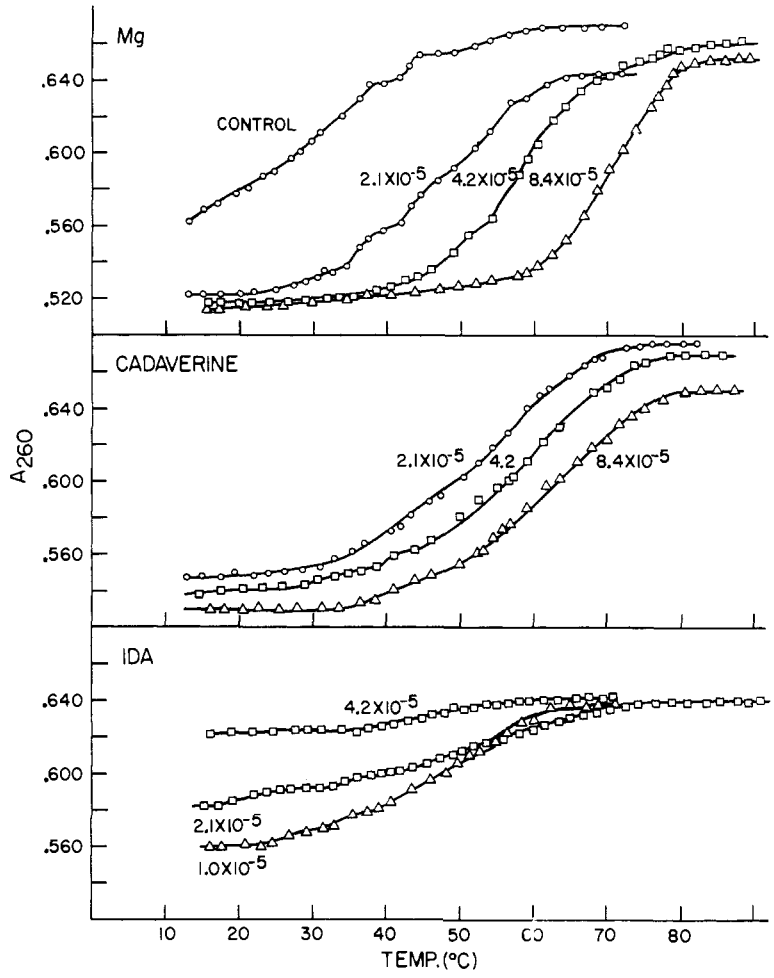


FIGURE 11: The effect of various cations on the thermal profile for s-RNA from *E. coli*.

As with most other agents tested, including a variety of metal ions (Dove and Davidson, 1962; Mahler and Mehrotra, 1962; Mandel, 1962; Mehrotra and Mahler, 1964; Nandi *et al.*, 1965), the extent of stabilization is enhanced for DNA molecules rich in adenine and thymine residues. The present studies allow one to define with a little more precision just what is meant by "enhanced stabilization," for we see (Figures 3, 4, 9, 10) that the enhanced shift in T_m (ΔT_m) is due to a change in slope of the T_m *vs.* r plot, and *not* to a decrease in r_{EP}. Therefore, it appears likely that what is affected is the *strength* of the interaction between ligand and DNA (hence the stability of the complex) and not the *number* of potential binding sites (or interacting groups on the polymer). Quite similar considerations also appear to govern the labilizing interaction, whenever it exists.

As far as answering the crucial last question concerning mechanism is concerned, some answers can be given although many aspects of the problem still remain obscure. First, regarding stabilization: the definite stoichiometries, the ligand specificities observed, the changes in hyperchromicity and transition width, the effects on hydrodynamic properties, and the dialysis experiments all tend to make explanations in terms of nonspecific environmental counterion effects much less attractive than the alternative one which postulates formation of definite complexes between the helix and the ligand. The complex, although more stable, in the sense of requiring higher temperature for half (or complete) denaturation, cannot be identical with that obtained in the presence of sodium ions at high concentrations or even stoichiometric amounts of Mg or cadaverine. Its optical properties are different and it melts over a much wider temperature range. From these properties one might postulate a stable but somewhat distorted helix with the bases tilted or at least no longer aligned in the same manner as they were in the native Watson–Crick–Wilkins B conformation, a complex in which short regions can now melt out without necessarily affecting the stability of other regions some distance removed. The steroid molecules are probably not intercalated (Lerman, 1961, 1963, 1964) into the helix: intercalation requires planar ring systems and steroids are puckered, not planar.

Which then are the groups on the helix involved in

complex formation? Phosphate residues are implicated, at least in part, in any interaction between a polymeric phosphate diester and dications. The fact that the diquaternary amine malouetine is the most effective compound so far tested would tend to rule out diamines as potential proton donors in hydrogen-bond formation. Furthermore, the similarities in both effective charge and stabilizing effects of all the divalent cations tested are striking. Finally, we return to the observed value of $r \approx 0.2$ found with both IDA and malouetine. Lyons and Kotin (1964, 1965a,b) have shown that at the mole ratios used here, the thermodynamic activity coefficient of the sodium ion in Na-DNA, γ_{Na^+}, ≈ 0.6, *i.e.*, that the effective negative charge of the polymer equals 0.6, while for the magnesium salt the values of the corresponding parameters equal approximately 0.06. The decrease in activity coefficient or effective negative charge therefore equals about 0.5. This reduction in charge decreases electrostatic repulsion drastically and leads to a shift in T_m for calf thymus DNA of about 60°. But this is also precisely the maximal increase in T_m observed at the end or equivalence points for *all* the stabilizing divalent organic cations tested here, regardless of structure. Therefore, we can say that maximal stabilization[4] of DNA will occur whenever its complex with a cation possesses an effective negative charge ≈ -0.05. This result obtains with the most effective steroidal diamine salts when about 0.4 equiv (0.2 m-le) has been bound to DNA; for cadaverine^{2+}, it requires 0.7 equiv and for Mg^{2+}, 1 equiv. Since $a_+ = \gamma_+ m_+$, where a_+, γ_+, and m_+ are the activities, activity coefficients, and molar concentrations of various cations, and equal stabilizing effectiveness requires equality of γ_+, a_+ for the three classes of cations therefore will be in the ratio of 0.20:0.35:0.50.

What about the labilizing interactions? In the preceding publication (Mahler and Dutton, 1964) we discussed a four-step mechanism which envisaged (a) formation of bridged complexes with the helix (stabilizing) at low ligand concentrations, (b) formation of unbridged "monovalent" $DNA^-\ldots R_3N^+-R'-{}^+NR_3$ complexes at high ligand concentrations, preferentially with denatured coiled regions, accompanied by (c) formation of micelles of stacked steroids molecules on the coils and followed by (d) phase separation and precipitation of the denatured DNA–steroidal amine complex. This is very similar to one suggested by Lyons and Kotin (1964, 1965a,b) who have shown that Mg added in large excess ($\geq 100\times$) leads to destabilization and precipitation of DNA in its denatured form and thus behaves qualitatively in a manner reminiscent of that of the steroidal diamines. As pointed out by these authors step b is inherently destabilizing by virtue of the fact that in going from $[(DNA^-)_2 \cdot ligand^{2+}]_{helix}$ (with, as we have just seen, net charge close to zero or slightly negative) to $2[DNA^{1-} \cdot ligand^{2+}]_{coil}$ we form a positively charged polymer beset with electrostatic repulsion. The much greater efficiency of steroidal diamines in producing destabilization as compared to Mg^{2+} is probably due to two effects. First, they shift equilibrium b further toward complex formation, just as they were more effective in forming the complexes in a. Second, while micelle formation is dependent solely on base stacking in the case of the Mg complexes, it is cooperatively enhanced by the stacking of steroid molecules on top of one another (or perhaps on top of bases), a process quite feasible when examined by means of space filling models. The proposed mechanism also accounts for two important experimental observations, the requirement for two amino functions in the steroid nucleus as well as the inability of more highly substituted diamines than primary-tertiary to produce any labilization. Although monoamines can stack, they cannot produce charge reversal in forming the stacked complex. Conversely the spatial requirements of the added methyl groups in bitertiary and biquaternary amines are such as to preclude any effective stacking. We conclude, therefore, that steps b–d in our mechanism probably all act in concert, though not necessarily simultaneously, in order to produce effective destabilization. Finally one may raise the question whether in the present case also the denatured complexes are essentially intra- or intermolecularly cross-linked between phosphates as suggested by Lyons and Kotin. The latter alternative appears somewhat more plausible perhaps, since sedimentation coefficients of such complexes are unusually high and since precipitation of the complexes at low r (*e.g.*, at $r \approx 0.3$ for IDA) are strongly dependent on the concentration of DNA added. We leave open for the moment the question of the extent of *strand* separation or denaturation in the presence of labilizing or for that matter stabilizing concentrations of diamine.

To the possible implications of these interactions discussed earlier (Mahler and Dutton, 1964) we now add three questions: (1) Steroidal diamines have now been shown to be able to displace histones from nucleohistones; can they, therefore, activate heretofore inactive nucleoprotein complexes for transcription? (2) The complexes of DNA (and to a certain extent of s-RNA) with steroidal diamines appear to possess rather peculiar structures; can these peculiarities produce mistakes or interruptions in the transcription or translation of the genetic message? (3) If steroidal diamines are able to interact in such a highly unusual fashion with nucleic acids, is it not possible that they may be capable of analogous interactions with other negatively charged entities arranged in a definite spacial relation to each other, for instance in, or on, a membrane? Can their well-established effects on the nervous system [*e.g.*, Khuong-Huu-Lainé and Pinto-Sconamiglio (1964) for malouetine as a curarizing agent] perhaps be explained in this fashion?

[4] We assume here that in this range of concentrations there is either no significant interaction between ligand and denatured (regions in) DNA, or that at least there is no great difference in stability of such complexes with different ligands.

Acknowledgment

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