INTERACTIONS OF HORMONAL STEROIDS WITH NUCLEIC ACIDS, I. A SPECIFIC REQUIREMENT FOR GUANINE

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Abstract.—Under equilibrium conditions testosterone, progesterone, estradiol, and corticosterone bind to denatured but not to native DNA. Among synthetic polynucleotides, steroids bind only to guanine containing polymers: poly dG, poly G, poly UG, poly AG, and "denatured" poly dG:dC; however, they do not bind to rRNA, sRNA, TMV-RNA, poly CG, or native poly dG:dC. These data indicate a preferential affinity for guanine residues and for single-stranded regions of polynucleotides. That different functional groups of the purine are involved in the associations with different steroids is indicated by the observation that only estradiol binds to poly I. Restrictions are also imposed by structural attributes of the steroids: an α-substitution at C-17 reduces binding, polyhydroxylated steroids bind poorly or not at all.

Various classes of small molecules, such as dyes, carcinogens, and antibiotics, bind to nucleic acids. In each instance, binding is a function of the structure of the polymer as well as of the ligand. There is generally a preference for the helical form of DNA but affinity may also extend to RNA, as in the case of ethidium bromide.[1] Different mechanisms of binding exist for different ligands. For example, the principal mode of binding of acridines[2] involves intercalation between adjacent base pairs; mitomycin[3] appears to cross-link the two DNA strands; the chromophore of actinomycin[4] is intercalated between base pairs, while the peptide chains interact with the DNA backbone. Some of these ligands exhibit a base preference, such as actinomycin for guanine;[5] others appear to be less selective, but there are no examples of requirement of unique base sequences for binding. The biological effects of some of these classes of small molecules can be correlated with their nucleic acid interactions: in the case of the antibiotics these are of a general inhibitory nature, such as impaired transcription or replication.

Steroids fall into a somewhat different category, in that the naturally occurring hormonal steriods perform essential control functions in relation to normal biological processes such as development and differentiation. It might be expected, therefore, that any relevant interactions between steroids and nucleic acids would show a higher degree of specificity with respect to both steroid and polymer structures. Weak binding of steroids to bases, mononucleosides, and mononucleotides has been demonstrated[6] by partition methods. Ts'o and Lu[7] reported the binding of steroids and aromatic hydrocarbons to denatured calf thymus DNA: they proposed that these associations depend mainly on hydrophobic forces. In the present study, measurements made at equilibrium suggest that steroids bind only to single-stranded regions of polynucleotides. Restrictions are imposed by structural attributes of both steroid and polymer. It is

inferred from studies with homopolymers that those steroids which bind to nucleic acids exhibit a strict requirement for guanine residues.

Materials and Methods.—Nucleic acids: Native DNA was isolated from bovine spleen and from *Pseudomonas aeruginosa* by phenol methods,[8] using sodium triisopropylnaphthalene sulfonate[9] as detergent. These preparations contained less than 1% protein[10] and gave hyperchromic shifts of 34 and 42%, respectively, on thermal denaturation in HMP[7] buffer (0.0025 *M* Na_2HPO_4, 0.005 *M* NaH_2PO_4, 0.001 *M* Na_2EDTA, pH 6.8).

Denatured DNA was obtained by heating the DNA solutions in HMP at 100°C for 15 min, followed by rapid cooling on ice. Residual hyperchromicities after this treatment were found to be about 30% for bovine spleen DNA and about 20% for *Pseudomonas aeruginosa* DNA. Concentrations of DNA were determined spectrophotometrically on the native form in HMP, on the basis $E^{1\%}_{1cm} = 200$ at 260 mμ. Ribosomal RNA (rRNA) was isolated from the livers of C3H mice by the method of Parish and Kirby,[9] yeast soluble RNA (sRNA) was obtained from Sigma Chemical Company, and tobacco mosaic virus RNA (TMV-RNA) was generously supplied by Dr. R. J. Young. On thermal denaturation in HMP these RNA preparations gave hyperchromic shifts of 23, 20, and 20%, respectively.

Deoxyribopolymers: The double-stranded, alternating copolymer, poly dAT, and the double-stranded, duplex polymer, poly dG:dC, were obtained from Miles Laboratories. After heat "denaturation" these polynucleotides exhibited loss of viscosity but little or no residual hyperchromicity.[11] Single-stranded polydeoxyribonucleotides were obtained from Biopolymers, Inc., and their concentrations were determined spectrophotometrically in HMP on the basis of quoted ϵ_{max}: poly dA ($\epsilon_{m\ 275m\mu} = 8.6.10^3$), poly dC ($\epsilon_{m\ 270\ m\mu} = 7.2.10^3$), poly dT ($\epsilon_{m\ 267\ m\mu} = 8.7.10^3$), and poly dG ($\epsilon_{m\ 252\ m\mu} = 10^4$).

Ribopolymers: Polyribonucleotides were obtained from Miles Laboratories, and their concentrations were calculated spectrometrically on the basis of the following ϵ_{max}: poly A ($\epsilon_{m\ 257\ m\mu} = 10.5.10^3$), poly C ($\epsilon_{m\ 267\ m\mu} = 6.5.10^3$), poly U ($\epsilon_{m\ 260\ m\mu} = 9.2.10^3$), poly G ($\epsilon_{m\ 252\ m\mu} = 9.0.10^3$), poly I ($\epsilon_{m\ 248\ m\mu} = 10^4$), poly AG ($\epsilon_{m\ 260\ m\mu} = 12.7.10^3$), poly UG ($\epsilon_{m\ 260\ m\mu} = 10^4$), poly CG ($\epsilon_{m\ 260\ m\mu} = 6.8.10^3$).

Radiochemicals: The following radioactive steroids of high specific activity were obtained from the New England Nuclear Corporation: testosterone-1,2-H^3 (50 c/mM), 17β-estradiol-6,7-H^3 (50 c/mM), 17α-testosterone-1,2-H^3 (30 c/mM), progesterone-1,2-H^3 (50 c/mM), 17α-OH-progesterone-7α-H^3 (50 c/mM), corticosterone-1,2-H^3 (15 c/mM), hydrocortisone-1,2-H^3 (15 c/mM). Ecdysone-H^3 (9 c/mM) and 25-deoxy-ecdysone-H^3 (10 c/mM) were kindly supplied by Dr. W. Hafferl. All steroids were taken into solution in HMP from methanol, followed by evaporation of methanol under nitrogen at ambient temperature. In all cases radiochemical purity was checked by thin-layer chromatography in hexane:ethyl acetate:methanol (9:9:2).

Experimental procedures: Equilibrium dialysis[12] was carried out for 48 hr or longer in polymethylmethacrylate cells of 100, 200, or 1000 μl in volume on each side, separated by membranes prepared from Visking dialysis tubing. The latter was pretreated successively with Na_2EDTA, hot $NaHCO_3$ 5%, and ethanol 80%. Polymers and steroids were dissolved in HMP or HMP–0.5 *M* NaCl, as indicated. The polymer solution (0.2–3 mg/ml) was introduced by microsyringe into one side of each cell, the steroid solution (10^{-9} M–$2.5.10^{-8}$ *M*) into the opposite side. Dialysis cells were agitated continuously on a horizontal shaker at 4°C. Determination of bound and free steroid concentrations was effected by radioassay on aliquots of 10, 20, or 50 μl removed by microsyringe from each side of the cell after equilibrium was reached. Radioactivity was assayed by liquid scintillation spectrometry with a dioxane phosphor and ranged from 1,500 to 10,000 cpm in each aliquot. Initial kinetic experiments, carried out in the presence and absence of polymer, showed that dialysis equilibrium of each steroid was attained in less than 48 hr. On this basis a 48-hr equilibration time was routinely used, but in assays where little or no binding was detectable, dialysis was continued beyond 72 hr. Other control experiments showed that in the presence of any given polymer, the distribution of the steroid

at equilibrium was the same if the steroid was introduced into the dialysis cells on the same side as the polymer.

All experiments were carried out with a range of steroid concentrations and in certain cases a range of concentrations of the polymer. In most instances three or more experiments were performed, each in quadruplicate, for determination of nK values. After each dialysis, absence of measurable degradation of the polymers into dialyzable nucleotides was checked by UV measurements on aliquots from each side of the dialysis cell. Polymer degradation, or polymer loss due to binding to cell walls or membranes, was negligible. Some small loss of steroids due to binding to cells or membranes was measurable.[7]

Analysis of binding equilibria: The calculation of the apparent binding constant is based on the mass action law.[13] Since the actual number of binding sites is not known, values for nK have been calculated on the basis:

$$nK(M^{-1}) = \frac{[\text{Complex}]}{[\text{Free steroid}]\cdot[\text{free nucleotide units}]},$$

where n is the number of binding sites per nucleotide unit, and K is the binding constant of the steroid to the nucleotide binding site. In these experiments we are below saturation,[14] and the number of binding sites occupied relative to the total number of nucleotide units is small, so that for practical purposes

$$nK(M^{-1}) = \frac{[\text{Bound steroid}]}{[\text{Free steroid}]\cdot[\text{nucleotide units}]}.$$

Results.—Binding of steroids to DNA: Equilibrium dialysis of a variety of steroids with native bovine spleen DNA showed no detectable binding (Table 1). With denatured DNA, however, measurable binding occurred with all of these same steroids except hydrocortisone, ecdysone, and 25-deoxyecdysone. This suggests that only single-stranded DNA or DNA with a less-ordered structure binds steroids and thus restricts the types of interaction which can occur. Comparison of nK values for testosterone and its 17α derivative, for progesterone and its 17α-OH derivative, and for corticosterone and hydrocortisone (= 17α-OH-corticosterone) demonstrated that the introduction of an α-substitution on C-17 of the steroid decreased the apparent binding constants. These data suggest the possibility that binding is to some extent dependent on the total hydrophobicity of the steroid molecule: highly hydroxylated steroids showed little or no binding.

Binding of steroids to deoxyribopolymers: Estradiol and testosterone, used as model steroids, did not show measurable binding with the native synthetic

TABLE 1. *Relative affinity of various steroids for bovine spleen DNA.**

Steroid	$nK(M^{-1})$ Native DNA	$nK(M^{-1})$ Denatured DNA
Estradiol	0†	68
Testosterone	0	124
17α-Testosterone	0	25
Progesterone	0	252
17α-OH-Progesterone	0	45
Corticosterone	0	28
Hydrocortisone	0	0
Ecdysone	0	0
25-Deoxyecdysone	0	0

* Equilibrium dialysis in HMP-0.5 *M* NaCl.

† A difference in cpm between the two sides of the dialysis cell of less than 3% of the total radioactivity measured was within the limits of error of the technique and in such cases the nK value has been scored as 0 in this and subsequent tables.

TABLE 2. *Affinity of two steroids for double-stranded deoxyribopolynucleotides.**

Steroid	$nK(M^{-1})$ Native poly dAT	Denatured poly dAT	Native poly dG:dC	Denatured poly dG:dC
Estradiol	0	0	0	83
Testosterone	0	0	0	53

* Equilibrium dialysis in HMP. The number of steroids examined was restricted by the limited amount of each polymer available.

polymers, poly dAT and poly dG:dC (Table 2). This finding is in keeping with their absence of binding to native DNA (Table 1). "Denaturation" of these polymers resulted in measurable binding of both steroids to poly dG:dC but not to poly dAT. This "denaturation" led to little or no residual hyperchromicity but a dramatic loss of viscosity[11] which presumably leaves some residual disordered regions despite the general resumption of base-pairing on cooling. It is presumed that residual unpaired regions were responsible for the binding when observed. When the four standard homodeoxypolymers were tested with estradiol and progesterone as model steroids, poly dG exhibited binding, but poly dA, dC, and dT did not (Table 3). If these two steroids are representative, these data suggest a preferential affinity for guanine in the deoxy-series.

Binding of steroids to homoribopolymers: Estradiol, testosterone, progesterone, and corticosterone bound measurably to poly G but not to poly A, C, or U (Table 4). As was observed with DNA (Table 1), hydrocortisone and ecdysone did not show measurable binding to these homoribopolymers. The restriction of binding to guanine-containing polynucleotides was thus apparent in both deoxyribo- and ribo-series for those steroids examined: i.e., denatured DNA, "denatured" poly dG:dC, poly dG, and poly G. Poly I is 2-deaminated poly G and, as such, is a suitable model for examining further the chemical basis of the guanine specificity. The observation (Table 4) that binding to this polymer occurred with estradiol but not with testosterone and progesterone suggests that the 2-amino group of guanine is necessary for the association of the latter two steroids with poly G, whereas other groups are presumably involved in the binding of estradiol.

Equilibrium dialysis of steroids with RNA and ribocopolymers: No binding was measurable with three model steroids—estradiol, testosterone, and progesterone —to yeast sRNA, mouse liver rRNA, or TMV-RNA (Table 5). In these experiments possible degradation of RNA during dialysis was rigorously examined and excluded in each assay. Binding was observed with the ribocopolymers UG and AG but not with CG (Table 5). The lower the proportion of guanine residues in the copolymer, the lower was the nK value observed. In the case of poly CG, which contained a large excess of cytosine versus guanine residues, the absence of detectable binding could be due to extensive complementary base-pairing.

TABLE 3. *Affinity of two steroids for homodeoxyribopolynucleotides.**

Steroid	$nK(M^{-1})$ Poly dA	Poly dG	Poly dC	Poly dT
Estradiol	0	422	0	0
Progesterone	0	314	0	0

* Equilibrium dialysis in HMP. The number of steroids examined was restricted by the limited amount of each polymer available.

TABLE 4. *Affinity of several steroids for homoribopolynucleotides.**

Steroid	$nK(M^{-1})$				
	Poly A	Poly G	Poly I	Poly C	Poly U
Estradiol	0	298	197	0	0
Testosterone	0	223	0	0	0
Progesterone	0	1212	0	0	0
Corticosterone	0	155	—	0	0
Hydrocortisone	0	0	—	0	0
Ecdysone	0	0	—	0	0

* Equilibrium dialysis in HMP.

Influence of ionic strength and DNA source: Denatured *Pseudomonas aeruginosa* DNA (67% G + C) in HMP gave higher nK values for testosterone and progesterone than did denatured bovine spleen DNA (42% G + C) but did not give significantly different nK values for estradiol (Table 6). Increased ionic strength (HMP-0.5 M NaCl) increased the nK values of testosterone and progesterone with both DNA's, but the increment, due to high salt, was much more marked in the case of the bovine spleen DNA (Table 6). Again, the effect of increased ionic strength on estradiol binding was small or absent. If it is assumed that the number of binding sites is not limiting, the effect of salt could imply the preference of a certain degree of ordered structure for optimal binding sites for testosterone and progesterone even though single-stranded DNA was apparently required. Similar studies have not been carried out with poly G, due to the limited solubility of the polymer at high salt concentrations.

Discussion.—It is evident that some hormonal steroids will bind to certain polynucleotides under carefully defined conditions. Several important features of such interactions emerge from the present data:

(1) No detectable binding of any steroids was observed to occur with highly ordered double-stranded deoxyribopolymers. Where it did occur, binding to denatured DNA or to homopolynucleotides could indicate a requirement for a less-ordered structure of the polymer or for a more hydrophobic environment. It could also indicate a requirement for binding sites involving reactive groups of the bases which are not available in double-stranded structures. This observation is in contrast to the binding of many ligands where an ordered, double-stranded polymer structure is a prerequisite. Many of these latter ligands comprise, in whole or in part, relatively planar ring structures, such that intercalation between nearest neighbor bases is possible. Of the steroids, even estradiol, with a planar aromatic ring A, did not bind detectably to double-stranded polynucleotides.

(2) Those steroids which were observed to bind to polynucleotides in both the ribo- and deoxyribo-series exhibited a preferential affinity for guanine residues.

TABLE 5. *Affinity of three steroids for RNA and ribocopolynucleotides.**

Steroid	$nK(M^{-1})$					
	sRNA	rRNA	TMV-RNA	Poly UG†	Poly AG†	Poly CG†
Estradiol	0	0	0	193	21	0
Testosterone	0	0	0	78	21	0
Progesterone	0	0	0	173	72	0

* Equilibrium dialysis in HMP.

† Poly UG = 1:1, poly AG = 2:1, poly CG = 3:1. No binding was observed with poly CG even when the polymer was preheated at 100° for 10 min and cooled on ice before dialysis.

TABLE 6. *Relative affinity of three steroids for denatured bovine spleen and Pseudomonas aeruginosa DNA's in different ionic strengths.*

Steroid	$nK(M^{-1})$			
	Bovine Spleen DNA		*Ps. aeruginosa* DNA	
	Low salt*	High salt*	Low salt*	High salt*
Estradiol	50	68	56	58
Testosterone	60	124	102	135
Progesterone	70	252	243	278

* Low salt = HMP; high salt = HMP − 0.5 *M* NaCl.

The unique abilities of poly dG and poly G among the homopolymers (except the special case of poly I) to bind these steroids is consistent with the higher nK values observed with denatured DNA having the higher G + C content. However, if DNA binding sites are not limiting here, these higher nK values may reflect the presence of more idealized binding sites, based on differences in polymer conformation (*vide infra*). Although guanine residues appear to be required for binding of the steroids, this does not necessarily imply that random guanine residues will function as binding sites, nor that only guanine residues form the preferred binding sites on copolymers. In addition, the comparison of binding data with poly I and poly G indicates that different functional groups of the purine residue are required for binding estradiol, on the one hand, and for binding testosterone and progesterone, on the other. This does not necessarily mean that the same functional groups are involved in the interactions with testosterone and progesterone.

(3) Restrictions on binding are also imposed by the structures of the steroids. The nature and orientation of the substituent at C-17 greatly influenced the magnitude of the nK values. A substituent in the 17α position reduced the nK values; in the case of hydrocortisone no binding was apparent. The α-orientation of a substituent at C-17 is presumably energetically less favorable than the β-orientation for steroid-nucleotide interaction; hindrance might be expected if the α-surface of the steroid is oriented toward the polynucleotide. On the other hand, any hydrogen bonding between polymer and steroid involving the carbonyl on C-20 (progesterone, corticosterone) is expected to be weakened by intramolecular hydrogen bonding with a hydroxyl situated at C-17[15] (17α-OH-progesterone, hydrocortisone). Net hydrophobicity is apparently also important: the more hydrophilic steroids bearing several hydroxyl groups showed little or no binding. Other structural attributes of the steroids are important, such as the presence of a carbonyl or a hydroxyl group at C-3, of a hydroxyl group at C-17, or a carbonyl group at C-20.[14]

(4) Values of nK for the binding of steroids to denatured DNA were generally higher at higher salt concentrations. Under these conditions the degree of base stacking is enhanced, as the repulsion between neighboring phosphates is minimized.[16] This situation might well provide an energetically more favorable fit for the steroid molecules at their binding sites. Currently available evidence[11] indicates that poly G and poly dG have considerable secondary structure, although its extent and nature is not understood in detail; presumably, both homopolymers have sufficient unpaired regions to account for steroid binding. However, precise interpretation of the structural requirements for this binding must await further study.

(5) From the homoribopolymer data, binding of certain steroids to single-stranded RNA could be expected to occur. However, binding was not observed with yeast sRNA, mouse liver rRNA, and TMV-RNA, in general agreement with the observations of Ts'o and Lu.[7] All three classes of RNA in aqueous solution at moderate salt concentrations have significant degrees of ordered secondary structure, which might severely restrict the number of potential binding sites. Guanine residues especially might be largely involved in base-pairing: this is certainly true for most of the guanine residues in surmised secondary structures of transfer RNA molecules whose primary sequences are known.[17] That binding of steroids to RNA should be possible under conditions where secondary structure is minimal is suggested by the binding data with ribocopolymers.

Thus, there, are indications that a guanine requirement, certain imprecisely defined ordered structural requirements of single-stranded regions of polynucleotides, and certain steroid structural restrictions govern the binding of steroids to polynucleotides. Examination of binding isotherms indicates that the free energy of binding is of the order of 7–10 kcal/mole of steroid bound.[14] Model building shows that intercalation of a steroid molecule between adjacent bases is unlikely, except in the above-mentioned case of ring A of estradiol. The construction of space-filling (Corey-Pauling-Koltun) models indeed suggests the possibility that a steroid molecule may interact with a short nucleotide sequence: if the bases are closely stacked, this could be a trinucleotide. In the case of progesterone, for example, this would permit hydrogen bonding between the C-3 and C-20 carbonyl groups and the 2-amino groups of guanine moieties, if the latter occupied positions 1 and 3 of the trinucleotide sequence. Additional contributions would presumably come from forces of the van der Waals type.

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