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Interactions of Hormonal Steroids with Nucleic Acids. II. Structural and Thermodynamic Aspects of Binding*

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ABSTRACT: Structural attributes governing associations of estradiol, progesterone, and testosterone with polyguanylic acid and denatured deoxyribonucleic acid in aqueous buffer have been determined using equilibrium dialysis as an assay method. Structural substitution of the steroids indicates that in each case two functional groups are necessary for binding to guanine residues in polyguanylic acid: the 3- and 20-keto groups of progesterone, the 3- and 17-hydroxyl groups of estradiol, and the 3-keto and 17-hydroxyl groups of testosterone. These observations, the fact that these functional groups can act as proton donors or acceptors, and the minimal effect of slightly protic solvents on the binding of the parent steroids to polynucleotides suggest that two hydrogen bonds are formed in each case between functional groups of steroid and purine. Values for free energy of binding of 7–10 kcal/mole of steriod bound, derived from binding constants corresponding to the maximum slopes of binding isotherms, are consistent with the formation of two hydrogen bonds. While the relevant functional groups of guanine residues are not defined in all cases, studies with polyinosinic acid and hydroxymethylation of the 2-amino group indicate that this group is essential to the binding of progesterone and testosterone but not of estradiol. Alteration of binding properties by factors affecting polynucleotide conformation and the finding that the number of binding sites corresponding to maximum binding constants is less than 1/10,000 nucleotide residues demonstrate the critical role of polymer geometry in the provision of optimal binding sites. Construction of space-filling molecular models indicates that these hormonal steriods may interact with short, single-stranded nucleotide sequences.

As shown by equilibrium dialysis in aqueous buffer, the hormonal steriods progesterone, estradiol, testosterone, and corticosterone bind to denatured but not to native DNA (Cohen and Kidson, 1969b). Analysis of steroid binding to synthetic polynucleotides revealed a specific requirement for guanine residues and, in accord with their binding to DNA, a preferred affinity for single-stranded regions. Although these steroids share a common specificity for guanine, only estradiol was found to bind to poly I, suggesting that different functional groups of the nucleotide base are involved in these associations. Conformation of the polynucleotides appeared to influence binding: conditions associated with increased base stacking (high salt) enhanced the binding of progesterone and testosterone to denatured DNA but did not appreciably affect the binding of estradiol.

Binding was also found to depend upon steroid structure: a 17α-OH substituent reduced binding; steroids such as hydrocortisone and ecdysone, bearing several hydroxyl groups, did not bind measurably. These data suggest that the nature of the functional groups and net hydrophobicity of the steroids may influence interactions with polynucleotides.

In the present studies we examine further some of the structural requirements for associations between three steroids (estradiol, testosterone, and progesterone) and polynucleotides. Evidence is presented which suggests that these associations involve the formation of hydrogen bonds between given groups of steroid and guanine residues in the polynucleotide.

Materials and Methods

DNA. Pseudomonas aeruginosa DNA was isolated and denatured as described previously (Cohen and Kidson, 1969b). DNA concentration was determined spectrophotometrically on the native form in HMP buffer (Ts'o and Lu, 1964) on the basis $E^{1\%}_{1\,cm}$ 200 at 260 mμ.

Ribopolymers. Poly G and poly I were obtained from Miles Laboratories, Inc. Their concentrations were determined spectrophotometrically on the basis of the following ϵ_{max} poly G (ϵ_{252} 9.10^3) and poly I (ϵ_{248} 10^4).

Radiochemicals. The following radioactive steroids of high specific activity were obtained from the New England Nuclear Corp.: [1,2-^{3}H]testosterone (50 Ci/mmole), [6,7-^{3}H]estradiol-

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17β (50 Ci/mmole), [1,2-^{3}H]progesterone (50 Ci/mmole), and [6,7-^{3}H]estrone (50 Ci/mmole). Dr. W. Hafferl kindly supplied [10,11-^{3}H]3-methoxyestrone (15 Ci/mmole). In all cases radiochemical purity was checked by thin-layer chromatography in hexane–ethyl acetate–methanol (9:9:2, v/v). Radioactivity was assayed using a Packard radiochromatogram scanner. Other substituted radioactive steroids were prepared as follows.

Preparation of [1,2-^{3}H]3-Desoxytestosterone. I. [1,2-^{3}H]ANDROST-4-EN-17-OL-3-ONE ETHYLENE THIOKETAL. [1,2-^{3}H]Testosterone (20 μCi) was treated for 15 min with 10 μl of ethane dithiol (Baker) in 0.5 ml of acetic acid containing *p*-toluenesulfonic acid monohydrate (Matheson Coleman and Bell) according to the method of Ralls and Riegel (1954). The reaction mixture was then diluted with water and extracted three times with ether. Thin-layer chromatography (hexane–ethyl acetate, 9:1, v/v) of the crude extract revealed a single radioactive band with an R_F value corresponding to that of a nonradioactive sample prepared under the same conditions and identified by its nuclear magnetic resonance spectrum.

II. BIRCH REDUCTION OF [1,2-^{3}H]ANDROST-4-EN-17β-OL-3-ONE ETHYLENE THIOKETAL. The thioketal prepared above (15 μCi) was treated with liquid ammonia in the presence of lithium in ether–ethanol (1:1, v/v) for 15 min at $-80°$. After evaporation of excess ammonia, addition of one-half volume 1 N HCl and extraction by $CHCl_3$, thin-layer chromatography (hexane–ethyl acetate, 3:1, v/v) revealed a single radioactive band with an R_F value corresponding to that of a nonradioactive sample of androst-4-en-17β-ol (3-desoxytestosterone) prepared by the same method and identified by its melting point, infrared, and nuclear magnetic resonance spectra.

Preparation of [1,2-^{3}H]3-Desoxyprogesterone. I. [1,2-^{3}H]-PREGN-4-EN-20α-OL-3-ONE ETHYLENE THIOKETAL. [1,2-^{3}H]-Pregn-4-en-20α-ol-3-one (50 μCi of 50 Ci/mmole), kindly provided by Dr. Adela Zeitlin, was treated with ethane dithiol under the same conditions as for testosterone. On thin-layer chromatography the ether extract (45 μCi) revealed a single radioactive peak with an R_F value corresponding to that of a nonradioactive sample of the thioketal prepared by the same technique and identified by its melting point and nuclear magnetic resonance spectrum.

II. BIRCH REDUCTION OF [1,2-^{3}H]PREGN-4-EN-20α-OL-3-ONE ETHYLENE THIOKETAL. The thioketal band eluted from the thin-layer plate (30 μCi) was treated, extracted, and purified as described above for the testosterone derivative and was found to be identical chromatographically with a nonradioactive sample of pregn-4-en-20α-ol.

III. JONES OXIDATION OF [1,2-^{3}H]PREGN-4-EN-20α-OL. The eluted band from part II (25 μCi) was treated with 0.1 ml of Jones' reagent in 0.5 ml of acetone for 15 min at room temperature. Ether extraction gave a single radioactive band with an R_F value corresponding to that of a nonradioactive sample of pregn-4-en-20-one prepared by the same method and identified by its melting point and its nuclear magnetic resonance spectrum.

Preparation of [6,7-^{3}H]3-Methoxyestradiol. Reaction of [6,7-^{3}H]estradiol with dimethyl sulfate (Mallinckrodt) in methanolic KOH (1 M) gave the 3-methyl ether. On thin-layer chromatography the ether extract gave a single band with an R_F value corresponding to that of a nonradioactive sample prepared by the same method.

Reaction of DNA and Poly G with Formaldehyde. DNA or poly G, 0.5 mg/ml in HMP containing 6 M formaldehyde (Fischer), was heated in a sealed tube at 100° for 15–30 min, followed by rapid cooling on ice. Excess formaldehyde was removed by dialysis for 16 hr. Before and after dialysis both treated polymers showed the same red shifts in their ultraviolet spectra, 6 mμ in the case of DNA and 4 mμ in the case of poly G.

Spectroscopy. Ultraviolet spectra were measured with a Zeiss PMQ II spectrophotometer. Optical rotatory dispersion spectra were recorded with a JASCO spectropolarimeter (ORD-UV5 model) using a cell of 1-cm path length. Nuclear magnetic resonance spectra were recorded with a Varian HA100 spectrometer at 100 Mc operating in the frequency sweep mode.

Buffers and Solvents. HMP (0.0025 M Na_2HPO_4–0.005 M NaH_2PO_4–0.001 M Na_2EDTA, pH 6.8) was used as the standard buffer in most experiments. Experiments which examined the effect of protonation of guanine on binding were carried out in 0.01 M sodium citrate–HCl at pH values stated in the text, the pH being adjusted with HCl. In experiments which examined the influence of organic solvents on binding, methanol (Merck), ethylene glycol (Mallinckrodt), or dimethyl sulfoxide (Baker) were each used at a concentration of 20% in the phosphate buffer, adjusted so that the final molarity was equivalent to HMP.

Measurement of Binding Parameters. Binding data have been expressed previously in terms of nK (M^{-1}) (Cohen and Kidson, 1969b). Calculation of binding constants, K, can be made if it is possible to approach saturation of polynucleotide binding sites within the solubility limits of the steroids in aqueous buffer. These limits were found to be slightly greater than 10^{-5} M for estradiol, progesterone, and testosterone in HMP buffer.

Equilibrium dialysis was carried out as described previously (Cohen and Kidson, 1969b) using polymethylmethacrylate cells of 100 μl each side, separated by pretreated Visking dialysis membrane. Solutions of each polymer were used at concentrations of about 2×10^{-3} M in nucleotide residues, with a series of steroid concentrations ranging from 10^{-11} to 10^{-5} M. The polymer solution was introduced by microsyringe into one side of the cell, the steroid solution into the opposite side, and dialysis cells were agitated continuously on a horizontal shaker at 4°. After equilibrium was reached (48 hr; Cohen and Kidson, 1969b) concentrations of bound and free steroid were determined by radioassay of 10-, 20-, or 50-μl aliquots removed by microsyringe from each side of the cell. Radioactivity was assayed by liquid scintillation spectrometry using a dioxane phosphor and ranged from 300 to 10,000 cpm in each aliquot.

For determination of the binding constant, K, of the steroid to the nucleotide binding site and of the number of binding sites, n, per nucleotide unit, the method of Scatchard (1949) was applied. According to the mass action law, $B/F = Kn - KB$, where B refers to the number of steroid molecules bound per nucleotide unit at a free concentration, F, of steroid. If each binding site were random, independent, and equivalent, a Scatchard plot of B/F *vs.* B would yield a straight line, whose ordinate intercept is nK, whose abscissa intercept is n and whose slope is $-K$. Where there is a series of different binding sites, the slope $(B/F)/B$ at a particular value of B is still an indication of the weighted, average association constant prevailing at that B value (Blake and Peacocke, 1968). In the

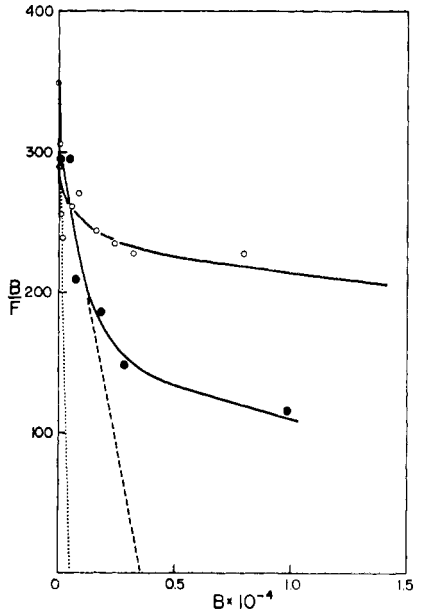


FIGURE 1: Scatchard plots of binding of testosterone to poly G and denatured *Ps. aeruginosa* DNA by equilibrium dialysis. Conditions, 4°, 48 hr in HMP buffer, pH 6.8. (—●—) Poly G and (—○—) denatured *Ps. aeruginosa* DNA.

present experiments, maximum binding constants have been calculated on the basis of the maximum slope of the binding isotherm.

Where binding studies have employed substituted steroids with a given polymer, n is presumed to be potentially the same as for the parent steroid, permitting a direct comparison of nK values at a single steroid concentration. The free energy of binding, ΔG, was calculated from the maximum values for K for each steroid with a given polymer.

Results and Discussion

Binding Isotherms. Figure 1 shows typical biphasic binding isotherms obtained by Scatchard treatment of the association between steroid and polynucleotide at equilibrium. From the shape of the curve it seems likely that more than one type of binding exists, even for the homopolymer poly G. Data derived in this manner for the binding equilibria of progesterone, testosterone and estradiol with poly G are listed in Table I. The number of binding sites n derived from the maximum slope, $-K$, was found to be low in each instance. In the case of poly G only about 1/10,000 to 1/30,000 guanine residues appeared to function as binding sites corresponding to the maximum value of K. Even considering the high degree of ordered structure of this polymer and the likelihood that a large proportion of guanine residues is probably involved in hydrogen-bonded, multistrand structures (Pochon and Michelson, 1965), these data may well reflect the nonrandomness of guanine binding sites. The maximum binding constants K of the three steroids to a nucleotide binding site were of the order of 10^6–10^7 (Table I). These correspond to negative values for the free energy of binding of about 7–8 kcal/mole of bound steroid.

With denatured *Pseudomonas aeruginosa* DNA the binding

TABLE I: Values of K^a and n Derived from Scatchard Analysis of Binding Equilibrium with Poly G in HMP Buffer.

Steroid	n	K (M^{-1})
Testosterone	3.5×10^{-5}	8.6×10^6
Progesterone	1.7×10^{-4}	7.3×10^6
Estradiol	1.3×10^{-4}	2.5×10^6

[a] Maximum binding constants were calculated on the basis of the maximum slope of the binding isotherm.

isotherms were similarly biphasic but somewhat different in shape, as shown for testosterone in Figure 1. At very low steroid concentrations scattering of points occurred due to decreased accuracy of radioassay. However, reproducible best-fit curves were obtained. Here the number of binding sites per nucleotide unit, n, corresponding to the maximum slope, $-K$, was about 3.2×10^{-6} and the maximum binding constant, K, was about 1.2×10^8 M^{-1}. This binding constant corresponds to a negative value of the free energy of binding of about 10 kcal/mole of bound steroid.

Other types of binding of these steroids to poly G or to DNA have not been further investigated here although it is evident from the binding isotherms that they do occur. It is apparent, however, that they will have lower binding constants and that they will involve considerably higher numbers of binding sites per nucleotide unit.

Solvent Effects. In order to evaluate the possible contribution of hydrophobic forces to the association of steroids with both poly G and denatured DNA, we have investigated the binding equilibrium in the presence of organic solvents. Weakly protic solvents, in reducing the dielectric constant of the medium, should stabilize hydrogen bonds while being active in disrupting hydrophobic forces (Singer, 1962; Sage and Singer, 1962). Ethylene glycol and methanol, both in this category, and a nucleophilic solvent, dimethyl sulfoxide, were used. At standard concentrations of steroid and polymer, the addition of 20% methanol or dimethyl sulfoxide to the HMP buffer reduced the nK values to some extent without abolishing the binding of estradiol, testosterone, and progesterone to the polymers (Table II). In most cases little or no effect was observed with ethylene glycol. Binding isotherms of the steroids with poly G in HMP–20% methanol were similar to those in HMP alone except for a slight shift corresponding to the small change in nK (Figure 2). From Figure 2, the values for n for testosterone and progesterone, respectively, with poly G were 3.4×10^{-5} and 1.3×10^{-4} M^{-1} while the values for K were 5.2×10^6 and 6.2×10^6 M^{-1}. These values are not very dissimilar to those in HMP alone (Table I). These observations suggest that if hydrophobic forces, including van der Waals interactions and the aggregation tendency of nonpolar groups due to strong water–water interactions of the solvent (Sinanoglu and Abdulnur, 1964), contribute to some extent to the binding, they are not the only forces involved. Effects of the organic solvent on the structure of the polymer cannot, of course, be distinguished from its effects on the interaction between the steroid and the polymer. Known destabilizing effects of high concentrations of organic solvents on polynucleo-

TABLE II: Solvent Effects on Binding of Steroids by Denatured DNA and Poly G.

Steroid	Solvent	nK (M^{-1}) DNA[a]	nK (M^{-1}) Poly G
Estradiol	HMP	65	298
	HMP–20% MeOH	54	245
	HMP–20% ethylene glycol	65	278
	HMP–20% dimethyl sulfoxide	20	94
Progesterone	HMP	185	800
	HMP–20% MeOH	170	530
	HMP–20% ethylene glycol	203	790
	HMP–20% dimethyl sulfoxide	145	433
Testosterone	HMP	102	223
	HMP–20% MeOH	70	187
	HMP–20% ethylene glycol	70	234
	HMP–20% dimethyl sulfoxide	61	183

[a] DNA = denatured *Ps. aeruginosa* DNA.

tide structure (Fasman *et al.*, 1964, 1965; Adler *et al.*, 1967) restrict the amount of information which can be derived from studies of this kind.

Influence of pH. Using standard concentrations of poly G and of each steroid, in 0.01 M sodium citrate–HCl buffer, binding was shown to depend upon pH (Table III). Although there was a decrease in nK for testosterone, binding of all three steroids persisted at pH 5.0 but was abolished or very low at pH 2.2. At this pH protonation of N-7 of the purine ring occurs (Pochon and Michelson, 1965). At an ionic strength of 0.15 M in sodium ions, the structure of poly G has been reported to be stable, as judged by optical rotatory dispersion (Ulbricht *et al.*, 1966). In the present studies, however, it has been found that the stable conformation is dependent upon the ionic strength. In the 0.01 M sodium citrate–HCl buffer used, a marked change in conformation of poly G was observed on optical rotatory dispersion (Figure 3), as judged by altered rotatory strength of the Cotton effects in the regions of 220, 250, 270, and 300 mμ. These changes were reversible on returning to pH 6.8 (Figure 3) and, in accord with the observations of Ulbricht *et al.* (1966), were prevented by increasing the ionic strength at pH 2.2 to 0.15 M with NaCl. Although precise interpretation of the altered optical rotatory dispersion of poly G is not possible, protonation of the gua-

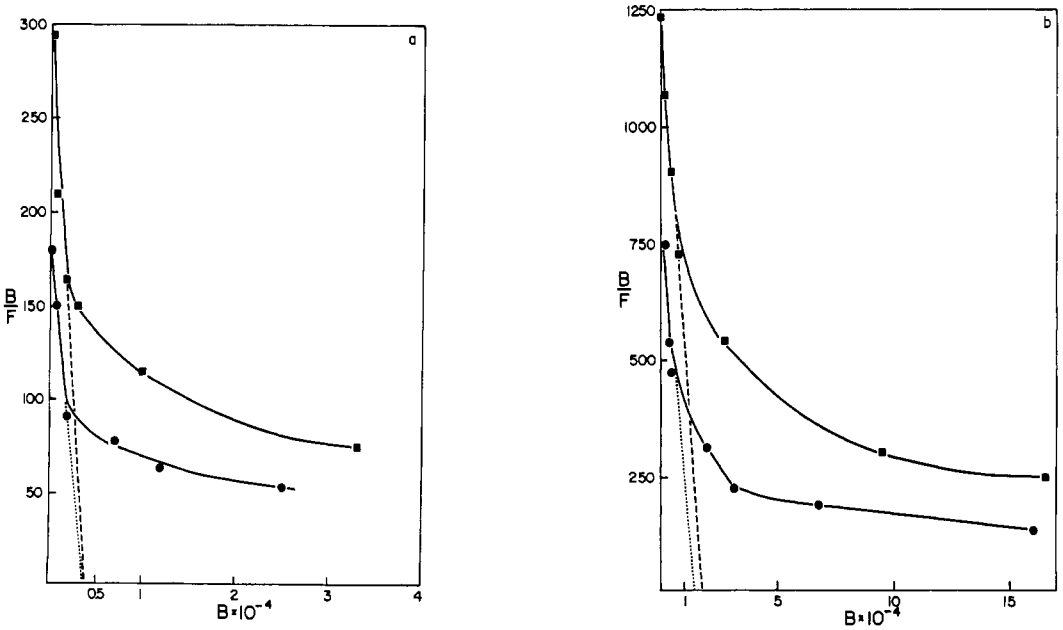


FIGURE 2: Scatchard plots of steroid binding to poly G in the absence and presence of 20% methanol. Equilibrium dialysis at 4° for 48 hr. (a) Binding of testosterone to poly G in HMP and in HMP–20% methanol. (—■—) HMP and (—●—) HMP–20% methanol. (b) Binding of progesterone to poly G in HMP and in HMP–20% methanol. (—■—) HMP and (—●—) HMP–20% methanol.

TABLE III: Effect of pH on Steroid Binding to Poly G.[a]

Steroid	nK (M^{-1}) pH 6.8	pH 5.0	pH 2.2
Testosterone	176	115	0
Progesterone	750	859	17
Estradiol	280	296	20

[a] Equilibrium dialysis in 0.01 M citrate–HCl.

nine residues would tend to cause increased repulsion between neighboring bases and in so doing would distort the conformation of the polymer and hence presumably of binding sites. These data can be correlated with the previous observation (Cohen and Kidson, 1969b) that the binding affinities of testosterone and progesterone (and to a much lesser extent of estradiol) to denatured DNA are enhanced by high ionic strength, where base stacking is increased.

Role of the 2-Amino Group of Guanine Residues. I. STUDIES WITH POLY I. Poly I is 2-deaminated poly G and, as such, is a suitable model for examining any contribution of the 2-amino group of guanine to the binding of steroids to poly G. Previously it was found that estradiol, but not progesterone or testosterone, binds to poly I (Cohen and Kidson, 1969b), suggesting 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. In the present studies, examination of the binding isotherm for the association of estradiol with poly I (Figure 4) showed a curve comparable with that with poly G. The maximum binding constant, K, was about 2.5×10^6 M^{-1} and the corresponding value of n was about 1.0×10^{-4} M^{-1}. The precise structure of poly I under these conditions (0.01 M sodium ion) is not known, but it is likely to be partly ordered (Rich, 1958; Sarkar and Yang, 1965).

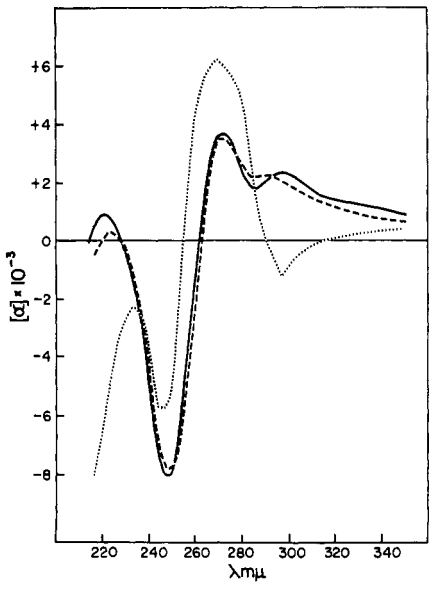


FIGURE 3: Optical rotatory dispersion of poly G in 0.01 M sodium citrate–HCl buffer. (———) pH 6.8, (· · · ·) pH 2.2, and (– – – –) dialyzed from pH 2.2 to 6.8 before spectroscopy. The specific rotation, [α], is expressed in deg ml/dm g of poly G.

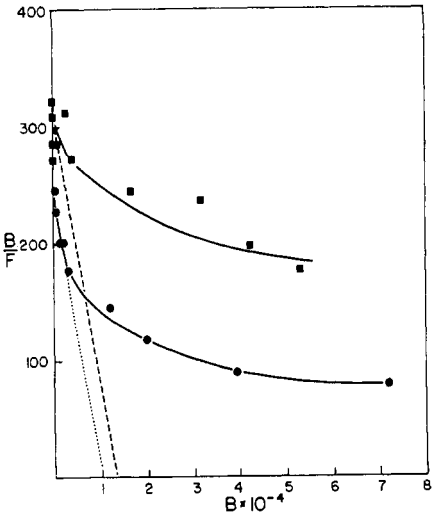


FIGURE 4: Scatchard plots of binding of estradiol to poly G and poly I. Equilibrium dialysis at 4° for 48 hr in HMP buffer, pH 6.8. (—●—) Poly I and (—■—) poly G.

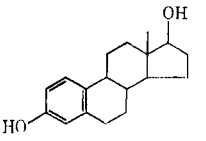
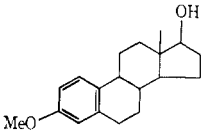
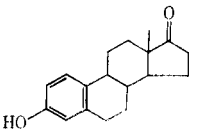
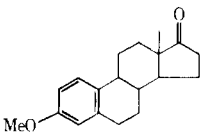
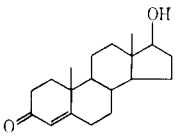
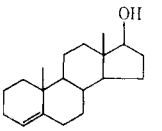
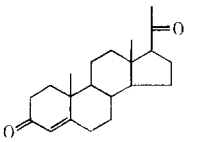
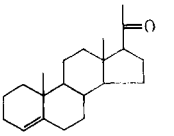
II. HYDROXYMETHYLATION OF DNA AND POLY G. Reaction of nucleic acids with formaldehyde leads to hydroxymethylation of exocyclic amino groups (Grossman *et al.*, 1961; Stollar and Grossman, 1962). At room temperature, amino groups involved in conventional base pairing are not susceptible to electrophilic attack (Haselkorn and Doty, 1961) but at higher temperatures most react with formaldehyde. Reaction of *Ps. aeruginosa* DNA with 6 M formaldehyde at 100° followed by removal of excess formaldehyde abolished the binding of testosterone and progesterone (Table IV) while the nK for estradiol was partially reduced. Reaction of poly G with formaldehyde in the same manner decreased the nK values for testosterone and progesterone while that for estradiol increased (Table IV). With DNA, denaturation and reaction are almost complete at 100°. Denaturation of poly G involves a progressive, noncooperative transition which is not complete at this temperature (Pochon and Michelson, 1965). Denaturation of poly G in the presence of formaldehyde may lead to an opening of the structure without ensuring total hydroxymethylation of the amino groups. Incomplete hydroxymethylation (or partial reversibility of the reaction)

TABLE IV: Binding of Steroids to Formaldehyde-Reacted Polymers.

	DNA[a]	HCHO-Reacted DNA[a]	nK (M^{-1}) Poly G	HCHO-Reacted Poly G
Testosterone	102	0	223	145
Progesterone	243	0	1212	411
Estradiol	56	30	298	600

[a] DNA = Denatured *Ps. aeruginosa* DNA.

TABLE V: Effects on Binding to Polynucleotides of Structural Modification of Steroids.

Solvent	Steroid		nK (M^{-1}) DNA[a]	Poly G	Poly I
HMP	Estradiol		58	298	197
HMP	Estradiol-3-methyl ether		60	190	0
HMP	Estrone		61	352	60
HMP-20% MeOH					0
HMP	Estrone-3-methyl ether		33	220	34
HMP-20% MeOH					0
HMP	Testosterone		102	223	0
HMP	3-Desoxy-testosterone		55	60	0
HMP-20% MeOH			0	0	
HMP	Progesterone			1212	0
HMP	3-Desoxy-progesterone			52	
HMP-20% MeOH				80	

[a] Denatured *Ps. aeruginosa* DNA.

would leave more potential binding sites available in the homopolymer than in DNA, if more than one guanine residue is involved in a binding site (*vide infra*). In addition, cross-linking of adenine residues in DNA (Freifelder and Davison, 1963) could alter the binding properties and spurious binding might occur with the $NHCH_2OH$ group in the case of testosterone and progesterone.

Influence of Substituents at C-3 and C-17 of the Steroid Molecules. I. MODIFICATION OF TESTOSTERONE STRUCTURE. By a two-step procedure [1,2-^{3}H]desoxytestosterone was prepared from [1,2-^{3}H]testosterone (Materials and Methods). Loss of the 3-keto group resulted in decreased nK values with both denatured DNA and poly G (Table V) although some residual binding was present. Since the hydrophobicity of the 3-desoxy derivative is enhanced, this interaction could be purely hydrophobic in nature; complete elimination of binding by the addition of 20% methanol to HMP buffer during dialysis confirmed this view (Table V), and permits the conclusion that the 3-keto group is involved in binding of testosterone. The marked reduction in nK resulting from the change from α to β orientation of the 17-hydroxyl group previously observed (Cohen and Kidson, 1969b) strongly suggests that

this functional group is also involved in the interaction.

II. MODIFICATION OF ESTRADIOL STRUCTURE. Modification of the phenolic function, of the 17β-hydroxyl group or of both hydroxyls altered the binding properties of this steroid (Table V). Conversion of the 3-hydroxyl into the methyl ether eliminated the binding to poly I but not to poly G. Since methylation does not imply loss of hydrogen bonding capacities, this could mean that guanine residues can provide the methoxy with a hydrogen-bond donor absent in inosine, presumably the 2-amino group. Conversion of the 17β-hydroxyl into a carbonyl group (estrone) greatly reduced the magnitude of interaction with poly I: residual binding was eliminated by addition of 20% methanol, suggesting that its nature is mainly hydrophobic. The same trend was observed on modification of both hydroxyls (3-methoxyestrone: Table V). These data suggest that both 3- and 17-hydroxyl groups are involved in the association of estradiol with model polynucleotides.

III. MODIFICATION OF PROGESTERONE STRUCTURE. Removal of the 3-keto group from [1,2-^{3}H]progesterone ([1,2-^{3}H]3-desoxyprogesterone) resulted in a marked decrease in the observed *nK* with poly G (Table V). Residual binding could not be abolished by the presence of 20% methanol in the buffer. However, this concentration of solvent was probably insufficient to reduce the largely hydrophobic character of this interaction, since a considerable amount of the 3-deoxy derivative was observed to bind to the dialysis cell walls (and/or membrane) even in the presence of methanol. We have previously reported a marked decrease in binding affinity with denatured DNA when a 17α-hydroxyl group was introduced into the progesterone molecule, this decrease probably being due to intramolecular hydrogen-bond formation (Cohen and Kidson, 1969b). These observations together demonstrate that in the case of progesterone both the 3- and 20-keto groups are involved in the binding of this steroid to polynucleotides.

Although hydrophobic forces are involved, several observations together suggest that hydrogen bonds play an important role in these associations of steroids with polynucleotides: the effect on binding of substitution of steroid functional groups, examination of the function of the 2-amino group of guanine, the effects of slightly protic solvents, and the magnitude of the free energy of binding. Further, the finding that for each of the three steroids, estradiol, testosterone, and progesterone, two functional groups (either proton donor or acceptor) are together involved suggests that two hydrogen bonds are formed between steroid and polynucleotide: free-energy values of 7–10 kcal/mole of steroid bound are compatible with this view. If two hydrogen bonds are formed, two guanine residues would be necessary to bind one steroid molecule. This is possible if the steroid molecule associates with a short nucleotide sequence.

In view of the role of the 2-amino group of guanine, it may reasonably be assumed that this substituent acts as a proton donor in hydrogen bonds with the 3-keto group of testosterone and with the 3- or 20-keto groups of progesterone. The 17-hydroxyl group of testosterone could presumably act as a proton donor in forming a hydrogen bond with either the 6-carbonyl group or the nitrogen at position 3 in guanine. The 3- or 17-hydroxyl groups of estradiol could, for example, each form a hydrogen bond with the 6-carbonyl group of a guanine residue.

The basis for the guanine specificity inferred from studies with homopolymers (Cohen and Kidson, 1969b) is not entirely clear, since other bases carry functional groups potentially suitable as proton donors or acceptors in hydrogen bonds. The reason for this specificity could reside in factors other than the availability of chemically adequate functional groups. One possible explanation may lie in the conformation of a given nucleotide sequence. The effect of high salt in enhancing steroid binding to denatured DNA (Cohen and Kidson, 1969b), the effect of protonation of guanine residues in poly G in decreasing or eliminating steroid binding and the low numbers of binding sites reported here indicate that there is an optimal polymer conformation which provides favorable binding site configuration. The construction of space-filling, Corey–Pauling–Koltun molecular models illustrates the probable role of base stacking. For example, the sequences GpGpG or dGpdGpdG can interact with progesterone or testosterone by the formation of hydrogen bonds between the relevant functional groups of the steroid and those of the terminal guanine residues, provided that the three bases are closely stacked. As judged from the models, the same degree of stacking is apparently not favorable for estradiol binding. Little is known about the conformation of particular short nucleotide sequences in a polynucleotide. However, the presence of bases other than guanine in or adjacent to a nucleotide sequence which forms a potential steroid binding site would obviously change its geometry. There is at present no knowledge about the types of nucleotide sequences which might form steroid binding sites on copolymers such as single-stranded regions of DNA or RNA.

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