Molecular Interactions of Progesterone Analogues with Rabbit Uterine Cytoplasmic Receptor*

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Binding energies of progesterone analogues with single modifications were calculated from their affinities for the cytosolic receptor of rabbit uterus. The effects of individual substituents were analyzed in terms of hydrogen bonds, van der Waals’ forces, and hydrophobic interactions. Binding to the receptor is attributed to hydrogen bonds involving the ketones at carbons 3 and 20, and van der Waals’ interactions at carbons 2, 4, 7, 9, 12, 18, and 19 at which positions the separation of the steroid from the receptor appears to be about 0.1 nm or less. Greater separation occurs at carbons 6, 11, 14, 15, 16, and 21. The receptor probably has a hydrogen acceptor approximating the 11β position of the bound steroid. The enthalpy of binding of the progesterone molecule is about 26 kcal/mol but on the basis that the two hydrogen bonds contribute about 6 kcal/mol and each of the van der Waals’ attractions about 1 kcal/mol, the sum of the individual bonds totals only about 20 kcal/mol. The difference of 6 kcal/mol is attributed to intrareceptor bonds that are established after a change in receptor conformation is initiated by progesterone binding. This change in conformation fixes the steroid in its protein niche and retards dissociation. We speculate that this alteration in conformation is related to “activation” and possibly other functions of the complex.

Progesterone’s action on the uterus is mediated by specific, high affinity binding to a cytoplasmic receptor. The relative binding energies of various unlabeled progestational and other compounds can be calculated from equilibrium competition experiments. Furthermore, the binding energy of the steroid molecule and the receptor protein macromolecule presumably represents the sum of the binding energies of individual atoms involved in hydrogen bonding, induced dipolar van der Waals’ forces, and hydrophobic interactions. In an attempt to identify the sites on the steroid molecule responsible for the intermolecular interactions with the receptor protein, we have determined systematically the relative binding energies of progesterone analogues which differ from progesterone by a single substituent or by another simple modification.

Other investigators have measured the relative binding strengths of progesterone and other progestational agents to mammalian receptors in attempts to correlate binding with the molecular structure of progesterone (Fig. 1) (e.g. Kontula et al., 1973; Smith et al., 1974; McGuire et al., 1974; Kontula et al., 1975). Our study differs in that we have compared compounds that have single modifications or substitutions permitting interpretation of steric and electronic interactions at specific sites on the progestosterone molecule. The analogues studied and their sources are listed in Table II.

MATERIALS AND METHODS

[1,2,6,7-3H]Progesterone† (103.7 Ci/mmol), [17α-methyl-3H]17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione (87.0 Ci/mmol), and unlabeled R5020 were purchased from New England Nuclear. Nonradioactive progesterone (P) was purchased from Steraloids and estradiol-17β from Sigma. Sources of other steroids are cited in Table I. 4-Pregnen-3-one was synthesized in our laboratory by Oppenauer oxidation of 4-pregnen-3-ol (Neustaeder, 1963). Purification was achieved by melting point, thin layer chromatography, and ultraviolet and infrared spectroscopy.

All studies were performed at 0 °C and pH 8.0 in 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid buffer containing 1.5 mM EDTA disodium salt, 12 mM monothioglycerol, and 20% glycerol. Free steroids were removed with a suspension of 0.5% charcoal and 0.05% dextran in buffer. Radioactivity of 0.4- and 0.6-ml samples were measured by liquid scintillation counting in 5 ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene. Protein concentrations were measured by the method of Bradford (1976), modified so that the final dye solution contained 0.25 g of Coomassie brilliant blue G-250 per liter of methanol, o-phosphoric acid (85%), and distilled water in the proportions 1:2:17 (v:v:v). Cytoplasmic progesterone receptors were prepared from the uteri of immature New Zealand White rabbits as previously described (Seeley et al., 1980). The cytosol was dialyzed for 24 h against buffer containing 5% charcoal to remove endogenous steroids.

Competition Experiments—Binding energies were calculated from equilibrium competition experiments using R5020 as the standard. Various concentrations (0–200 nM) of unlabeled compounds were incubated for 24 h with 5 nM tritiated R5020 in 0.4 ml of a 0.3 mg/ml solution of cytosol with and without an excess of unlabeled R5020. The unbound tritiated R5020 was removed with charcoal/dextran and the concentration of specifically bound radioactivity R5020 was calculated by the method of Blondeau and Robel (1975).

Data Analyses—The concentration of progesterone receptor, Qo, and the equilibrium dissociation constants, Kd, values for R5020 receptor and progesterone-receptor interactions were calculated from Scatchard plot analysis (Scatchard, 1949) and the Kd values determined from the regression lines (Fig. 2) were in agreement with the values determined by direct linear plot (Essential and Cornish-Bowden, 1974) to within 7%.

The data from the competition experiments are plotted in Fig. 3. The fraction (B/U)/(B/U)max is shown as a function of the logarithm of competitor concentration where (B/U) is the ratio of bound to

† The abbreviations used are: progesterone (P), 4-pregnen-3,20-dione; R5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; estradiol, 1,3,5(10)-estratriene-3,17β-diol.

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The accuracy of the measured ratio, $\rho = [C]/[R5020]$, of competitive inhibitor concentration to R5020 concentration. In addition to statistical errors, nonspecific binding could affect the accuracy by reducing the concentration of ligand that can interact with the receptor. No error is introduced in the ratio, $\rho$, if the nonspecific binding is a constant percentage of the total ligand added. An error, $\Delta \rho$, would be introduced if the percentage of nonspecific binding varied from ligand to ligand. Nonspecific binding of R5020 and of progesterone in crude cytosol is typically about 3% of the total ligand added. If other progesterone analogues have nonspecific binding less than 10% of the total ligand, then the estimated error in calculating binding energy differences would be approximately $(RT \Delta \rho)/\rho$, which is less than 0.05 kcal/mol.

The use of the competition method for estimating binding energy differences is further validated by comparing energies obtained by this technique with energies calculated from equilibrium binding constants derived from Scatchard plots and direct linear plots. The dissociation constants determined from Scatchard plots are $K_d^{R5020} = 0.53$ nm and $K_d^{[H]R5020} = 1.32$ nm (Fig. 2). These constants correspond to the energy differences, $\Delta G^*$, between the unbound and steroid-bound states of the receptor: $\Delta G^{R5020} = -11.59$ kcal/mol and $\Delta G^{[H]R5020} = -11.10$ kcal/mol. The difference in binding energies, $\delta \Delta G^*$, between progesterone binding and R5020 binding to the receptor is calculated to be $-0.49$ kcal/mol. Similarly, the $\delta \Delta G^*$ derived from direct linear plots

Validation of Energy Difference Calculation — The accuracy of this method for calculating binding energy differences depends upon
is \(-0.56\) kcal/mol. From experiments that measure the competition for the receptor binding sites between tritiated R5020 and unlabeled progesterone or unlabeled R5020, the difference in binding energies of \(\Delta G^*\) is \(-0.51\) kcal/mol. Therefore, the agreement between these three methods is within 10%, confirming the reliability of the competition technique.

Of the 82 compounds that we have studied, the relative affinities of 15 between the steroid and receptor have been measured previously by Kontula et al. (1975). Energies derived from our measurements correlate well with those recalculated from the data of Kontula et al. (Fig. 4); the slope is 1.13 and the coefficient of determination is 0.93.

RESULTS AND DISCUSSION

General Interactions between Progesterone and the Receptor—We have shown previously that low energy bonds, totaling about 26 kcal/mol, are formed between the progesterone molecule and the receptor protein (Seeley et al., 1980); this rules out covalent or strong ionic bonding. A positive entropy change upon dissociation indicates that the orientations of the progesterone bound to the receptor are restricted.

General Interactions between Progesterone and the Aqueous Medium—The small enthalpy change measured for the association of progesterone and the receptor (3 kcal/mol) is probably due to bonding between progesterone and water in the buffer (Seeley et al., 1980). The low energy implies that, on the average, only one hydrogen bond per progesterone molecule is formed with water. Alternatively, this energy could be due partially to the change in the hydrogen bond strengths between water molecules as they reorient upon removal of the nonpolar progesterone molecule (Frank and Evans, 1945). The removal of progesterone from the aqueous medium should also be accompanied by an increase in entropy due to the hydrophobic effect of reorienting the water molecules in the absence of the progesterone molecule (Arnett et al., 1969). The measured entropy change is negative, however, which indicates that a highly specific orientation of progesterone relative to the receptor binding site is required for the formation of the progesterone-receptor complex (Seeley et al., 1980).

The importance of steroid-water interactions is apparent also from comparisons of binding energies of progesterone analogues with methyl and hydroxyl substituents. A hydroxyl substituent reduces receptor binding relative to a methyl substituent for paired analogues at nine positions (Table I). The mean energy difference is 1.7 kcal/mol. The spaces occupied by methyl and hydroxyl groups are comparable so the hydrogen bonds broken between the oxo groups and water and the bonds formed between the oxo groups and the receptor when progesterone is bound. The enthalpy of the oxo groups' interactions with water is about \(-3\) kcal/mol. If we ignore the entropy difference between progesterone and deoxyprogesterone, the \(-3\) kcal/mol free energy reflects the difference in enthalpies of bonds broken between the oxo groups and water and the bonds formed between the oxo groups and the receptor when progesterone is bound. The enthalpy of the oxo groups' interactions with water is 3 kcal/mol (Seeley et al., 1980). Therefore, the enthalpy of the oxo groups interaction with the receptor is about \(-6\) kcal/mol. These values are consistent with the hypothesis that each of progesterone's oxo groups forms one hydrogen bond with a hydrogen donor on the receptor.

Typical enthalpies of bond formation between a hydrogen donor and a carbonyl group range between \(-2\) and \(-5\) kcal/mol (Murthy and Rao, 1968). In general, the strengths of the bonds increase in direct proportion to the electronegativity of the hydrogen donor (\(O > N > S\)). Because the ranges of enthalpies for each type of hydrogen donor overlap considerably, identification of the hydrogen donors on the receptor cannot be determined from the enthalpy sum of \(-6\) kcal/mol.

Conformational Changes in the Basic Pregnenone Structure—Reduction of the double bond between carbons 4 and 5 yields two diastereomers: 5a-pregnane-3,20-dione and 5b-pregnane-3,20-dione. The 5a-isomer has a 3-oxo orientation that is similar to the 1\(\alpha\), 2\(\beta\)-half chair conformation of progesterone and its binding affinity for the receptor is approximately that of progesterone. The 3-oxo group on the 5b-isomer is oriented toward the \(\alpha\)-side and its relative binding energy is 2 kcal/mol. This change indicates that a hydrogen bond between the 3-oxo group of the 5b-isomer and the receptor is disrupted.

A double bond between carbons 1 and 2 of the progesterone molecule increases the polarizability of the conjugated carbonyl group at carbon 3 and flattens the A ring between carbons 10, 1, and 2. The increased polarizability would strengthen the hydrogen bond between the 3-oxo group and the hydrogen donor of the receptor. The relative affinity is decreased, however. The change in the 3-oxo position relative

\[\Delta G^* = \Delta H^* - T\Delta S^*\]

\(\Delta G^*\) is the difference in Gibbs free energy between an initial state and a final state of a reaction (e.g. in the initial state the steroid and the receptor are unbound in the final state, the steroid and receptor are bound to form a complex). Enthalpy, \(\Delta H^*\), is the energy required to break bonds or release when bonds are formed. Entropy, \(\Delta S^*\), is a measure of the randomness of a system. They are related by the equation: 

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TABLE I

Differences between the binding energies of hydroxyl- and methyl-substituted analogues

| Position | Differences in relative binding energy (\(\Delta G^*\)) | kcal/mol |
|----------|-----------------------------------------------------|----------|
| 2x       | >0.94                                               |          |
| 4        | >2.04                                               |          |
| 6a       | >1.53                                               |          |
| 6p       | >2.34                                               |          |
| 7a       | >1.96                                               |          |
| 15b      | 1.06                                                |          |
| 16a      | >1.96                                               |          |
| 16p      | 0.30                                                |          |
| 17a      | >3.15                                               |          |
| Mean     | >1.71                                               |          |

Effects of Structural Modifications of Progesterone on Relative Binding Energies—Among the modifications made were: 1) removal of oxo groups, 2) changes in the basic pregnene structure by reduction of the double bond at carbon 4 or introduction of additional double bonds, 3) removal of the angular methyl groups at carbons 10 and 13, and 4) addition of hydrophobic methyl groups, hydrophilic hydroxyl groups (many of which are discussed above), or halides. The effects are tabulated in Table II.

Removal of Oxo Groups—Loss of the 3-oxo group or 20-oxo group reduces affinity for the receptor by a relative binding energy of 1.2 kcal/mol and 1.7 kcal/mol, respectively. Thus, the sum of the oxo groups' contributions to the free energy of binding is about \(-3\) kcal/mol. If we ignore the entropy difference between progesterone and deoxyprogesterone, the \(-3\) kcal/mol free energy reflects the difference in enthalpies of bonds broken between the oxo groups and water and the bonds formed between the oxo groups and the receptor when progesterone is bound. The enthalpy of the oxo groups' interactions with water is 3 kcal/mol (Seeley et al., 1980). Therefore, the enthalpy of the oxo groups interaction with the receptor is about \(-6\) kcal/mol. These values are consistent with the hypothesis that each of progesterone's oxo groups forms one hydrogen bond with a hydrogen donor on the receptor.
gated carbonyl group at carbon 3 and flattens the B ring. The bond moderately decreases affinity for the receptor. This 1 kcal/mol energy difference the 3-oxo group, but the binding energy increases by 0.69 kcal/mol. This difference may result from steric effects around the 6 and 7 carbons or from conformational changes elsewhere in the steroid molecule.

Modification of the B/C ring junction by an 8-9 double bond moderately decreases affinity for the receptor. This additional double bond has little effect on the orientation of the 3-oxo group, but the binding energy increases by 0.69 kcal/mol.

| Source | Relative binding energy (ΔG') | Compound | Relative binding energy (ΔG') | Source |
|--------|-------------------------------|----------|-------------------------------|--------|
| a      | Progesterone                  | 4-Pregnene-3β,20β-diol | >3.00 | n      |
| b      | Halide substituents           | 6α-Fluoro-4-pregnene-3,20-dione | -0.06 | e      |
| c      | 17α-Fluoro-4-pregnene-3,20-dione | 0.18 | e      |
| d      | 17α-Bromo-4-pregnene-3,20-dione | 0.28 | e      |
| e      | 21-Fluoro-4-pregnene-3,20-dione | 0.54 | e      |
| f      | 21-Iodo-4-pregnene-3,20-dione | 0.60 | n      |
| g      | 2α-4-Pregnene-3,20-dione | 0.63 | e      |
| h      | 6β-Fluoro-4-pregnene-3,20-dione | 0.65 | e      |
| i      | 2α-4-Fluoro-4-pregnene-3,20-dione | 0.68 | e      |
| j      | 21-Difluoro-4-pregnene-3,20-dione | 0.72 | t      |
| k      | 6β-Chloro-4-pregnene-3,20-dione | 1.45 | s      |
| l      | Dehydroprogestogens           | 4,9(11)-Pregnadiene-3,20-dione | 0.04 | e      |
| m      | 4,11-Pregnadiene-3,20-dione | 0.08 | s      |
| n      | 4,14-Pregnadiene-3,20-dione | 0.15 | g      |
| o      | 4,8-Pregnadiene-3,20-dione | 0.44 | e      |
| p      | 4,15-Pregnadiene-3,20-dione | 0.49 | e      |
| q      | 4,6-Pregnadiene-3,20-dione | 0.69 | e      |
| r      | 1,4-Pregnadiene-3,20-dione | 0.59 | s      |
| s      | 4,16-Pregnadiene-3,20-dione | 1.58 | u      |
| t      | Acetoxyprogestogens           | 1α-Hydroxy-4-pregnene-3,20-dione acetate | 0.54 | e      |
| u      | 4-Hydroxy-4-pregnene-3,20-dione acetate | 0.60 | g      |
| v      | 17β-Hydroxy-4-pregnene-3,20-dione | 3.00 | 1      |
| w      | 12α-Hydroxy-4-pregnene-3,20-dione | 3.00 | 1      |
| x      | 7β-Hydroxy-4-pregnene-3,20-dione acetate | 3.00 | 1      |
| y      | Miscellaneous                | 9α-Fluoro-11β-hydroxy-4-pregnene-3,20-dione | 0.13 | e      |
| z      | 17α-Methoxy-4-pregnene-3,20-dione | 0.25 | d      |
| {      | 9α-Fluoro-4-pregnene-3,11,20-trione | 0.44 | e      |
| | 16α-Methyl-4,9-pregnadiene-3,20-dione | 0.54 | g      |
| | 13β-Ethyl-17β-ethylthio-17-hydroxy-gona-4,9,11-trien-3-one | 0.59 | v      |
| | 5α-Pregna-3β,20β-dione | 0.77 | a      |
| | 17α-Bromo-6α-methyl-6-pregnene-3,20-dione | 0.89 | w      |
| | 4-Pregnene-3,11,20-trione | 1.78 | e      |
| | 12α-Methyl-4,9-pregnadiene-3,20-dione | 1.35 | l      |
| | 4,9,11-trien-3-one | 1.09 | x      |
| | 5α-Pregna-11β-3,20-dione | 1.35 | l      |
| | 17α-Bromo-6α-methyl-6-pregnene-3,20-dione | 0.89 | w      |
| | 4,9,11-trien-3-one | 1.78 | e      |
| | 12-Methylene-4-pregnene-3,20-dione | 1.79 | g      |
| | 4-Pregnene-3,11,12-trione | 2.13 | a      |
| | 20β-Hydroxy-4-pregnene-3,11-dione | 3.00 | d      |
| | 6α-Methyl-4,16-pregnadiene | >3.00 | e      |

| Sources | a, purchased from Steraloids, Inc.; b, synthesized in our laboratories from 5-pregnene-3β,20β-dione purchased from Steraloids, c, gift from Ortho Research Foundation; d, gift fr. Sanofi Steelco, Merck Institute; e, gift from John Babcock, Upjohn Co.; f, gift from Dr. M. Fasler, Schering Corp.; g, gift from Dr. Josef Fried, Squibb Institute for Medical Research; h, gift from Dr. Seymour Bernstein, Lederle Laboratories, i, gift from Farmacia Co., k, gift from Dr. M. Ehrenstein, University of Pennsylvania, l, gift from Dr. Victor Drill and Dr. Raphael Pappo, Searle Co.; m, purchased from New England Nuclear Co.; n, purchased from Elite Chemical Co.; o, gift from Dr. Richard Edgren, Wyeth Institute for Medical Research; p, gift from Dr. Robert Kroc, Warner-Lambert Research Institute; q, purchased from Sigma Chemical Co.; r, gift from Dr. Robert Gaunt, Ciba Pharmaceutical Co.; s, gift from Syntex Laboratories; t, gift from Dr. Maurice Fecht, Research Institute for Medicine and Chemistry; u, purchased from L. Light Co.; v, gift from Reussel-UCLAF; w, gift from Dr. Charles Engel, University of Toronto; x, purchased from Biochemical Research Co.

### Table I

**Progesterone Analogue-Receptor Binding**

| Compound | Relative binding energy (ΔG') | Source |
|----------|-------------------------------|--------|
| 4-Pregnene-3β,20β-diol | >3.00 | n |

**Hydroxyl substituents**

| Compound | Relative binding energy (ΔG') | Source |
|----------|-------------------------------|--------|
| Dihydroxy substituents | 5α-Pregna-3β,20β-diol | >2.97 | a |

**Addition of a double bond between carbons 6 and 7 of progesterone also increases the polarizability of the conjugated carbonyl group at carbon 3 and flattens the B ring. The additional double bond has little effect on the orientation of the 3-oxo group, but the binding energy increases by 0.69 kcal/mol. This difference may result from steric effects around the 6, 7, and 8 carbons or from conformational changes elsewhere in the steroid molecule.**
modification flattens the molecule, introduces some strain into the C ring, and reorients the axial hydrogen on carbon 11 to a pseudoaxial conformation. However, double bonds between carbons 9 and 11 or between 11 and 12 have little effect on the affinity of the steroid. This relationship is interesting because 9(11)-dehydro steroids have decreased biological activity, whereas 11-dehydroprogesterone is quite potent.

Modification of the D ring by double bonds at carbon 14, 15, or 16 increases the relative binding energy, $\Delta G^\circ$, by 0.15, 0.44, and 1.98 kcal/mol, respectively. Thus, the closer the unsaturation is to the acetyl side chain, the greater the decrease in affinity. The effect of 16,17 unsaturation is great because the double bond conjugates with the 20-carbonyl group causing a planar conformation of carbons 16, 17, 20, and the 20-oxo group. Even though the polarizability of the carbonyl group is increased, the drastic reorientation of the side chain reduces binding by 2 kcal/mol, indicating that the hydrogen bond between the 20-oxo group and the receptor has been disrupted.

**Methyl Substituents on Progesterone**—A methyl substituent at a single location on the progesterone molecule provides a sensitive probe of the distance between the steroid surface and the receptor binding site at that location. This sensitivity is due to the highly distance-dependent forces between molecules at small separations. Attractive van der Waals forces from the methyl group causes a slight increase in binding, but, at an intermolecular carbon-carbon distance of about 0.4 nm they are counterbalanced by repulsive forces due to overlapping electronic orbitals of the two molecules. At the energetically optimal carbon-carbon separation of 0.4 nm, each carbon with its associated hydrogens typically contributes 0.5–1 kcal/mol to the binding energy of the progesterone molecule (Hirschfelder et al., 1964). If a hydrogen is replaced by a methyl group, the surface is extended an additional 0.1 nm, which may increase the overlap of electronic orbitals. The increased repulsion will decrease the binding energy by 1–3 kcal/mol. Therefore, an increase in relative binding energy, $\Delta G^\circ$, of more than 1.3 kcal/mol (which corresponds to a 10-fold decrease in relative affinity) indicates that the separation between progesterone and receptor surfaces is less than 0.1 nm. These regions are detected at the 2a, 2b, and 7b positions. Similarly, an increase in relative binding energy, $\Delta G^\circ$, of less than 0.4 kcal/mol (which corresponds to a 2-fold or less decrease in relative affinity) indicates a separation of at least 0.1 nm between the progesterone and receptor surfaces or flexibility of the receptor sufficient to accommodate the additional mass. These regions are detected at the 6a, 6b, 15b, and 17a positions. Intermediate values determined for 4a, 7a, and 16a-methyl substituents indicate a separation of approximately 0.1 nm. Other methyl substituents that may produce more complicated effects are discussed later.

**Removal of Angular Methyl Groups**—Removal of the angular methyl group from carbon 10 increases binding to the receptor protein over progesterone. This observation implies that the receptor surface is in close contact with the 19-methyl group, which displaces other atoms of the progesterone from their optimal binding orientations. However, removal of the 19-methyl group also changes the conformation of the progesterone molecule from the 15a,2a-half chair conformation to the 1a,2b-half chair conformation which might allow for better binding (Duax et al. 1980).

The decrease in binding measured for 18,19-dinor progesterone, as compared with 19-nor-progesterone, indicates that the 18-methyl contributes about 1 kcal/mol to the energy of binding between progesterone and the receptor. This value is typical for close van der Waals’ interaction of a methyl group.

Compared with 19-nor-progesterone, 13-ethyl-18,19-dinor-progesterone binds to the receptor somewhat more weakly; the difference in binding energies is about 0.5 kcal/mol. This difference confirms that the receptor binding site surface is close to carbon 18 of the progesterone molecule and that the additional methyl group on carbon 18 causes steric hindrance.

An unusual conformation is found in 19-nor-14b,17a-progesterone. The plane of the C/D rings in this analogue is cis instead of the normal trans orientation of progesterone. This conformation orients the 15, 16, and 17 region toward the α-face and changes the side chain orientation. The drastic configurational rearrangement decreases the affinity relative to progesterone; however, the energy change relative to its analogue, 19-nor-progesterone, is only 0.8 kcal/mol. From the methyl substitution data it is deduced that increased bulk at positions 15α and 17α does not greatly affect binding affinity; apparently, a shift in mass to the 16b position (in contrast to the 16β position) also does not greatly hinder binding. More important, the hydrogen bond between the 20-oxo group and the receptor is not totally disrupted by this unusual conformation.

**Modification of the A Ring**—Modification of the A ring must be considered in light of electronic effects on the carbonyl group as well as steric effects due to substituents. Analogues with substituents at the 2α position can affect binding by one or more mechanisms including 1) steric hindrance, 2) inductive effect of an electronegative substituent on the adjacent 3-carbonyl group which will reduce the strength of the hydrogen bond between that group and a hydrogen donor (Ringold et al., 1964). 3) Polarization of a substituent by a neighboring charge, which will produce an attractive force, 4) formation of an additional intermolecular hydrogen bond between the substituent and the receptor, and 5) formation of an additional intermolecular hydrogen bond between the substituent and the buffer. These interactions are summarized in Table III for substituents in order of increasing relative affinities.

In contrast to a methyl substituent at the 2α position, an iodo substituent decreases binding only moderately even though it extends the steroid surface 0.2 nm beyond that of the hydrogen it replaces and 0.1 nm beyond that of a methyl group. This suggests that the polarizability of iodine has an effect on binding that counteracts the steric effect. Thus, the receptor probably has a charge that can polarize the iodine and create an attractive force. The source of this charge might be anionic or a strongly polar group on the protein or, more likely, the hydrogen that forms the hydroxyl bond with the adjacent carbonyl group.

Thefluoro substituent, which has a van der Waals’ radius only slightly larger than hydrogen, should cause little steric hindrance. Unlike iodine, fluorine is strongly electronegative and can have an inductive effect on the adjacent carbonyl group. As a result, the strength of the hydrogen bond between the carbonyl group and the hydrogen donor on the receptor is reduced.

On the other hand, a fluoro group can form hydrogen bonds with hydrogen donors either on the receptor or in the buffer. Although quantitative assessment of each contribution is not possible, in sum the relative affinity is moderately reduced by this substituent.

In addition to steric and inductive effects, a hydroxy substituent or carbon 2 probably forms an intramolecular hydrogen bond with the 3-carbonyl group which would interfere with that group’s intermolecular hydrogen bond with the receptor. It is also possible that the hydroxyl substituent would form an additional hydrogen bond with the receptor if a hydrogen donor were properly located to interact with the hydroxyl oxygen. Although this hypothetical hydrogen bond
with the receptor is speculative, the hydroxyl substituent most likely forms hydrogen bonds with the buffer. Thus, the combination of these effects reduces the relative affinity of this analogue more than that of other hydroxyl analogues.

A methyl substituent at the 2β position reduces binding more than at the 2α position. Steric interference of the 2β methyl group with the receptor may be responsible for the decrease, but steric interaction with the angular methyl on carbon 10 must also be considered. Interaction of the 2β substituent with carbon 19 causes an inversion of the A ring to a 1β,2α-half chair conformation with the substituent being placed in the equatorial orientation (Patashnik et al., 1963). This inversion disrupts the 4-en-3-one conjugation which reduces the hydrogen bond strength and, along with the steric interference, greatly reduces the affinity of this analogue.

On carbon 4, an acetoxy substituent reduces binding moderately. The acetoxy group extends about 0.3 nm beyond the van der Waals’ radius of hydrogen which suggests that separation of the progesterone and receptor surfaces at this location is about 0.3 nm. The acetoxy group, which has two electronegative oxygens, has an inductive effect on the 3-carbonyl which should decrease the relative affinity of this analogue more than the 4-methyl analogue (Ringold et al., 1964). Because this decrease is not detected experimentally, neither the carbonyl of the acetoxy group forms an additional, weak hydrogen bond with the receptor or the acetoxy group does not produce the same inductive effect as other electronegative substituents.

In contrast to methyl and acetoxy substitution, hydroxylation of carbon 4 greatly reduces binding. An intramolecular hydrogen bond between the 4-hydroxy group and the 3-oxo group may interfere with the receptor protein-3-oxo-hydrogen bond formation. Furthermore, the electronwithdrawn oxygen of the hydroxy group may have an inductive effect on the adjacent carbonyl group to reduce hydrogen bond strength, similar to the effect shown by electronwithdrawn substituents on carbon 2. Alternatively, 4-hydroxy-4-pregnene-3,20-dione could exist as three other tautomers—pregnan-3,4,20-trione, 3,4-dihydroxy-2,4-pregnadien-20-one, or 3-hydroxy-2-pregnene-4,20-dione, each of which could disrupt the hydrogen bond formed by the carbonyl group. By spectroscopy, however, the 4-en-4-ol structure has been established as the predominant tautomer (Fieser and Fieser, 1959).

Modifications of the B Ring—At the axial 6β position, a hydroxyl substituent causes a large decrease in binding. The decrease may be the result of an inductive effect of the electronegative oxygen on the 3-carbonyl group. As Ringold et al. (1964) have shown, a strong inductive effect exists for electronwithdrawn substituents at the axial 6β position while equatorial 6α substituents have weaker inductive effects.

Fluoro and chloro substituents at the equatorial 6α position have little effect on binding. At the 6β position, however, a fluoro substituent causes an increase in relative binding energy of about 0.6 kcal/mol and a chloro substituent causes an increase of about 1.4 kcal/mol. The decreased affinity of the chloro analogue as compared to the methyl analogue, as discussed above, implies that the progesterone-receptor separation is between 0.1 nm and 0.15 nm. Strongly electronwithdrawn fluoro group, which is smaller than a methyl group, probably acts through an inductive effect on the 3-carbonyl rather than by steric interference at the 6β position.

An acetoxy group at 7β decreases binding below the sensitivity of our assay. This indicates that the separation between the progesterone surface at carbon 7 and the binding site is less than 0.3 nm.

Modification of the C Ring—Modifications at carbon 11 of many steroid molecules are important in regulating steroidal effects (Raynaud et al., 1973; Kelly et al., 1977). Of interest is 11β-hydroxyprogesterone which binds to the receptor more strongly than other hydroxylated analogues including 11α-hydroxyprogesterone. Presumably, the 11β-hydroxy interacts with the receptor through a hydrogen bond.

An oxo group on carbon 11 reduces binding to an extent typical of hydrogen bonding in the buffer. The reduction is not due to flattening of the steroid C ring, since introduction of a double bond on either side of carbon 11 has a negligible effect on binding.

Binding of the 11-oxo and 11β-hydroxyl analogues is increased by the presence of fluorine at the adjacent 9α position. The fluoro substituent stabilizes the 1α,2β-half chair conformation in the A ring, which might increase binding (Duax et al., 1976). The strongly electronwithdrawn fluorine also has an inductive effect at carbon 11. In particular, the hydrogen donor characteristics of an 11β-hydroxyl substituent are increased and the hydrogen donor characteristics of an 11-carbonyl group are diminished. Thus, if the 11β-hydroxyl group forms a hydrogen bond with a hydrogen acceptor on the receptor, the additional 9α-fluoro substituent would strengthen that bond. Conversely, if the 11-carbonyl group does not form a hydrogen bond with a hydrogen donor on the receptor, the additional 9α-fluoro substituent would still weaken the hydrogen bonds of the ketone with the buffer and there would still be an increase in affinity for the receptor. Consequently, the data for the 11β-hydroxyl and 11-carbonyl analogues and their 9α-fluoro derivatives suggest that a hydrogen acceptor exists on the receptor near the 11β position of bound progesterone.

Modifications of the D Ring—Modification of the D ring can have local effects or can effect the orientation of the 17β-side chain. Change in side chain orientation can drastically
reduce the binding affinity of the 20-carbonyl as evidenced by the effects of the 16,17 unsaturation modification discussed previously. A methyl substituent at the 16β position produces a 1.8 kcal/mol increase in relative binding energy. This increase probably results from steric interference with the 20-oxo group, which changes the orientation of the 17β-side chain from its normal position. In contrast, a hydroxyl at the same position imposes the relative binding energy of 0.3 kcal/mol more than the methyl analogue. Apparently, the hydroxyl group produces effects other than steric interaction or typical hydrogen bond formation with water. Because of its location on progesterone, the 16β-hydroxyl differs from other hydroxyls in that it can form an intramolecular hydrogen bond with the 20-oxo group. That intramolecular bond can stabilize the orientation of the 17β-side chain and possibly interfere with simple hydrogen bond formation between the hydroxyl analogue and the buffer. This observation further supports the hypothesis that 16β methylation reduces binding by sterically affecting the 17β-side chain orientation.

Of the methyl substituents which we have investigated, the only one at 17α increases the affinity of progesterone for its receptor. This increase could result from van der Waals' interaction between the 17α-methyl and the receptor binding site, which would indicate a methyl-sized hydrophobic pocket in the binding site. It could also result from the 17α-methyl steric interaction with the 17β-side chain which could reorient the 20-oxo group to a more favorable hydrogen bond geometry. The methyl hydrophobic pocket hypothesis is unlikely, because a methoxy substituent can also be accomodated at 17α with only a slight decrease in affinity. An acetoxy substituent does interfere with binding, however, which suggests that the distance between the progesterone and the receptor binding site surfaces at this point must be about 0.3 nm. Apparently, the receptor has no charge concentration at this point because halide substituents have only a slight effect on the binding affinity.

A hydroxyl substituent at this position has two major effects: 1) it increases the number of hydrogen bonds formed in the buffer and 2) it changes the orientation of the 17β-side chain including the 20-carbonyl group (Duax et al., 1976). Each of these effects reduces the affinity for the receptor; together, these effects result in a large decrease in affinity.

**Modification of the 17β-Side Chain—Reduction of the 20-carbonyl to a hydroxyl group yields two isomers. The 20α-hydroxyl isomer produces an increase in relative binding energy of 2.1 kcal/mol; the 20β-hydroxyl isomer produces an increase of 1.6 kcal/mol. These results are consistent with a decrease in strength of the hydrogen bond upon reduction of the 20-ketone to a hydroxyl group. Although the oxygen of the 20-hydroxyl group can still act as a hydrogen acceptor, the differences in orientation between 20-hydroxyl and 20-carbonyl groups, or other factors, could account for the decreased affinity.

An iodo substituent on carbon 21 increases the relative binding energy 0.6 kcal/mol. Iodine, which has low electronegativity and therefore has little inductive effect on the 20-carbonyl group, probably causes either steric interference with the receptor or a change in orientation of the side chain. If there is no steric interference, then the separation between the receptor and carbon 21 of the bound progesterone is about 0.2 nm.

Fluoro substituents on carbon 21 act on the 20-carbonyl group much as the fluoro substituent on carbon 2 acts on the 3-carbonyl group. Electronegative fluoro substituents moderately reduce binding by an inductive effect to diminish the hydrogen bond strength of the 20-carbonyl group. One fluoro substituent increases the relative binding energy by 0.5 kcal/mol, while two fluoro substituents further increases the energy to 0.7 kcal/mol.

**Interpretations—** It appears that progesterone binds to its receptor at two hydrophilic sites and multiple hydrophobic sites. The carbonyls at positions 3 and 20 form hydrogen bonds with hydrogen donors on the receptor. At positions 6, 11, 14, 15, 16, 17, and 21, the distance between the bound steroid and the receptor protein exceeds 0.1 nm. At positions 2, 4, 7, 9, 12, 18, and 19, the separation appears to be about 0.1 nm or less. Also, the receptor may contain a hydrogen acceptor at the 11β position of the bound steroid. Other regions (carbons 1 and 8) were not explored because suitable analogues were not available.

The regions of separation of the hormone from its receptor are predominantly located at the "lower edge" of the molecule (Fig. 1). This is consistent with the view that the binding site of the receptor is a narrow cleft into which progesterone fits and binds. The negative entropy measured for the association of progesterone and the receptor also supports this narrow cleft hypothesis, i.e. progesterone must have a specific orientation relative to the receptor in order to bind.

The carbonyls at position 3 and 20 contribute about 6 kcal/mol to the enthalpy of binding. Other contributions are made by van der Waals' interactions, of which each contributes about 1 kcal/mol. If we assume that all of the surface of the progesterone molecule, except those regions of separation of the steroid and the receptor protein, has a charge concentration at this point, the quantum mechanical transmission coefficient of the dissociating progesterone-receptor complex is unity, may be in error. Yet, a decrease in the transmission coefficient by even a factor of 10 would reduce the discrepancy by only 0.5 kcal/mol. Possibly, the value of 1 kcal/mol for individual van der Waals' interaction may be error. This suggestion is unlikely because an experimental error of over 30% in the values listed by Hirschfelder et al. (1964) for these interactions would be required to remove the discrepancy. We postulate that the additional enthalpy measured by dissociation experiments is due to intrareceptor interactions that occur after progesterone is bound. Thus, 20 kcal/mol of enthalpy can be attributed to progesterone-receptor interactions and the remaining 6 kcal/mol can be attributed to intramolecular bonds formed in the receptor after progesterone is bound. One can speculate that an induced conformational change in the receptor closes the cleft, trapping the progesterone molecule, and that the conformation of the receptor is stabilized by the formation of low energy bonds within the receptor. Those intrareceptor bonds would have to be broken in order for the progesterone molecule to dissociate from the receptor.

This model contrasts with the one proposed by Duax et al. (1978, 1980) which suggests that interactions of the receptor with the A ring are primarily responsible for the high affinity of progesterone. It is hard to reconcile the measured enthalpy of binding with the energy derived from interactions restricted to the A ring unless a covalent bond is involved.

On the other hand, there are similarities between our model...
and those proposed by Westphal's laboratory for progesterone binding to guinea pig plasma progesterone-binding globulin (Blanford et al., 1978) and for cortisol binding to human plasma corticosteroid-binding globulin (Mickelson and Westphal, 1980). For the progesterone-binding globulin, we and Blanford et al. (1978) propose bonding by hydrophobic groups along the "upper edge" of the steroid molecule and hydrogen bonding at the 3-carbonyl. In addition, there is evidence for a hydrogen acceptor on both the plasma binding globulin and the uterine cytoplasmic receptor which may interact with the carbon 11 region of the steroid. The progesterone-binding globulin model and our model differ, however, in relationships at the 6 and 16 regions, as well as at the 20-carbonyl, which we propose as a hydrogen acceptor. Finally, we present data for a van der Waals' interaction at carbon 7, a region that they did not study.

Although the model for corticosterone-binding globulin is not presented in as much detail as that for the progesterone-binding globulin, it also shares features with our model for the progesterone receptor. In particular, both models propose hydrogen bonding at the 3-carbonyl and the 20-carbonyl, as well as a hydrogen acceptor on the binding protein near position 11. The corticosterone-binding globulin model differs from ours at carbons 17 and 21, regions where Mickelson and Westphal (1980) propose weak hydrophilic bonds.

Of particular interest is the demonstration of Mickelson and Westphal's (1980) that the enthalpy of binding increases by about 20 kcal/mol when the temperature is increased from 4 °C to 37 °C, while the entropy changes from positive to negative. They speculate without elaboration that the changes may be due to a structural change in the protein. Their analysis may be extended to consider that at the lower temperature, the protein may be in a different conformation primarily due to hydrogen bond formation. At the higher temperature the binding site of the protein is easily accessible through low energy internal bonds, possibly salt bridges, to fix the steroid in place. Whether this conformational change of the protein is related to the process of "receptor activation" is speculative. Correlation with biological activity or inhibition of biological activity will be topics for a future publication.

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