Johnson Research Foundation, University of Pennsylvania, where luminescence experiments were performed.

References

Ansevin, A. T., Hnilica, L. S., Spelsberg, T. C., and Kehm, S. L. (1971), *Biochemistry 10*, 4793.
Bryan, S. E., and Bright, J. E. (1973), *Toxicol. Appl. Pharmacol. 26*, 109.
Bryan, S. E., and Hayes, E. F. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21*, 21.
Carrabine, J. A., and Sundaralingam, M. (1971), *Biochemistry 10*, 292.
Chalkley, R., and Jensen, R. H. (1968), *Biochemistry 7*, 4380.
Chanda, S. K., and Cherian, G. (1973), *Biochem. Biophys. Res. Commun. 50*, 1013.
Chauveau, J., Moule, Y., and Rouiller, C. (1956), *Exp. Cell Res. 11*, 317.
Dische, Z. (1930), *Mikrochemie 8*, 4.
Dorne, B., and Hirth, L. (1970), *Biochemistry 9*, 119.
Eisinger, J., and Lamola, A. A. (1971), Excited States of Proteins and Nucleic Acids, Steiner, R. F., and Winryb, I., Ed., New York, N. Y., Plenum Press, p 138.
Gruenwedel, D. W., and Davidson, N. (1966), *J. Mol. Biol. 21*, 129.
Henson, P., and Walker, I. O. (1971), *Eur. J. Biochem. 22*, 1.
Izatt, R. M., Christensen, J. J., and Rytting, J. H. (1971), *Chem. Rev. 71*, 439.
Jarrell-Ash (1970), Atomic Absorption Analytical Applications, No. Hg-1, Fisher, Jarrell-Ash, Waltham, Mass.
Jensen, R. H., and Chalkley, R. (1968), *Biochemistry 7*, 4388.
Li, H. J., and Bonner, J. (1971), *Biochemistry 10*, 1461.
Lindsfedt, G. (1970), *Analyst 95*, 264.
Luck, G., and Zimmer, C. (1971), *Eur. J. Biochem. 18*, 140.
Manning, D. C. (1970), *At. Absorption Newslett. 9*, 97.
Nandi, U. S., Wang, J. C., and Davidson, N. (1965), *Biochemistry 4*, 1687.
Purkey, R. M., and Galley, W. C. (1970), *Biochemistry 9*, 3569.
Shih, T. Y., and Lake, R. S. (1972), *Biochemistry 11*, 4811.
Simpson, R. T. (1972), *Biochemistry 11*, 2003.
Slayter, H. S., Shih, T. Y., Adler, A. J., and Fasman, G. D. (1972), *Biochemistry 11*, 3044.
Truong, T., Bersohn, R., Brumer, P., Luk, C. K., and Tao, T. (1967), *J. Biol. Chem. 242*, 2979.
Wacker, W. E. C., and Vallee, B. L. (1959), *J. Biol. Chem. 234*, 3257.
Wagner, T., and Spelsberg, T. C. (1971), *Biochemistry 10*, 2599.
Wagner, T. E., and Vandergrift, V. (1972), *Biochemistry 11*, 1431.
Weber, G., and Teale, F. W. J. (1965), *Proteins, 2nd Ed., 3*, 472.
Weinryb, I., and Steiner, R. F. (1970), *Biochemistry 9*, 135.
Yamane, T., and Davidson, N. (1961), *J. Amer. Chem. Soc. 83*, 2599.
Yasmineh, W. G., and Yunis, J. J. (1970), *Expt. Cell Res. 59*, 69.

# Interaction between Steroids and a Uterine Progestogen Specific Binding Macromolecule†

John L. McGuire,* Charlene D. Bariso, and Arvin P. Shroff

ABSTRACT: Forty-five steroidal compounds, derivatives of both progesterone and testosterone, have been studied for their ability to bind to the rabbit uterine progestogen receptor. Introduction of a hydroxyl group in the 17$\alpha$, 16$\alpha$, 6$\alpha$, 11$\alpha$, or 14$\alpha$ position precipitously reduces the affinity of progesterone for the macromolecule, whereas introduction in the 11$\beta$ position has no effect. If the polarity of the hydroxyl groups is altered by acylation, binding affinity is not depressed. When an alkyl group is added at C-6$\alpha$ or C-16$\alpha$, binding affinity increases, whereas a $\beta$-alkyl group at C-16 prevents binding. Although the C-3 ketone is not required for binding affinity, introduction of a bulky thioketal group at C-3 completely prevents binding. Reduction of the $\Delta^{4,5}$ double bond of progesterone to yield a compound with A/B trans juncture has no effect on binding affinity, but the isomer with a nonplanar A/B cis juncture possesses lower binding affinity. Removal of C-19 from progesterone results in a slightly greater binding affinity for the uterine macromolecule. The two modifications of testosterone which most dramatically increase binding affinity are removal of the C-10 methyl group and introduction of an alkyl group in the 17$\alpha$ position. In the latter case, binding affinity increases as the electron-donating power of the group is increased. The *d* isomer of norgestrel binds, whereas the *l* isomer does not bind to the uterine progestogen receptor.

It has been reported that a protein which specifically binds progesterone and progestational drugs exists in the high-speed supernatant fraction of mammalian uteri (Milgrom *et al.*, 1970; Rao and Wiest, 1971; McGuire and DeDella, 1971; McGuire and Bariso, 1971, 1972). This macromolecule, termed a receptor, appears to be target tissue specific (McGuire and Bariso, 1972) and a positive correlation exists between the protein's affinity for steroids *in vitro* and the progestational activity of those steroids *in vivo* (McGuire *et al.*[1]). Although the physiological role of this binding protein is unknown, the

† From the Molecular Biology Section and Division of Chemical Research, Ortho Research Foundation, Raritan, New Jersey 08869. *Received August 31, 1973.*

[1] J. L. McGuire, C. D. Bariso, B. S. Fuller, R. E. Homm, J. Settepani, G. O. Allen, and J. P. DeVanzo, submitted for publication to *Eur. J. Pharmacol.*

above findings support the contention that this macromolecule may play a pivotal role in the biochemical mechanism of action for progesterone and progestational drugs.

The objective of the present study was to define some of the steric requirements for steroid binding to the rabbit uterine progestogen receptor in order to gain insight into the chemical interaction between steroid and macromolecule.

## Materials and Methods

[1,2-$^3$H]Progesterone (50 Ci/mmol) was purchased from the New England Nuclear Corporation. The radiopurity of this compound was determined by thin layer chromatography; all radioactivity was found in a single peak characteristic of progesterone. Cholesterol, progesterone, 17$\alpha$-acetoxyprogesterone, and $\Delta^5$-pregnene-3,20-dione were purchased from Steraloids and found to be pure by thin layer chromatography.

Medroxyprogesterone, medroxyprogesterone acetate, and 17$\alpha$-hydroxy-6$\alpha$-methylprogesterone were donated by the Upjohn Co.; chlormadinone and 19-norprogesterone were donated by Syntex Laboratories, Inc. *dl*-Norgestrel, *d*-norgestrel, *l*-norgestrel, and WY-4355 were donated by Wyeth Laboratories. These compounds were used without further purification. All of the other compounds were obtained pure from our Organic Chemistry Division.

Compounds were tested for the ability to displace bound [$^3$H]progesterone from the rabbit uterine progestogen specific binding macromolecule by the same competitive binding assay used earlier in this laboratory and laboratories of others (McGuire and DeDella, 1971; McGuire and Bariso, 1972). Uteri from ten New Zealand white rabbits weighing no more than 2 kg each were finely minced and washed for 1 hr in Tris-HCl buffer (pH 8.0) at 4° before homogenizing in $^2/_5$ vol of the same buffer (0.01 M Tris-HCl buffer (pH 8.0), containing 0.001 M EDTA and 0.25 M sucrose) at 4°. The homogenate was first centrifuged at 12,000*g* for 15 min and the resulting supernatant was then centrifuged at 273,000*g* for 1 hr. The protein concentration was 12.2 mg/ml. Rabbits of this weight are mature but not yet producing large quantities of progesterone.

Analogs of both progesterone and testosterone were studied for their abilities to bind to the rabbit uterine progestogen receptor at the same molar concentration as that of progesterone which displaces 50% of bound [$^3$H]progesterone. Reaction mixtures consisting of, in order, (a) 0.2 ml of buffer (0.01 M Tris-HCl buffer (pH 8.0), containing 0.001 M EDTA, 0.25 M sucrose, and 25,000 cpm of [$^3$H]progesterone per ml), (b) $0.8 \times 10^{-10}$ M nonradioactive steroids where called for, and (c) 50 $\mu$l of final uterine supernatant (cytosol fraction), were incubated at 4° for 16 hr. Studies have demonstrated that this protein is stable for at least 16 hr (McGuire and Bariso, 1972). After incubation, bound steroid was separated from unbound steroid by adding 1.0 ml of activated charcoal suspension (0.25% Norit A and 0.0025% dextran, w/v, in buffer). The samples were incubated for 15 min at 4° after gentle mixing and immediately centrifuged at 2500 rpm for 10 min. The supernatants were quantitatively extracted with toluene phosphor, and the radioactivity was measured. Data were expressed as the depression of bound [$^3$H]progesterone/50 $\mu$l of uterine cytosol from control levels (*i.e.*, competition by $0.8 \times 10^{-10}$ M of competitor for [$^3$H]progesterone binding).

A Beckman Model L-265-B ultracentrifuge was used for centrifugation, and a Packard Model 3376 Tri-Carb liquid scintillation spectrophotometer was used for all radioactivity determinations. An external standard was used to correct for quenching.

## Results

Binding data reported here actually represent the depression of bound [$^3$H]progesterone by a molar concentration of compounds equal to that quantity of progesterone which displaces 80% of the isotope, *i.e.*, the $ED_{80}$. Previous studies indicated that depression of bound hormone induced by this molar concentration of unlabeled compound is an indicator of relative binding affinity. Strong binders are indicated by a high numerical index and weak binders by a low numerical index. Control data for these experiments were: [$^3$H]progesterone, 910 ± 130 cpm; [$^3$H]progesterone + progesterone, 180 ± 50 cpm (80% depression); [$^3$H]progesterone + cholesterol, 1000 ± 90 cpm (no depression).

All compounds in Table I are structurally related to progesterone (**2**). Introduction of a hydroxyl group in the 17$\alpha$ (**4**), 16$\alpha$ (**28**), 6$\alpha$ (**29**), 11$\alpha$ (**17**), or 14$\alpha$ (**30**) position precipitously reduces the ability of progesterone to bind. On the other hand, if the hydroxyl group is introduced in the 11$\beta$ position (**18**) there appears to be no effect on binding affinity. An exception to this general trend appears, however, when a hydroxyl is placed in the 6$\beta$ position (**31**).

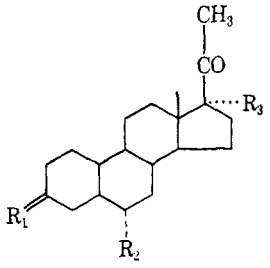
These findings suggest to us that interaction between protein and steroid around rings C and D is on the $\alpha$ face of the steroid. We propose that the $\alpha$-hydroxyl groups may hinder binding to the protein due to repulsion by a similarly charged species on the protein. This supposition is strengthened by our finding that if the polarity of the hydroxyl group is altered by acylation as in compounds **5**, **19**, **25**, and **26**, binding affinity is not depressed. If an alkyl group is added on the $\alpha$ side of the steroids at C-6 or C-16 or at both positions, binding increases (**9**, **20**, and **22**), whereas a $\beta$-alkyl group in the 16 position (**15**) prevents binding. We cannot explain this latter observation unless the alkyl group interferes with binding by C-20 and C-21.

The C-3 position also appears to be important in steroid binding as shown by substitutions made at this carbon. Disubstitution at C-3 by a hydroxyl and a methyl group (**11**) slightly reduced binding affinity, possibly due to the unfavorable ratio of 3$\alpha$ and 3$\beta$ hydroxyl substitutions. Introduction of a bulky thioketal group at C-3 (**7** and **12**) completely prevents adsorption. It will be noted that the $\Delta^5$ isomer of progesterone (**23**) binds equally as well as does progesterone until the 3-keto is replaced by a 3-hydroxyl (**1**), causing a precipitous drop in binding.

Several additional observations are evident from the data. First, reduction of the double bond of progesterone yields a compound with an A/B trans juncture (**16**) maintaining the planar steroid structure. This does not reduce binding affinity. However, the other isomer, *i.e.*, a compound with a nonplanar A/B cis juncture (**21**), definitely possesses lower binding affinity. These findings clearly indicate the need for a planar steroid structure. Secondly, removal of C-19 from progesterone (**24**) results in slightly greater binding affinity. This may suggest some adsorption of steroid to protein around the A and B rings on the $\beta$ surface of the steroid. Finally, introduction of unsaturation at C-6 (**8**, **10**, **13**, **14**) does not significantly alter the ability of the steroid to bind to the protein.

The class of compounds in Table II can be broadly divided into derivatives of testosterone and 19-nortestosterone. The two modifications of testosterone (**32**) which most dramatically increase binding affinity are, first, removal of the C-10 methyl

TABLE I: Effect of Nonradioactive Steroids on Binding of [$^3$H]Progesterone.[a]



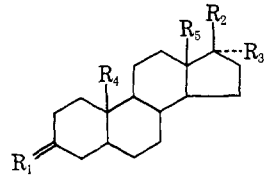
| Compd No. | Unsat. | $R_1$ | $R_2$ | $R_3$ | Others | Depression (%) |
|---|---|---|---|---|---|---|
| **1** | 5 | OH | | | | 10 |
| **2** | 4 | O | | | | 80 |
| **3** | 4 | O | | OH | | 0 |
| **4** | 4 | O | $CH_3$ | OH | | 20 |
| **5** | 4 | O | $CH_3$ | OAc | | 85 |
| **6** | 4 | | $CH_3$ | OH | | 0 |
| **7** | 4 | $SCH_2$ $SCH_2$ | $CH_3$ | OAc | | 10 |
| **8** | 4,6 | O | Cl | OAc | | 85 |
| **9** | 4 | O | $CH_3$ | | $C_{16\alpha}CH_3$ | 80 |
| **10** | 4,6 | O | CH | OAc | $C_{16}CH_3$ | 85 |
| **11** | 4 | HO, $CH_3$ > | $CH_3$ | OAc | | 40 |
| **12** | 4 | $SCH_2$ $SCH_2$ | $CH_3$ | | $C_{16\alpha}CH_3$ | 0 |
| **13** | 4,6 | O | Cl | OAc | 1,2-$CH_2$ | 85 |
| **14** | 4,6 | O | | OAc | | 80 |
| **15** | 4 | O | | | $C_{16\beta}CH_3$ | 15 |
| **16** | | O | | | 5$\alpha$-H | 80 |
| **17** | 4 | O | | | 11$\alpha$-OH | 25 |
| **18** | 4 | O | | | 11$\beta$-OH | 75 |
| **19** | 4 | O | | OAc | | 85 |
| **20** | 4 | O | | | 16$\alpha$-$CH_3$ | 75 |
| **21** | | O | | | 5$\beta$-H | 45 |
| **22** | 4 | O | $CH_3$ | OH | | 30 |
| **23** | 5 | O | | | | 80 |
| **24** | 4 | O | | | 19-Nor | 90 |
| **25** | 4 | O | | Opr | | 30 |
| **26** | 4 | O | $CH_3$ | Opr | | 85 |
| **27** | | O | | | 5$\alpha$-H, 12-keto | 75 |
| **28** | 4 | O | | | 16$\alpha$-OH | 0 |
| **29** | 4 | O | | | 6$\alpha$-OH | 7 |
| **30** | 4 | O | | | 14$\alpha$-OH | 20 |
| **31** | 4 | O | | | 6$\beta$-OH | 2 |

[a] By the cytosol fraction of rabbit uteri following incubation for 16 hr at 4°. Data are based on ten trials for each compound and are expressed as the per cent depression of bound [$^3$H]progesterone in control experiments.

group (**36** and **37**) to produce a 19-nor compound and, secondly, introduction of an alkyl group in the 17$\alpha$ position (**33**, **43**, and **44**). In the latter case, binding affinity increases as the electron donating power of the group is increased. A chloroethynyl group in the 17$\alpha$ position (**42** and **45**) is no less active than the ethynyl group.

Finally, certain other clear findings emerge from our data.

TABLE II: Effect of Nonradioactive Steroids on Binding of Progesterone.[a]



| Compd No. | Unsat. at C | $R_1$ | $R_2$ | $R_3$ | $R_4$ | $R_5$ | Others | Depression (%) |
|---|---|---|---|---|---|---|---|---|
| **32** | 4 | O | OH | H | $CH_3$ | $CH_3$ | | 5 |
| **33** | 4 | O | OH | CH | $CH_3$ | $CH_3$ | | 50 |
| **34** | 4 | O | OH | C≡CH | $CH_3$ | $CH_3$ | | 70 |
| **35** | 4 | O | OH | C≡CCH | $CH_3$ | $CH_3$ | 6$\alpha$-$CH_3$ | 80 |
| **36** | 4 | O | OH | H | H | $CH_3$ | | 60 |
| **37** | 4 | O | OH | C≡CH | H | $CH_3$ | | 80 |
| **38** | 5(10) | O | OH | C≡CH | H | $CH_3$ | | 80 |
| **39** | *dl* 4 | O | OH | C≡CH | H | $C_2H_5$ | | 80 |
| **40** | *l* 4 | O | OH | C≡CH | H | $C_2H_5$ | | 0 |
| **41** | *d* 4 | O | OH | C≡CH | H | $C_2H_5$ | | 90 |
| **42** | 4 | O | OH | C≡CCl | H | $C_2H_5$ | | 90 |
| **43** | 4 | O | OH | $CH_3$ | H | $CH_3$ | | 65 |
| **44** | 4 | O | OH | $C_2H_5$ | H | $CH_3$ | | 75 |
| **45** | 4 | O | OH | C≡CCl | H | $CH_3$ | | 80 |

[a] By the cytosol fraction of rabbit uteri following incubation for 16 hr at 4°. Data are expressed as the per cent depression of bound [$^3$H]progesterone in control experiments.

First, a change in the double bond from the $\Delta^4$ position (**34**) to $\Delta^5$ (**10**) does not appear to have any significant affect on binding (**38**). Some reservation must be made with regard to this observation, however, because many tissues contain enzymes capable of carrying out the isomerization of $\beta,\alpha$-unsaturated ketone, and one would like to be certain that this transformation had not occurred *in vitro*. Secondly, norgestrel (**39**) exists as a mixture of the *d* and *l* isomers. The *l* isomer (**40**) does not bind, whereas the *d* isomer (**41**) retains binding activity.

## Discussion

All of the progestogens known today are steroids which, with few exceptions, fall into two broad classes: derivatives of progesterone (pregnenes) and derivatives of 19-nortestosterone (estrenes). Members of each are active in the classical Clauberg assay (Clauberg, 1930). The compounds we have used in these studies are analogs of these progestogens. We have studied their binding affinity for a uterine progestogen specific binding protein (Milgrom *et al.*, 1970; Rao and Wiest, 1971; McGuire and DeDella, 1971; McGuire and Bariso, 1971, 1972) in an attempt to define some of the steric requirements which a steroid must fulfill in order to bind to that protein and to gain some insight into the nature of this interaction between steroid and binder. Our interpretations assume that, at the concentration used in these studies, [$^3$H]-progesterone binds to a single macromolecular species in the high-speed supernatant fraction. Studies reported to date are consistent with this hypothesis (McGuire and Bariso, 1972).

The fact that 19-nor compounds, especially estrenes, bind

more strongly than do compounds possessing C-19 suggests to us that partial adsorption to the protein around rings A and B may be on the $\beta$ side of the steroid. In general, however, our data more clearly indicate that the $\alpha$ side of the steroid adsorbs to the binder. In order for this to occur, the steroid apparently must be planar and there must be no hydroxyl groups on the $\alpha$ side of the steroid. Electron density around C-6 and C-16 caused by introduction of either a double bond or an alkyl group increases adsorption. Our data indicate that the ketone at C-3 is not essential for binding, but if present will aid in adsorption. A bulky substitution at C-3 prevents this adsorption. $\beta$ substitutions do not appear to play a part in adsorption in most cases. All of these observations appear true for both pregnenes and estrenes. In the latter class of compounds, however, increasing the electron density around C-17 also results in increased binding affinity.

Previous studies have demonstrated that progesterone binding is of a noncovalent type since the steroid is completely extractable with organic solvents (McGuire and DeDella, 1971; McGuire and Bariso, 1971). Since most of the substitutions in steroids which we observe to result in changes in binding affinity are those which interact primarily with hydrogens, we assume steroid–protein binding involves hydrogen binding, hydrophobic binding, and van der Waal's forces. The energy of binding then would be the result of these three forces.

In his classical paper on the structure–activity relationships of steroids, Ringold (1961) proposed certain steric requirements necessary for progestational activity

It would be premature to conclude that the hypothetical receptor to which Ringold referred is the protein studied in our experiments. However, two points argue in favor of such a possibility. The progesterone binding macromolecule is found in tissues which respond to progestogens (McGuire and DeDella, 1971). Further, it appears to bind only compounds which are progestational in nature (McGuire *et al.*[1]). Definitive evidence indicating whether or not the uterine progestogen binder studied in these experiments is actually a pharmacological receptor must await confirmation of the biochemical role played by this protein in the mechanism of progesterone action.

## Acknowledgments

The authors are indebted to many individuals for the substances tested. General gifts of *dl*-norgestrel, *d*-norgestrel, *l*-norgestrel, and WY-4355 were made by Dr. Richard Edgren of Wyeth Laboratories; Dr. R. L. Bergstrom of G. D. Searle and Co. supplied oxandrolone. Dr. John Babcock of The Upjohn Co. donated medroxyprogesterone and medroxyprogesterone acetate, and 19-norprogesterone was given to us by Dr. James D. Mutch of Syntex Laboratories, Inc. Dr. S. Liao of the University of Chicago donated a sample of cyproterone acetate. Drs. Irving Scheer, Joseph Settepani, Allen Hirsch, and George Karmas of the Organic Chemistry Division at Ortho Research Foundation provided many of the compounds used in this study. Many of the concepts presented in this paper and earlier papers are the result of stimulating discussions with Dr. John P. DaVanzo. Finally, thanks are due Mrs. Barbara Fuller for her excellent technical assistance in these studies.

## References

Clauberg, C. (1930), *Zentraol. Gynaekol. 54*, 2757.

McGuire, J. L., and Bariso, C. D. (1971), *Pharmacologist 13*, 286.

McGuire, J. L., and Bariso, C. D. (1972), *Endocrinology 89*, 468.

McGuire, J. L., and DeDella, C. E. (1971), *Endocrinology 88*, 1099.

Milgrom, E., Atger, M., and Baulieu, E. E. (1970), *Steroids 16*, 741.

Rao, B. R., and Wiest, W. G. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30* (3), 1213.

Ringold, H. J. (1961), Mechanism of Action of Steroid Hormones, Villee, C. A., and Engel, L. L., Ed., Elmsford, N. Y., Pergamon Press.