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BINDING OF STEROIDS TO UTEROGLOBIN

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SUMMARY

The capacity of 41 different steroids to compete with progesterone for binding to reduced rabbit uteroglobin was used to determine the structural requirements in the steroid molecule relevant for its interaction with the active site of the protein. The binding of titrated estradiol-17β and testosterone was also studied directly, and both steroids were shown to bind to the same site as progesterone, although with lower affinity.

There is a hydrophobic interaction of the protein with the α-side of the steroid molecule. Although minor modifications in the progesterone molecule can interfere with binding, the active site of uteroglobin appears to be able to accommodate different configurations of both the ring A and the side chain, including the phenolic A ring of estrogens and the C-17α-ethynyl side chain of some synthetic steroids. With the exception of estrogens, there is a good correlation between the ability of various steroids to bind to uteroglobin and their progestational activity.

INTRODUCTION

During the pre-implantation phase of pregnancy the composition of the uterine luminal fluid exhibits hormonally dependent characteristic changes, supposed to be involved in the capacitation of the blastocyst for implantation and normal development [1, 2]. In the rabbit, the protein composition of the uterine secretion changes dramatically during the preimplantation phase, and uterus-specific proteins dominate the gel electrophoretic pattern [3–5]. During the last two days of the pre-implantation period one of these proteins, which has been called uteroglobin [4] or blastokinin [5], accounts for up to 40–50% of the total protein content of the uterine luminal fluid. Although its function has not yet been established, antibodies against uteroglobin appear to be able to prevent implantation [6], and, together with other uterine proteins, uteroglobin seems to favor blastocyst development [5, 7]. Since uteroglobin has been detected within the blastocyst [4, 8, 9] it seems reasonable to assume a functional role of this protein during pre-implantation. On the other hand, the synthesis and secretion of uteroglobin is markedly enhanced by progesterone [10, 11] and the purified protein binds radioactive progesterone with relatively high affinity [12–14]. If a relationship is established between these two sets of observations it will be conceivable as a working hypothesis, that uteroglobin is a mediator for some action of steroids on the blastocyst. In fact, it has been reported that the complex of uteroglobin and progesterone rather than the protein alone could be required for blastocyst development [15]. It seemed therefore relevant to study the capacity of different steroids to compete with progesterone for the binding to uteroglobin as this could probably lead to a control of implantation. In this paper I present a series of competition studies of this type, as well as data on the binding of other steroids to uteroglobin.

EXPERIMENTAL

[1,2-^{3}H]-progesterone, specific radioactivity 48 Ci/mmol, [7-^{3}H]-progesterone, specific radioactivity 24 Ci/mmol, [6,7-^{3}H]-estradiol-17β, specific radioactivity 49.3 Ci/mmol, and [1,2-^{3}H]-testosterone, specific radioactivity 40 Ci/mmol, were obtained from New England Nuclear Inc. Ovalbumine and most of the non-radioactive steroids were obtained from Sigma Chemicals Inc. Some of the synthetic steroids were kindly provided by Schering AG, Berlin. Activated charcoal was purchased from Mallinckrodt Inc.

Normal female rabbits of various breeds with an average weight of 3 kg were used for the purification of uteroglobin [14]. The uterine fluid was collected 5 days after the intramuscular injection of 30 I.U. of HCG (Primogonyl, Schering AG), and gel filtration chromatography on Sephadex G 100 was performed as previously described [14]. For some experiments additional purification of uteroglobin was achieved by column chromatography on carboxy-methylcellulose. The purity of the uteroglobin was tested by polyacrylamide gel electrophoresis and immunoelectrophoresis [14].

Binding assay. Measurement of [^{3}H]-progesterone binding to uteroglobin was based on the adsorption of the free steroid to activated charcoal coated on to Dextran-500 (Pharmacia, Sweden), followed by separation from protein-bound steroid in the centrifuge [14]. The conditions of the test were optimized for the low uteroglobin concentrations required in our competition experiments. It was found necessary to add an inert protein to the incubation mixture in order to prevent inactivation or adsorption of uteroglobin to the charcoal. In the presence of ovalbumin

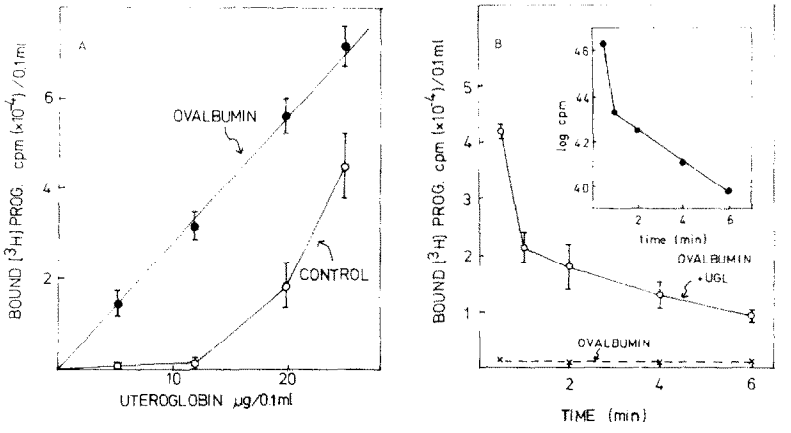


Fig. 1. Influence of ovalbumin on the charcoal binding assay.

(A) Different amounts of uteroglobin were incubated at 0°C for 60 min with 10^{-7} M [^{3}H]-progesterone (specific radioactivity 29 Ci/mmol in 100 µl Tris–saline buffer alone or in the presence of ovalbumin (final concentration 2 mg/ml). The concentration of protein-bound radioactivity was determined by treatment with Dextran-coated charcoal for 1 min as described in Methods. (○- —○) Uteroglobin; (● ——●) uteroglobin plus ovalbumin. The data represent the average and standard deviations of 4 determinations.

(B) Aliquots (100 µl) of Tris-saline buffer containing ovalbumin or ovalbumin plus uteroglobin (200 and 10 µg respectively) were incubated at 0°C for 60 min with 10^{-7} M [^{3}H]-progesterone (specific radioactivity 29 Ci/mmol). Dextran coated charcoal (100 µl) was then added, and the concentration of protein-bound radioactivity was determined at various time intervals thereafter. (×- - -×) ovalbumin; (○- ○) ovalbumin plus uteroglobin. The data represent mean and, when indicated, standard deviation of 4 determinations.

The *inset* shows a half logarithmic representation of the average data for the assay containing uteroglobin and ovalbumin.

(2 mg/ml), progesterone binding was linearly dependent on the amount of uteroglobin within the concentration range studied (Fig. 1a). Ovalbumin neither binds progesterone nor changes the affinity of uteroglobin for the steroid (data not shown). The extent and the kinetics of adsorption of progesterone to charcoal were unaffected by the presence of ovalbumin (Fig. 1b). Under these conditions, after 1 min incubation with charcoal, the dissociation of progesterone from uteroglobin follows rapid apparent first order kinetics with a half time of about 4 min at 0–4°C.

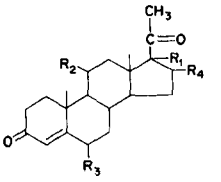
The competition experiments were performed in duplicate as follows: 10 µl of the radioactive steroid (usually at a concentration of 10^{-6} M) in 20 mM Tris–HCl, pH 7.5, containing 0.1 M NaCl, were mixed in conical centrifuge tubes with 10 µl of the competing steroid (10 different concentrations) in the same buffer. At the highest concentrations addition of ethanol was required to dissolve some of the steroids, and in these cases appropriate controls were performed to ensure that the concentration of ethanol used did not change progesterone binding to uteroglobin. To the dissolved steroids 80 µl of a solution of uteroglobin and ovalbumin (100 µg/ml and 2 mg/ml respectively) in the same buffer were added and mixed. The protein solution was previously treated at 37°C for 15 min with 5 mM dithioerythritol (DTE), as this treatment is essential for quantitative binding [14]. Incubation of the steroids with the protein solution were at 0–4°C for 60 min, after which 100 µl of Dextran-coated charcoal (3,75 g activated charcoal and 375 Dextran-500 swollen in 100 ml Tris–saline buffer), were added while holding the tube on a vortex mixer for 5 s. After standing for 60 s in the ice-bath the tubes were centrifuged at 3000 ***g*** for 1 min in a refrigerated centrifuge. Radioactivity was determined in 100 µl aliquots of the supernatant by mixing thoroughly with 5 ml Bray's solution [16]. Appropriate blanks were performed containing only ovalbumine and DTE. These values, which did not differ from the buffer blanks, were subtracted from all experimental values. The variation of the duplicate determinations was considerable (up to 40%), but apparently was compensated by the large number of concentrations tested. Thus the values of the relative affinity calculated in two separate experiments did not usually differ by more than 20%.

RESULTS

The results of a competition experiment using [^{3}H]-progesterone as the radioactive steroid and progesterone, testosterone or estradiol-17β as the non-radioactive competitors are depicted in Fig. 2. This graphic representation is used to determine the concentration of competitor which reduces the binding of [^{3}H]-progesterone by 50%. From these values ($(S)_{50\%}$) the relative affinity (R.A.) of the different steroids is calculated:

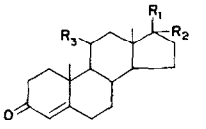
$$\text{R.A.} = \frac{(P)_{50\%}}{(S)_{50\%}} \times 100$$

Table 1.



Nr.	Trivial name	R_1	R_2	R_3	R_4	Relative affinity (n)
I.1	17α-Hydroxyprogesterone	α-OH	—	—	—	8.5 (4)
I.2	17α-Acetoxyprogesterone	α—O—C(=O)—CH_3	—	—	—	48.0 (3)
I.3	16β-Methylprogesterone	—	—	—	β—CH_3	1.0 (2)
I.4	16α-Methylprogesterone	—	—	—	α—CH_3	4.6 (2)
I.5	11β-Hydroxyprogesterone	—	β—OH	—	—	5.0 (2)
I.6	11α-Hydroxyprogesterone	—	α—OH	—	—	5.7 (2)
I.7	11-Ketoprogesterone	—	=O	—	—	9.3 (2)
I.8	16-Dehydroprogesterone	—	—	—	16-ene-	1.5 (2)
I.9	6α,16α-Dimethylprogesterone	—	—	α—CH_3	α—CH_3	15.6 (1)
I.10	6α-Methyl-17α-Hydroxyprogesterone	α—OH	—	α—CH_3	—	47.0 (1)

Table 2.



Nr.	Trivial name	R_1	R_2	R_3	Relative affinity (n)
II.1	21-Hydroxyprogesterone	—C(=O)—CH_2OH	—	—	32.4 (3)
II.2	17α,21-Dihydroxyprogesterone	—C(=O)—CH_2OH	α—OH	—	0.8 (2)
II.3	20β-Dihydroprogesterone	—C(OH)(H)—CH_3	—	—	5.0 (3)
II.4	20α-Dihydroprogesterone	—C(H)(OH)—CH_3	—	—	6.6 (2)
II.5	Cortisol	—C(=O)—CH_2OH	α—OH	β—OH	0.0* (3)
II.6	Cortisone	—C(=O)—CH_2OH	α—OH	=O	0.0* (3)
II.7	Corticosterone	—C(=O)—CH_2OH	—	β—OH	0.0* (2)
II.8	Testosterone	—OH	—	—	7.2 (4)
II.9	Androstendione	=O	—	—	5.7 (3)

* These compounds increase binding.

where $(P)_{50\%}$ is the concentration of non-radioactive progesterone which reduces to half the binding of [^{3}H]-progesterone.

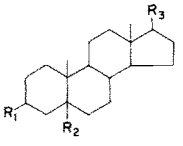
The relative affinities of a number of different steroids are given in Table 1 to 4, together with the structural formula common to the various compounds included in each particular table.

In Table 1 the effect of various substituents on the 4-pregnen-3,20-dione nucleus are listed. A hydroxyl group in α-position at C-17 markedly reduces binding (I.1), and this effect is partially counteracted by an α-directed non-polar substituent at C-6 (I.10). An acetoxy rest at C-17α reduces the affinity by half (I.2), whereas substituents at C-16, including a double bond between C-16 and C-17, markedly reduce the affinity (I.3, I.4, I.8). A hydroxyl group at C-11, both in α- or β-position lowers the competing efficiency 20-fold (I.5, I.6), and an oxo group 10-fold (I.7).

Table 2 depicts the effect on the relative affinity of substitutions in the structure of the side chain at C-17, alone or in combination with other substituent a C-17 and C-11. A hydroxyl group at C-21 alone reduces binding to 1/3 (II.1), whereas in combination with another hydroxyl group at C-17α it almost completely abolishes binding (II.2). Reduction of the oxo function at C-20 to either the α or β hydroxyl derivatives has a very marked effect on binding (II.3 and II.4), as does the elimination of C-20 and C-21 and its substitution by either a hydroxyl or a keto group (II.8 and II.9).

The binding of testosterone was also studied using the radioactive steroid, and the affinity for uteroglobin was shown to be less than one-tenth that of progesterone (data not shown). Progesterone was more efficient than testosterone in competing with [^{3}H]-testosterone for binding to uteroglobin (Fig. 3).

Table 3.



Nr.	Trivial name	R_1	R_2	R_3	Relative affinity (n)
III.1	Pregnandione	=O	β—H	—C(=O)—CH_3	24.1 (2)
III.2	5α-Pregnandione	=O	α—H	—C(=O)—CH_3	222.0 (3)
III.3	Androstandione	=O	α—H	=O	0.1 (2)
III.4	Androstanolone	=O	α—H	—OH	0.1 (2)
III.5	Pregnanolone I	β—OH	β—H	—C(=O)—CH_3	41.3 (2)
III.6	Pregnanolone II	α—OH	β—H	—C(=O)—CH_3	35.0 (3)
III.7	3β,5α-Pregnanolone	β—OH	α—H	—C(=O)—CH_3	160.0 (3)
III.8	Pregnenolone	β—OH	5-ene	—C(=O)—CH_3	20.6 (3)
III.9	Pregnandiol III	β—OH	α—H	—C(OH)(H)—CH_3	2.5 (1)
III.10	Pregnandiol IV	β—OH	α—H	—C(H)(OH)—CH_3	3.5 (1)
III.11	Pregnandiol V	α—OH	β—H	—C(OH)(H)—CH_3	<0.1 (1)

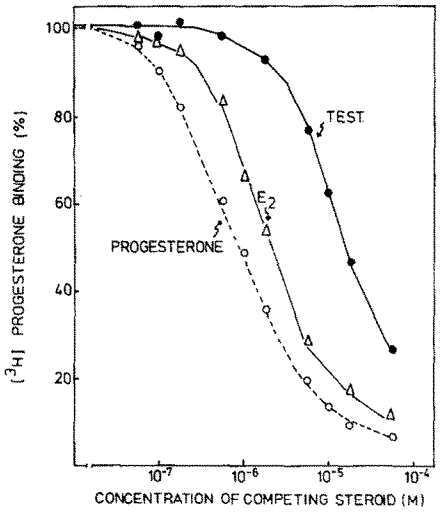


Fig. 2. Effect of competing steroids on the binding of [^{3}H]-progesterone to uteroglobin. A mixture of uteroglobin (0.1 mg/ml) and ovalbumin (2 mg/ml) was treated with 5 mM DTE and incubated with 10^{-7} M [^{3}H]-progesterone (specific radioactivity 48 Ci/mmol) alone or in the presence of different concentrations of non-radioactive progesterone (○——○), estradiol-17β (△——△) or testosterone (●——●). The amount of protein-bound radioactivity was then determined by the charcoal assay as described in Methods. The results are expressed as per cent of the control values obtained in the absence of competing steroids. Blank values without uteroglobin have been subtracted in each case. The points represent an average of 4 determinations.

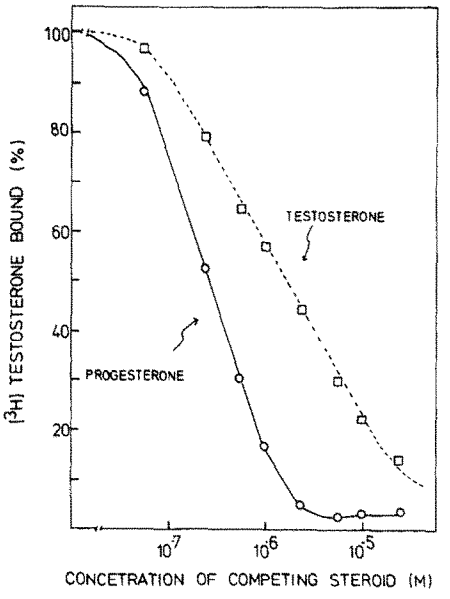


Fig. 3. Effect of progesterone and testosterone on the binding of [^{3}H]-testosterone to uteroglobin. The experimental conditions were identical to those of Fig. 2, except that [^{3}H]-testosterone (specific radioactivity 40 Ci/mmol) was used as the radioactive steroid. The competing steroids were progesterone (○——○), and testosterone (□---□). The points represent the average of six determinations.

Testosterone acts as a competitive inhibitor of [^{3}H]-progesterone binding, as shown in the double reciprocal plot of Fig. 4.

Natural glucocorticoids (II.5, II.6, II.7) do not compete at all and even slightly enhance the binding of [^{3}H]-progesterone to uteroglobin. In the case of cortisone and corticosterone this enhancement was about 50% at a 100-fold excess of the non-radioactive steroid.

The influence on the relative affinity of structural changes in the A-ring, alone or in combination with substitutions in the side chain, is summarized in Table 3. Reduction of the C4-C5 double bond leading to the 5-β arrangement reduces the affinity to one fifth (III.1); whereas the 5-α configuration has more than twice the affinity of progesterone (III.2). These two isomers differ markedly in the relative orientation of ring A and ring B and in respect to the direction of 3-oxo group.

Similar changes of the A ring, in compounds where the side chain has been substituted by a keto or a hydroxyl group, completely abolish binding (III.3 and III.4).

The C-3-oxo group can be replaced by a hydroxyl group, either in α or β position without affecting the affinity to a large extent (III.5, III.6 and III.7). Pregnenolone, with the double bond located between C-5 and C-6 and the C-3-oxo group replaced by a C-3 β-OH (III.8), still preserves one-fifth of the binding

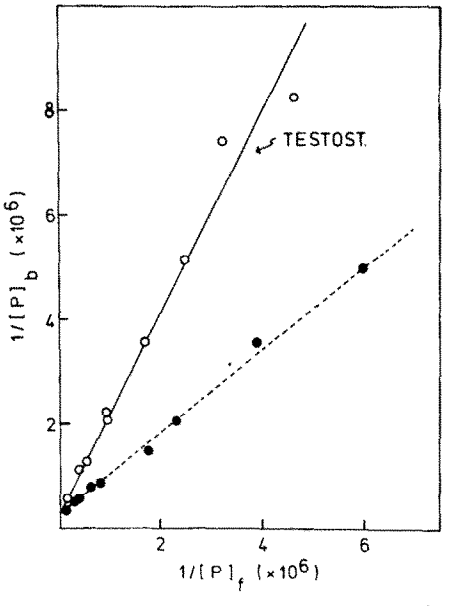
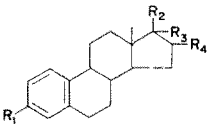


Fig. 4. Effect of testosterone on the binding of [^{3}H]-progesterone to uteroglobin. A solution of uteroglobin in Tris-saline buffer (100 μg/ml) was incubated with 5 mM DTE at 37°C for 15 min and used for the binding assays. Of this solution 90 μl aliquots were incubated at 30°C for 30 min with 5 μl of [^{3}H]-progesterone, to which either 5 μl ethanol (●---●) or 5 μl of 8×10^{-4} M testosterone in ethanol were added (○——○). The specific radioactivity of [^{3}H]-progesterone was 1 Ci/mmol and the final concentrations used ranged from 0.5 to 10 μM. After the incubation, the amount of progesterone bound to uteroglobin was determined by the charcoal technique (see Methods). The figure represents a double reciprocal plot of the concentration of free progesterone $(P)_f$ against the concentration of uteroglobin-bound progesterone $(P)_b$.

Table 4.



Nr.	Trivial name	R_1	R_2	R_3	R_4	Relative affinity (n)
IV.1	Estradiol-17β	β—OH	β—OH	—	—	53.1 (8)
IV.2	Estradiol-17α	β—OH	α—OH	—	—	1.0 (1)
IV.3	Estrone	β—OH	=O	—	—	37.0 (2)
IV.4	Estriol	β—OH	β—OH	—	α—OH	42.0 (4)
IV.5	Mestranol*	β—O—CH_3	β—OH	α—C≡CH	—	70.0 (3)

* 17α-Ethynyl-1,3,5(10)-estratrien-3,17β-diol-3-methyl ether.

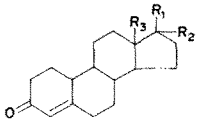
affinity of progesterone indicating that there is a certain degree of freedom in the structure of the A-ring which is compatible with binding.

The three pregnandiols tested are all poor binders, specially that having the β-configuration at C-5 (III.9, III.10 and III.11).

The competing efficiency of steroids with a phenolic A-ring is shown in Table 4. Estradiol-17β is the most active competitor among natural estrogens (IV.1, IV.3, IV.4). The 17α-isomer has very low activity (IV.2), whereas mestranol, a synthetic compound with a α-C≡CH at position C-17 is even more active than estradiol-17β. The binding of estradiol-17β to uteroglobin was also directly studied using the tritiated steroid. The association constant calculated using the charcoal procedure was approximately $1 \times 10^6\ M^{-1}$ at 4° C. The variability of results was larger with [^{3}H]-estradiol than with [^{3}H]-progesterone suggesting a faster rate of dissociation. Non-radioactive progesterone was more efficient than estradiol in competing against [^{3}H]-estradiol for binding to uteroglobin. As in the case of testosterone, the competition between the two ovarian steroids was of competitive nature, although the number of estradiol molecules bound per molecule of uteroglobin at saturation was always much lower than unity.

Table 5 summarizes the competing efficiency of various 19-nor steroids. The 19-nor derivatives of progesterone and testosterone have less than half the relative affinity of the original compounds (V.1 and V.2). An ethynyl group in position C-17α increases binding (V.3) as does acetylation in the C-17 β-position (V.6). The affinity is further increased by the substitution of methyl group at C-12 by an ethyl group (V.4). The highest competing activity of all tested compounds was shown by norethynodrel, which has a double bond between C-5 and C-10 (V.5). This compound acts as a competitive inhibitor of progesterone

Table 5.



Nr.	Trivial name	R_1	R_2	R_3	Relative affinity (n)
V.1	19-Nor-progesterone	β—C(=O)—CH_3	—	—CH_3	40.0 (2)
V.2	19-Nor-testosterone[1]	β—OH	—	—CH_3	3.1 (2)
V.3	Norethisterone[2]	β—OH	α—C≡CH	—CH_3	16.0 (3)
V.4	D-Norgestrel[3]	β—OH	α—C≡CH	—CH_2—CH_3	62.7 (2)
V.5	Norethynodrel[4]	β—OH	α—C≡CH	—CH_3	357.0 (2)
V.6	Norethisterone acetat[5]	β—O—C(=O)—CH_3	α—C≡CH	—CH_3	29.0 (3)

[1] 17β-Hydroxy-4-estren-3-one.
[2] 19-Nor-17β-hydroxy-17α-pregn-4-en-20-yn-3-one.
[3] 19-Nor-18ethyl-17β-hydroxy-17α-pregn-4-en-20-yn-3-one.
[4] 17α-Ethynyl-17β-hydroxy-5(10)-estrene-3-one.
[5] 19-Nor-17β-hydroxy-17α-pregn-4-en-20-yn-3-one-17-acetate.

binding to uteroglobin, as determined by double reciprocal plot (data not shown).

DISCUSSION

The results presented above do not allow a complete analysis of the interaction of the steroid molecule with uteroglobin but certain aspects of this interaction are quite obvious. I summarize below the structural features of steroid molecules relevant for binding to uteroglobin, and try to establish a correlation between these features and the progestational activity of the different steroids. I also compare the structural requirements for steroid binding to uteroglobin and to the progesterone receptor and other progesterone-binding serum proteins.

The α,β-unsaturated carbonyl system of ring A can be substituted, even by a phenolic ring without the binding being affected markedly. Reduction of the α,β-unsaturated carbonyl system to the 5α-dihydroprogesterone (5α-pregnan-3,20-dione), which preserves the A/B *trans* configuration, even increases binding, whereas the A/B *cis* 5β-isomers and pregnenolone are poor binders, indicating the importance of the steric relations between rings A and B. It is interesting to note that 5α-pregnan-3,20-dione is also the principle metabolite bound to nuclear and cytosol receptor of chick oviduct after injection of [^{3}H]-progesterone, and that this metabolite induces avidin synthesis *in vivo*, whereas the 5β-isomer is inactive in this respect [17, 18].

Norethynodrel, which has a double bond between C-5 and C-10, will have the C-3-oxo group slightly tilted towards the β-side, and was the best competitor in our studies. This synthetic steroid has been shown to bind to the endometrium progesterone receptor in the rat and humans [19].

Introduction of hydroxyl groups at position 11,17 or 21 markedly reduces binding, indicating the hydrophobic nature of the interaction of uteroglobin with the steroid. A hydroxyl group at C-21 reduces the affinity to one-third that of progesterone, in good correlation with the effect on progestational activity [20]. A C-17α hydroxyl group also interferes with binding, reducing the affinity to less than one-tenth that of progesterone, whereas a C-17α acetoxy group halves the affinity. These results also agree with the effect on progestational activity of both substitutions.

The hydrophobic nature of the interaction of the α-side of the steroid molecule with uteroglobin is confirmed by the positive effect on binding of a methyl group at C-6 in α-position, and by the very effective binding observed with synthetic derivatives containing the 17α-C≡CH side chain. The negative influence on binding of a methyl substituent at position C-16 is probably due to steric hindrance.

Interestingly, the 20β-hydroxy-4-pregnen-3-one derivative which can induce avidin in chicken oviduct [17] does not bind to uteroglobin. The same is true for the 20α-isomer, which has a relatively high affinity for human uterine receptor [18].

Glucocorticoids not only do not compete with progesterone but increase its binding to uteroglobin. These steroids however do not interact strongly with the protein as shown by a lack of spectral quenching in the ultraviolet absorption of the α,β unsaturated carbonyl system, which is characteristic for the binding of steroids to various proteins (21, unpublished results).

The fact that the structure of the side chain in natural compounds is very important for binding, but can be substituted by the α-C≡CH, indicates that the steroid binding site of uteroglobin can accommodate two different types of side chains. This seems also to be the case for progesterone receptors, as these synthetic compounds are very potent progestational agents.

Taken together, the results presented here suggest a good correlation between the progestational activity of various steroids and their ability to bind uteroglobin. The main exception to this rule is the relatively high affinity of estrogens. This may be related to the antagonist effect of estrogens and progesterone, but may also reflect the fact that estrogens are able to induce partially the synthesis and secretion of uteroglobin [11].

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