Interaction of Progestins with Steroid Receptors in Human Uterus

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(Received 23 May 1978)

Norethindrone (17 β -hydroxy-19-nor-17 α -pregn-4-en-20-yn-3-one) and norethindrone acetate (17 β -acetoxy-19-nor-17 α -pregn-4-en-20-yn-3-one) interfered to a varying degree, by competitive inhibition, with the binding of progesterone and oestradiol to respective cytoplasmic receptors in the human uterus. Progesterone binding to 4S macromolecule was saturable and co-specific for progestins. Competitors like norgestrel (17 β -hydroxy-18-methyl-19-nor-17 α -pregn-4-en-20-yn-3-one), 19-norprogesterone, medroxyprogesterone acetate (17 α -acetoxy-6 α -methylpregn-4-ene-3,20-dione) and compound R₅₀₂₀ (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione) possessed higher binding affinities for the progestin receptor. The dissociation constant (K_d) for the progesterone-receptor interaction was 0.6–1.6 nM and the receptor concentration ranged between 6600 and 8200 sites/cell. Norethindrone and norethindrone acetate competed for the progesterone receptor with inhibition constants (K_i) of 6.8 and 72 nM respectively. Gradient displacement and competitive-receptor assays indicated that norethindrone acetate-binding affinity for progestin receptor was approximately one-tenth that of norethindrone and progesterone. The progestins also inhibited oestradiol binding to 4.6S oestrogenic receptor by 8–12%, involving interaction at the oestradiol-binding site with a calculated K_i value of 0.5–0.8 μ M. The competitive interaction of progestins with steroid receptors may be of putative importance in explaining the progestin action at the target site.

Steroid-hormone receptors are the primary recognizing and regulatory molecules, functioning as biological transducers at the target site. Since uterine steroid-hormone receptors are indispensably implicated in the action of progesterone and oestradiol (Baulieu, 1975; Janne *et al.*, 1975; O'Malley & Schrader, 1976), any alteration in the receptor-binding properties by other compounds could modulate the processes controlled by these hormones. Previous work has shown that synthetic progestins like norethindrone (17 β -hydroxy-19-nor-17 α -pregn-4-en-20-yn-3-one) and norethindrone acetate (17 β -acetoxy-19-nor-17 α -pregn-4-en-20-yn-3-one) bind specifically to a uterine macromolecule with characteristics similar to the progesterone receptor (Laumas & Kasid, 1977). These findings prompted us to investigate whether binding of these progestins occurred at the progesterone-binding site (with inhibition kinetics of competitive type), or that the progestins interacted by binding at some other site on the receptor molecule. Further, these progestins have also been reported to interfere with the binding of oestradiol to the oestrogenic target tissues in rat (Van Kordelaar *et al.*, 1975). However, no quantitative data are available. As progestin-receptor interaction may be significant in the understanding of progestin action and consequently the pharmacological manipulation of cellular functioning, this interaction needed further eluci-

dation. The present work embodies the qualitative and quantitative evaluation of the interaction of norethindrone and norethindrone acetate with the receptor proteins for progesterone (pregn-4-ene-3,20-dione) and oestradiol (oestra-1,3,5(10)-triene-3,17 β -diol) in the human uterus.

Materials and Methods

Radioactive steroid

[15,16-³H]Norethindrone (sp. radioactivity 57 Ci/mmol) and [15,16-³H]norethindrone acetate (sp. radioactivity 57 Ci/mmol) were gifts from Schering A.-G. (Berlin, West Germany) and purified on LH-20 columns with toluene/methanol (17:3, v/v) as solvent. [1,2-³H]Progesterone (sp. radioactivity 48 Ci/mmol) and [6,7-³H]oestradiol-17 β (sp. radioactivity 40 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). The radiochemical purity of the steroids was confirmed by t.l.c. with chloroform/acetone (9:1, v/v) as solvent for progesterone and chloroform/ethyl acetate (4:1, v/v) for oestradiol.

Human uterine tissue

Normal uterine tissue was obtained from women undergoing hysterectomy operations because of the following conditions: myomata uteri, adenomyosis

and prolapsus uteri. The subjects were in the reproductive-age group (26–40 years) with normal menstrual cycles and without any hormone treatment. Immediately after hysterectomy, the uterus was cut longitudinally, adequate samples were taken for histological examination, and the pieces of tissue were chilled on ice. Further processing of samples commenced immediately at 0–2°C.

Preparation of cytosol fractions

After thorough washing, the uterine tissue was finely minced and homogenized in buffer A (0.01 M-Tris/HCl/0.0015 M-disodium EDTA, pH 7.4), containing 0.012 M-thioglycerol, 20% (v/v) glycerol and 1 μ M-cortisol (11 β ,17 α ,21-trihydroxypregn-4-ene-3,20-dione). The homogenate was centrifuged at 105000g for 1 h at 4°C to obtain the cytosol (supernatant) fraction. For binding assays, the cytosol was diluted with buffer A (10 mg of protein/ml of cytosol) and used fresh.

Sucrose-density-gradient centrifugation

Cytosol was analysed for binding by centrifugation on linear 5–20% (w/v) sucrose gradients, prepared in buffer A containing 10% glycerol with a Beckman gradient former. A sample (200 μ l) of cytosol was incubated with a 10 nM solution of 3 H-labelled steroid in the presence or absence of a 100-fold excess of unlabelled steroid for 1 h at 0°C and layered on top of the gradients. Centrifugation was carried out at 50000 rev./min for 15 h at 4°C in an SW 56 rotor. Bovine serum albumin (4.4 S) and human γ -globulin (7.1 S) were run as reference proteins in the estimation of sedimentation coefficients by the method of Martin & Ames (1961). Fractions (2 drops; 130 μ l) were collected and A_{280} was recorded by using a ISCO gradient fractionator.

Quantification of 3 H-labelled steroid-binding sites and determination of dissociation constant (K_d) (Scatchard analysis)

Cytosol samples (100 μ l) were incubated with increasing concentrations of 3 H-labelled steroid (0.2–12.5 nM) for 12–16 h at 0°C. The samples were assayed for receptor-bound radioactivity by using the dextran/charcoal adsorption technique (Korenman, 1975). The binding data were analysed as described by Scatchard (1949), and the correction for specific binding was made as described by Chamness & McGuire (1975). For each assay, the relationship between the bound to free ratio and the concentration bound (nM) was subjected to linear regression analysis by using a Hewlett–Packard model 9810A calculator. The instrument was programmed to

provide a correlation coefficient (r), slope (representing $1/K_d$) and the concentration of binding sites (pmol/mg of protein), where the number of binding sites per receptor molecule was assumed to be one. Receptor concentration per cell was obtained from the concentration per mg of DNA by the expression (mol of receptor-binding sites/mg of DNA) $\times$ (mg of DNA/cell) $\times$ Avogadro's number. DNA content was assumed to be 6 pg/cell as the diploid amount of DNA in human cells (Vendrely, 1955).

Non-specific (non-saturable) binding was measured in parallel incubation mixtures where a 100-fold molar excess of the respective unlabelled steroid was added. It was subtracted from the total binding to obtain the specific saturable binding.

Determination of the type of inhibition (Lineweaver–Burk plots)

Cytosol samples were incubated with increasing concentrations (0.25–10 nM) of 3 H-labelled steroid alone, and also in the presence of a constant concentration of inhibitor (competitor). The assay protocols were as follows: set A, cytosol labelled with [3 H]progesterone with norethindrone and norethindrone acetate as competitors; set B, cytosol complexed with [3 H]norethindrone with progesterone and norethindrone acetate as competitors; set C, cytosol labelled with [3 H]norethindrone acetate with progesterone and norethindrone as competitors; set D, cytosol labelled with [3 H]oestradiol with norethindrone and norethindrone acetate as competitors.

After equilibrium was attained (12–16 h), the samples were assayed for receptor-bound radioactivity by the dextran/charcoal technique. At each experimental point, control tubes with only labelled steroids at different concentrations were incubated to give the uncompetited values. All values were corrected for blank. The double-reciprocal plots of the specific-binding data were adapted from classic Lineweaver–Burk (1934) analysis. The best-fitted lines were drawn by the least-squares method. The dissociation constant (K_d) was calculated either from the slope and ordinate intercept or from the negative abscissa intercept. The number of binding sites were interpreted from the ordinate intercept.

To increase the specificity, all incubation solutions with labelled progestins also contained non-labelled 1 μ M-oestradiol. Similarly all incubation solutions with labelled oestradiol also contained unlabelled 1 μ M-progesterone.

Determination of inhibition constants (K_i)

The calculated K_i values of various progestins were obtained either by using the equation for competitive inhibition or directly from Dixon plots (Dixon & Webb, 1960).

Determination of relative binding affinity

The relative binding affinities of various steroids was evaluated by their ability to compete with saturating amounts of any one of the following ^3H -labelled steroids, progesterone, norethindrone or norethindrone acetate, for binding to cytosol progesterin receptor.

Samples (100 μl) of cytosol were added to assay tubes containing 0.5 nM ^3H -labelled steroid in 100 μl of buffer A and 100 μl of buffer A alone or containing various concentrations (0.01–100 ng) of unlabelled steroid competitors. The separation of bound from free radioactive steroid was carried out at 0–2°C by using the dextran/charcoal adsorption technique.

Each assay point was determined in duplicate. All values were corrected for blanks (incubation solutions without cytosol treated with dextran/charcoal). A plot of percentage of radioactivity bound (c.p.m.) against the logarithm of the mass of the competing ligand added gave a competition curve for each ligand. The molar concentration of each competitor required to decrease the binding of ^3H -labelled progesterin by 50% was calculated from these plots. The effectiveness of the competitor was calculated as described by Smith *et al.* (1974).

Protein and DNA determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. DNA was determined by the diphenylamine method as described by Burton (1956) with calf thymus DNA as standard.

Results

Mediation by progestins of the displacement of ^3H -labelled steroid from receptor sites on density gradients

^3H Progesterone displacement. When cytosol was labelled *in vitro* with ^3H progesterone (10 nM) and centrifuged on a density gradient (Fig. 1) the peak of radioactivity was invariably localized in the 4.1–4.3 S region. More than 90% of the total binding measured was stereospecific for progestins. Unlabelled progestins, when added simultaneously, decreased progesterone binding, although with varying degree of efficiency. Nevertheless, as the concentration of unlabelled progestins increased, the binding of ^3H progesterone decreased in the 4S region, indicating that the binding was saturable. Binding of ^3H progesterone was completely abolished when a 100-fold excess of progestin was added.

^3H Norethindrone/norethindrone acetate displacement. The sedimentation profile of cytosol proteins complexed with ^3H norethindrone or ^3H norethindrone acetate (results not shown) demon-

strated the complex sedimenting with a mean S value of 4.2 ± 0.2 . The binding of ^3H progesterin was completely inhibited by concomitant addition of a 100-fold molar excess of potent progestins. However, oestradiol and cortisol showed no displacement of progesterin binding.

^3H Oestradiol displacement. As shown in Fig. 2, ^3H oestradiol was specifically bound to a 4.6S protein. Unlabelled oestradiol (100-fold excess) distinctly displaced the ^3H oestradiol from the binding sites. However, norethindrone and norethindrone acetate (100-fold excess) inhibited ^3H oestradiol binding to the extent of 8–12% only.

Competitive interaction of progestins with ^3H -labelled steroid-receptor complex

Determination of binding constants (Scatchard analysis). The concentration of the specific-binding sites with corresponding dissociation constants (K_d)

Fig. 1. Competitive displacement of ^3H progesterone bound to cytoplasmic receptor (sucrose-gradient analysis). Uterine cytosol was incubated with ^3H progesterone (10 nM, 2 h, 0°C) alone (●) or together with norethindrone (10 nM, ▲; 100 nM, ▼; 1 μM , △) or norethindrone acetate (10 nM, ■; 100 nM, □; 1 μM , ○). The samples were analysed for receptor-bound ^3H progesterone on linear 5–20% (w/v) sucrose gradients by centrifugation at 50000 rev./min for 15 h at 2–4°C. The broken line shows the migration of cytosol protein after centrifugation. Arrows denote positions of bovine serum albumin (BSA) and human γ -globulin (HG). The horizontal arrow indicates the direction of sedimentation.

for progesterone-, norethindrone- and norethindrone acetate-receptor interaction are summarized in Table 1. The binding in the competed tubes (with a 100-fold molar excess of the test steroid) was designated as non-specific and the difference between the non-competitive (without a 100-fold molar excess) and competitive binding was defined as specific or receptor binding. The number of receptor binding sites was almost the same for all the progestins, but the K_d values were 8–10 times higher for norethindrone acetate as compared with norethindrone and progesterone.

Competitive inhibition of progesterone binding by norethindrone/norethindrone acetate (Lineweaver-Burk plots). Binding of progesterone in the presence of norethindrone and norethindrone acetate has been graphically (double-reciprocal plots) represented in Figs. 3(a) and 3(b) respectively. The progestins caused no inhibition of progesterone binding to non-specific sites, but there was significant inhibition of progesterone binding to the specific or displaceable sites, particularly at lower progesterone concentration. However, this effect tended to decrease at higher concentrations of progesterone. Since the interaction with the ordinate remained essentially constant (at the maximum-binding points) in the presence of the competing steroids, the patterns were for typical competitive inhibition. The calculated number of progesterone-binding sites was 260 fmol/mg of protein (6600 molecules/cell) and the K_d value was in the range 0.61–0.63 nM. Conversely, when uterine proteins were labelled with different concentrations of either [3 H]norethindrone (results not shown) or [3 H]norethindrone acetate in the presence of indicated concentrations of unlabelled progesterone (Fig. 4a) or unlabelled norethindrone (Fig. 4b), a typical competitive inhibition was consistently observed. The concentration of progestin receptor was calculated to be about 6000 sites/cell.

Determination of inhibition constants (K_i) (Dixon plots). The K_i values of norethindrone (Fig. 5a) and norethindrone acetate (Fig. 5b) for progesterone receptor were 6.8 and 72 nM respectively. In other

Fig. 2. Competitive displacement of [3 H]oestradiol bound to cytoplasmic receptor (sucrose-gradient analysis)

Uterine cytosol was incubated with [3 H]oestradiol (5 nM, 2 h, 0°C) alone (●) or together with a 100-fold excess of norethindrone (■), norethindrone acetate (▲) or oestradiol (○). The samples were analysed on 5–20% sucrose gradients (50000 rev./min, 15 h, 2–4°C). Arrows denote positions of bovine serum albumin (BSA) and human γ -globulin (HG). The horizontal arrow indicates the direction of sedimentation.

Table 1. Comparison of the binding constants of uterine cytosol receptors for different steroids

Cytosol samples were incubated with increasing concentrations (0.2–12.5 nM) of 3 H-labelled steroid for 12 h at 0–2°C and assayed for bound steroid by the dextran/charcoal adsorption technique. Protein normalized Scatchard plots were prepared and the concentration of binding sites and equilibrium dissociation constant (K_d) were calculated by using the equation $[\text{bound}/\text{free}] = 1/K_d \times [(\text{binding sites}) - (\text{bound})]$. Results are means $\pm$ s.e.m. for the numbers of tissue samples (from day 13–15 of menstrual cycle) processed, indicated in parentheses.

Steroid	Receptor-binding site concentration		
	(fmol/mg of cytosol protein)	(molecules/cell*)	(K_d) (nM)
Norethindrone (5)	331 $\pm$ 102	8340 $\pm$ 2570	1.3 $\pm$ 0.3
Norethindrone acetate (9)	280 $\pm$ 67	7060 $\pm$ 1690	12.9 $\pm$ 3.1
Progesterone (5)	326 $\pm$ 83	8200 $\pm$ 2090	1.6 $\pm$ 0.4
Oestradiol (3)	265 $\pm$ 60	6680 $\pm$ 1500	0.5 $\pm$ 0.1

* DNA content assumed to be 6 pg/cell.

Fig. 3. Competitive inhibition of [^3H]progesterone binding to uterine cytosol receptor by norethindrone (a) and norethindrone acetate (b)

The cytosol was incubated with increasing concentrations (0.25–10 nM) of [^3H]progesterone alone ($i = 0$) or together with the indicated concentration of inhibitor (i). The specific bound radioactivity was analysed by the dextran/charcoal adsorption technique. The double-reciprocal plots represent the best-fitted lines drawn by linear regression analysis.

Fig. 4. Competitive inhibition by progesterone (a) and norethindrone (b) of [^3H]norethindrone acetate binding to uterine cytosol receptor

The cytosol was incubated with increasing concentrations (0.2–10 nM) of [^3H]norethindrone acetate alone ($i = 0$) or together with indicated concentrations of inhibitor (i). Specifically bound [^3H]progesterin was determined by the dextran/charcoal adsorption technique. The best-fitted lines were drawn by linear regression analysis.

Fig. 5. Determination of inhibition constants of norethindrone (a) and norethindrone acetate (b) for the progesterone receptor of uterine cytosol

Uterine cytosol was incubated with [3 H]progesterone at either 0.5 nM (Δ) or 1.5 nM ($\bullet$) in the absence or presence of various concentrations of inhibitors. The specifically bound [3 H]progesterone was analysed by the dextran/charcoal technique. The horizontal line drawn at $1/n$, where $n = 260$ fmol/mg of protein, represents the number of progesterone-binding sites in the uterine cytosol.

experiments, where cytosol receptor was complexed with [3 H]norethindrone acetate, the K_i values were 0.28 nM for norethindrone (Fig. 6a) and 0.24 nM for progesterone (Fig. 6b), indicating high competitive efficiencies of norethindrone and progesterone compared with norethindrone acetate for binding to progestin receptor.

Interaction of progestins with oestradiol receptor

Fig. 7 represents the double-reciprocal plots of [3 H]oestradiol binding in the absence or presence of (a) norethindrone and (b) norethindrone acetate. The plots suggested competitive inhibition of oestradiol binding by progestins, with average K_i values of $0.5 \mu\text{M}$ for norethindrone and $0.8 \mu\text{M}$ for norethindrone acetate. The specific number of oestradiol-binding sites in the absence of progestin was approx. 7000 sites/cell and the K_d value of oestradiol interaction was calculated to be 0.61 – 0.64 nM.

Ligand specificity of [3 H]progestin–receptor interaction

The ligand affinities for progestin-binding proteins have been summarized in Table 2. For each competitor, relative binding affinity was indexed by its ability to compete separately with saturating amounts of [3 H]progesterone, [3 H]norethindrone or [3 H]norethindrone acetate (relative affinity in each case taken as 100). Most of the steroids tested exhibited similar relative competitive indices for progesterone- and norethindrone-binding proteins. Potent progestins competed with an affinity either higher than [norgestrel (17 β -hydroxy-18-methyl-19-nor-17 α -pregn-4-en-

Fig. 6. Determination of inhibition constants of norethindrone (a) and progesterone (b) for norethindrone acetate receptor of uterine cytosol

Uterine cytosol was incubated with [3 H]norethindrone acetate at either 0.3 nM (Δ) or 1.8 nM ($\bullet$) in the absence or presence of various concentrations of inhibitors. Other details were similar to those given in the legend to Fig. 5 ($n = 240$ fmol/mg of progestin).

Fig. 7. Inhibition of [3 H]oestradiol binding by norethindrone (a) and norethindrone acetate (b) in the human uterine cytosol

The cytosol was incubated with increasing concentrations of [3 H]oestradiol (0.2–5 nM) alone ($i = 0$) or together with different concentrations (200 nM, Δ ; 500 nM, $\circ$) of progestins. Specifically bound [3 H]oestradiol was determined by the dextran/charcoal adsorption technique. The best-fitted lines were drawn by using the method of least squares.

20-yn-3-one), 19-norprogesterone, medroxyprogesterone acetate (17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione)] or comparable with [compound R₅₀₂₀ (17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione), megestrol acetate (17 α -acetoxy-6-methylpregna-4,6-diene-3,20-dione)] those for progesterone and norethindrone. Compound R₂₃₂₃ (13-ethyl-17-hydroxy-18,19-dinor-17 α -pregna-4,9,11-trien-20-yn-one), chlormadinone acetate (17 α -acetoxy-6-chloropregna-4,6-diene-3,20-dione) and 17 α -acetoxyprogesterone competed with relative affinity values ranging between 40 and 60. Steroids like 5 α -pregnane-3,20-dione, 11 β -hydroxyprogesterone, norethynodrel (17 α -ethynyl-17-hydroxyoestr-5(10)-en-3-one) and 19-

Table 2. Relative binding affinities of the human uterine progesterin receptor towards different ligands
Samples (100 µl) of cytosol were incubated (12h, 0-2°C) with 0.5 nM-[³H]progesterone, [³H]norethindrone or [³H]norethindrone acetate in the absence or presence of various concentrations (0.01-100ng) of unlabelled competitors. The receptor-bound radioactivity was determined by the dextran/charcoal absorption technique. The competing ability of each competitor was estimated relative to each ³H-labelled progesterin. Values given represent the means ± s.e.m. (n = 3).

Competitor	Δ	Substituents			Binding affinities relative to:		
		17α	17β	Other	Progesterone	Norethindrone	Norethindrone acetate
d-Norgestrel	4	C≡CH	OH	18-CH ₃ , 19-nor	162 ± 12	157 ± 11	1760 ± 76
19-Norprogesterone	4	—	—	19-nor	131 ± 13	151 ± 14	1750 ± 147
Medroxyprogesterone acetate	4	OAc*	—	6α-CH ₃	127 ± 9	114 ± 8	1450 ± 138
Progesterone	4	—	—	—	100	103 ± 11	950 ± 76
Norethindrone	4	C≡CH	OH	19-nor	98 ± 9	100	1250 ± 96
Compound R ₅₀₂₀	4,9	CH ₃	—	21-CH ₃ , 19-nor	87 ± 6	83 ± 9	900 ± 160
Megestrol acetate	4,6	OAc	—	6-CH ₃	82 ± 7	71 ± 5	950 ± 39
Compound R ₂₃₂₃	4,9,11	C≡CH	OH	13-C ₂ H ₅ , 18,19-dinor	67 ± 6	61 ± 7	625 ± 47
Chlormadinone acetate	4,6	OAc	—	6-Cl	49 ± 4	53 ± 6	550 ± 52
17α-Acetoxyprogesterone	4	OAc	—	—	52 ± 5	39 ± 4	350 ± 47
5α-Pregnane-3,20-dione	—	—	—	5α-H	32 ± 4	28 ± 3	400 ± 47
Norethynodrel	5(10)	C≡CH	OH	19-nor	31 ± 3	24 ± 4	430 ± 29
11β-Hydroxyprogesterone	4	—	OH	11β-OH	29 ± 4	27 ± 3	320 ± 30
19-Nortestosterone	4	—	OH	19-nor	16 ± 2	27 ± 2	250 ± 17
20β-Hydroxyprogesterone	4	—	—	20β-OH	16 ± 2	12 ± 2	180 ± 19
Norethindrone acetate	4	C≡CH	OAc*	19-nor	11 ± 2	8 ± 2	100
5β-Pregnane-3,20-dione	—	—	—	5β-H	7 ± 1	5 ± 1	85 ± 9
20α-Hydroxyprogesterone	4	—	—	20α-OH	3 ± 1	4 ± 1	50 ± 6
17α-Hydroxyprogesterone	4	OH	—	—	4 ± 1	2 ± 1	65 ± 8
11α-Hydroxyprogesterone	4	—	—	11α-OH	2 ± 1	2 ± 1	—
Oestradiol	1,3,5(10)	—	OH	3-OH	<1	8 ± 2	17 ± 2
Testosterone	4	—	OH	—	<1	12 ± 2	16 ± 2
Cortisol	4	OH	—	11,21-OH	<1	<1	8 ± 2

* Ac = acetate.

Fig. 8. Correlation of binding affinities of progesterone receptor with norethindrone receptor (Δ) and progesterone receptor with norethindrone acetate receptor ($\bullet$) in human uterine cytosol

Each point represents triplicate assays. The best-fitted lines were drawn by linear regression analysis. Abbreviations used: P-R, progesterone receptor; ENT-R, norethindrone receptor; ENTA-R, norethindrone acetate receptor.

nortestosterone (17 β -hydroxy-19-norandrost-4-en-3-one) had relative affinity varying between 16 and 32. However, the relative affinity of various competitors for norethindrone acetate receptor indicated poor receptor affinity of the progestin. All potent progestins were 4–18 times more effective than norethindrone acetate in binding to the uterine progestin receptor. Oestrogens, testosterone and cortisol showed little or no competition for progestin receptors.

Fig. 8 illustrates the correlation of relative binding affinities of progesterone receptor with norethindrone receptor and progesterone receptor with norethindrone acetate receptor. High correlation within the receptor affinities was noted. The correlation coefficients (r) between the receptor specificities were as follows: progesterone against norethindrone, $r = 0.98$; progesterone against norethindrone acetate, $r = 0.95$. The difference between the two correlations observed was not significant ($P < 0.05$).

Discussion

The present results convincingly demonstrate that in the human uterus progestins like norethindrone and norethindrone acetate are bound to the progesterone-recognition site, but with varying degrees of inhibition and binding constants. From

this significant steroid–protein interaction, the action of different progestins in the human uterus via a common receptor is conceivable. Further, these progestins were also found to interfere with the binding of oestradiol to oestradiol receptor.

Studies on density-gradient centrifugation indicated that the uterine receptor, which migrated as a 4.1–4.3S macromolecule, bound progestins in general rather than the physiological ligand progesterone. Previous work by McGuire & DeDella (1971), Murugesan & Laumas (1973), Verma & Laumas (1973), Terenius (1974), Kontula *et al.* (1975) and Feil *et al.* (1976), demonstrating progestin-mediated displacement of progesterone binding to uterine receptor, supports our observations. However, no data are available to further characterize the type of inhibition obtained with the progestins. Our results involving competitive-receptor assays, which have been evaluated by the double-reciprocal plots, unequivocally demonstrate the existence of a competitive mechanism between the progestins and progesterone for the same binding site on the receptor. The interpretation becomes more plausible in that a putative intracellular receptor has been characterized for norethindrone and norethindrone acetate, with physicochemical and kinetic behaviour very similar to that of uterine progesterone receptor (Laumas & Kasid, 1977). Further, these progestins also inhibited oestradiol binding to oestrogenic receptor, but only to the extent of 8–12%. The decrease in the uptake of [3 H]oestradiol in the presence of norethynodrel (Laumas *et al.*, 1972), chlormadinone acetate (Rosner *et al.*, 1972) and norethindrone/norethindrone acetate (Van Kordelaar *et al.*, 1975) has been well demonstrated. In the present study, this progestin–oestradiol interaction was investigated at the receptor level, giving adequate attention to the specific and non-specific binding. It appeared that these progestins inhibited oestradiol binding by competitively modulating the receptor activity.

Receptor affinity is of prime importance in studies of kinetics and biological profile of the drugs. The displacement efficiency on density gradients and the relative binding data with [3 H]progesterone, [3 H]norethindrone or [3 H]norethindrone acetate to tag the progestin receptor suggested that norethindrone had receptor-binding affinity comparable with progesterone, but markedly higher than norethindrone acetate. The kinetic constants (K_d and K_i) further indicated that norethindrone acetate affinity for progestin receptor was approximately one-tenth that of norethindrone or progesterone, which is contrasted by the uniformity in receptor concentration (7060–8340 molecules/cell; Table 1) for all the three progestins, when quantified concurrently in the cytosol samples. Probably, the discrepancy on the lack of correlation of receptor-binding affinity and the high biological potency of norethindrone acetate could be explained

by the low dissociation rate of progestin-receptor interaction ($t_{\frac{1}{2}} \sim 110$ –130 min; A. Kasid & K. R. Laumas, unpublished work) and a high conversion into norethindrone (with strong receptor affinity).

Ligand specificity data yielded some valuable information on the structural requirements for efficient binding to progestin receptor, and supported the earlier observations by Liao *et al.* (1973), Smith *et al.* (1974) and Kontula *et al.* (1975). In general a 3-oxo-4-ene structure is required for effective binding. Modifications of the planar structures of the A/B ring of steroid decreased the binding significantly. The orientation of the 17 β -hydroxy group and as a consequence its hydrogen-bond geometry and the directional interaction of the 17-ethynyl group were directly linked to the binding mechanism of progestin on the uterine receptor. The absence of a C-19 methyl group (19-norsteroids) facilitated the binding considerably. However, the introduction of an additional 18-methyl group in d-norgestrel showed dramatic enhancement in the binding. The esterification of the 17 α -hydroxy function leads to a substantial increase in the binding affinity. However, the introduction of an acetate group at the 17 β -position disrupted the steroid binding considerably.

The conclusive evidence in the present paper that the progestins bind to the progesterone-binding site on the receptor, and also inhibit the oestradiol binding, is of considerable significance. It is well known that oestradiol prepares the uterus for progesterone action, and progesterone is necessary for the implantation. Studies with a single silastic implant (Bhatnagar *et al.*, 1975; Srivastava & Laumas, 1975) indicate that norethindrone acetate released in micro quantities does not appear to interfere with ovulation in the earlier months, but suppresses ovulation during the later months of use. The contraceptive effect, when ovulation is not suppressed, could probably result from the progestin action at some other site, like uterus. Thus the interaction of these progestins with the receptors for progesterone and oestradiol, either by simple competition or by change in the relative spatial orientation at the active site, would change the receptor availability, leading to modulation of hormone-dependent changes. This would critically influence uterine 'receptivity' to hormonal stimuli and thus account for the contraceptive action.

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