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In Vitro and in Vivo Enhancement of Progesterone Binding to the Uterine Progesterone Receptor by Cortisol†

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ABSTRACT: It was found that, in the presence of high concentrations of cortisol (7×10^{-7} M), the apparent K_D for progesterone binding to the rat uterine progesterone receptor decreased when compared with the calculated K_D determined without cortisol. Dilution of the cytosol showed a similar effect. The decrease in the apparent K_D was observed when the amount bound was determined by the dextran coated charcoal (DCC) adsorption method but not when binding was assayed by equilibrium dialysis. A mathematical model has been derived for the analysis of progesterone binding to the rat uterine progesterone receptor which can explain the above results. The model takes into account that the concentration of free progesterone at equilibrium (as calculated from total minus bound) is overestimated due to the binding of progesterone to low affinity material. Since this low affinity binding largely dissociates during treatment with DCC, it is not detected as bound. We show that several predictions of the model are confirmed using the rat uterine progesterone receptor. Specifically, adding cortisol to the in vitro incubations increases the amount of bound progesterone when the reactions are carried out at nonsaturating concentrations of [^{3}H]progesterone. This effect of cortisol is explained by postulating that cortisol displaces [^{3}H]progesterone from the low affinity sites, thus resulting in a higher concentration of free progesterone available for binding to the receptor. In vivo effects of cortisol on progesterone action were also examined. The inhibition by progesterone of replenishment of the rat uterine estrogen receptor was used as a bioassay for progesterone. Simultaneous injections of cortisol and progesterone resulted in greater inhibition than injections of progesterone alone. This potentiation by cortisol was dose dependent. We postulate that the potentiation is due to an increase in the level of unbound plasma progesterone because of its displacement from plasma proteins by cortisol.

In the determination of equilibrium constants for the binding between steroids and proteins, it is generally recognized that equilibrium dialysis is the most accurate method (King and Mainwaring, 1974a). For routine determinations or large numbers of samples, however, dialysis becomes unwieldy, and thus many investigators employ nonequilibrium methods (e.g., methods employing DCC,[1] gel filtration, or hydroxylapatite) to remove free from bound steroid. These methods, though quicker and simpler than equilibrium dialysis, result in a loss

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[1] Abbreviations used: K_D, equilibrium dissociation constant: E_2, estradiol-17β; ER, the complex between estradiol-17β and its receptor; DCC, dextran-coated charcoal; CBG, corticosteroid binding α-globulin; R 5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; sc, subcutaneously; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TE buffer, 0.01 M Tris-HCl-0.0015 M EDTA (pH 7.4); TTG buffer, 0.05 M Tris-HCl-25% glycerol-2.4 mM thioglycerol-0.02% sodium azide-1 mM phenylmethanesulfonyl fluoride (pH 7.4).

of the equilibrium between the reactants during the subsequent assay for the amount of bound steroid.

In cytosol preparations from target tissues, labeled steroid binds not only to receptors, which are characterized by high affinity, low capacity binding, but also to sites of low affinity and high capacity. A major portion of that low affinity binding dissociates during the separation of free from bound steroid. The remaining low affinity binding can be determined by measuring the binding of labeled steroid in the presence and absence of excess unlabeled competitor. The difference between the two determinations is a measure of the high affinity, low capacity binding (i.e., specific binding). The concentration of free steroid at equilibrium can then be calculated by subtracting the specifically bound steroid from the total steroid added initially. The concentration of free steroid calculated in that manner, however, is overestimated for it ignores the binding of steroid to the low affinity sites. Depending on the concentration and K_D of those sites, the correction to the calculated concentration of free steroid can be quite significant. Though numerous mathematical analyses have been reported on equilibrium steroid binding to one or more high affinity sites in the presence of low affinity binding (Scatchard, 1949; Rosenthal, 1967; Baulieu and Raynaud, 1970; Rodbard and Feldman, 1975, Chamness and McGuire, 1975), they do not directly take into consideration the necessity for that correction.

We report herein the mathematical treatment of a model for steroid binding that stipulates: (1) a homogeneous class of high affinity sites, (2) the presence of a higher concentration of low affinity sites, and (3) that the assay measures only the high affinity binding. We show that, in the determination of the K_D for the rat uterine progesterone receptor by the DCC adsorption method, several predictions of the mathematical model can be verified experimentally. Finally, we show that the implications of that analysis are not restricted to in vitro considerations but that we can also show effects in vivo which are readily explained by this model.

Experimental Procedure

Materials. Estradiol-17β-6,7-^3H (47.9 Ci/mmol) and progesterone-1,2-^3H (55.7 Ci/mmol) were from New England Nuclear, Boston, Mass. R 5020- and R 5020-6,7-^3H (51.4 Ci/mmol) were a generous gift from Dr. J. P. Raynaud, Centre de Recherches, Roussel UCLAF, Raminville, France. The labeled steroids were tested for purity by thin-layer chromatography. Cortisol, diethylstilbestrol, progesterone, thioglycerol, and phenylmethanesulfonyl fluoride were from Sigma Chemical Co., St. Louis, Mo. Hydrocortisone sodium phosphate (50 mg/mL) for injection was from Merck, Sharpe, and Dohme, West Point, Pa. Progesterone for injection (Proluton 50 mg/mL) was from Schering Corp., Renilworth, N.J. Dextran T-70 was from Pharmacia Inc., Piscataway, N.J., and charcoal (Norit A) was from Pfanstiehl Laboratories, Inc., Waukegan, Ill. and used after extensive washing with 1 N HCl, H_2O, 1 N NaOH, and finally H_2O until the wash showed a neutral pH.

Animals. Sprague-Dawley rats were obtained from the Hormone Assay Labs, Chicago, Ill. Steroids were injected subcutaneously in a volume of 0.1 mL of sesame oil (estradiol-17β and progesterone) or 0.9% saline (hydrocortisone sodium phosphate). To stimulate the synthesis of progesterone receptors (Feil et al., 1972), ovariectomized rats were injected for 3–5 days with 5 μg of estradiol-17β and killed by decapitation 24 h after the last injection.

Tissue Preparation. Uterine cytosol (105 000g supernatant) was prepared as described previously (Saiduddin and Zassenhaus, 1977). Estradiol-17β receptors were assayed in TE buffer (0.01 M Tris-HCl–0.0015 M EDTA, pH 7.4) and progesterone receptors were assayed in TTG buffer (0.05 M Tris-HCl–25% glycerol–2.4 mM thioglycerol–0.02% sodium azide–1 mM phenylmethanesulfonyl fluoride, pH 7.4). Protein was determined by the method of Lowry (Lowry et al., 1951).

Cytosol Receptor Assay. The DCC (1% activated charcoal–0.05% dextran T-70 in the appropriate buffer) adsorption method described by Korenman (Korenman, 1968) and as modified by us (Saiduddin and Zassenhaus, 1977) was used to assay estradiol-17β and progesterone receptors. The latter were assayed in polystyrene tubes in a total volume of 0.40 mL containing: 0.25 mL of cytosol, 0.05 mL of [^{3}H]progesterone or [^{3}H]R 5020 at the concentrations indicated in the figure legends, 0.05 mL of cortisol (100 ng) where specified, and 0.05 mL of TTG buffer. To $\frac{1}{2}$ of the incubation tubes, 0.05 mL of 100-fold unlabeled competitor (progesterone or R 5020) was substituted for the TTG buffer to determine the nonspecific binding. All incubations were carried out at 4 °C for 20 h.

Equilibrium Dialysis. Dialysis tubing was boiled 5 min in TE buffer and soaked overnight at 4 °C in TTG buffer. Aliquots (1 mL) of the cytosol were pipetted into dialysis bags and then dialyzed in triplicate against 50 mL of TTG buffer containing a fixed concentration of 0.59×10^{-9} M [^{3}H]progesterone and unlabeled progesterone ranging from 0 to 41.5×10^{-9} M. When cortisol was included in the dialysate, it was present at 7.65×10^{-7} M. After 30 h with shaking at 4 °C, during which time equilibrium was established, one-half of the dialyzed cytosol (0.5 mL) was extracted immediately with 3 mL of toluene–isoamyl alcohol (19:1) for determination of the total amount of bound steroid; to the remaining 0.5 mL, an equal volume of DCC was added for determination of the high affinity binding as per the cytosol receptor assay. The concentration of free steroid was calculated from aliquots of the dialysate. In all cases more than 90% of the labeled steroid added initially could be accounted for after dialysis. Moreover, degradation of the progesterone after 30 h was less than 15% as determined by thin-layer chromatography.

Results

Model. The model assumes the presence of two homogeneous classes of steroid binding proteins (R_1 and R_2) in a cytosol preparation, of which binding to only one class is detected after steroid adsorption with DCC.

Definitions. B_1 is the concentration of bound steroid which is detected; B_2, concentration of bound steroid which is not detected; F_a, concentration of free steroid at equilibrium; F_t, concentration of free steroid added initially (i.e., total steroid); F_c, concentration of free steroid as calculated from $F_c = F_t - B_1$; K_1 and K_2, equilibrium dissociation constants for R_1 and R_2; $R_1°$ and $R_2°$, initial concentrations of R_1 and R_2.

At equilibrium steroid binding to the detectable sites can be described by eq 1, the familiar equation for the Scatchard plot:

$$B_1/F_a = B_1(-1/K_1) + R_1°(1/K_1) \quad (1)$$

But in practice F_c is used to construct the Scatchard plot. From the definition of F_c and the equation for the conservation of mass,

$$F_t = F_a + B_1 + B_2 \quad (2)$$

it can be shown that:

$$F_a = F_c - B_2 \quad (3)$$

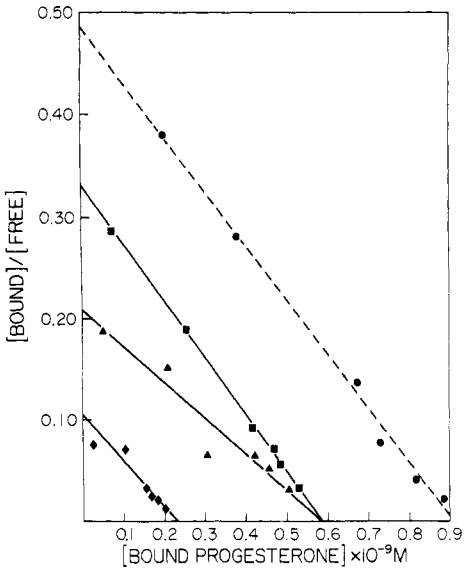

FIGURE 1: Scatchard plots of [^{3}H]progesterone binding to the rat uterine progesterone receptor as determined by equilibrium dialysis and the DCC adsorption method. Cytosol was prepared to a final protein concentration of 1.7 mg/mL (equivalent to 52 mg wet weight/mL). (A) (●- -●) Determination by equilibrium dialysis of the high affinity binding. Correction for nonspecific binding was made by subtracting from each point the binding determined with 250-fold excess unlabeled progesterone (150×10^{-9} M). (B) (■—■) High affinity binding in the presence of 7.65×10^{-7} M cortisol as determined by DCC adsorption. The concentration of [^{3}H]progesterone ranged from 0.35 to 16.9×10^{-9} M. (C)(▲—▲)As for B, except that no cortisol was present in the incubations. (D) (♦—♦) As for C, except that the cytosol was diluted beforehand 1:2.5 with TTG buffer. The best straight line through the points was determined by linear regression analysis. K_D's were calculated from the slopes of the Scatchard plots and are: (A) equilibrium dialysis, 1.86×10^{-9} M; (B) DCC adsorption method with cortisol, 1.76×10^{-9} M; (C) DCC adsorption method without cortisol, 2.81×10^{-9} M; (D) DCC adsorption method using 1:2.5 diluted cytosol, 2.19×10^{-9} M.

Substituting this expression for F_a into eq 1 and rearranging, we can derive an expression for B_1/F_c:

$$\frac{B_1}{F_c} = B_1(-1/K_1)\left(\frac{F_a}{F_a + B_2}\right) + R_1^\circ(1/K_1)\left(\frac{F_a}{F_a + B_2}\right) \quad (4)$$

At equilibrium, B_2 is defined by:

$$B_2 = \frac{R_2^\circ F_a}{K_2 + F_a} \quad (5)$$

This expression for B_2 can be substituted into eq 4 to derive the general equilibrium equation describing the Scatchard plot when calculated using F_c:

$$\frac{B_1}{F_c} = B_1(-1/K_1)\left(\frac{1}{1 + [R_2^\circ/(K_2 + F_a)]}\right) + R_1^\circ(1/K_1)\left(\frac{1}{1 + [R_2^\circ/(K_2 + F_a)]}\right) \quad (6)$$

Steroid binding to R_2 represents the low affinity, high capacity binding which dissociates during DCC treatment and is considered nonsaturable. Mathematically this means that, under the usual conditions for determining the Scatchard plot via the DCC adsorption method,

$$k_2 \gg F_a \quad (7)$$

Under that assumption eq 6 can be simplified and results in an equation describing a linear Scatchard plot:

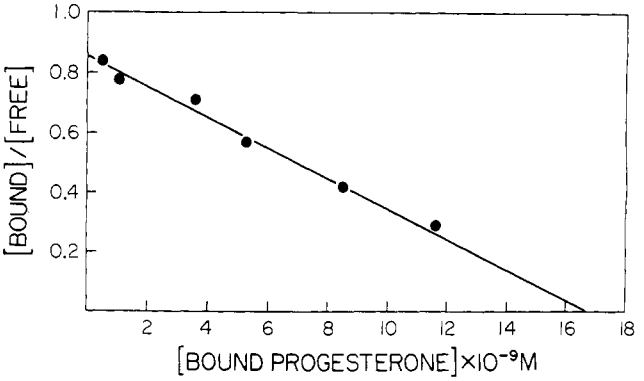

FIGURE 2: Scatchard plot of the binding of [^{3}H]progesterone to low affinity sites as determined by equilibrium dialysis. The same cytosol preparation and dialysis conditions as described in Figure 1 were employed. Total [^{3}H]progesterone binding (i.e., binding to sites of low and high affinity) was measured by counting 0.5-mL aliquots of the dialyzed cytosol. The low affinity binding was calculated by subtracting from each point the specific, high affinity binding as determined in Figure 1.

$$\frac{B_1}{F_c} = B_1(-1/K_1)\left(\frac{1}{1 + [R_2^\circ/K_2]}\right) + R_1^\circ(1/K_1) \times \left(\frac{1}{1 + [R_2^\circ/K_2]}\right) \quad (8)$$

From eq 8 we see that the calculated apparent K_D is overestimated when determined via the DCC adsorption method and that the magnitude of that error is defined by:

$$\text{apparent } K_D = K_1\left[1 + \left(\frac{R_2^\circ}{K_2}\right)\right] \quad (9)$$

Several predictions are suggested by eq 8.

(a) At high concentrations of free steroid, B_1/F_c approaches 0 and thus the extrapolated value for the total concentration of measurable binding sites is R_1°.

(b) If labeled steroid binding to R_2 could be eliminated, then the calculated K_D for the high affinity binding should decrease by a factor of $[1 + (R_2^\circ/K_2)]$ when compared with the calculated K_D without elimination of the low affinity binding.

(c) Dilution of the cytosol should result in a decrease in the calculated K_D since the correction factor $[1 + (R_2^\circ/K_2)]$ now assumes a lower value due to the decrease in R_2°.

(d) Determination of the K_D by equilibrium dialysis should be unaffected by the presence or absence of labeled steroid binding to R_2 since a true determination of F_a can be made. Moreover, the calculated K_D by equilibrium dialysis should be the lowest obtainable and be equal to that determined under condition b above.

Our model postulates that cortisol, by eliminating or at least greatly reducing [^{3}H]progesterone binding to low affinity sites, increases the concentration of free progesterone available for binding to the receptor. As shown in Figure 1, several predictions of the model are borne out experimentally. First, the extrapolated values for the total concentration of specific binding sites (0.94 pmol/mL cytosol) are nearly identical whether determined with or without cortisol. The absence of a reduction in that value with cortisol also indicates that CBG is not a significant contaminant of the cytosol preparation. Secondly, adding cortisol to the reaction mixture or incubating with diluted cytosol decreases the apparent K_D. Finally, the K_D as determined by equilibrium dialysis is nearly equal to the K_D as calculated by the DCC adsorption method with cortisol present. That is what would be expected since in both cases an

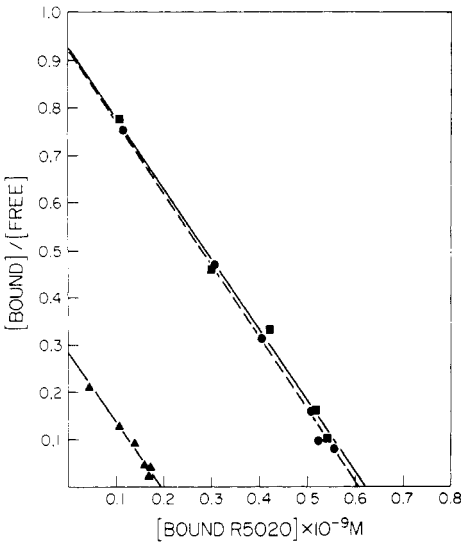

FIGURE 3: Scatchard plots of [^{3}H]R 5020 high affinity binding with 7.65×10^{-7} M cortisol (■---■, $K_D = 0.67 \times 10^{-9}$ M), without cortisol (●- -●, $K_D = 0.66 \times 10^{-9}$ M), and without cortisol employing 1:3 diluted cytosol (▲ ▲, $K_D = 0.69 \times 10^{-9}$ M), as determined by DCC adsorption. The same cytosol preparation as described in Figure 1 was used. The concentration of [^{3}H]R 5020 ranged from 0.26 to 7.2×10^{-9} M.

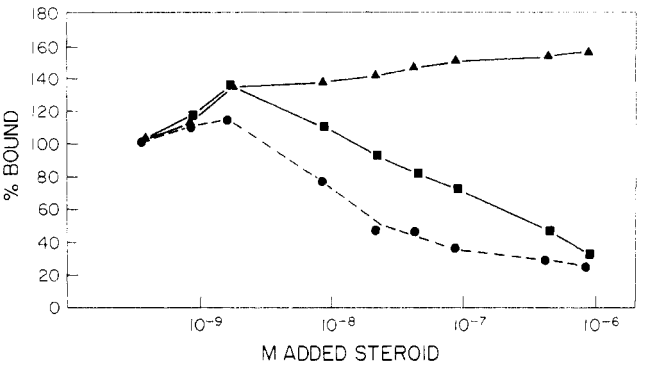

FIGURE 4: Competition curves for [^{3}H]progesterone binding to the progesterone receptor. Cytosol was prepared to a final protein concentration of 2.23 mg/mL (equivalent to 63 mg wet weight/mL) and incubated in 8×10^{-9} M [^{3}H]progesterone plus cortisol (▲—▲), progesterone (■---■), or R 5020 (●- -●). The concentration of bound [^{3}H]progesterone in the control with no competitor added was 1.09 pmol/mL.

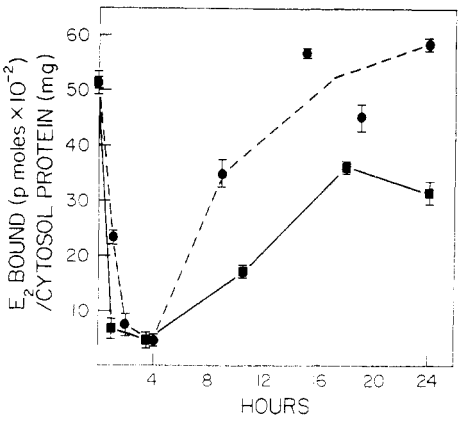

FIGURE 5: Replenishment of the E_2 receptor in immature rats with and without progesterone injections. Rats were injected beginning at 22 days of age with 2.5 μg of E_2 for 2 days. On the third day they were injected with 2.5 μg of E_2 only (●- -●) or 2.5 μg of E_2 + 0.25 mg of progesterone + 2.0 mg of hydrocortisone (■---■). Rats were killed at the times indicated after the last injection (3 rats were used per time point).

accurate determination of the concentration of free steroid can be made. Moreover, the apparent K_D by equilibrium dialysis was unaffected by the presence or absence of cortisol; with cortisol the K_D equaled 1.71×10^{-9} M and without cortisol it equaled 1.80×10^{-9} M (data not shown).

Figure 2 presents the Scatchard plot for the low affinity binding (from the same cytosol preparation used to calculate the high affinity binding in Figure 1) as determined by equilibrium dialysis in the absence of cortisol. Excess cortisol in the dialysate reduced that binding by greater than 90% (data not shown). From eq 8 a correction factor $[1 + (R_2°/K_2)]$ can be calculated from the low affinity binding. Dividing that factor into the apparent K_D for the high affinity binding in the absence of cortisol gives a corrected K_D that should be in close agreement with that determined by equilibrium dialysis or when low affinity binding is eliminated. From Figure 2, $R_2°/K_2$ equals 0.86 which results in a correction factor of 1.54 ($1 + \{[0.25$ mL of cytosol/0.4 mL total volume$] \times 0.86\}$). Dividing this into the apparent K_D determined via the DCC adsorption method in the absence of cortisol (Figure 1) gives a corrected K_D of 1.82×10^{-9} M. Similarly, for the diluted cytosol a correction factor of 1.22 is calculated which gives a corrected K_D of 1.80×10^{-9} M. These values for the corrected K_D are in reasonable agreement with the K_D determined by equilibrium dialysis or by the DCC adsorption procedure in the presence of cortisol (Figure 1).

Since R 5020, a synthetic progestin, binds to progesterone receptors with a K_D lower than progesterone but apparently does not bind avidly to CBG to which progesterone does bind (Horowitz and McGuire, 1975), we examined the influence of cortisol on the binding of [^{3}H]R 5020 to the uterine progesterone receptor. As shown in Figure 3, adding cortisol to the incubation mixture or diluting the cytosol had no effect on the apparent K_D. According to our model, that suggests that in this receptor preparation the binding of R 5020 to low affinity sites is characterized by a negligible $R_2°/K_2$. In Figure 4 are presented competition curves between [^{3}H]progesterone at a nonsaturating concentration and unlabeled progesterone, R 5020, and cortisol. In agreement with the relative K_D's, R 5020 is a more effective competitor than progesterone. Also, it can be seen that the enhancement of progesterone binding by cortisol is greatest at a concentration of cortisol equal to about 7×10^{-7} M.

The foregoing results suggest that the presence of cortisol in vitro increases the concentration of free progesterone by reducing its low affinity binding. Whether a similar effect can be observed in vivo was also studied. It has been shown that progesterone in vivo inhibits in a dose-dependent manner the replenishment of the uterine cytosol E_2 receptor following an injection of E_2 (Hsueh et al., 1976). Thus, assaying ER 24 h after a simultaneous injection of E_2 and progesterone is in effect a biological assay for progesterone. Figure 5 shows a typical curve for replenishment of the ER with and without the simultaneous injection of 0.25 mg of progesterone. In adult rats, the effect of injecting cortisol along with E_2 and progesterone is shown in Table I. Cortisol alone had no effect on the replenishment of the ER, but potentiated the action of progesterone. Figure 6A shows that this potentiation by cortisol is dose dependent. In prepuberal rats (22 days of age), however, no potentiation by cortisol could be shown (Figure 6A), though for both adult and prepuberal rats a dose-response curve for the inhibition of ER replenishment by progesterone could be demonstrated (Figure 6B). The results suggest that the po-

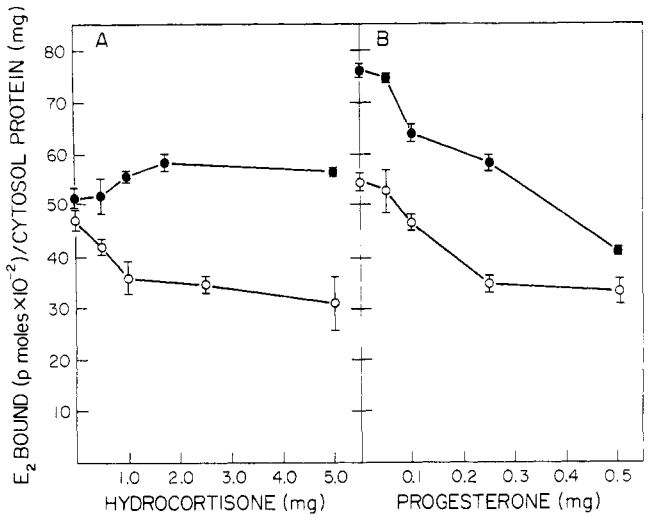

FIGURE 6: Replenishment of the E_2 receptor in adult and prepuberal (22 days old) rats after injection of varying doses of progesterone or hydrocortisone. Adult rats (4-6 months) were ovariectomized and 4 days later injected with 2.5 μg of E_2 daily for 2 days. On the 3rd day they were injected with 2.5 μg of E_2, 0.25 mg of progesterone, and increasing concentrations of hydrocortisone (panel A, O—O), or 2.5 μg of E_2, 2 mg of hydrocortisone, and increasing concentrations of progesterone (panel B, O—O). Prepuberal rats were injected for 3 days with 2.5 μg of E_2 daily. On the fourth day they were injected with 2.5 μg of E_2, 0.25 mg of progesterone, and increasing amounts of hydrocortisone (panel A, ●—●) or 2.5 μg of E_2, 2.0 mg of hydrocortisone, and increasing amounts of progesterone (panel B, ●—●). All rats were killed 24 h later and the E_2 receptors assayed in the uterine cytosol.

TABLE I: In Vivo Effect of Cortisol on the Progesterone Inhibition of Estradiol Receptor Replenishment in the Rat Uterine Cytosol.

Treatment[a]		[^{3}H]E_2 bound
Progesterone	Cortisol	% of control[b]
−	−	100
−	+	102 ± 5
+	−	70 ± 7
+	+	51 ± 4

[a] One week after ovariectomy rats (4-6 months old) were given estradiol (2.5 μg daily, sc) for 3 days. On the 3rd day they also received one injection of cortisol (3 mg) and/or progesterone (0.25 mg). They were killed 24 h later. ER in uterine cytosol was measured by DCC adsorption method. [b] Compilation of four experiments with 2 rats per treatment per experiment. Controls varied between 40 and 65 × 10^{-2} pmol of [^{3}H]E_2 bound/mg of cytosol protein.

tentiation by cortisol in vivo is analogous to its in vitro effects insofar that simultaneous injections of cortisol and progesterone result in higher levels of unbound plasma progesterone.

Discussion

Calculations of the apparent K_D for steroid-receptor interactions assume that all reactants are at equilibrium (King and Mainwaring, 1974a). Since the addition of DCC disturbs the established equilibrium, however, those calculations may not be accurate. Our model takes into consideration the nonequilibrium conditions imposed at the termination of incubation by the addition of DCC. An equilibrium equation (eq 8) is derived which predicts that the apparent K_D for the high affinity sites is overestimated and that any action that reduces low affinity binding (e.g., dilution of the cytosol or competing

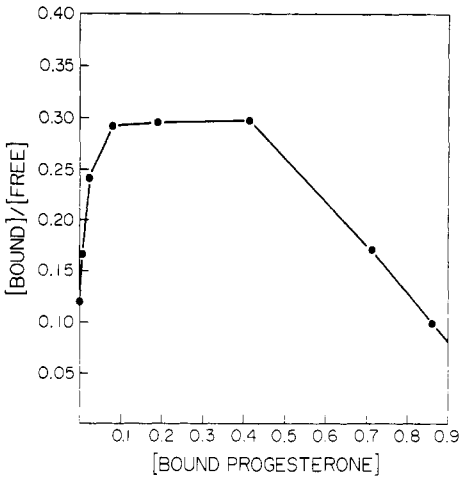

FIGURE 7: Scatchard plot of [^{3}H]progesterone binding to the uterine progesterone receptor with decreasing concentrations of cortisol. Cytosol was prepared to a final protein concentration of 1.42 mg/mL (equivalent to 43 mg wet weight/mL). The concentration of [^{3}H]progesterone ranged from 0.02 to 8.7 × 10^{-9} M; the concentration of cortisol was 100 × that of [^{3}H]progesterone. [^{3}H]Progesterone binding was determined by the DCC adsorption method.

away the labeled steroid from the low affinity sites) decreases the calculated K_D.

We have shown that several predictions of the model are verified experimentally with regards to the rat uterine progesterone receptor. Specifically, the addition of cortisol, by greatly reducing the binding of [^{3}H]progesterone to low affinity sites, increases the concentration of free progesterone and thus leads to higher concentrations of bound progesterone at nonsaturating concentrations of [^{3}H]progesterone. Other investigators have published, though not emphasized, the same observation with respect to the addition of cortisol (Feil et al., 1972; McGuire and DeDella, 1971; McGuire and Bariso, 1972). In determining whether cortisol competed with [^{3}H]-progesterone for binding to the progesterone receptor, a greater amount of [^{3}H]progesterone binding was observed in the presence of cortisol than in its absence.

Our model also predicts, and the results confirm, that in the absence of cortisol the apparent K_D for the progesterone receptor decreases as dilution of the cytosol increases. As the cytosol is diluted so is the concentration of the low affinity sites, which in turn decreases the binding of [^{3}H]progesterone to those sites. This might be a partial explanation for the variation in the published K_D's for the same receptor from the same tissue (King and Mainwaring, 1974b). Considerable variation in the concentration and K_D of the low affinity sites might be expected with changes in buffer, metabolic state, and type of tissue.

Occasionally a downward concavity in the shape of the Scatchard plot at low concentrations of steroid is attributed to positive cooperativity (Rodbard and Feldman, 1975). Though degradation of the free receptor (King and Mainwaring, 1974a) or loss of a fraction of the high affinity binding during the assay (Swillens and Dumont, 1975) would also demonstrate that, our model shows that a similar observation arises from the nonequilibrium conditions of the DCC adsorption assay. An extreme example of such an artifact can be induced experimentally (Figure 7) by constructing a Scatchard plot from an experiment in which the concentrations of cortisol and progesterone decrease in parallel (e.g., by keeping the concentration of cortisol fixed at 100-fold that of progesterone). It should be stated that dissociation of the low affinity

binding during DCC treatment is not the only explanation for results consistent with our model. Any procedure that removes from detection a class of binding sites (either of high or low affinity) that are present in the incubation mixture might result in data amenable to analysis based upon the general form of the equilibrium equation (eq 6).

Our in vivo evidence suggests that simultaneous injections of cortisol and progesterone result in the displacement of progesterone by cortisol from one or more species of plasma binders. The presumed rise in free plasma progesterone, therefore, is seen as a potentiation of progesterone action by cortisol. In vivo the displacement of progesterone need not necessarily be from low affinity sites, as appears to be the case in vitro, but could well be from high affinity sites such as CBG. Displacement from CBG as the cause for the potentiation by cortisol might explain why no potentiation was observed in prepuberal rats. It has been reported that immature rats have a lower level of plasma CBG than adult rats (Milgrom et al., 1971).

Finally, the model suggests a relatively simple way to estimate the magnitude of the required correction to the K_D as determined via the DCC adsorption method. The correction factor is equal to $1 + (R_2^\circ/K_2)$ and from eq 5 it can be seen that:

$$\frac{B_2}{F_a} = \frac{R_2^\circ}{K_2 + F_a} \tag{10}$$

Since at low concentrations of free steroid $K_2 \gg F_a$ for the low affinity binding, eq 10 can be approximated by:

$$\frac{B_2}{F_a} = \frac{R_2^\circ}{K_2} \tag{11}$$

As described under Experimental Procedure, B_2/F_a can be measured by equilibrium dialysis and thus the correction factor can be calculated by simply determining B_2/F_a at a single concentration of free steroid.

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