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The Binding of Progesterone, R-5020 and ORG-2058 to Progesterone Receptor*

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Abstract—*The binding of two synthetic progestins, R-5020 and ORG-2058 and a natural progestin, progesterone to progesterone receptors of human myometrium is examined. The receptor, which sediments at 4S in a sucrose density gradient, binds all three progestins with high affinity* (K_D *values for R-5020, ORG-2058 and progesterone are* 4.1×10^{-10}, 1.2×10^{-10} *and* 3.8×10^{-10} mole/l *respectively). However, competition studies using various concentrations of unlabelled steroids indicate that the synthetic steroids bind to the progesterone receptor with higher specificity than does progesterone itself.*

It is suggested that because of its high specific activity, its similar receptor binding kinetics to the natural hormone and its higher specificity for the progesterone receptor, R-5020 is the most suitable of the three progestins for use in progesterone receptor assays.

INTRODUCTION

MANY laboratories assay estrogen receptors in human breast cancers in order to determine an appropriate form of therapy for the patient. Of those women with estrogen receptors in their tumours 60% will respond to endocrine manipulative therapy, while in the absence of receptors the response rate is less than 10% [1]. To improve the discrimination between hormone-sensitive and hormone-insensitive breast cancers, the use of other hormone receptor assays has been suggested [2]. Early results suggest that the progesterone receptor assay may achieve this [3], but this assay has the problem of considerable non-specific binding. However, this problem may be overcome either by using the natural hormone, progesterone, in combination with excess cortisol [4], or by using synthetic steroids which are claimed to have greater specificity for the receptor protein [3, 5].

This study examines the binding properties of progesterone and of two synthetic progestins, R-5020 and ORG-2058, to the progesterone receptor protein of human myometrium. In addition to these kinetic studies, progesterone and R-5020 are used to measure progesterone receptors in human myometrium.

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MATERIALS AND METHODS

The labelled progesterone used in this study was 1, 2, 6, 7-^{3}H-progesterone (Amersham Searle; SA 85 Ci/mmole). Labelled and unlabelled ORG-2058 (17-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione; SA = 19.2 Ci/mmole) was kindly supplied by the Organon Company, Oss, The Netherlands. R-5020 (17,21-dimethyl-19-nor-4,9-pregnadiene-3, 20-dione; SA = 86 Ci/mmole) was obtained from New England Nuclear.

Premenopausal myometrium was obtained fresh from the operating theatre, immediately chilled on ice, sectioned into 500 mg aliquots and snap-frozen in liquid nitrogen for storage for up to 2 weeks at −70°C. At the time of assay the tissue was again placed in liquid nitrogen and powdered in a chilled stainless steel chamber by sudden percussion. The powder, with 3 ml Tris-buffer (Tris, 10 mM; EDTA, 1.5 mM; pH 7.4) was homogenized at 4°C with an Ultra Turrax homogenizer (Janke and Kunkel) with 4 × 15 sec runs with 45 sec of cooling between runs. After centrifugation of the homogenate at 20,000 rev/min for 1 hr, the supernatant cytosol fraction was separated off with a Pasteur pipette and assayed within 3 hr. Protein concentration of the

cytosol fraction was measured by the method of Lowry *et al.* [6].

Assays for progesterone receptor using ^{3}H-progesterone were based on the method of Pichon and Milgrom [4]. Assays using ^{3}H-R-5020 and ^{3}H-ORG-2058 were as described by Janne *et al.* [5]. In the assays using synthetic progestins, a 100-fold excess of unlabelled ORG-2058 was used to distinguish between specific and non-specific binding.

To assess the effectiveness of dextran-coated charcoal (charcoal 2.5 g; dextran T-70, 250 mg; glycerol, 50 ml; Tris-buffer, 450 ml) in removing unbound hormone from solution, cytosol fraction (100 μl) was incubated for 4 hr at 4°C with each progestin (in 100 μl Tris-buffer containing 20% glycerol). At the end of the incubation, 200 μl of the charcoal solution was added. After various incubation times at 4°C, the mixture was centrifuged and a 200 μl aliquot of the supernatant counted for tritium.

Several concentrations of competitors were used to assess the binding specificity and affinity of the various progestins. The competitors used were estradiol-17β (E_2), diethylstilboestrol (DES), dihydrotestosterone (17β-hydroxy-5α-androstan-3-one, DHT), testosterone (T), progesterone (P), 17-hydroxyprogesterone (17-P), 20β-hydroxy-4-pregnen-3-one (20-P), hydrocortisone (C) and ORG-2058 (ORG). The unlabelled steroid in ethanol was added to each incubation tube and evaporated under a stream of nitrogen. To this was added 100 μl of cytosol fraction and 100 μl of the labelled progestin in Tris-buffer containing 20% glycerol to give a final concentration of the unlabelled steroid of 10, 50, 100, 500, or 1000 nM. After 16 hr at 4°C, the reaction was stopped by addition of dextran-coated charcoal. Following centrifugation, an aliquot of the supernatant was counted for tritium.

Rates of association and dissociation of each progestin with the progesterone receptors were estimated using the technique outlined by Janne *et al.* [7].

Sedimentation properties of the ^{3}H-progestin-receptor complexes incubated with and without a 100-fold excess of unlabelled progestin were analysed in a 5–20% gradient of sucrose in Tris-glycerol buffer. Gradients were centrifuged for 18 hr at 4°C in an SW4OTi rotor on a Beckman L5-50 ultracentrifuge. Using top-harvesting (by displacement with 40% sucrose solution), 25 fractions of 12 drops each were collected and counted for tritium.

RESULTS

1. *Efficiency of dextran–charcoal in removing unbound hormone*

Dextran-coated charcoal effectively removes unbound progestin from cytosol fractions within 5 min at 4°C (Fig. 1). However over a 2 hr incubation with charcoal, progesterone is stripped from its binding sites indicating that careful timing is required in progesterone receptor assays using both progesterone and charcoal. Over the 2 hr incubation period, the synthetic steroids remained bound to receptor protein.

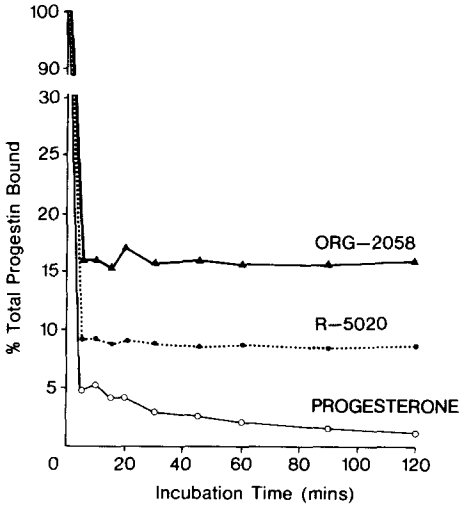

Fig. 1. Graph of bound progestin in cytosol fraction (expressed as a percentage of total progestin added) against time of incubation with dextran-coated charcoal. The ^{3}H-progestin (66 fmole) was incubated with human myometrial cytosol fraction prior to charcoal addition and centrifugation.

2. *Specificity of receptor-binding*

In order to assess specificity of binding to the receptor, each ^{3}H-progestin was incubated with myometrial cytosol fraction in the presence of various concentrations of other steroids. For the synthetic steroids (Fig. 2a), and for progesterone (Fig. 2b), low concentrations of progesterone were not as effective as ORG-2058 in competing for receptor sites. At concentrations of 1000 nM, they were equally competitive. At low concentrations, none of the other steroids tested were effective competitors for binding of R-5020 or ORG-2058 to the receptor sites.

The relatively weak affinity of ^{3}H-progesterone for receptor sites was apparent since a low concentration (10 nM) of estradiol-17β, diethylstilboestrol, 20β-hydroxy-4-pregnen-3-one and dihydrotestosterone was able to compete for binding to the receptor protein.

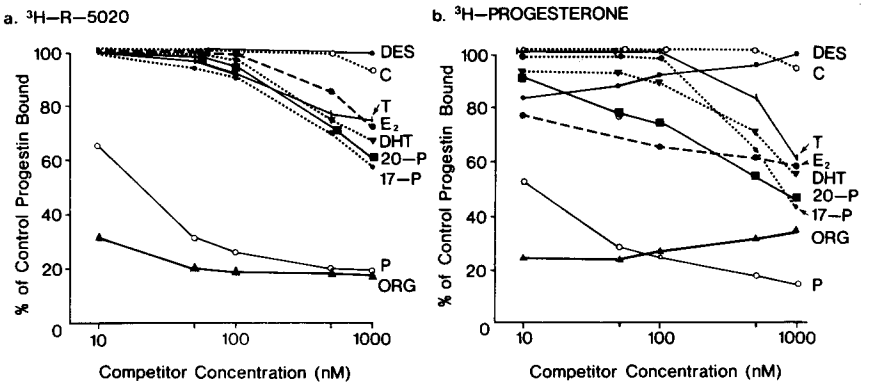

Fig. 2. Effect of addition of unlabelled steroids on binding of ^{3}H*-progestins to receptor proteins of human myometrium. Results for* ^{3}H*-ORG-2058 were identical to those of* ^{3}H*-R-5020. Concentrations of competitor used were* 10, 50, 100, 500 *and* 1000 nM. *Details of incubations and abbreviations are found in the text. Results on the ordinate are expressed as percentage of original added* ^{3}H*-progestin bound after charcoal treatment.*

3. *Association and dissociation rates*

Results of association and dissociation studies of each progestin with progesterone receptor obtained from human myometrium are shown in Fig. 3. Association and dissociation rate constants calculated from Fig. 3(b) and (d) respectively are summarized in Table 1.

The values obtained for association and dissociation constants are in good agreement with those obtained by Janne, Kontula and Vikko [5]. They indicate that the synthetic progestin R-5020 more closely approximates the binding kinetics of the natural hormone than does ORG-2058. This latter steroid appears to have a very high association rate

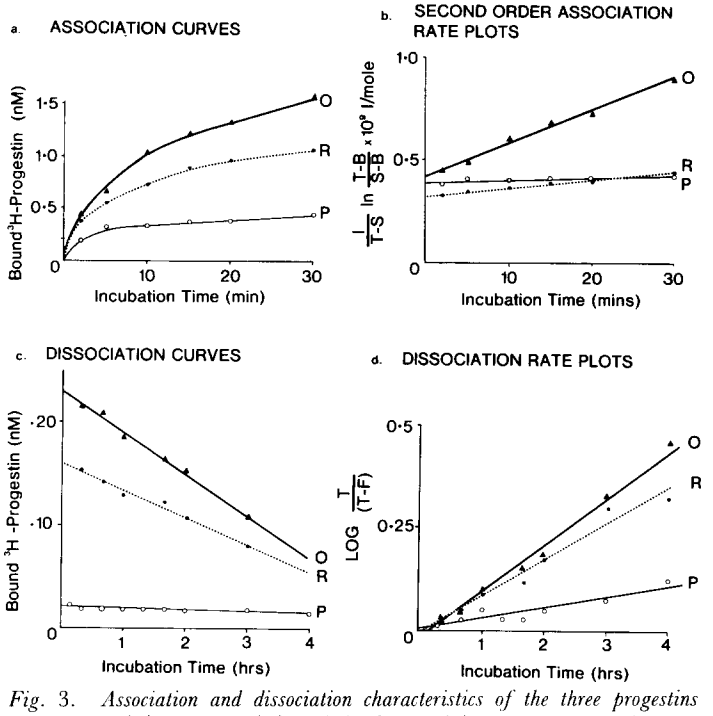

Fig. 3. Association and dissociation characteristics of the three progestins progesterone (P), R-5020 (R) and ORG-2058(0). Association results were obtained by incubating 600 fmole *of each* ^{3}H*-progestin with myometrial cytosol fraction at* 4°C *for various times before addition of dextran-coated charcoal and centrifugation. An aliquot of supernatant was counted to determine bound progestin. Dissociation results were obtained by incubating* ^{3}H*-progestin with cytosol fractions for* 16 hr *at* 4°C, *removing unbound hormone with charcoal, adding a* 100*-fold excess of the unlabelled progestin and incubating at* 4°C *for various times. Addition of dextran-coated charcoal stopped the reaction. After centrifugation an aliquot of the supernatant was counted to determine bound progestin in each tube.* T = *total hormone added* (nM); S = *concentration of binding sites* (nM); B = *bound* ^{3}H*-progestin at each time interval* (nM); F = *free* ^{3}H*-progestin at each time interval* (nM).

Table 1. *Summary of binding kinetics for the three progestins*

	R-5020	ORG-2058	Progesterone
Assoc^N rate (1/mole sec^{-1})	5.95×10^4	26.6×10^4	1.92×10^4
Dissoc^N rate (sec^{-1})	2.45×10^{-5}	3.12×10^{-5}	7.33×10^{-6}
Assoc^N constant (1/mole)	2.4×10^9	8.5×10^9	2.6×10^9
Dissoc^N constant (mole/l)	4.1×10^{-10}	1.2×10^{-10}	3.8×10^{-10}

Experimental details are outlined in the text. Values were obtained from Fig. 3(b) and (d).

constant compared with that of the other two progestins.

4. *Sucrose density gradient ultracentrifugation*

The binding of each progestin to the progesterone receptor of premenopausal human myometrium was further characterised through sucrose density gradient centrifugation. For each progestin, the receptor sedimented at 4S, the binding in this peak being inhibited by addition of a 100-fold excess of unlabelled hormone. Only results for progesterone binding are shown in Fig. 4 as results for the synthetic progestins are similar.

Scatchard plots [8], from which both binding site numbers and dissociation constants could be calculated (Fig. 5b). For one such cytosol fraction, the ^{3}H-progesterone assay measured 404 fmole ^{3}H-progesterone bound/mg cytosol protein, with a dissociation constant of 4.16×10^{-9} mole/l. Using ^{3}H-R-5020 for the same cytosol fraction, values of 417 fmole ^{3}H-R-5020 bound/mg cytosol protein, and 8.91×10^{-10} mole/l were obtained. The two assays consistently measure similar levels of receptor sites in the same cytosol fraction, but give dissociation constants which are higher than those obtained in the kinetic studies. This

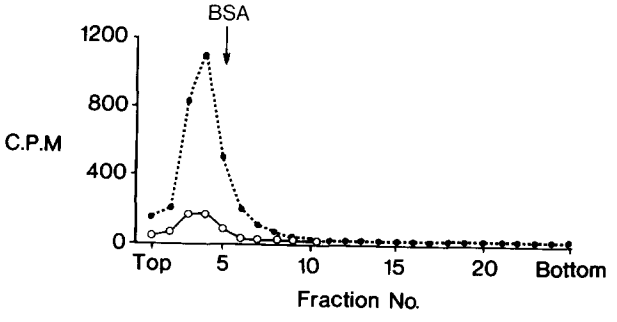

Fig 4. *Sucrose density gradient centrifugation of progesterone receptor. Cytosol and* ^{3}H-*progesterone* (600 fmole) *were incubated either with* (○——○) *or without* (●- - -●) *a* 100-*fold excess of unlabelled progesterone. Following incubation with a charcoal pellet and centrifugation,* 300 μl *of the supernatant was layered on a* 5–20% *sucrose density gradient and centrifuged* 18 hr *at* 4°C *on a SW* 40 *Ti rotor at* 40,000 rev/min. *Bovine serum albumin was run as a standard.*

5. *Progesterone receptor assay of human myometrium*

Because of its low specific activity (19.2 Ci/mmole), ORG-2058 was not used for assays of progesterone receptor. Tissues were therefore assayed for progesterone receptors using ^{3}H-progesterone and ^{3}H-R-5020. Assays of human plasma showed no specific binding sites for either R-5020 or progesterone (Fig. 5a), suggesting that both assays discriminate well between specific and non-specific binding of the progestin.

Assays of premenopausal human myometrial cytosol fraction found specific progesterone receptors, allowing the construction of unexplained observation has been made by other workers [5].

DISCUSSION

The three progestins R-5020, ORG-2058 and progesterone bound to the specific receptor for progesterone in human myometrial cytosol fraction, the receptor sedimenting characteristically for premenopausal myometrium at 4S in a sucrose density gradient [5]. However, the kinetics studies indicated that ORG-2058 had the fastest rates of association with and dissociation from the receptor, while progesterone had the slowest rates.

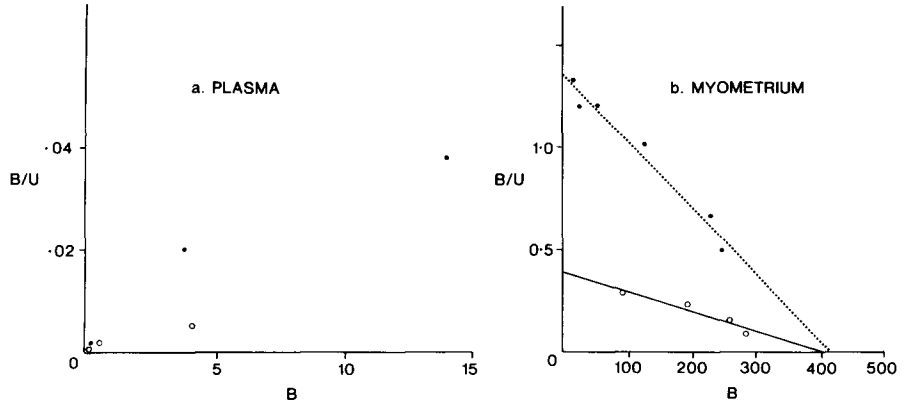

Fig. 5. Scatchard plots for progestin binding in human female plasma and human myometrial cytosol fraction. Incubation conditions are described in the text. Results were obtained using ^{3}H-*progesterone* (○) *or* ^{3}H-*R*-5020 (●). *B = bound* ^{3}H-*progestin* (fmole/mg *cytosol protein*), *U = unbound* ^{3}H-*progestin* (fmole/mg *cytosol protein*).

Competition studies between the radiolabelled progestins and various concentrations of unlabelled steroids revealed that the synthetic progestins bound to the receptor with higher specificity than did the natural hormone. At low concentrations (10 nM), only the synthetic hormone or the natural hormone were able to compete significantly with either R-5020 or ORG-2058. However, at the same concentration (10 nM), estradiol-17β, diethylstilboestrol, dihydrotestosterone, and 20β-hydroxy-4-pregnen-3-one were able to compete significantly with ^{3}H-progesterone. At higher concentrations of competitor, the displacement of ^{3}H-progesterone from the binding sites was even greater. Binding of synthetic progestins was affected much less by changes in concentrations of competing steroids.

Both the synthetic hormone, R-5020 and the natural hormone, progesterone have been used successfully in the measurement of progesterone receptors in human myometrium. The values obtained with either hormone are comparable. However, in using the natural hormone it is necessary to include an excess of cortisol in each assay tube to overcome non-specific binding [4].

These studies indicate that the synthetic progestins are preferable to the natural hormone, progesterone, in assays for progesterone receptor. Both are more stable in their binding to receptor during charcoal incubation, and their receptor binding is affected less by other steroids. Of the two synthetic progestins, ORG-2058 and R-5020, R-5020 is preferable because of its higher specific activity and its similar binding kinetics to the natural hormone.

The synthetic steroid, ^{3}H-R-5020 now is being used routinely in this laboratory to assay for progesterone receptors in human mammary gland tumours.

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