J. Clin. Chem. Clin. Biochem.
Vol. 23, 1985, pp. 265–276

^{3}H-Labelled RU 38486: Characterization of Binding Sites in Human Uterine Cytosol

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(Received July 10/November 29, 1984))

Summary: The behaviour of the antifertilizing synthetic steroid RU 38486 towards human uterine progestin receptor was investigated. RU 38486 competed in the same order of magnitude as progesterone for the [^{3}H]R 5020 binding site of progestin receptor, whereas R 5020 was unable to compete against [^{3}H]RU 38486. This apparent contradiction could be explained by means of HPLC-chromatography. HPLC-chromatography with an anion exchange column (MonoQ, Pharmacia, Uppsala, Sweden) showed that [^{3}H]RU 38486 forms at least two stable complexes with uterine cytosol, on one hand with serum albumin, which presents almost 90% of bound radioactivity, and on the other hand with the two native progestin receptor forms, corresponding to 4 S and 8 S receptor forms in sucrose density gradient analysis.

Whether reduced binding of salt-activated RU 38486 receptor complexes to DNA-cellulose is due to reduced activation is still uncertain and remains to be further investigated.

^{3}H-Markiertes RU 38486: Charakterisierung der Bindungseigenschaften in Cytosol aus Humanuterus

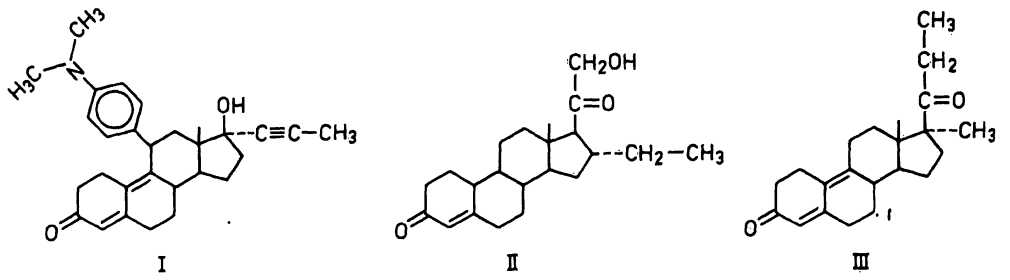
Zusammenfassung: Das synthetische Antifertilisierungshormon RU 38486 wurde hinsichtlich seiner Bindungseigenschaften am Progesteron-Rezeptor aus Humanuterus untersucht. RU 38486 kompetiert in der gleichen Größenordnung wie Progesteron um den [^{3}H]R 5020 Bindungsbezirk am Progesteron-Rezeptor, dagegen ist kaltes R 5020 nicht in der Lage, [^{3}H]RU 38486 aus seinen Bindungsbezirken im Uteruscytosol zu verdrängen. Diese scheinbare Widersprüchlichkeit findet ihre Erklärung in der HPLC-Analyse der Bindungsverteilung von [^{3}H]RU 38486 in Uteruscytosol über eine MonoQ-Anionenaustauscher-Säule. [^{3}H]RU 38486 bildet zwei stabile Komplexe, zum einen mit Serum-Albumin, das etwa 90% der eingesetzten Radioaktivität bindet, zum anderen mit den nativen Rezeptorformen, die in der Saccharose-Dichtegradienten-Analyse sich als 4 S- und 8 S-Rezeptorspecies darstellen.

Ob die verminderte Bindung salzaktivierter [^{3}H]RU 38486-Rezeptor-Komplexe an DNA-Cellulose die Ursache für die antigestagene Wirkung dieses synthetischen Steroids ist, bedarf weiterer Klärung.

Introduction

RU 38486 (structure see scheme 1) is the first synthetic steroid possessing a greater affinity for the progestin receptor in different animal target tissues than progesterone, but without exhibiting any agonist progestin activity (1–3). Moreover, at large doses this steroid is capable of antagonizing the effects of progesterone in vivo, a property that is manifested in the interruption of the luteal phase of the menstrual cycle and of early pregnancy in women.

There are some indications that the potent antagonist effect of RU 38486 is related to the essential biochemical steps following progestin receptor occupancy which lead to the biological response i. e.



I II III

Scheme 1. Chemical structure and chemical nomenclature of
I RU 38486: 17β-hydroxy-21-methyl-11β(4-dimethylaminophenyl)-19-nor-4,9 pregnadiene-20-yne-3-one
II Org 2058: 16α-ethyl-19-nor-21-hydroxy-4-pregnene-3,20-dione
III R 5020, promegestone: 17α, 21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione

activation of the cytosolic progestin receptor and the nuclear translocation. Like other antihormones (e. g. some antiglucocorticoids which have been described as effective in vitro) (4–8) a possible mechanism of the antifertility effect of RU 38486 could be in forming a less stable complex with the cytosolic receptor, which is unable to translocate properly into the nucleus and trigger a progesterone response.

In view of the hypothesis described above, the following investigation was undertaken. The aim was to present a comparison of biochemical and physical properties of the complexes which the human uterine progestin receptor forms with the well-established progestins progesterone, R 5020 and Org 2058 on the one hand and with RU 38486 (structure and nomenclature see scheme 1) on the other hand.

Moreover, since binding to polyanions has been taken as a test for activation, the binding of the "salt-activated" RU 38486- and R 5020-progestin receptor complexes to DNA-cellulose was compared.

Materials and Methods

Steroids and reagents

[^{3}H]R 5020 (specific activity 3219 GBq/mmol), [^{3}H]dihydrotestosterone (specific activity 1872 GBq/mmol), [^{3}H]aldosterone (specific activity 2960 GBq/mmol) and unlabeled R 5020 were obtained from New England Nuclear (Boston, USA). [^{3}H]Cortisol (specific activity 1739 GBq/mmol), [^{3}H]Org 2058 (specific activity 1554 GBq/mmol), [^{3}H]dexamethasone (specific activity 3034 GBq/mmol) and unlabeled Org 2058 were purchased from the Radiochemical Centre (Amersham UK). Radioinert and tritiated RU 38486 (specific activity 1850 GBq/mmol) were a generous gift from Roussel-Uclaf Laboratories. All these steroids were kept in benzene-ethanol (85/15, vol/vol) at 4 °C and checked regularly for purity by thin-layer chromatography.

Nonradioactive steroids for competition experiments were obtained from Sigma Chemical Company (St. Louis, USA) and not further purified. All solvents used were obtained from Merck (Darmstadt, FRG) and were Uvasol grade.

Preparation of tissue samples

Human uteri were obtained after hysterectomy on the following indications: myomata uteri and prolapsus uteri. Immediately after hysterectomy an adequate sample was sent for histological examination and the rest was washed in ice-cold saline to remove mucous and blood. Then the tissue specimens were snap frozen in liquid nitrogen and stored at −80 °C until use.

For cytosol preparation, uterine tissues were minced and homogenized with an Ultra Turrax at 0–4 °C in ice-cold buffer consisting of 10 mmol/l KH_2PO_4, 10 mmol/l K_2HPO_4, 1.5 mmol/l EDTA, 3 mmol/l NaN_3, glycerol, volume fraction 0.1, pH 7.5. The homogenates were then centrifuged at 105000 *g* at 4 °C for 30 min. The supernatant was taken as cytosol. Rat liver and kidney cytosols from adrenalectomized female Wistar rats (150–200 g) were prepared in the same way as the uterine cytosol.

For HPLC (high performance liquid chromatography) cytosol was prepared in ice-cold Tris/HCl-EDTA-buffer (20 mmol/l Tris/HCl, 1.5 mmol/l EDTA, pH 7.5).

Rate of association of the [^{3}H]RU 38486 uterine receptor complex

Aliquots (0.1 ml) of uterine cytosol were incubated with 0.1 ml aqueous solutions of [^{3}H]RU 38486 (final concentration 8 nmol/l) for various time intervals (as indicated in fig. 1) at 4 °C (total binding). Nonspecific binding was determined using [^{3}H]RU 38486 together with a 200-fold molar excess of unlabeled RU 38486 or R 5020 under identical incubation conditions. All incubations were carried out in triplicate. At the time intervals indicated, the unbound steroid was removed by the addition of 0.5 ml aliquots dextran-coated charcoal suspension (5 g/l Norit A, 0.5 g/l Dextran T400 in 10 mmol/l K_2HPO_4, 10 mmol/l KH_2PO_4, 1.5 mmol/l EDTA, 3 mmol/l NaN_3 and glycerol, volume fraction 0.1, pH 7.5). The tubes were incubated for 10 min at 4 °C, followed by 10 min centrifugation at 1500 *g*. Supernatant (0.5 ml) was placed in scintillation vials, followed by the addition of 10 ml scintillation cocktail, then counted for radioactivity.

Time-dependent dissociation of the [^{3}H]RU 38486 uterine receptor complex in the presence of dextran-coated charcoal suspension

Aliquots (0.1 ml) of human uterine cytosol were incubated overnight at 4 °C with 0.1 ml aqueous solutions of [^{3}H]RU 38486 (final concentration 8 nmol/l) ± 200-fold excess of unlabeled RU 38486 or R 5020. After incubation for 16 h at 4 °C, 0.5 ml of dextran-coated charcoal were added and the tubes were incubated under gentle shaking at 4 °C for the following time intervals: t = 5, 15, 30, 45, 60, 90 min and 2, 4, 7 h. This was followed by centrifugation and determination of protein-bound radioactivity as described above.

Titration of specific steroid binding sites

The binding constants and receptor capacities were determined by multipoint titration using *Scatchard* analysis of the data (9). The crude cytosol in phosphate/EDTA/NaN_3/glycerol-buffer, pH 7.5 was incubated for 16 h at 0–4°C with various concentrations of ^{3}H-labeled R 5020 and RU 38486 over the range which should saturate the receptors (0.5–8 nmol/l). Additional cytosols were incubated with tritiated ligands in the presence of a 200-fold excess of unlabeled ligands, in order to determine the extent of nonspecific binding. Unbound ligand was removed from receptor-associated ligand by using dextran-coated charcoal.

Specificity of the receptor binding

The tubes for competition assays were prepared as follows: to each tube 50 µl of ^{3}H-labeled steroids (in phosphate/EDTA/NaN_3/glycerol-buffer, pH 7.5) were pipetted to give a final concentration of 32 nmol/l. Then aliquots of 50 µl, containing the various competitors (in phosphate/EDTA/NaN_3/glycerol-buffer, pH 7.5) at six different concentrations (10^{-10} – 10^{-5} mol/l) were added. Finally, to each tube 100 µl of cytosol were added, and after gentle shaking the tubes were incubated overnight at 4°C. The reactions were terminated by the addition of 0.5 ml dextran-coated charcoal. After 10 min incubation under gentle shaking the tubes were centrifuged for 10 min at 1500 *g*. Aliquots (0.5 ml) of the supernatant were transferred to scintillation vials, and after addition of 10 ml scintillation cocktail they were counted for radioactivity. All determinations were carried out in triplicate. The min^{-1}-values were expressed as % binding, taking the values for the tubes containing only ^{3}H-ligands as 100%.

Serum binding

A pool of human pregnancy serum was stripped of endogeneous steroids with dextran-coated charcoal. Aliquots of this serum pool in a dilution of 1:10 with phosphate/EDTA/NaN_3/glycerol-buffer, pH 7.5 were incubated in triplicate for 60 min at room temperature with 8 nmol/l [^{3}H]cortisol (for determination of cortisol binding globulin) or [^{3}H]dihydrotestosterone (for characterization of sex hormone binding globulin) in the presence of increasing amounts of the competitors listed in tab. 1. After incubation the unbound steroid was removed with dextran-coated charcoal. Aliquots of the supernatant (0.5 ml) were then counted for radioactivity.

High performance liquid chromatography (HPLC)

Aliquots (2.5 ml) of human uterine cytosol (10–15 g/l) were incubated with [^{3}H]RU 38486, [^{3}H]Org 2058 or [^{3}H]21-dehydro-Org 2058, respectively, to yield a final concentration of 32 nmol/l (total binding), or together with a 200-fold molar excess of unlabeled steroids (unspecific binding) for 16 hours at 0–4°C.

For details of the synthesis of [^{3}H]16α-ethyl-19-nor-4-pregnene-3,20-dione-21-al ([^{3}H]21-dehydro-Org 2058), see *Heubner* et al. (10) and *Manz* et al. (11).

In another preparation 10 mmol/l sodium molybdate was added to the homogenization buffer, and cytosol was incubated as described above. Aliquots (1 ml) of each incubation mixture were heat activated for 30 min at 37°C. Aliquots (500 µl) were filtered through a Millex GV 0.22 µm filter unit (Millipore, Neu-Isenburg, F.R.G.), then injected onto the column.

Chromatography was carried out on the Pharmacia HPLC system (FPLC, Pharmacia, Uppsala, Sweden) fitted with a prepacked Mono Q column (high efficiency anion exchanger based on monodisperse 10 µm spheres, Pharmacia, Uppsala, Sweden). The column was equilibrated in Tris/HCl-EDTA-buffer at 20°C and eluted at a flow rate of 2 ml/min with a NaCl gradient to 1 mol/l. Protein was monitored at 280 nm. Fractions (500 µl) were collected and radioactivity was measured by liquid scintillation counting (Ready-Solv HP, Beckman, Munich, F.R.G.).

Sucrose density gradient analysis

In 0.5 ml aliquots of all incubation mixtures, described in the paragraph on HPLC, unbound steroid was removed with dextran-coated charcoal. Aliquots of 0.2 ml were then layered on top of 5–30% linear sucrose gradients. The gradients were centrifuged for 2.5 h at 4°C at 404000 *g* in a vertical rotor (Beckman VTi65). The gradients were collected from the bottom in 40 fractions. The gradients were standardized with FITC-labeled bovine serum albumin (M_r = 67000) and FITC-labeled IgG (M_r = 150000) (Behring Werke, Marburg, F.R.G.).

Assay of receptor binding to DNA-cellulose

Cytosol fractions were incubated with 32 nmol/l ^{3}H-labelled steroid alone or with a 200-fold molar excess of the respective unlabeled steroid for 16 h. Activation of steroid receptor complex was accomplished by adding a solution of 2 mol/l NaCl in phosphate/EDTA/NaN_3/glycerol-buffer, pH 7.5 to a final concentration of 0.4 mol/l NaCl. This mixture was kept for 40 min at 4°C. Now 100 µl activated cytosols were diluted with 700 µl buffer and were incubated for 20 min at 4°C with a DNA-cellulose pellet (Sigma Munich, F.R.G.) from 200 µl of a 25% slurry of DNA-cellulose. After centrifugation for 1 min at 2000 *g* the samples were washed three times in cold buffer and the final pellet was taken for determination of radioactivity. All determinations were carried out in triplicate.

Protein determination

The protein concentrations were determined with the BioRad protein assay kit (BioRad, Richmond, USA) using bovine serum albumin as a standard.

Results

Time studies of [^{3}H]RU 38486 uptake show a rapid increase in the amount of specific binding during the first 4 to 5 hours (fig. 1). After saturation, the amount of specifically bound [^{3}H]RU 38486 remains almost constant up to 20 hours at 0–4°C. In contrast to authentic RU 38486, a 200-fold molar excess of nonradioactive R 5020 did not reduce total [^{3}H]RU 38486 binding (especially during the first 4 hours).

The dissociation of [^{3}H]RU 38486 from the uterine progestin receptor, measured by the displacement of labeled steroid with dextran-coated charcoal treatment at 0–4°C at various times, showed a biphasic or two-component first order dissociation curve (fig. 2). The [^{3}H]RU 38486 dissociation rate constant of the first, slower dissociating component, k_{-1}, was linear with an k_{-1} of $3.61 \cdot 10^{-5}$ s^{-1} (tab. 1) or only 13% of k_{-2}. The dissociation rate constant of the second, more rapid component, k_{-2}, was $27.5 \cdot 10^{-5}$ s^{-1}. The biphasic dissociation curve was corrected for "receptor" lability or inactivation by measuring the specifically bound [^{3}H]RU 38486 in the absence of nonradioactive steroid during the 0–4°C incubation.

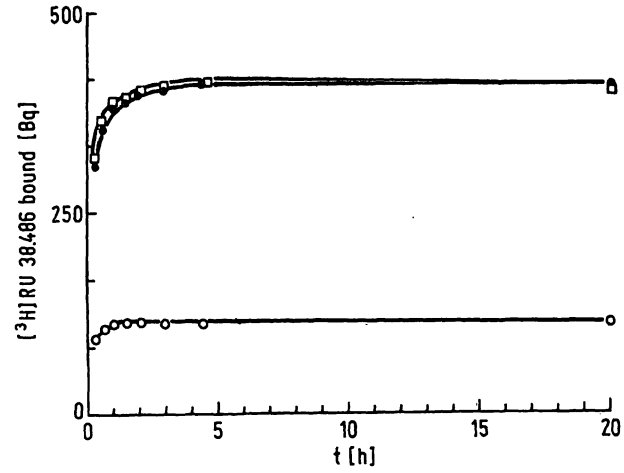


Fig. 1. Time course of association of [^{3}H]RU 38486 with the human uterine progestin binding protein.
● Total binding, ○ nonspecific binding ([^{3}H]RU 38486 plus a 200-fold excess of RU 38486), □ nonspecific binding ([^{3}H]RU 38486 plus a 200-fold excess of R 5020).

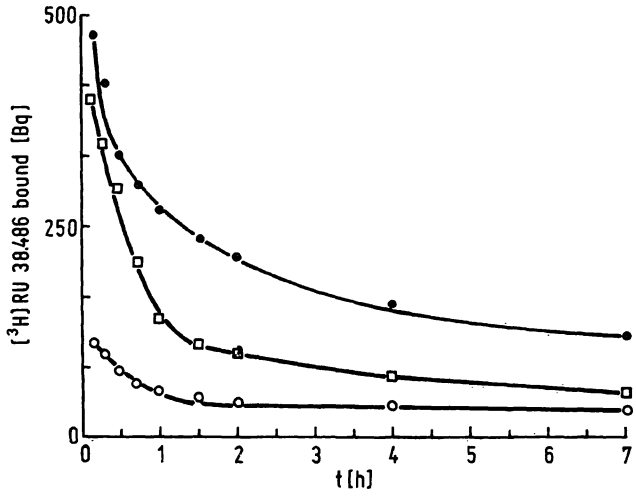


Fig. 2. Time course of dissociation of [^{3}H]RU 38486-progestin binding protein complex of human uterus in the presence of dextran-coated charcoal:
● total binding, ○ nonspecific binding ([^{3}H]RU 38486 plus a 200-fold excess of RU 38486), □ nonspecific binding ([^{3}H]RU 38486 plus a 200-fold excess of R 5020).

Tab. 1. Kinetic parameters of binding of [^{3}H]labeled steroids to human uterine progestin receptor.

Ligand	k_{-1} (s^{-1})	Half-life (min)	k_{-2} (s^{-1})	Half-life (min)
[^{3}H]R 5020	$3.6 \cdot 10^{-5}$	320	–	–
[^{3}H]Progesterone	$21.3 \cdot 10^{-5}$	54	–	–
[^{3}H]Cyproterone acetate	$17.8 \cdot 10^{-5}$	65	–	–
[^{3}H]RU 38486	$3.61 \cdot 10^{-5}$	321	$27.5 \cdot 10^{-5}$	42

There was no binding loss after a 4 h incubation, but 6% inactivation after 7 h.

Moreover, it was the purpose of this study to compare the kinetic properties of binding of [^{3}H]RU 38486 with those of radiolabeled R 5020, progesterone and cyproterone acetate, typical, well-characterized progestins. All these three steroids show, in contrast to RU 38486, single first order dissociation curves (fig. 3). [^{3}H]R 5020 forms more stable complexes with uterine progestin receptor than radiolabeled progesterone or cyproterone acetate, which confirms earlier observations (12, 13).

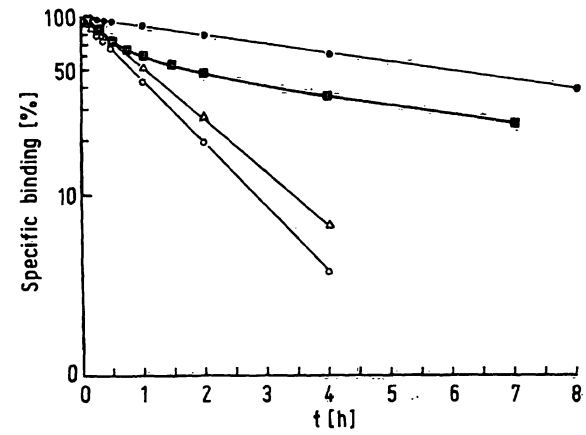


Fig. 3. Determination of the half-life of the [^{3}H]RU 38486 (□—□), [^{3}H]R 5020 (●—●), [^{3}H]progesterone (○—○) and [^{3}H]cyproterone acetate (△—△) binding protein complexes of human uterine cytosol.

Figure 4 shows competition between various steroids and labeled [^{3}H]R 5020 and [^{3}H]RU 38486 for binding to crude uterine cytosol.

As anticipated, Org2058, R 5020, progesterone and RU 38486 compete well with [^{3}H]R 5020 for the progestin binding sites of uterine cytosol. The order of potency was: Org2058 > R 5020 > progesterone > RU 38486. Some competition can also be seen with dexamethasone and cortisol. Most interesting, however, is the fact that neither progestins, androgens, oestrogens nor glucocorticoids have any effect on [^{3}H]RU 38486 binding.

Results concerning the specificity of binding to progestin, glucocorticoid, oestrogen and mineralocorticoid receptors in cytosol of typical target tissues as well as to cortisol binding globulin and sex hormone binding globulin in diluted pregnant serum have been summarized in table 2. They are expressed as relative binding affinities, i.e., as the relative concentration of test compound and radioinert ligand required to displace 50% of bound radioligand from its binding sites.

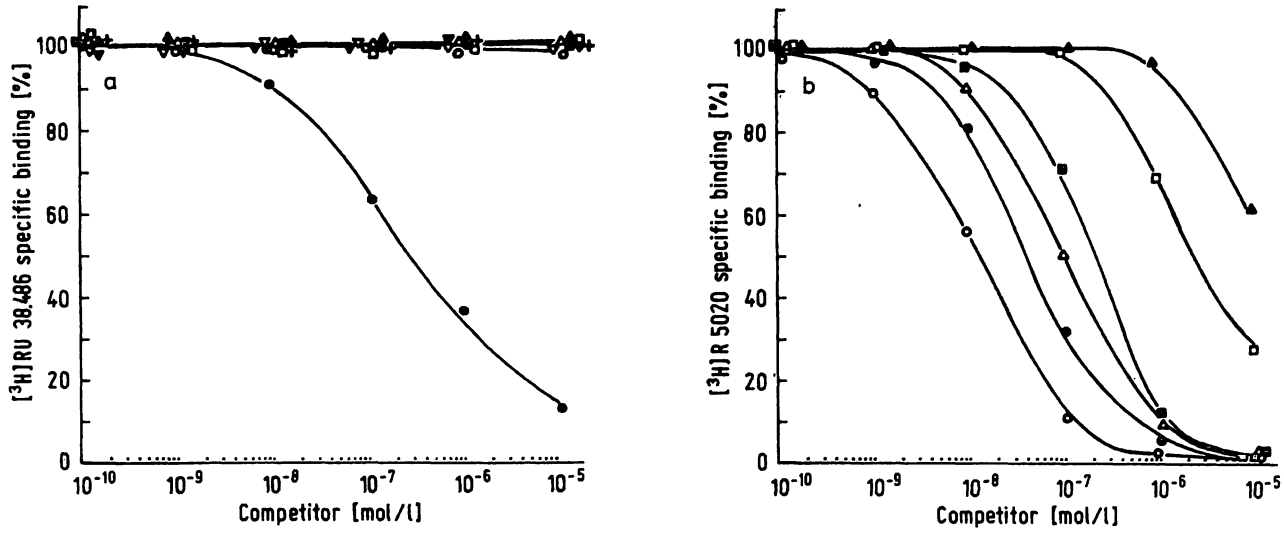


Fig. 4. Specificity of the binding of [^{3}H]RU 38486 and [^{3}H]R 5020 to the cytoplasmic progestin receptor in human uterus.

a) [^{3}H]RU 38486
- ○ RU 38486
- ○ R 5020
- △ Org 2058
- □ Progesterone
- ▲ Cortisol
- ▽ Dexamethasone
- ▼ Oestradiol
- □ Testosterone
- \+ Nortestosterone

b) [^{3}H]R 5020
- ● R 5020
- ○ Org 2058
- △ Progesterone
- ■ RU 38486
- □ Dexamethasone
- ▲ Cortisol

Tab. 2. Relative binding affinites of various steroids for binding to human uterine progestin ([^{3}H]R 5020 or [^{3}H]RU 38486) and oestrogen ([^{3}H]oestradiol) receptors, rat liver glucocorticoid receptor ([^{3}H]dexamethasone), rat kidney aldosterone receptor ([^{3}H]aldosterone) as well as human corticosteroid binding globulin and sex hormone binding globulin. n.d. = not determined

Ligand	Relative binding affinity values (%). Progesterone receptor [^{3}H]R 5020	Progesterone receptor [^{3}H] RU 38486	Gluco-corticoid receptor [^{3}H]Dexa-methasone	Oestradiol receptor [^{3}H] Oestradiol	Mineralo-corticoid receptor [^{3}H] Aldosterone	Cortisol binding globuline [^{3}H]Cortisol	Sex hormone binding globuline [^{3}H]dihydro-testosterone
RU 38486	25	100	45	<0.01	70	<0.01	<0.01
Progesterone	40	<0.01	0.2	<0.01	10	13	<0.01
R 5020	100	<0.01	7	<0.01	53	<0.1	<0.01
Org 2058	350	<0.01	0.7	<0.01	27	<0.1	<0.01
Nortestosterone	95	<0.01	0.7	<0.01	n.d.	<0.1	8
Norethisterone	70	n.d.	<0.1	<0.01	10	0.9	50
Medroxyprogesterone acetate	115	n.d.	29	<0.01	160	<0.1	<0.1
Chlormadinone acetate	25	n.d.	n.d.	<0.01	n.d.	<0.1	<0.1
17α-Hydroxy-progesterone	0.8	n.d.	0.8	<0.01	40	40	<0.01
Dexamethasone	3	<0.01	100	<0.01	400	<0.01	<0.01
Cortisol	<1	<0.01	18	<0.01	70	100	<0.01
Oestradiol	0.8	n.d.	<0.01	100	<0.1	<0.01	7
Diethylstilb-estrol	<0.1	n.d.	<0.01	45	<0.1	<0.01	<0.01
Testosterone	0.9	<0.01	<0.01	<0.1	17.5	10	n.d.
Dihydro-testosterone	0.9	n.d.	<0.01	<0.1	n.d.	0.3	100
Aldosterone	0.2	n.d.	1.25	<0.01	100	6	<0.01

RU 38486 does not compete with [^{3}H]dihydrotestosterone and [^{3}H]cortisol for serum binding. Moreover, this compound has no effect on [^{3}H]oestradiol binding, but shows a definite influence on [^{3}H]R 5020, [^{3}H]dexamethasone and [^{3}H]aldosterone binding.

Figure 5 compares the titration of specific binding sites in crude cytosol of human uterine tissue with [^{3}H]R 5020 and [^{3}H]RU 38486. Increasing concentrations of labeled steroids were incubated with cytosol either with or without a 200-fold molar excess of radioinert R 5020 or RU 38486 for 16 hours at 0–4 °C, then assayed by the dextran-coated charcoal technique to determine specific binding. *Scatchard* analysis of these data gave only a linear plot for [^{3}H]R 5020. For this particular steroid, the specific R 5020 binding protein displayed comparable apparent dissociation constants (K_d) under two different dextran-coated charcoal incubation conditions: 2.08 · 10^{-9} mol/l with 4 h dextran-coated charcoal treatment, and 2.6 · 10^{-9} mol/l with 5 min dextran-coated charcoal stripping. The binding capacities were calculated as 400 fmol/mg protein and 645 fmol/mg protein, respectively. In contrast to these results, [^{3}H]RU 38486 was unable to saturate specific binding sites of crude cytosol preparations up to a concentration of 8 nmol/l. This result shows a striking similarity to those obtained by sucrose gradient centrifugation and FPLC-chromatography as described below.

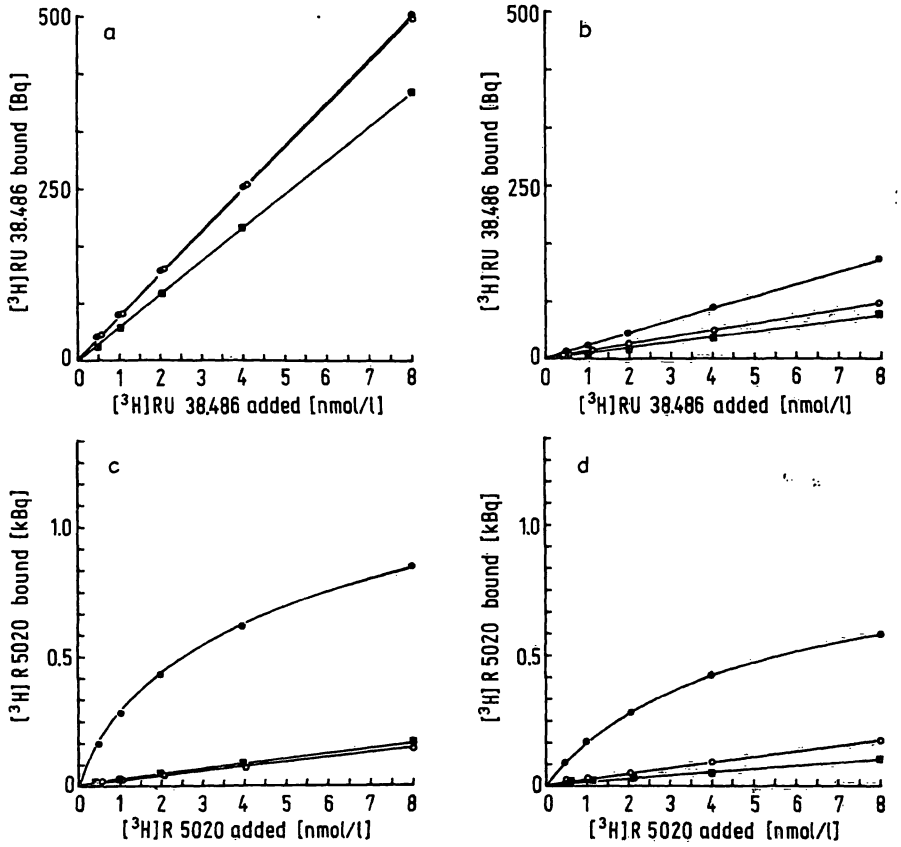


Fig. 5. Titration of [^{3}H]RU 38486 or [^{3}H]R 5020 binding sites in cytosol from human uterus. (For details see "Material and Methods").

a) [^{3}H]RU 38486
5 min dextran-coated charcoal treatment
● [^{3}H]RU 38486
○ + 200-fold R 5020
■ + 200-fold RU 38486

b) [^{3}H]RU 38486
4 h dextran-coated charcoal treatment
● [^{3}H]RU 38486
○ + 200-fold R 5020
■ + 200-fold RU 38486

c) [^{3}H]R 5020
5 min dextran-coated charcoal treatment
● [^{3}H]R 5020
○ + 200-fold R 5020
■ + 200-fold RU 38486

d) [^{3}H]R 5020
4 h dextran-coated charcoal treatment
● [^{3}H]R 5020
○ + 200-fold R 5020
■ + 200-fold RU 38486

Centrifugation of [^{3}H]Org2058 labeled human myometrial cytosol through a 5–30% sucrose gradient revealed two binding components which sedimented in the 4 S and 8 S region (fig. 6). After heat activation in the absence of molybdate only the component in the 4 S region was detected. When the cytosol preparation was labeled in vitro with [^{3}H]21-dehydro-Org2058 (a highly potent affinity label, which labels covalently the steroid binding site of the progestin receptor by formation of intermediate stable azomethine protein complexes (10, 11), and investigated on a density gradient, two [^{3}H]labelled steroid macromolecule complexes with sedimentation rates of about 4 S and 8 S (in the cold and in the presence of molybdate) were observed (fig. 7). Nonradioactive Org 2058 displaced [^{3}H]21-dehydro-Org2058 only from the 8 S binding protein, not from the 4 S region.

Sucrose gradient centrifugation analysis of the [^{3}H]RU 38486-labeled cytosol of human uterus showed that the RU 38486 binding components migrated as two peaks sedimenting in the 4 S and 8 S regions (fig. 8). A 200-fold molar excess of nonradioactive RU 38486 or Org2058 reduced only the 8 S peak; competition in the 4 S region was only observed in the presence of RU 38486.

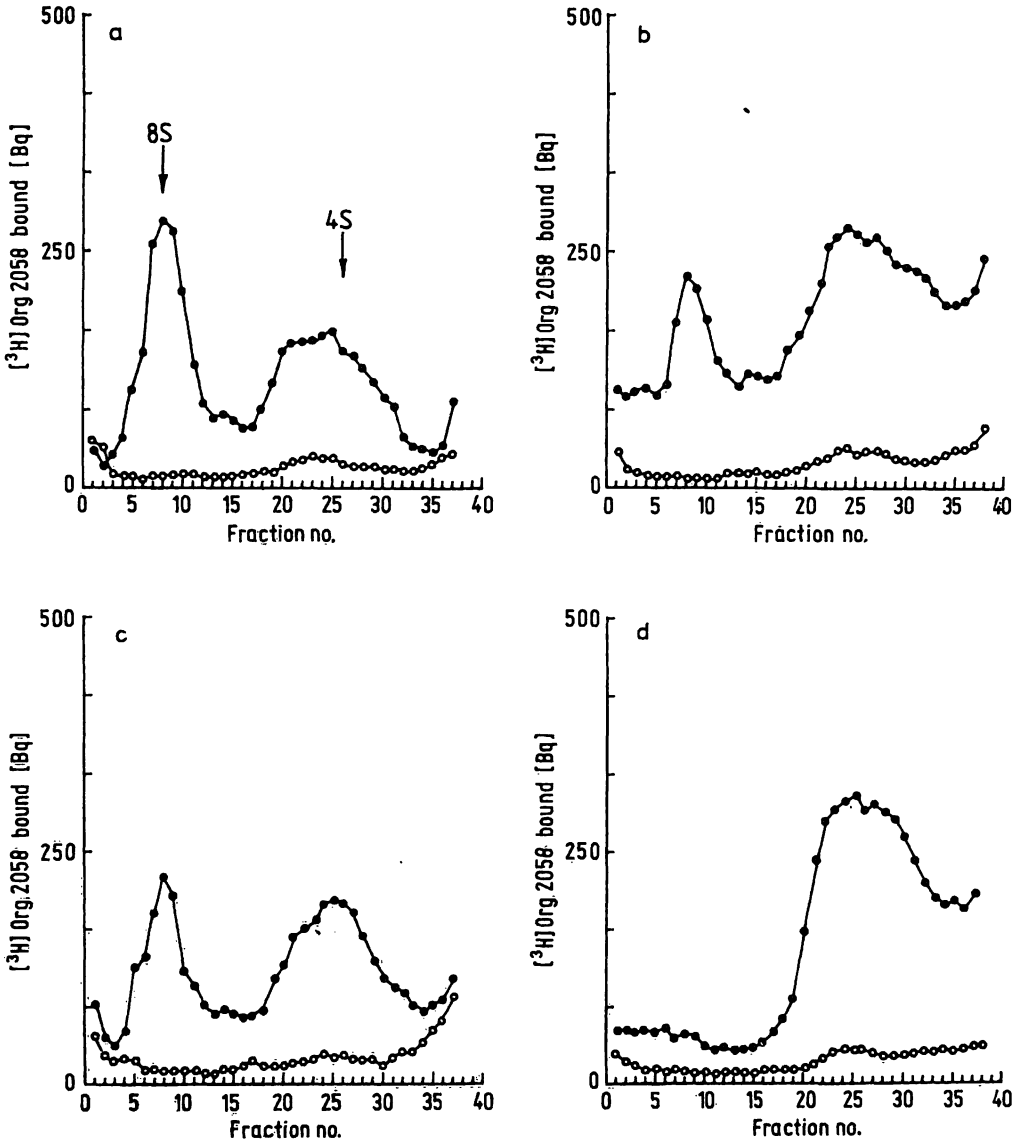


Fig. 6. Sucrose density gradient analysis of [^{3}H]Org 2058 binding protein complexes on 10–35% linear sucrose density gradients under low salt conditions with (a, b) and without (c, d) sodium molybdate at different incubation temperatures (a, c 4 °C; b, d 37 °C). Arrows indicate positions of 4 S and 8 S marker proteins.
●—● [^{3}H]Org 2058; ○—○ + 200 fold Org 2058.

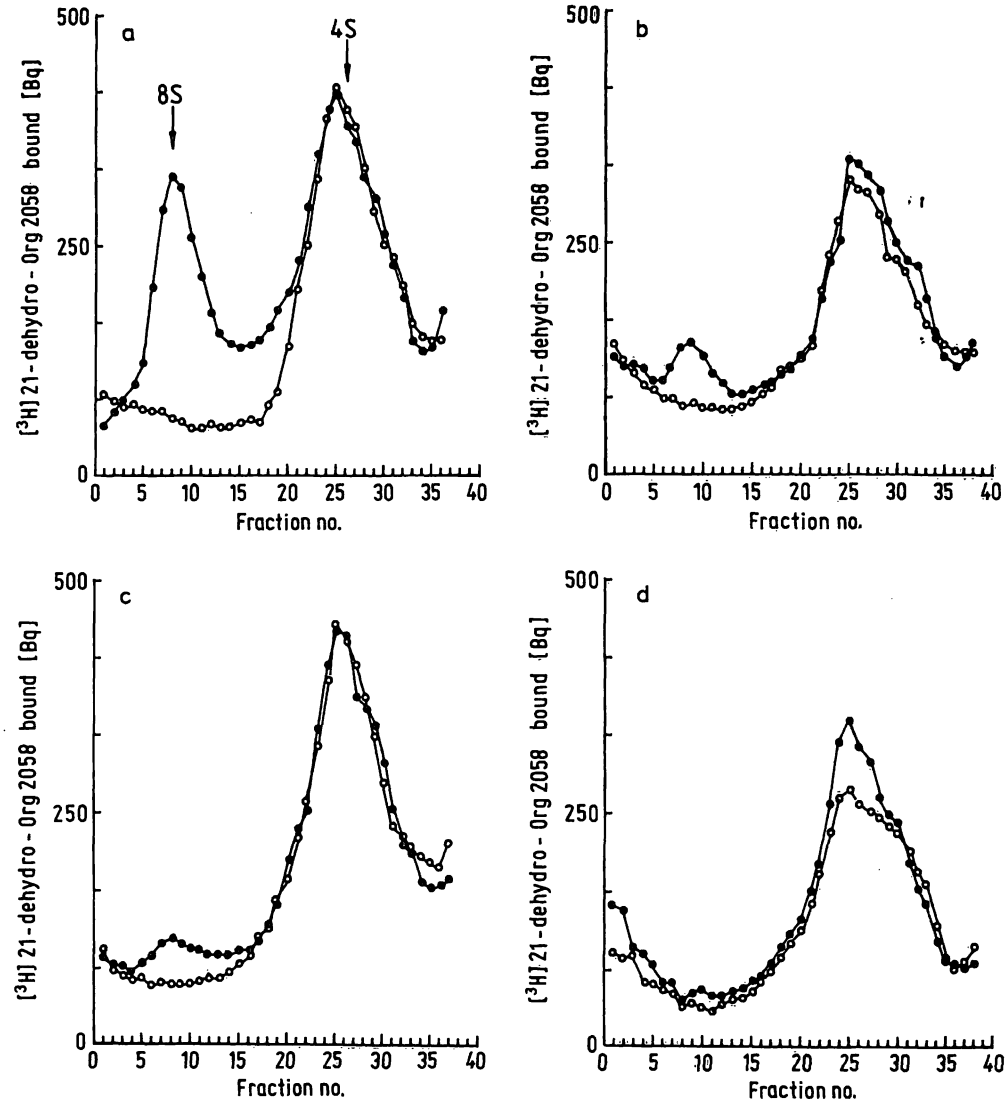


Fig. 7. Influence of incubation temperature (a, c 4 °C; b, d 37 °C) and sodium molybdate (a, b with; c, d without) on the sedimentation profiles of [^{3}H]21-dehydro Org 2058 binding components of human uterine cytosol. Arrows indicate positions of 4 S and 8 S marker proteins.
●—● [^{3}H]21-dehydro-Org 2058; ○—○ + 200 fold Org 2058.

The results in figure 9 and 10 illustrate HPLC of differently labeled uterine progestin receptors on a Mono Q anion exchange column. Free steroid was eluted with the washing buffer. Independent of the [^{3}H]labelled steroid used, the specifically labeled proteins eluted as peak A and B (approximately 0.075 and 0.13 mol/l NaCl). Peak B was not detectable after heat activation (20 min, 37 °C) and is probably identical with the 8 S binding protein in sucrose density gradient analysis (data will be published in detail elsewhere). The nonspecifically labeled protein (peak C) eluting at 0.22 mol/l NaCl was found in all preparations and showed identical behaviour to serum albumin (LC-partigen albumin immunodiffusion plates, Behring-Werke, Marburg, F.R.G., fig. 11). A comparison of [^{3}H]Org2058 and [^{3}H]RU 38486 labeled cytosol shows that the unspecifically bound radioactivity, eluting with albumin (peak C), is more than 10-fold higher for [^{3}H]RU 38486 and represents almost 90% of labeled protein. This radioactivity cannot be suppressed by a 200-fold molar excess of Org2058 but is reduced by about 30% with a 200-fold molar excess of RU 38486. In the case of [^{3}H]Org2058, however, unspecific binding, eluting with albumin, represents approximately 10% of bound radioactivity and the overall unspecific binding is less than 50% of bound radioactivity.

The important biological property of any steroid hormone receptor is its ability to bind DNA after transformation. Therefore, the DNA-binding ability of the uterine progestin receptor was tested. Cytosols

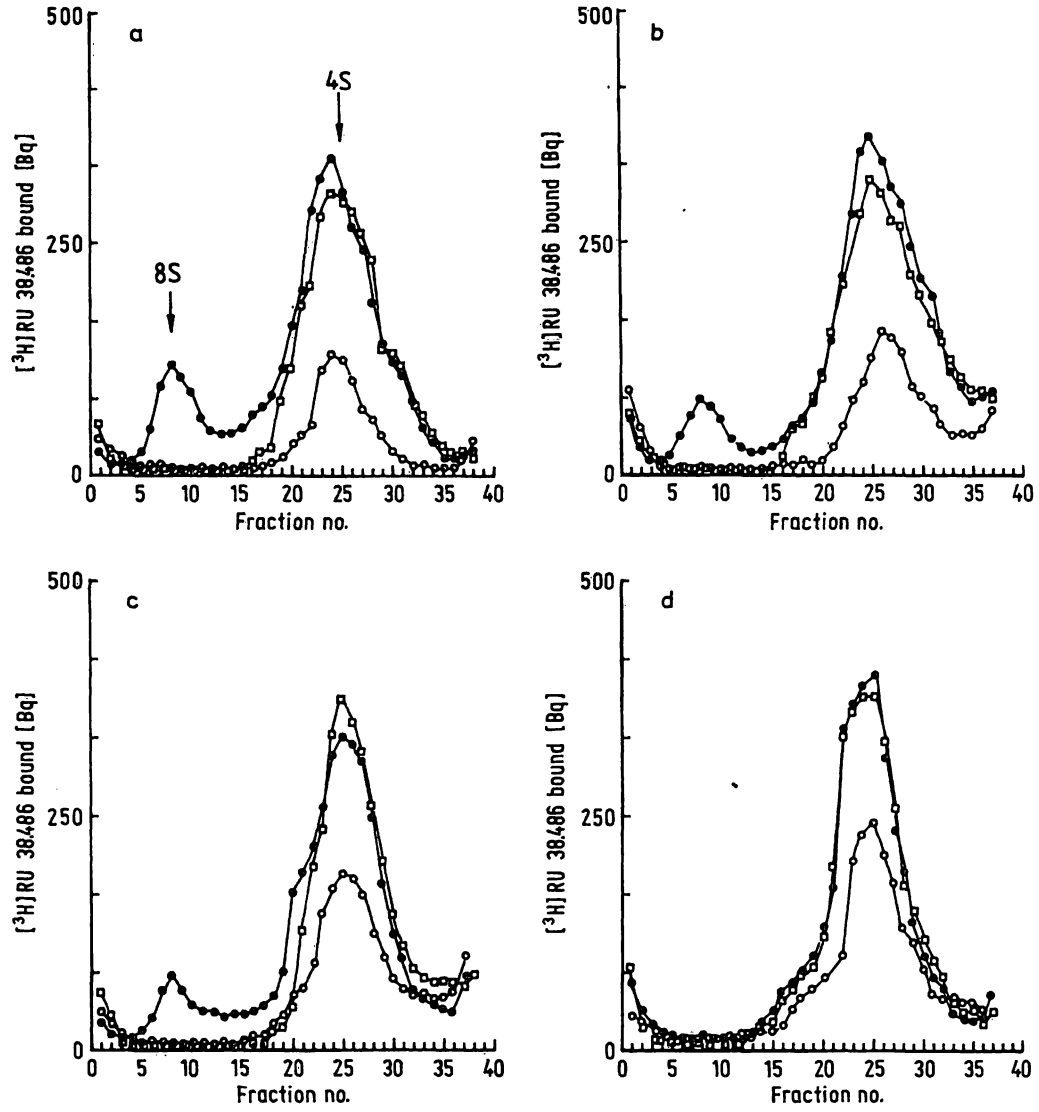


Fig. 8. Sedimentation profiles of [^{3}H]RU 38486 binding proteins in cytosol of human uterine tissue under conditions described under "Material and Methods". Arrows indicate positions of 4 S and 8 S marker proteins.
a, b with; c, d without sodium molybdate; a, c 4 °C; b, d 37 °C
●—● [^{3}H]RU 38486; ○—○ + 200-fold RU 38486; □—□ + 200-fold Org 2058.

were either labeled with 32 nmol/l of [^{3}H]R 5020 or [^{3}H]RU 38486 with or without a 200-fold molar excess of cold authentic steroids. Bound radioactivity and DNA-cellulose binding were then determined.

Table 3 shows that the complex of [^{3}H]RU 38486 with the progestin receptor is far less capable of binding to DNA than the complex of progestin receptor formed with [^{3}H]R 5020.

Tab. 3. Binding of steroid-progestin receptor complexes to DNA-cellulose.
The table compares the binding of nonactivated and salt-activated complexes of progestin receptor with the known progestins [^{3}H]R 5020 and [^{3}H]Org 2058 and complexes formed by [^{3}H]RU 38486 in human uterine cytosol. For experimental details see "Materials and Methods".

	R 5020/Org 2058			RU 38486		
	Bq/100 µl cytosol	Binding to DNA-cellulose non-activated	salt-activated	Bq/100 µl cytosol	Binding to DNA-cellulose non-activated	salt-activated
Total binding	1670/1330	67/33	183/153	1170	38	75
+ 200-fold R 5020/or Org 2058	250/ 183	42/11	27/ 12	1250	25	25
+ 200-fold RU 38486	233/ 175	25/10	27/ 10	1020	27	27

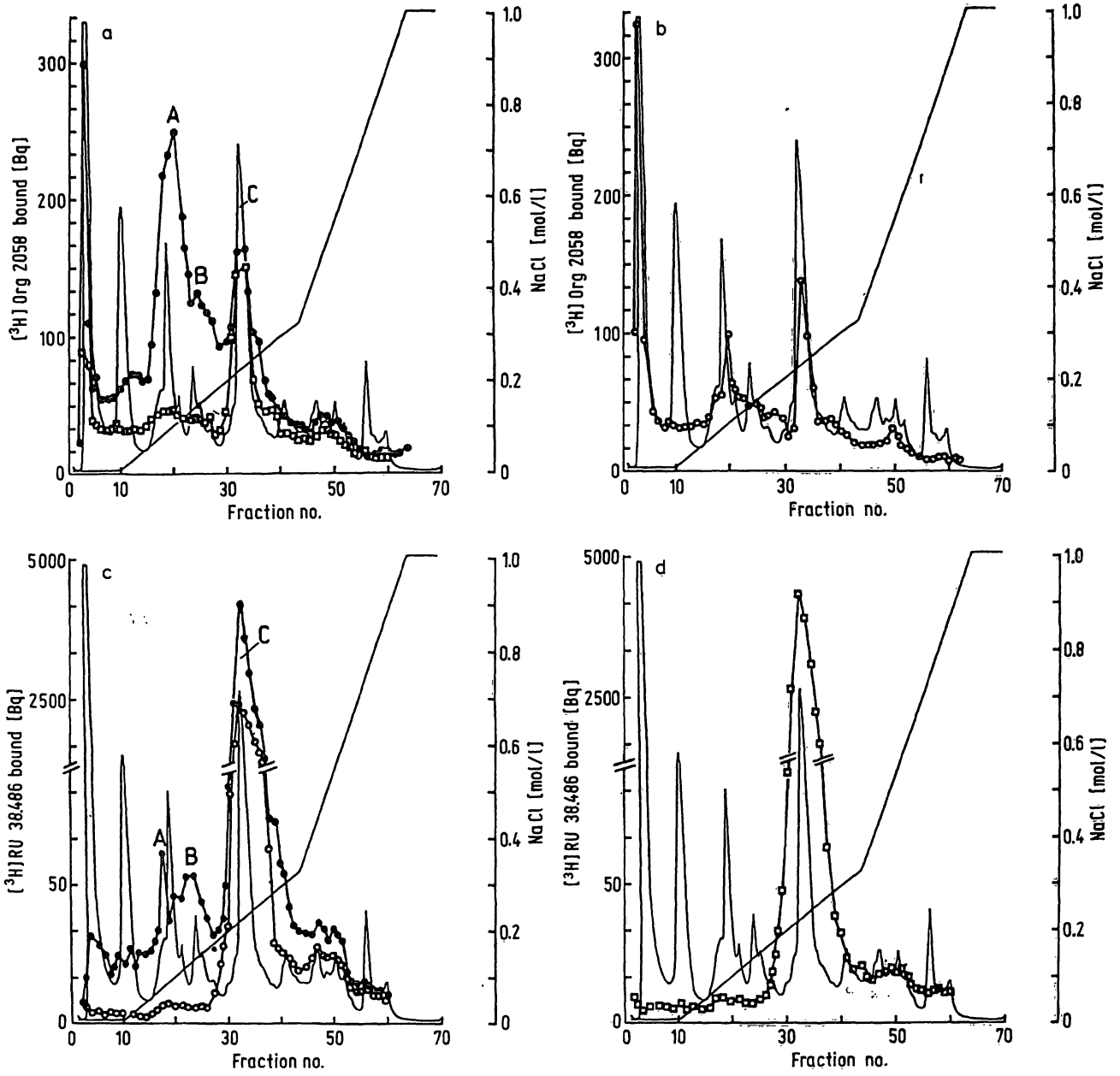


Fig. 9. Analysis of human uterine progestin receptor by HPLC on a MonoQ anion exchange column. High speed supernatant which had been exposed to (a, b) [^{3}H]Org 2058 or (c, d) [^{3}H]RU 38486 (32 nmol/l) with (□) or without (●) nonradioactive competitor was injected (500 µl) onto the column. Elution was performed as described under "Material and Methods". Absorbance determinations at 280 nm and NaCl-gradients are shown by drawn lines.
a, b [^{3}H]Org 2058: ●—● [^{3}H]Org 2058; □—□ + 200-fold Org 2058; ○—○ + 200-fold RU 38486.
c, d [^{3}H]RU 38486; ●—● [^{3}H]RU 38486; ○—○ + 200-fold RU 38486, □—□ + 200-fold Org 2058

Discussion

These observations suggest that the highly potent antifertilizing synthetic steroid RU 38486 (1–3) forms at least two stable complexes with crude uterine cytosol, one apparently with albumin (peak C), and the other with the native progestin receptor forms (peaks A and B). This was clearly demonstrated by HPLC-chromatography analysis of [^{3}H]RU 38486 binding in uterine cytosol preparations.

The binding of [^{3}H]RU 38486 to peak C, which has a mobility identical to serum albumin, is of high capacity. More than 90% of the added labeled steroid was bound in this fraction, but only partial competition for binding was shown by unlabeled RU 38486, and as anticipated, none was shown by Org 2058 which is normally characterized by a lack of albumin binding.

On the other hand unlabeled RU 38486 competed in the same order of magnitude as progesterone for the [^{3}H]R 5020 binding site of human uterine progestin receptor, indicating that this compound is a potent progestin with respect to receptor binding.

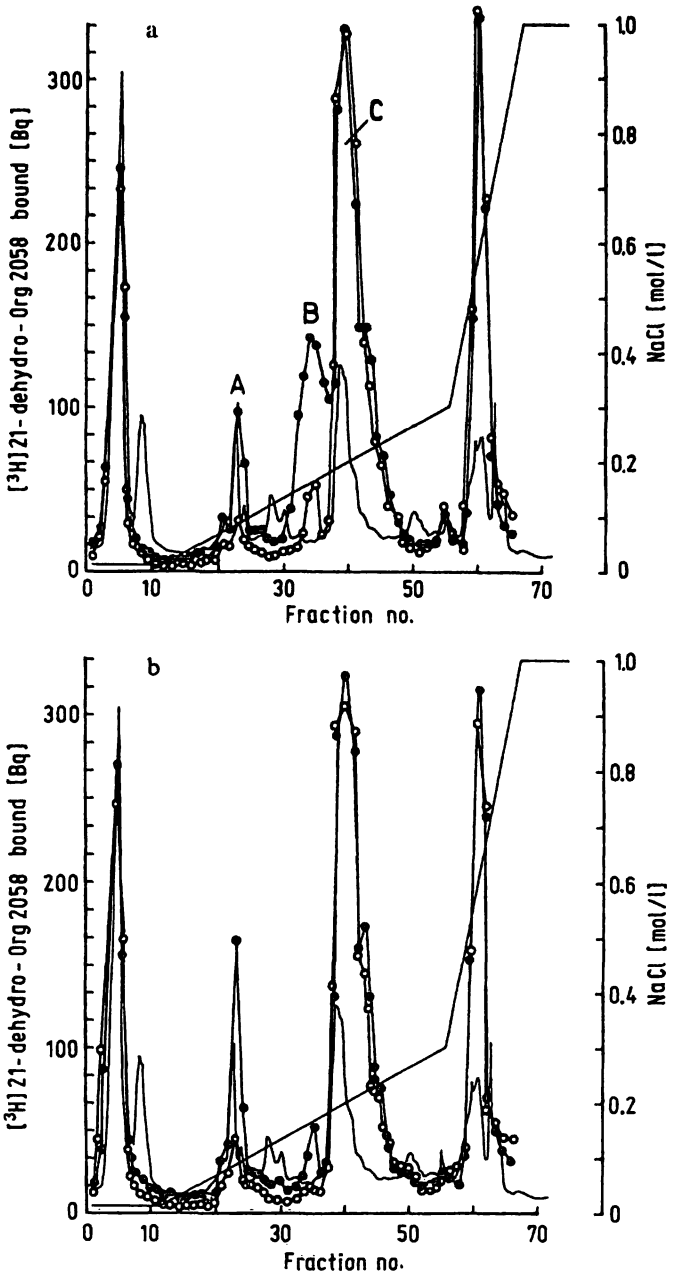


Fig. 10. Analysis of human uterine progestin receptor by HPLC on a MonoQ anion exchange column. High speed supernatant which had been exposed to [^{3}H]21-dehydro-Org 2058 (32 nmol/l) with (○) or without (●) nonradioactive competitor was injected (500 µl) onto the column. Elution was performed as described in detail under "Material and Methods". Absorbance determination at 280 nm and NaCl-gradients are shown by drawn lines. a) with b) without molybdate
●—● [^{3}H]21-dehydro-Org 2058, ○—○ + 200-fold Org 2058

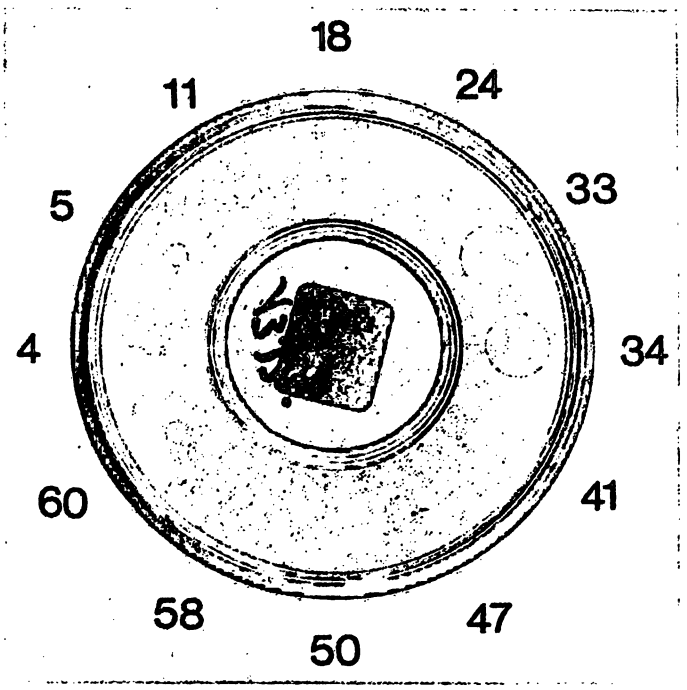


Fig. 11. Analysis of various fraction numbers of HPLC elution scheme (fig. 9 and 10) according to human serum albumin contamination using LC-partigen albumin immunodiffusion plates (Behring Werke, Marburg, F.R.G.). Only fraction no. 33 and 34 showed specific albumin precipitation.

It seems obvious that the dimethylaminophenyl side group on carbon 11 is responsible for the high capacity binding to serum albumin, because a free electron pair can interact with free carboxy groups of aspartic and glutamic acids of albumin, which act as proton donors.

The question of whether RU 38486 exerts its antihormonal action via the well established receptor interactions or via modified actions cannot be answered at the moment. Different modes of action, such as interaction with carboxyl groups within or apart the steroid binding side, changing the protein conformation or sealing the binding site, could prohibit either binding of naturally occurring steroids or translation of the steroid receptor complexes into the nucleus.

The reduced binding of salt-activated RU 38486-receptor complexes to DNA-cellulose is not nessarily due to reduced activation but might also reflect the distribution of binding equilibria between albumin and receptor, thus reducing the availability of added RU 38486 for the progestin receptor. On the other hand, it was demonstrated for a broad spectrum of antiglucocorticoids that their antihormonal action is due to the formation of labile cytoplasmic steroid receptor complexes with a decreased or absent capacity for translocation (5,14–18). This phenomenon is not general but tissue-specific. Carboxamide derivatives of dexamethasone, for example, are antiglucocorticoids in hepatoma cells (19), but are potent agonists in human peripheral lymphocytes (20).

RU 38486 not only exerts pronounced antifertilising effects, it is also a potent antiglucocorticoid (21). But in contrast to other known antiglucocorticoids, like carboxamide derivatives of dexamethasone, RU 38486 forms stable complexes with the glucocorticoid receptor protein under low temperature conditions. These are destabilized only after heat activation, thus prohibiting an effective nuclear translocation (22).

To what extent an RU 38486-"albumin"-complex, as presented in this study, behaves like a receptor within the target cell is unknown; its ability to translocate into the nucleus and its possible nonspecific interaction with the receptor binding site on the level of chromatin, thus acting as an antihormone, is entirely speculative but under investigation.

Finally, the high binding capacity of RU 38486 to albumin could lead to an enormous intra- and extracellular accumulation of this steroid thus forming a pool of steroid, which cannot be metabolized but can be released slowly. A long lasting hormonal impulse of a single dose of RU 38486, as demonstrated in in-vivo experiments in rats, keeps the animal infertile for a long time.

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