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THE SUBCELLULAR DISTRIBUTION OF ENDOGENOUS PROGESTERONE IN PREGNANT RAT MYOMETRIUM

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SUMMARY

The concentration of endogenous progesterone in the nuclear, mitochondrial, microsomal and cytosolic fractions of rat myometrium was determined during the last trimester of pregnancy and up to 20 h post partum. Progesterone was determined by a radioimmunoassay technique developed for this study.

The cytosolic concentration of progesterone in the myometrium was constant from the 14–16th days of pregnancy (9–11 pmol of progesterone/mg cytosolic protein) and began to decrease after the 16–17th day. The cytosolic concentration of progesterone became low on the 21st day of pregnancy (0·5–1·0 pmol/mg protein) and was practically the same on the 22nd day and 20 h post partum. The decrease in the concentration of endogenous progesterone in the myometrial cytosol was inversely correlated to foetal weight from the 18th to the 21st day of pregnancy.

The concentration of endogenous progesterone in the microsomal, mitochondrial and nuclear fractions of rat myometrium was lower than that in the cytosol on the 14th day of pregnancy. Thereafter the concentrations of endogenous progesterone in these particulate fractions approached that of the cytosol and the amounts in the microsomal fraction exceeded the cytosolic concentration after the 18th day. Mitochondrial progesterone reached cytosolic concentrations on the 18th day of pregnancy and nuclear on the 22nd day. The concentration of progesterone decreased steeply in all fractions during the last 4 days of pregnancy and was low in every fraction on the 21st day (one day before parturition).

The present results show that the ratios of endogenous progesterone concentrations in the 4 subcellular fractions studied do not vary much on a given day of pregnancy but these ratios change gradually during the last trimester of pregnancy.

INTRODUCTION

In the pregnant rat plasma progesterone concentrations fall markedly one day before parturition [1, 2]. If the animals are hypophysectomized on day 12 of pregnancy this decline in plasma progesterone concentration is not as pronounced as in intact rats and pregnancy is prolonged [1]. There exists a close relationship between plasma progesterone concentrations and the maintenance of pregnancy in the rat. Progesterone is essential for the maintenance of pregnancy but the principal progesterone metabolites in myometrium, 5α-pregnane-3,20-dione and 3α-hydroxy-5α-pregnan-20-one [3, 4] are ineffective in this respect [5].

The concentration of progesterone in the whole uterus of pregnant rats decreases in parallel with the plasma concentration [2]. The subcellular distribution and metabolism of injected [^{14}C]-progesterone in pregnant rat myometrium has been studied [3]. However, the relationship between endogenous cytoplasmic progesterone concentrations and the cytoplasmic progesterone binding-proteins of high affinity [6–9] has not been investigated in the pregnant rat.

The aim of the present study was to determine the concentration of endogenous progesterone in the subcellular fractions of rat myometrium during the last trimester of pregnancy. Progesterone was determined using a radioimmunoassay method developed for their particular study.

MATERIALS AND METHODS

Chemicals

Analytical grade diethylether, acetone and ethyl acetate (Merck, Darmstadt, Germany) were distilled before use. Analytical grade petroleum–ether, b.p. 30–60°C (Mallinckrodt Chemical Works, St. Louis, Mo., U.S.A.) was used as supplied. Neutral alumina, grade I (Merck) was deactivated with 3% water and used for column chromatography. Activated charcoal Norit A (neutral pharmaceutical grade) was obtained from

Amend Drug & Chemical Co., N.Y. 10010, U.S.A. The charcoal was washed several times with distilled water before use, the finest particles were decanted away with the washings and the charcoal was dried at room temperature. Dextran T 70, M.W. 70,000, was obtained from Pharmacia, Uppsala, Sweden. The medium used for tissue preparation was 25 mM Tris–HCl buffer containing 1·3 mM EDTA and 250 mM sucrose, pH 7·4 (TES buffer). Radioimmunoassay was performed in 0·133 mM borate buffer pH 8·0 containing 0·02% gelatin.

Antiserum

The sheep antiserum (batch no S-257 2) for the radioimmunoassay was kindly supplied by Dr G. E. Abraham, University of California, Los Angeles, U.S.A. The antigen used was deoxycorticosterone-21-hemisuccinate coupled to human serum albumin. The antiserum was stored in 0·1 ml aliquots at −70°C and was diluted in the borate buffer pH 8·0 for use in the radioimmunoassay.

Steroids

The purity of the nonradioactive progesterone (Ikapharm, Ramat-Gan, Israel) was checked by gas–liquid chromatography on two columns (SE-30 and XE-60). The purity of [1, 2, 6, 7-^{3}H]-progesterone (S.A. 81·1 Ci/mmol) (New England Nuclear Corporation, U.S.A.) was checked every 4th month by thin layer chromatography (solvent system, chloroform–acetone 9:1 v/v) and was 94–96% during the study.

Animals

Sperm-positive female Sprague–Dawley rats were used. The day vaginal sperm was detected was designated day zero of pregnancy and the following day, day 1. The rats thus delivered on the 22nd day of pregnancy.

Preparation and fractionation of myometrium

The rats were killed by decapitation. The uterus was liberated and immersed in ice-cold TES buffer. The rats usually carried 9–14 fetuses and 4–6 of these (usually 5) were immediately weighed if the duration of pregnancy was 18 days or more. The average weight of the fetuses was calculated. Preparation and fractionation of myometrium was performed using a modification of a method described previously [3]. Throughout this procedure the samples were kept ice-cold. The endometrial tissue and implantation sites were scraped off with a knife. The sample was washed with TES buffer, weighed and cut into 1–2 mm pieces with a scissor. The sliced tissue was washed twice with 5 vol. (wt./vol.) of TES buffer after which a 20% homogenate was prepared in a glass homogenizer fitted with a Teflon pestle. The homogenate was filtered through cheesecloth to remove any remaining unbroken tissue and the filtrate centrifuged at 1000 *g* for 10 min. The supernatant thus obtained was centrifuged at 10,000 *g* for 15 min and the supernatant thus obtained was further centrifuged at 105,000 *g* for 60 min. The 1000 *g* and 10,000 *g* pellets were washed by suspending them in 2·0 ml of TES buffer with the aid of a glass rod and were immediately recentrifuged. The 105,000 *g* supernatant (the cytosol) was decanted and the tube and the surface of the 105,000 *g* pellet rinsed with TES buffer. The three pellet fractions and the cytosol were stored at −20°C until assayed. The 1000 *g* fraction contains most of the nuclei, the 10,000 *g* fraction contains most of the mitochondria and the 105,000 *g* pellet contains the microsomes [3]. These particulate fractions will be referred to as the nuclear, mitochondrial and microsomal fraction, respectively.

Determination of progesterone concentration

For the determination of endogenous progesterone concentration each pellet was suspended in 2·0 ml of TES buffer with the aid of a homogenizer. One and half ml was taken for extraction and 0·5 ml was used for protein assay which was performed by the method of Lowry *et al.* [10]. Samples of cytosol (1·0–2·0 ml) were always analyzed in duplicate. The internal standard (~7000 d.p.m. of [1, 2, 6, 7-^{3}H]-progesterone) was pipetted in 25 μl of ethanol into the extraction tubes, the sample was added and the tubes were mixed for 30 sec on a vortex mixer and then incubated for 5 min at 35°C. After mixing for another 30 sec the tubes were allowed to stand for 30 min at room temperature in order to achieve equilibration of the internal standard with the endogenous steroid. The samples were then extracted twice with 5 vol. of diethylether ethyl acetate (2:1, v/v) for 5 min in a horizontal shaker. The extract was evaporated to dryness under a stream of nitrogen and the dry residue was dissolved in 1·0 ml of petroleum ether.

Chromatography on alumina

Chromatography on alumina microcolumns was performed as described in detail previously [11]. The sample was added to the column in petroleum ether and the fraction containing progesterone was eluted with 12·0 ml of 15% (v/v) ethyl acetate in petroleum ether.

Radioimmunoassay (RIA)

The progesterone containing eluate was evaporated to dryness under a stream of nitrogen and then dis-

solved in borate buffer pH 8·0 as follows: 1·0 ml of borate buffer was added to the tubes containing the dry sample, the tubes were mixed on a vortex mixer for 60 sec, incubated at 35°C for 10 min and then mixed again for 60 sec. For recovery estimations 0·4 ml of the sample was taken for liquid scintillation counting and 2–4 fractions were simultaneously taken for RIA. The volume of the unknown samples for RIA varied from 50–250 μl depending on the expected progesterone content. Standards for RIA (0–1000 pg of progesterone) were dissolved in borate buffer containing 1% ethanol (v/v). The volume of samples and standards was adjusted to 250 μl with borate buffer and 100 μl of antiserum diluted 1:650 was added. Finally, 28,000 d.p.m. of [^{3}H]-progesterone was added in 50 μl of borate buffer (ethanol 1%) and the tubes were mixed for 2 min on a rotamixer. After incubation overnight at +6°C the tubes were placed in an ice-water bath, 300 μl of ice-cold Dextran coated charcoal was added (0·5% Norit A and 0·05% Dextran T 70) and the tubes mixed. The reaction time with dextran-coated charcoal was 15 min. The charcoal suspension adsorbed at least 98% of unbound steroid. The samples were then centrifuged at 12,000 *g* for 2 min at +6°C after which 400 μl samples were taken from the supernatant into liquid scintillation counting vials to determine the amount of bound steroid.

Correction of method blank

In every analysis 2–3 1·0 ml samples of TES buffer were taken through the procedure for the determination of the method blank. After extraction and chromatography the blank samples were dissolved in borate buffer in the same way as the unknowns and this "blank borate buffer" was used in the RIA standard curve instead of pure buffer. Thus standard curves which accounted for the effect of the blank were obtained. The amount of progesterone in the unknown samples was read from the standard curve containing blank buffer.

Calculations

Results were plotted on a semilogarithmic scale with c.p.m. of [^{3}H]-progesterone bound plotted against pg of progesterone (Fig. 1). The amount of progesterone in the samples was read from the standard or blank curves and the values were corrected for procedural losses. The final results are expressed as pmol of progesterone per mg protein in the subcellular fractions studied.

Measurement of radioactivity

Radioactivity was measured in a LKB Wallac liquid scintillation counter 81,000 (LKB Wallac Inc., Turku, Finland). 10 ml of Insta-Gel (Packard Instrument Co., Ill., U.S.A.) was used as the scintillation solution. No quenching corrections were made as standards and samples were always counted simultaneously and in identical media.

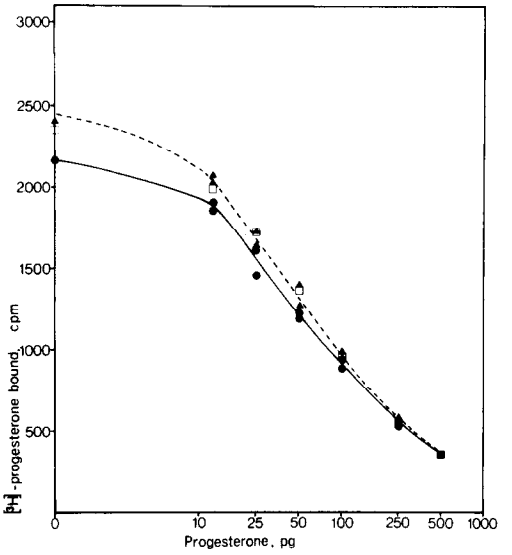

Fig. 1. Two RIA standard curves incorporating the effect of the method blank. ▲---▲: 50 μl of blank borate buffer. ●——●: 200 μl of blank borate buffer. □: no blank, volume adjusted with normal borate buffer. The RIA was performed as explained in the text.

Binding of progesterone by the nuclear and mitochondrial fractions in vitro

The progesterone binding capacity of the nuclear and mitochondrial fractions of pregnant rat myometrium was determined as follows: The washed nuclear and mitochondrial fractions from the myometrium of three pregnant rats were pooled. The pellets were suspended in TES buffer with the aid of a homogenizor and then each pool was divided equally into eight parts. Tracer [^{3}H]-progesterone (final concentration 2 nM) and nonradioactive progesterone in increasing concentrations (final concentration 0–2000 nM) was added to the fractionated pools after which the tubes were incubated for 1 h at +6°C. The final protein concentration was 1·6 and 1·2 mg/ml in the nuclear and mitochondrial tubes, respectively. After the incubation the tubes were centrifuged and duplicate samples of 0·1 ml were taken from the supernatants for liquid scintillation counting. The amount of steroid in the supernatants was considered as unbound and the percentage of bound steroid was then calculated.

RESULTS

Reliability of the RIA method

Method blank. The method blank varied slightly from analysis to analysis and was proportional to the volume of blank borate buffer in the RIA tubes (Fig. 1). The blank value was always negligible in the 100–1000 pg range and only affected the 0–100 pg part of the standard curve.

Sensitivity. The lower limit of sensitivity of the RIA method was 15 pg per RIA tube or 100 pg per sample originally extracted. The reliable range of the standard curve used was from 20–400 pg per RIA tube. The sensitivity was independant of the volume of sample taken for RIA as buffer blank effects were compensated for using blank curves.

Specificity. The chromatography on alumina [11] was used for the elimination of impurities and of steroids more polar than progesterone. The amount of progesterone measured in cytosolic samples was doubled when the chromatographic step was omitted. The antiserum used showed highest cross reaction with deoxycorticosterone (35%), 20α-hydroxy-4-pregnen-3-one (7%) and testosterone (5%) which are eliminated by the chromatographic step. The use of the antigen deoxycorticosterone-21-hemisuccinate, coupled at C-21, confers on the antiserum obtained highest specificity for ring A of the steroid molecule. The principal myometrial progesterone metabolites lack the 4-ene-3-one structure of progesterone [3, 4]. Interference in RIA may have been caused by 5α-pregnane-3,20-dione (cross reaction 4%), the primary dehydrogenation product of progesterone found in the myometrium [3, 4].

Accuracy and precision

The recovery of added [^{3}H]-progesterone was 72·9 ± 3·7% (mean ± S.D., n = 138). The accuracy of the procedure was investigated by adding two different amounts of progesterone to 1·0 ml samples of myometrial cytosol. The results are shown in Table 1. The coefficient of variation found in assays of progesterone in post partum and pregnancy myometrial cytosol is also shown in Table 1. The results show satisfactory precision of the method.

Weight of foetuses and duration of pregnancy

The weight of the foetuses increased linearly during the last 5 days of pregnancy (18th-22nd day) (Fig. 2). The difference between the mean foetal weights was statistically significant ($P < 0{\cdot}001$) from day to day and was independant on the number of foetuses in each rat. By weighing the foetuses the duration of pregnancy can be evaluated with greater accuracy during this period than when only the day of pregnancy is known.

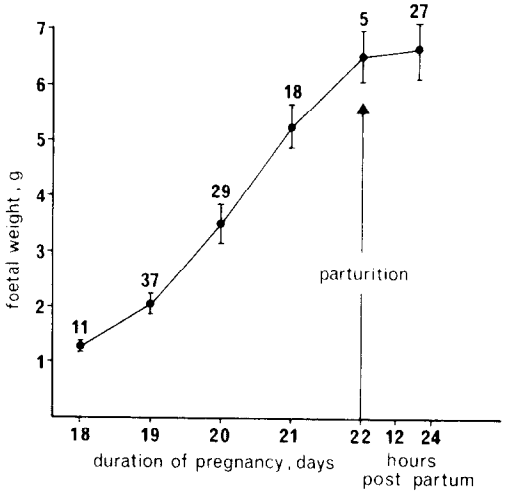

Fig. 2. Foetal weight and duration of pregnancy. Each determination was made by weighing 4–6 foetuses from each rat after the 18th day of pregnancy. The values are given as mean ± S.D. The figures on the top of the bars indicate the total number of foetuses weighed.

Table 1. Progesterone in myometrial cytosol

Sample analyzed	Progesterone added (pg)	Progesterone measured (pg)	n	Coefficient of variation (%)
Pooled myometrial cytosol, post partum rats	0	299 ± 52	10	17·4
Pooled myometrial cytosol, pregnant rats	0	1171 ± 100	11	8·5
Myometrial cytosol from different analyses	800	835 ± 154	25	18·4
Myometrial cytosol from different analyses	4000	4218 ± 471	15	11·2

RIA was performed as explained in the text. All samples were analyzed in duplicate. The coefficients of variation given represent the interassay variation of the method. The results given for "added progesterone determinations" have been corrected by subtracting the endogenous progesterone levels.

Endogenous progesterone in myometrial cytosol of late pregnancy

The decrease in the concentration of endogenous progesterone in the myometrial cytosol is inversely correlated to foetal weight from the 18th to the 21st day of pregnancy (Fig. 3). The correlation coefficient was −0·86. The greatest decrease in cytosolic progesterone takes place on the 20th day of pregnancy (from 4·2 to 2·0 pmol/mg protein) (Fig. 4). During and after the 21st day of pregnancy the concentration of endogenous progesterone decreases only slightly.

Endogenous progesterone in the subcellular fractions of myometrium

Figure 4 shows the distribution of endogenous progesterone among the 4 subcellular fractions of rat myometrium during the last trimester of pregnancy. During the 14-16th days of pregnancy the concentration of progesterone was rather constant in the cytosol and began to decrease after the 16–17th day. The decrease continued until the 21st day of pregnancy when low levels were reached.

The concentration of progesterone starts to decrease later in the other subcellular fractions than in the cytosol, namely during the 19–20th day of pregnancy. On the 21st day of pregnancy the concentration of progesterone is low in all fractions and the differences between the different fractions are negligible except that the microsomal fraction retains more progesterone per mg protein than any of the other fractions at all times after the 18th day of pregnancy. Statistically the difference in progesterone concentration between the microsomal and cytosolic fractions does not seem

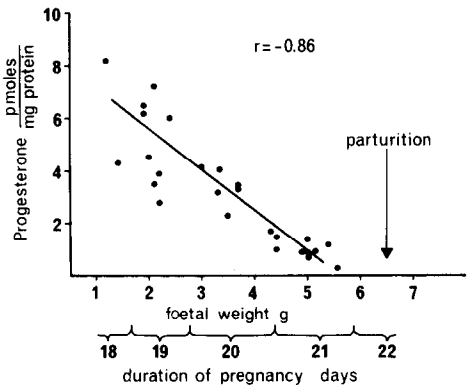

Fig. 3. Foetal weight and the concentration of progesterone in the cytosol of rat myometrium. The values were determined by RIA as explained in the text. The results are expressed as pmoles of progesterone measured per mg of cytosolic protein. The correlation coefficient was −0·86. The duration of pregnancy in days corresponding to the foetal weight given is shown underneath.

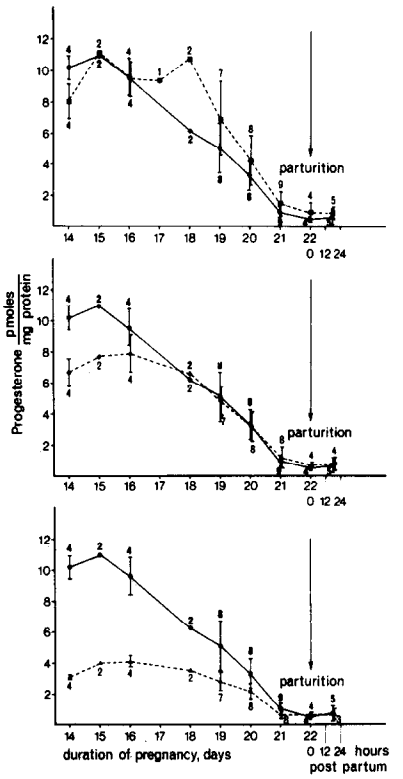

Fig. 4. The concentration of endogenous progesterone in the 4 subcellular fractions of rat myometrium during the last trimester of pregnancy. The values were determined by RIA as explained in the text. The values are given as mean ± S.D. and the figures shown indicate the number of rats used for the determination of each value. The results are expressed as pmol of progesterone per mg protein in the different subcellular fractions. ●——●: cytosol or 105000 *g* supernatant. ■---■: 105000 *g* pellet or microsomal fraction, ◆——◆: 10000 *g* pellet or mitochondrial fraction, ▲——▲: 1000 : or nuclear fraction.

significant ($P < 0{\cdot}05$ on the 19th day and $P < 0{\cdot}10$ on the 20th and 21st day of pregnancy).

The concentration of progesterone in the 3 pellet fractions relative to the cytosolic concentration is shown in Table 2. During the last third trimester of pregnancy the concentration of progesterone in each of these particulate fractions approached that of the cytosol and became higher than the cytosolic concentration in the microsomal fraction on the 18th day of pregnancy. The microsomal progesterone concentration remained higher and the mitochondrial concentration the same or slightly higher than the cytosolic concentration thereafter. The nuclear progesterone content was lower than the cytosolic concentration until the 22nd day of pregnancy when the values tended to equate. Post partum 20 h the concentration of progesterone was practically the same as on day

Table 2. The concentration of progesterone in the 3 particulate subcellular fractions of rat myometrium relative to the concentration of progesterone in the cytosol during the last third trimester of pregnancy

Day of pregnancy	n	Ratio to concentration in cytosol (%) (1000 *g*) nuclei	(10,000 *g*) mitochondria	(105,000 *g*) microsomes
14	3	30 ± 1	69 ± 7	78 ± 9
16	4	43 ± 7	83 ± 11	99 ± 6
18	2	55 ± 1	104 ± 9	168 ± 26
19	7	57 ± 14	97 ± 28	142 ± 46
20	8	70 ± 25	104 ± 36	139 ± 60
21	8	67 ± 27	123 ± 44	141 ± 51
22	4	116 ± 32	144 ± 46	184 ± 102
Hours post partum				
20	4	86 ± 20	123 ± 44	144 ± 35

The values are expressed as per cent of cytosolic concentration (mean ± S.D.). n indicates the number of rats used for the determination of each value.

22 just before parturition and the relative amounts in the 4 fractions were also the same. The relative percentages shown in Table 2 are of no significance on and after the 21st day because the absolute amount of progesterone in all fractions was low during and after the 21st day.

Binding of progesterone by the nuclear and mitochondrial fractions in vitro

The nuclear and mitochondrial fractions of pregnant rat myometrium both showed progesterone binding directly proportional to the steroid concentration over the 2–2000 nM concentration range studied. It was concluded that only nonspecific binding of high capacity and low affinity could be demonstrated in these particulate fractions using the technique employed.

DISCUSSION

The determination of the duration of pregnancy in rats is usually based on the detection of vaginal sperm during daily examination. Sometimes it is advantageous to know the exact duration of pregnancy more accurately than to a given day. In the present study it was shown that by weighing the foetuses of rats near term the duration of pregnancy could be assessed to within a matter of a few hours. Other parameters such as placental and uterine weights were also examined but they are not as suitable for this purpose as determination of mean foetal weight.

The concentration of progesterone in the whole uterus of pregnant rats has been determined previously [2]. The pattern of myometrial cytosol progesterone content during pregnancy which is now reported parallels that found in whole uterus. In the rat plasma progesterone concentration stays high until 2–3 days before parturition. Thereafter it begins to decrease [1, 2] reaching near minimal levels on the day before parturition (day 21 of pregnancy). According to these and earlier results the concentrations of progesterone in plasma and cytosol show no direct correlation except 2–3 days before parturition when they both decrease steeply. Cytosolic progesterone seems to be regulated, at least partly, by some means other than simple diffusion and distribution among different extra- and intracellular compartments.

The three subcellular pellet fractions were bathed in the cytosol during preparation and under *in vitro* conditions the nuclear and mitochondrial fractions were able to bind progesterone over a wide range of concentrations. However, the progesterone content of the three particulate fractions shows no direct correlation to the cytosolic progesterone concentration. The amount of progesterone in these particulate fractions relative to that in the cytosol was found to increase towards the end of pregnancy (Table 2). In our *in vitro* incubations of myometrial nuclear and mitochondrial fractions with [^{3}H]-progesterone we were unable to demonstrate any high affinity progesterone binding. It is probable that high affinity binding sites, if they exist, were masked by endogenous progesterone.

The heavy microsomes (centrifuged at 20,000 *g*) of late pregnancy rat myometrium have been reported to retain more injected [^{14}C]-progesterone per mole of nitrogen than other subcellular fractions [3]. In the present study, the microsomes were found to retain more endogenous progesterone per mg protein than the other fractions during the last five days of pregnancy (days 18–22). The microsomal pellet was not washed and recentrifuged as the nuclear and mitochondrial pellets were. It is probable that the concentration gradient resulting from resuspending the pellets in TES buffer decreases their progesterone content. The myometrial microsomes have high affinity progesterone binding components characterized by

rapid dissociation and exchange rate of bound steroid (Haukkamaa and Luukkainen, to be published). The washing step was therefore omitted because sedimentation at 105,000 *g* would have been very time consuming when compared to the rapid sedimentation of the heavier pellets. In fact it seems impossible to determine with certainty the true *in vivo* distribution of progesterone among the different subcellular compartments.

The concentration of cytosolic high affinity progesterone binding sites is low during the entire last third trimester of pregnancy [7,8] at the time when the concentration of progesterone is decreasing. However, the concentration of the nonspecific progesterone binding protein resembling corticosteroid binding globulin (CBG) [6, 12] begins to decrease 2–3 days before parturition [6, 8] and this decrease is simultaneous with the highest drop in cytosolic progesterone. Thus this CBG-like progesterone binder of high affinity might behave as a reservoir for cytosolic progesterone during pregnancy. This kind of progesterone binding protein resembling CBG has been found in human myometrium during pregnancy [8, 13] and in rabbit myometrium, also [14]. The change in the amount of progesterone in the different particulate fractions relative to cytosolic progesterone might be explained by speculating that while the CBG-like protein decreases to undetectable levels high affinity bound progesterone also decreases in the cytosol and therefore the relative progesterone content of the particulate fractions increases.

The concentration of cytosolic high affinity progesterone binding sites in pregnant rat myometrium has been reported to vary from 4–20 pmol/mg cytosolic protein by Davies and Ryan[7] and from 1–6 pmol/mg cytosolic protein in another study [8]. The concentration of endogenous progesterone in the cytosol is of the same order, 0·5–10 pmol/mg protein during the last trimester of pregnancy. This means that potentially all cytosolic progesterone can be bound to high affinity binding sites.

Milgrom *et al.*[15, 16] have recently shown that the cytosolic progesterone receptor of guinea-pig uterus is inactivated on binding its own ligand. These and previous results concerning the simultaneous existence of cytosolic progesterone and the cytosolic progesterone receptor in the myometrium of pregnant rats [8] are not in agreement with their findings. However, species differences may be great and moreover, it was shown [8] that the progesterone binding capacity was greater in nonpregnant than in pregnant rats which have high levels of endogenous progesterone.

The aim of this study was to determine the subcellular distribution of endogenous progesterone in pregnant rat myometrium. The results show that the ratios of endogenous progesterone concentrations in the subcellular fractions studied do not vary much on a given day of pregnancy but these ratios change gradually during the last trimester of pregnancy.

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