*Journal of Steroid Biochemistry*, 1977, Vol. 8, pp. 161–166. Pergamon Press. Printed in Great Britain

# $C_{21}$-STEROID BINDING PROTEINS AND PROGESTERONE LEVELS IN CHICKEN PLASMA DURING ONTOGENESIS

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(*Received* 19 *May* 1976)

SUMMARY

Protein system (CBG) binding with high affinity ($K_A 4°C \simeq 7 \times 10^8 M^{-1}$) and limited capacity $C_{21}$ steroids, could be detected as early as day 6 of incubation in the plasma of male and female chick embryos. Competitive experiments as well as apparent binding constants determination showed that this system is involved in the binding of corticosterone, progesterone, cortisone, cortisol and deoxycorticosterone. The corticosterone binding capacities increased from day 6 ($4.5 \pm 2.0 \times 10^{-9}$ M) to day 15 ($367 \pm 183 \times 10^{-9}$ M) and decreased almost three times between days 15 and 18. This decrease was confirmed one day before hatching (day 20). No sex difference in binding activities was apparent at any day of incubation.

Progesterone concentrations were determined by radioimmunoassay. No significant differences of the mean plasma progesterone concentrations were found between male and female embryos at no stages of incubation. The values increased from day 9 ($0.94 \pm 0.79$ ng/ml) to day 15 ($3.07 \pm 1.72$ ng/ml), and then stabilized to day 18 ($3.46 \pm 2.76$ ng/ml). Simultaneous decrease of $C_{21}$-steroid binders and stabilization of progesterone levels, indicate a relative increase of the unbound steroids during the last stage of incubation. This might be related either to target organ differentiation or perhaps to the onset of hatching.

## INTRODUCTION

The biological importance of specific steroid binding plasma proteins is currently under investigation and it is generally accepted that plasma proteins do not serve solely for direct steroid transport. The possibility that steroid binders are involved in the secretion of steroid hormone or afford protection for the steroid against red cell and liver metabolizing enzymes is mentioned by numerous authors [1, 2]. An indirect approach to the role of steroid binders in plasma, consists of delineating the interrelationships between different endocrine states and the variations of concentrations of steroid binders. The chick embryo developing is an interesting dynamic model for such studies.

In chick embryo plasma, it was assumed either for corticosterone alone [3] or for corticosterone, cortisol, cortisone and progesterone together [4] that their concentrations were increasing highly from day 9 or 10 to day 20 of incubation. In the latter study [4], no hormone was predominant throughout the embryonic development. And yet, Pedernera[5] found *in vitro* from adrenal glands sampled from embryos as young as 8 days that they were able to release corticosteroids. With regard to the presence of those steroids, to our knowledge, no information is available on the plasma protein binding $C_{21}$-steroids. The present investigation was undertaken in plasma of chick embryos, in order, firstly, to analyze the saturable binding of $C_{21}$-steroids, secondly to determine the date of first characterization of these specific proteins, and thirdly to follow their variations during development and according to the sex of embryos. Finally we measured the concentration of progesterone and we related it to the saturable binding proteins constant's values.

## MATERIALS AND METHODS

### *Animals and plasma preparation*

Chick eggs ($n \simeq 500$) of either with Leghorn or Hubbard breeds were incubated at 38°C in humid environment. Blood of 5 and 6-day-old embryos was collected by cardiac puncture with the help of a Pasteur capillary pipette. Blood samples were also taken from allantois blood vessels (arteries and veins) of 7, 8, 9, 10, 15, 18 and 20 day-old embryos. In order to sample blood from old embryos (20 days), they were previously opened at the beginning of the development; 3 ml albumen was removed and a "window" was made and sealed with scotch tape. The sexes of 5, 6, 7 and 8-day-old embryos were identified before blood collection by heterochromosomes examination according to a technique adapted by Gasc[6]; this

easy and innocuous technique does not disturb normal embryo development. Sex from 9–20 day-old embryos was observed after autopsy. Immediately after collection, sodium citrate (<0.5%) was added in blood samples. The blood was centrifuged (1500 *g* for 10 min) and plasma was decanted and stored at −20°C until assayed. Amniotic and allantois fluids (from 10 and 15-day-old embryos) were analyzed according to the same treatment.

*Steroids*

The radio inert (Roussel–Uclaf) and labelled (N.E.N.) steroids used were: [1,2-$^3$H]-corticosterone (50 Ci/mmol), [1,2,6,7-$^3$H]-progesterone (81 Ci/mmol), and [1,2-$^3$H]-progesterone (53 Ci/mmol). Steroids were shown to be homogeneous by t.l.c. Radioactivity was determined in a "Tricarb" Packard Scintillation spectrometer 3320 with external standardization. Each sample was dissolved in toluene based scintillator. Small volumes (0.5 ml) of aqueous solutions were counted in 10 ml of a mixture of PPO (5.5 g), POPOP (0.1 g), Triton X-100 (333 ml) toluene (667 ml) solution.

*The steroid–protein binding measurement*

1. *Equilibrium dialysis and binding parameters.* A 0.01 M-Tris–HCl buffer, pH 7.4 was used. Dialysis tubing was washed with distilled water and tris–HCl buffer. For all experiments 1 ml diluted whole plasma (1/3, 1/10, 1/20, 1/80) or 1 ml extra embryonic fluids was placed inside the bag and the radioactive steroid was placed outside the bag in 4 ml Tris–HCl buffer. In competitive experiments the unlabelled steroid was placed with the labelled steroid outside the bag. To each dialysing system were added various amounts of non-radioactive steroid ranging from $10^{-10}$–$10^{-7}$ M and a fixed amount ($1 \times 10^{-10}$ M) of labelled steroid. All binding experiments were performed at 4°C with stirring, for a set time of 48 h.

In some experiments, in order to remove most of the endogenous steroids, plasma was subjected to charcoal (Norit A) treatment [7]. The percentage of steroid binding was determined by the following equation: percentage bound = $100\ [1-(D.V_r)/(R.V_d)]$, where $R$ and $D$ are the total amounts of radioactivity present inside and outside the dialysis bag, respectively, and $V_r$ and $V_d$ are the corresponding volumes [8]. Apparent steroid binding constants, binding capacity ($N$) and constant of dissociation ($K_D$) were determined from results obtained by the equilibrium dialysis binding experiments, in which a fixed quantity of labelled steroid and various amounts of the unlabelled steroids were used. Estimation and statistical evaluation of binding parameters were analysed on the assumption that each binding system was independent, the sites of each system were identical, there was no cooperative effect, the system obeyed the law of mass action, and proteins did not affect the activity of steroids except by binding. These binding constants were calculated by a computational method (C II 10070 computer) [9] and by a graphic analysis according to Scatchard[10] as modified by Rosenthal[11]. Similar results were obtained by both methods. Unsaturable binding is determined by the product $K_{NS}\ N_{NS}$ where $K_{NS}$ is the association constant and $N_{NS}$ the molar concentration of binding sites in our experimental conditions.

2. *Polyacrylamide gel electrophoresis.* Analytical polyacrylamide gel electrophoresis [12] was done with 7.5% acrylamide gel containing $1 \times 10^{-9}$ M [$^3$H]-steroid in Tris-glycine buffer at pH 8.3. The plasma of female chick embryos 15-day-old was previously incubated with $5 \times 10^{-8}$ M [$^3$H]-steroid (during 12 h at 4°C) with or without $1 \times 10^{-5}$ M of unlabelled steroid and 10 µl was applied on the gel. Electrophoresis was carried out at 0°C for 120 min. with 1.5 mA per tube. The reference gel was stained with Bromophenol Blue. After electrophoresis, proteins bands were stained with Coomassie Blue. In order to estimate the mobility of [$^3$H]-steroids labelled plasma proteins, gels were cut into 1 mm slices which were put in counting vials. The relative mobility of proteins ($R_F$) could be calculated using the dye as a reference, Bromophenol Blue $R_F$ was referred to as 1.0.

*Steroid assays*

*Extraction.* Plasma (0.1–1.0 ml) was transferred to extraction tubes. To this sample, was added 40,000 d.p.m. [$^3$H]-progesterone-([$^3$H]-P) dissolved in 20 µl of ethanol. In all tubes the vol. was made up to 1 ml, if necessary, with bidistilled water. Aliquots containing the same amounts of [$^3$H]-progesterone were also dried in two scintillation counting vials for subsequent calculation of total recovery. One ml of bidistilled water, in duplicate, as a water blank was included in each series of assays. The samples were extracted with four vol. (4 ml) of diethylether. Extraction was accomplished by vigorous agitation on a vortex mixer for 30 seconds. After the plasma had settled and freezing the aqueous phase to −50°C in a bath of dry ice alcohol, the supernatant ether phase was poured out to a conical tube and evaporated to dryness.

*Chromatography.* The dry residue was redissolved in 0.1 ml of benzene–ethanol (95/5; v/v), which was applied to the top of "micro-column" (4 × 50 mm) filled with Sephadex LH-20 (Pharmacia-Uppsala-Sweden). When the sample had entered the column, solvent was carefully added and the collections of fraction was started: 0.6 ml was discarded and the following 0.5 ml containing Progesterone was recuperated. The elution pattern of progesterone compared to that of 17α-hydroxyprogesterone has been previously reported [13].

*Radioimmunoassay.* The progesterone eluate from the column was mixed and duplicate aliquots (one tenth of the eluate) containing about 3800 d.p.m. of [$^3$H]-progesterone were transferred to two assay tubes. One aliquot was also transferred to a small

tube filled with scintillation liquid in order to determine the total radioactivity in the assay tubes. The standard curve tubes were prepared with approximatively 3800 d.p.m. of [$^3$H]-P and increasing amounts (0–500 $\mu$g) of a solution of progesterone in ethanol. Samples and standards were dried and dissolved in 0.2 ml of diluted (1/5.000) antiserum and gently mixed on a vortex mixer before incubation (37°C, 30 min). This antiserum (no. 390) was raised in rabbits immunized with progesterone-11$\alpha$-hemisuccinate coupled to bovine serum albumin [14]. Separation of free from bound steroids was achieved as previously described [15]. A few modifications occurred: the toluene phase including unbound molecules is poured out into small polypropylene tubes filled with 3 ml of a scintillation solution (scintimix, Kochlight Laboratory). These tubes are then put into vials disposed in an Intertechnique SL 30 liquid scintillation counter (efficiency for tritium of 55% in this system).

In this study another antiserum (No. 390) than that used in the previous studies was used. The specificity of this new antiserum towards most of the steroids tested was quite similar to what was previously reported [16]. However cross reactions with the following steroids, 5$\alpha$-pregnane-3,20-dione (5$\alpha$-dihydroprogesterone) and 5$\beta$-dihydroprogesterone, was much less (no cross reaction respectively over 8 and 2%).

The limit of sensitivity was estimated as 0.10 ng/ml when taking into account mean blank value, the recovery and the vol. of aliquot of the sample. The coefficient of variation of results obtained by repeated determinations gives an estimation of intra-assay precision. It was calculated as 9%.

## RESULTS

### $C_{21}$ *Steroid–proteins interactions*

The binding of progesterone and corticosterone to plasma proteins of chick embryos was determined by equilibrium dialysis and polyacrylamide gel electrophoresis.

*Apparent binding constants.* Plasma of day 8.0 chick embryos binds progesterone and corticosterone with high affinity and limited capacity. The mean $\pm$ S.D. binding capacities ($N$) and dissociation constants ($K_D$) $\times$ $10^{-9}$M are: progesterone, $N = 29.8 \pm 7.4$; $K_D = 0.43 \pm 0.18$; corticosterone, $N = 27.1 \pm 11.8$; $K_D = 1.98 \pm 1.49$. The binding constants for these two steroids are not significantly different ($P > 0.05$). This suggests the existence of the same saturable binding proteins systems for progesterone and corticosterone in chick embryo plasma. The unsaturable binding of progesterone ($K_{NS}N_{NS} = 0.70 \pm 0.05$) and corticosterone ($K_{NS}N_{NS} = 0.035 \pm 0.030$) to plasma protein systems is however significantly different ($P < 0.01$). No saturable protein systems binding corticosterone were found in the amniotic and allantois fluids.

*Specificity studies.* The results of competition experiments with several steroids against the [$^3$H]-corticosterone binding by diluted plasma of chicks embryos 8.0-day-old, are shown in Table 1. The ability to displace [$^3$H]-corticosterone from high affinity binding sites is a property of cortisone, cortisol, deoxycorticosterone, progesterone and 17$\alpha$-hydroxyprogesterone. Less displacement by 5$\alpha$-pregn-3,20-dione and 5$\beta$-pregn-3,20-dione is observed. Testosterone, androstenedione and estradiol-17$\beta$ compete poorly.

*Polyacrylamide gel electrophoresis.* Plasma of 15 day chick embryos was incubated with [$^3$H]-corticosterone and fractionated using polyacrylamide gel electrophoresis. Corticosterone was found to bind to different protein systems. The relative mobility for the two major systems are 0.16 and 0.36. The [$^3$H]-corticosterone associated with the slower binder could be displaced by unlabelled corticosterone ($1 \times 10^{-5}$M)

Table 1. Competition between non-radioactive steroids and [$^3$H]-corticosterone for the high affinity binding sites in a pool of chick embryo plasma (8 days old). The concentration of [$^3$H]-corticosterone was $1 \times 10^{-9}$ M and competition was measured relative to the displacement achieved by non-radioactive steroids at three concentrations $10^{-9}$, $10^{-8}$ and $10^{-7}$ M. Binding of [$^3$H]-corticosterone ($1 \times 10^{-9}$ M) has a nominal value of 100; serum dilution: 1/20. (v/v).

| Non radioactive steroids \ Concentrations of non-radioactive steroids | $10^{-9}$ M | $10^{-8}$ M | $10^{-7}$ M |
|---|---|---|---|
| Corticosterone | — | 29 | 15 |
| Cortisone | 30 | 3 | 3 |
| Cortisol | 63 | 22 | 5 |
| Deoxycorticosterone | 67 | 20 | 2 |
| Progesterone | 71 | 30 | 2 |
| 17$\alpha$-hydroxyprogesterone | 59 | 20 | 4 |
| 5$\alpha$-pregn-3,20-dione | 65 | 43 | 11 |
| 5$\beta$-pregn-3,20-dione | 95 | 46 | 15 |
| Testosterone | 89 | 76 | 39 |
| Androstenedione | 100 | 85 | 72 |
| Estradiol-17$\beta$ | 100 | 100 | 82 |

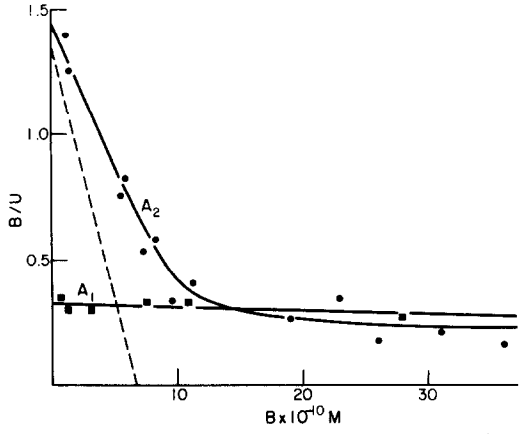


Fig. 1. Binding of corticosterone to chick embryo plasma [5-day-old embryos ($A_1$) and 7-day-old embryos ($A_2$)] as determined by equilibrium dialysis experiments with plasma diluted 1/3 for $A_1$ and 1/10 for $A_2$. The broken line represents the Scatchard-type plot as modified by Rosenthal[11]. B: concentration of bound corticosterone; U: concentration of unbound corticosterone.

while [$^3$H]-corticosterone bound to the faster protein could not. A similar pattern was obtained with [$^3$H]-progesterone.

*Influence of age and sex on the $C_{21}$-steroid binding activity*

The effects of physiological factors (age and sex) were evaluated on $C_{21}$ steroid binding plasma proteins activities.

From Scatchard plot analysis (Fig. 1), one can see that the saturable binding of corticosterone cannot be determined, in our experimental conditions, at day 5; specific binding occurred at day 7.

Table 2 shows separately for male and female embryos, the binding capacities for corticosterone between days 6 and 20 of incubation. Mean binding capacities were not significantly different between sexes at any stages ($P > 0.05$) and increased significantly from day 6 to day 15 ($P < 0.01$; Table 2). After this period of incubation, the mean value decreased ($P < 0.01$) and was at day 18 almost three times lower than at day 15 of incubation. Comparison of $K_D$ values in the plasma of both sexes embryos showed no significant difference between day 6 and day 20; the mean $K_D$ value for all determinations was $1.29 \pm 0.91 \times 10^{-9}$ M.

*Peripheral plasma progesterone concentration*

The progesterone concentrations for chick embryos plasmas are shown in Table 3. At the earliest ages examined (6 and 7 days of incubation), no detectable plasma progesterone was found. Progesterone level as early as 9 days of incubation was about 1 ng/ml and increased until 18 days. No significant differences of the mean plasma progesterone concentration were found between male and female embryos at neither of these stages of incubation. Comparison of mean progesterone values showed significant differences [$P < 0.05$ (males); $P < 0.01$ (females)] between plasma

Table 2. The mean (±S.D.) corticosterone-binding capacities (N) in the plasma of chick embryos. For all ages, each value was determined out of 2–20 individual samples, except on day 15 when larger blood samples are available, each determination corresponded then to one embryo

| Days of incubation | No. of determinations | Female | | Male | | No. of determinations |
|---|---|---|---|---|---|---|
| | | N × $10^{-9}$ M | S.D. | N × $10^{-9}$ M | S.D. | |
| 5 | | not detectable | | not detectable | | |
| 6 | 2 | 4.8 | — | 4.4 | — | 2 |
| 7 | 2 | 8.6 | — | 8.2 | — | 2 |
| 8 | 4 | 23.2 | 13.7 | 24.6 | 11.5 | 4 |
| 9 | 4 | 25.1 | 3.1 | 27.8 | 4.3 | 3 |
| 10 | 6 | 43.2 | 20.6 | 70.8 | 25.6 | 4 |
| 15 | 8 | 342 | 190 | 401 | 184 | 6 |
| 18 | 5 | 151 | 70.6 | 122 | 34.4 | 6 |
| 20 | 3 | 43.7 | 32.3 | 50.9 | 43.9 | 3 |

Table 3. The mean (±S.D.) plasma progesterone concentrations (ng/ml) in males and females chick embryos

| Days of incubation | Males | Mean | Females |
|---|---|---|---|
| 9 | 1.23 ± 1.04 ($n = 5$) | 0.94 ± 0.79* ($n = 10$) | 0.64 ± 0.35 ($n = 5$) |
| 15 | 3.62 ± 2.21 ($n = 10$) | 3.07 ± 1.72† ($n = 20$) | 2.53 ± 0.82 ($n = 10$) |
| 18 | 4.48 ± 3.09 ($n = 10$) | 3.46 ± 2.76 ($n = 19$)† | 2.33 ± 1.91 ($n = 9$) |

$n$ = number of measurements with 27 females and 20 males at 9 days of incubation, 10 females and 10 males at 15 days, 18 females and 22 males at 18 days.

*†Significantly different at $P < 0.05$.

samples of 9-day- and 15-day-old embryos, but no significant increase ($P > 0.05$) occurred between 15 days and 18 days.

## DISCUSSION

It was shown in this study that a protein system binding with high affinity and limited capacity $C_{21}$-steroids could be detected, as early as day 6 of incubation in the plasma of male and female chick embryos. Displacement studies as well as binding constants determinations showed that this protein system is involved in the binding of progesterone, corticosterone, cortisone, cortisol, deoxycorticosterone and 17α hydroxyprogesterone. This $C_{21}$ steroid binder in chick embryos does not differ from those identified from the plasma of chicken, laying hens and roosters as indicated by similar specificity, binding constants and electrophoretic pattern [17, 18]. When comparing our results with those published by Cochet and Chambaz [18] in chicken, the slowest moving [$^3$H]-corticosterone labelled fraction detected by polyacrylamide gel electrophoresis is characterized as a limited capacity binder, since [$^3$H]-corticosterone was displaced by an excess of unlabelled corticosterone and corresponds to CBG. Besides albumin and CBG, other protein (or proteins) which binds corticosterone was identified in chicken by analytical ion exchange chromatography [19]. The binding activity of this latest protein was classified as moderate by these authors.

The corticosterone binding activity in the plasma of chick embryos increased from day 6 to day 15. The values obtained at day 15 of incubation ($N = 367 \pm 183 \times 10^{-9}$ M) were much higher than those obtained with adult animals (in rooster plasma: $N = 131 \pm 67 \times 10^{-9}$ M; [17]). Between days 15 and 18, the binding level decreased almost three times and at day 18 ($N = 135 \pm 53 \times 10^{-9}$ M) the binding values were similar to those obtained in "standard" hens or in roosters [17]. Such a transient increase has already been reported in mammals such as a 3-day-old rat [20]. This decrease in binding activities of chick embryo plasma proteins is not to be related to the total protein concentration which in fact tends to increase (20%) during those last days of incubation [21, and unpublished results]. As in adult animals [17] no sex difference in binding activities was apparent during the embryo chick development.

Progesterone is secreted into the plasma of chick embryos at least as early as 9 days of incubation. Peripheral plasma progesterone concentration rose significantly in the growing embryo from 0.94 to 3.07 ng/ml at day 15 and then did not increase significantly any more. The mean values were similar for both sexes. The only chick embryos levels of progesterone published are those of Kalliecharan and Hall [4]; they are 2–5 times higher than ours. These authors used a Competitive Protein Binding method based on CBG and reported that specificity rested on the efficiency of the partition of corticosteroids and progesterone. The former were shown not to be contaminated by other steroids but no precision was given in regard to progesterone and it cannot be excluded that other steroids cross react with CBG, thus giving overestimated values.

Similarly to the progesterone pattern, three corticosteroids (cortisone, cortisol and corticosterone) were shown to exhibit changes of peripheral plasma concentration during development [4]. According to Kalliecharan and Hall[4] the combined concentration of these three corticosteroids increased significantly during the incubation period until day 17 (63 ng/ml) and then slightly decreased until day 20 (45 ng/ml).

Those peripheral plasma progesterone and corticosteroids changes might then be related to those of plasma proteins binding $C_{21}$-steroids. During a first period (9–15 days of incubation) plasma progesterone and corticosteroids appear to increase together with the $C_{21}$-steroid binder concentration. Such similar increment of steroids and binding proteins were reported in human pregnancy: the plasma $C_{21}$-steroids increase [22] and the CBG capacity is more than twice as much as in non-pregnant women [23]. According to Sandberg and Slaunwhite [24], the CBG-bound cortisol is biologically inactive as no clinical signs of hypercorticism can be observed. This hypothesis might also be applied to the chick embryo model: normal incubation occurring while both steroids and $C_{21}$-steroids binding proteins increase. Apparently chick embryos would be able to tolerate high $C_{21}$-steroid levels because of high concentrations of those binding proteins ($367 \pm 183 \times 10^{-9}$ M at day 15 for instance). At the late stages of incubation (after day 15) total plasma $C_{21}$ steroids concentrations seemed to be stabilized (or slightly lower for corticosteroids [4]) whereas plasma $C_{21}$-steroids binding proteins significantly decreased. This means that the unbound steroid concentration in plasma is in fact increasing at this period. If we admit that this unbound steroid fraction is biologically active agent, then this observation could suggest an essential role of those $C_{21}$-steroids on late tissular differentiation of target organs which is known to occur at the end of incubation [25] and perhaps on the onset of hatching.

*Acknowledgements*—We are grateful to J. Adeline and Dr. R. Scholler (Fondation de la Recherche en Hormonologie, Fresnes, France) for their generous gift of antisera (no. 390). We are also grateful to Pr. R. Ozon for his pertinent criticism in the course of this study.

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