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# ORIGIN OF TRANSCORTIN IN THE CHICK EMBRYO

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## SUMMARY

The synthesis of a transcortin-like molecule in liver of the chick embryo is demonstrated *in vitro*. Since no contamination by blood CBG occurred and no C-21 steroid binders were detected in the liver cytosol of 5-day-old embryos, liver of embryos at this stage was cultured in a synthetic medium without serum. The steroid binders were detected by equilibrium dialysis after a 4-day culture. Besides non-saturable binding, specific binding with $C_{21}$-steroids occurred in the liver cytosol (20%) and in the culture medium (80%). Determination of binding constants as well as competition experiments show that corticosterone and progesterone are bound to the culture medium and to the plasma CBG with the same affinity ($Ka_{4\ C}$: $5 \times 10^8\ M^{-1}$) and specificity. An effect of estradiol-17$\beta$ upon the secreted CBG synthesis was observed. Estradiol ($10^{-8}$ M) either alone or with thyroxine slightly increased CBG secretion in culture medium.

## INTRODUCTION

The ability of specific plasma proteins to bind circulating steroids with high affinity, limited capacity and great ligands specificity is now well established [1–3]. Corticosteroid binding globulin (CBG or transcortin) and sex steroid binding protein (SBP), the main proteins, are of physiological importance because they constitute the hormone pool from which free steroids dissociate and are available to receptor sites in steroid target cells [4]. Transcortin is present in the plasma of numerous vertebrates [5–7], and transcortin-like compounds are also detected in liver [8–12], adenohypophysis [13, 14], uterus and kidney [11], and other organs. The origin of these plasma and intercellular transcortin-like molecules remains unknown. It is generally assumed that steroid binders in plasma are synthesized in the liver [1, 14], although this has not been directly demonstrated yet. In a previous work [15, 16] we have shown that the CBG can be detected first in the circulating blood of 6-day-old male and female chick embryos, and that this binder has the same specificity, affinity and electrophoretic pattern in the embryo and the adult animal.

Since no contamination by blood CBG occurs in organs of 5-day-old embryos, the site of CBG synthesis can be detected. In this report, the hepatic synthesis of a transcortin-like molecule in chick embryo is demonstrated by means of organ culture. An effect of thyroxine and estradiol-17$\beta$ upon secreted CBG synthesis is further studied.

## MATERIALS AND METHODS

*Preparation and culture of liver explants.* Eggs of White Leghorn chick were incubated at 38.5°C in a humid environment. After 5, 7, 9, 13 or 15 days of incubation, embryos were removed from the eggs and the livers placed in a Petri dish containing the culture medium. Intact livers of 5-day-old embryo ($0.46 \pm 0.16$ mg) were cultivated; livers of 7-, 9-, 13- and 15-day-old embryos were cut in pieces of the same amount as 5-day-old liver. In 9-, 13- and 15-day-old embryos the outer part of the liver was cut in pieces and cultivated whereas the inner part was discarded. To remove part of the blood contained in the liver, explants were washed two times with culture medium.

Stainless steel grids with Millipore filters (GSWPO 1300) were placed in a 35 mm Petri dish containing 5 ml of culture medium and this set up was transferred into a 50 mm Petri dish. The medium was Dulbecco's modified Eagle Medium H.G. with L/Glucose and Glutamine, and without Sodium bicarbonate (ref. H 21; Gibco) to which 100 Units/ml of Penicillin G and 40 Units/ml of Streptomycin were added. No serum was added to the medium. Every grid received 20 5-day-old livers or an equivalent number of pieces from the 7-, 9-, 13- or 15-day-old livers.

Various anlages were used as controls: 32 mesonephros, 4 brains, 9 hearts, 36 limb buds, 9 allantois of 5-day-old embryos and 12 lungs and 6 hindguts of 7-day-old embryos were cultivated following the same method as the livers.

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Petri dishes with explants were placed in a humidified incubator with a circulating gas flow (air: 95%; $CO_2$: 5%) at 37°C for 4 days (livers, mesonephros, brains, hearts, limb buds and allantois of 5-day-old embryo; lungs and hindguts of 7-day-old embryos) or 3 days (livers of 7-, 9-, 13- and 15-day-old embryos).

Bresnik and Bürki[17] reported good results in cultivating fetal rat liver for some days by a very similar technique.

*Preparation of medium and cytosol.* After 3 or 4 days, culture medium were removed from the Petri dish and centrifuged (10 min, 3000 rev./min, at room temperature). To increase the concentration of protein the culture mediums were filtered on Amicon Filtermembranes (UM 20 E) at 4°C, and then were stored 4 to 48 h at the same temperature before using it for the steroid binding measurement.

Livers of 5- to 15-day-old embryos were homogenized with a glass-glass homogenizer in a Tris–KCl buffer (Tris 0.05 M; KCl 0.075 M; pH 7.4, HCl). The tissue/buffer ratio was 4 livers of 5-day-old embryos or an equivalent amount of liver for other stage embryos/1 ml Tris–KCl. This homogenate was centrifuged in a Spinco L2 50B (35 min, 110,000 *g*: 4°C), the sediment was discarded and the supernatant was used for the steroid binding measurement. Cultured liver and cytosols of mesonephros, brain, heart, limb buds, allantois, lung and hindgut were prepared according to the same technique.

*The steroid-protein binding measurement.* The radioinert (Roussel-Uclaf) and labelled (N.E.N.) steroids were used. [1,2,6,7,-$^3$H]-Corticosterone (S.A.: 82 Ci/mmol) was shown to be homogeneous by thin-layer chromatography. The protein binding of steroids was determined by an equilibrium dialysis method. Dialysis tubing was washed with distilled water and Tris-buffer. For all experiments, 1 ml cytosol or culture medium was placed inside the bag and the radioactive steroid was placed outside the bag in 99 ml Tris–KCl 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 $10^{-10}$ to $10^{-7}$ 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. The percentage of steroid binding was determined by the following equation: % 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 (Sandberg *et al.*, 1966). Steroid binding constants, binding capacity ($N$) and constant of association ($K_A$) 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. The binding constants were calculated by a computational method (C II 10070 computer; [19]) and by a graphic analysis according to Scatchard[20] as modified by Rosenthal [21]. Similar results were obtained by both methods. The binding index $K_{NS}N_{NS}$ of the non specific binding system (low affinity, relatively large number of binding sites) was determined. 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.

## RESULTS

### 1. *Secretion of corticosterone binders*

The binding of corticosterone was studied in cytosol of uncultured liver from 5 to 15-day-old chick embryos. No binding was observed with liver cytosol from 5- and 7-day-old embryos (Fig. 1), whereas significant binding occurred with 9-, 13- and 15-day-old embryos. After a 3- or 4-day culture, the corticosterone binding activity in culture medium of liver from 5- to 13-day-old embryos is significantly higher than in cytosol before culture. A lower activity is observed in the culture medium of liver of 15-day-old embryos. In all cases the corticosterone binding is displaced by unlabeled corticosterone ($10^{-6}$ M).

A corticosterone binding activity is detected in the liver cytosol of 5- and 7-day-old embryos after culture. This binding is five times lower than the one obtained in culture medium, and can be displaced by $10^{-6}$ M unlabeled corticosterone.

No corticosterone binding activity is detected in cytosol and culture medium of mesonephros, brain,

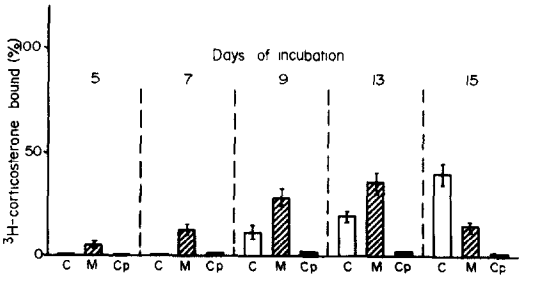


Fig. 1. Corticosterone binding activity in the cytosol before culture (C) and in the medium after culture (M) of the liver from 5- to 15-day-old chick embryos after a 3-4 day culture period. Cp: competition between [$^3$H]-corticosterone ($1 \times 10^{-10}$ M) and non-labeled corticosterone ($1 \times 10^{-6}$ M) for the high affinity binding sites in culture medium. Results in percent of [$^3$H]-corticosterone ($1 \times 10^{-10}$ M) bound in 1 ml of medium or cytosol prepared from 4 livers of 5-day-old embryo or an equivalent amount of liver for other stage embryos. Mean ± S.D. (three measurements).

heart, limb bud and allantois of 5-day-old embryos, lung and hindgut of 7-day-old embryos, cultured in the same conditions as liver.

### 2. $C_{21}$-steroid-protein interactions: Apparent binding constants and specificity

An equilibrium dialysis-Scatchard plot method was used to investigate corticosterone binding affinity. The association constant was determined in culture medium and liver cytosol of 5-day-old embryos after 4 days of culture. $K_a$ values for corticosterone are respectively $5 \times 10^8\ M^{-1}$ (Fig. 2) and $4 \times 10^8\ M^{-1}$ The unsaturable binding of corticosterone to culture medium ($K_{NS}N_{NS}$) is 0.07. The ability of progesterone, dexamethasone and estradiol-17$\beta$ to compete with [$^3$H]-corticosterone for binding sites in the culture medium of 7-day-old liver is shown in Fig. 3. Progesterone and dexamethasone strongly compete with [$^3$H]-corticosterone whereas estradiol-17$\beta$ does not.

### 3. Effect of hormones on the secretion of corticosterone binders

The effect of thyroxine and estradiol-17$\beta$ on corticosterone binders secretion in culture medium was measured. An increase in the secretion of steroid binders is observed with liver of 9 and 15-day-old embryos after a 3-day culture with estradiol-17$\beta$ ($10^{-8}$ M) (Fig. 4). Comparison of mean corticosterone binding values of controls and cultures with hormones (livers of days 9 and 15) showed significant differences ($P < 0.01$) with estradiol-17$\beta$ either alone or with thyroxine together in the culture medium, but no significant increase occurred with thyroxine alone. No effect was observed with liver of 7-day-old embryo cultured in the same conditions. In all cases, the [$^3$H]-corticosterone-binder system can be displaced by unlabeled corticosterone.

## DISCUSSION

The aim of this work was to detect the site of CBG synthesis in chick embryos. By the use of organ culture, synthesis of high affinity binding system for corticosterone and progesterone has been demonstrated in liver.

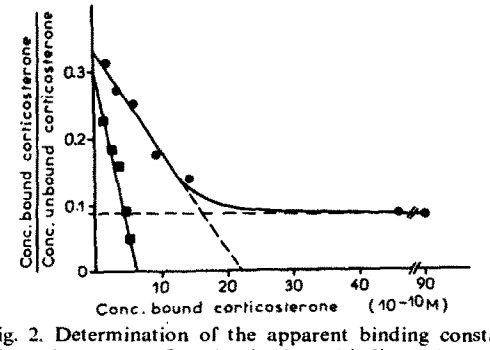


Fig. 2. Determination of the apparent binding constants of corticosterone (Scatchard plot). Binding of corticosterone to compounds secreted in the culture medium (concentration factor: 3.5) by liver of 5-day-old embryo, as determined by equilibrium dialysis experiments. Scatchard plot of total binding (O——O) and specific binding (Rosenthal's correction) (□——□).

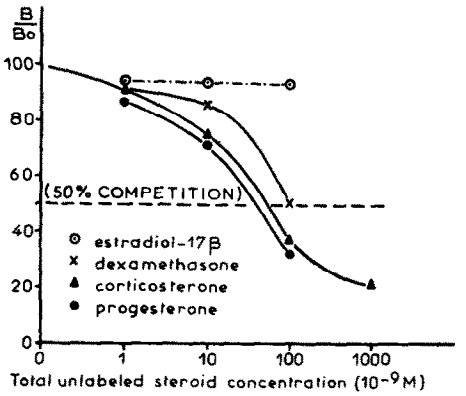


Fig. 3. Competition for the bound [$^3$H]-corticosterone in culture medium (concentration factor: 5) of liver of 7-day-old embryo as a function of unlabeled steroid concentration: $B/B_0$: ratio of labeled steroid bound in presence of unlabeled steroid, to the labeled steroid bound in absence of unlabeled steroid.

After culture, specific binding with $C_{21}$-steroids occurred in the culture medium of liver of 5-day-old chick embryo. Since in the 5-day-old chick embryo $C_{21}$-steroid binders cannot be detected in plasma (Martin *et al.*, 1977), neither in liver cytosol before culture (Fig. 1), there is no doubt that the binders have been synthesized in the liver and secreted in the culture medium.

Determination of binding constants as well as competition experiments show that corticosterone binds to the culture medium and to the plasma CBG of chick embryos [16], chickens and hens [22, 23] with the same affinity ($Ka_{4\,C}$ $5 \times 10^8\ M^{-1}$) and specificity. By contrast to human CBG [2] chicken CBG-like molecule binds to a synthetic fluorocorticoid (dexamethasone).

The existence of a corticosterone binding receptor in the high-speed supernatant fraction of the 16-day-

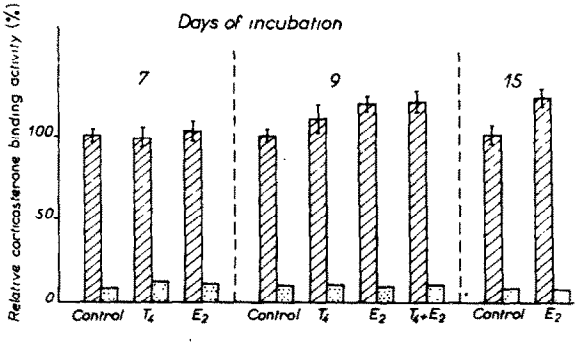


Fig. 4. *In vitro* effect of estradiol-17$\beta$ and thyroxine on corticosterone binding activity. Binding activity in the culture medium of liver explants (7-, 9- and 15-day-old chick embryo) after a 3-day culture. L. Thyroxine ($T_4$) ($3 \times 10^{-8}$ M) and/or estradiol-17$\beta$ ($E_2$) ($1 \times 10^{-8}$ M) were added to the culture medium. Binding of [$^3$H]-corticosterone ($1 \times 10^{-10}$ M) to the control culture medium has a nominal value of 100. Competition with $10^{-6}$ M unlabeled corticosterone for high affinity binding sites (column with points). Other conditions are described in materials and methods. Mean ± S.D. (three measurements).

old chick embryo liver was described by Tu and Moudrianakis[24]. It does not seem that the corticosterone binding substance synthesized by livers of 5-day-old embryos in organ culture may be the same receptor. In fact, the corticosterone binder described in our report is synthesized approximately at the same time as the CBG appears in the blood [16], the binder is secreted in the culture medium and its binding affinity and specificity are the same as those found with CBG.

Besides transcortin-like molecule (*i.e.* high affinity systems) synthesized by the liver, a low affinity binding corticosterone system was identified in the liver culture medium. This "non specific" system could be albumin as indicated by Grieninger and Granick[25] in a monolayer culture of chick embryo liver cells.

As far as we know, no information concerning the CBG regulation in chick is available. The large but reversible increase of CBG levels during ontogenesis of chick embryo [16, 26] suggests a hormonal control. Estrogens and thyroid hormones, detected in the plasma of chick embryos by Ozon[27] and Thommes *et al.*,[28] could play, as in human [1], an important role in this control. Carinci *et al.*[29], have demonstrated that estradiol-17$\beta$ induces in primary cultures of chick embryo liver the *de novo* synthesis of the yolk protein phosvitin. In the present work, it is shown that estradiol-17$\beta$ either alone or with L-Thyroxine together, slightly increase the secretion of CBG in culture medium of liver of 9- and 15-day-old chick embryos. No effect is observed with the 7-day-old embryo liver.

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