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TRANSCORTIN: A CORTICOSTEROID-BINDING PROTEIN OF PLASMA—XII. IMMUNOLOGIC STUDIES ON TRANSCORTIN IN GUINEA-PIG TISSUES

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SUMMARY

Guinea-pig tissues were tested for the presence of macromolecules antigenically related to transcortin by the use of anti-guinea-pig transcortin rabbit sera. By counterimmunoelectrophoresis, transcortin was detected in liver homogenates in all fractions obtained by differential centrifugation at 700, 35,000 and 200,000 *g*. Transcortin was also detected by immunoelectrophoresis in sonified liver, uterus, and kidney, but not in adrenal or spleen; and also by radioimmunoassay, with the highest transcortin concentration being in liver, followed by uterus and kidney, and the lowest concentration in adrenal and spleen. By chromatography of the 27,000 *g* supernatants of sonified liver or uterus on sucrose density gradient, a progesterone-binding peak was obtained that was close to and ahead of the standard peak of bovine serum albumin and produced precipitin lines by counterimmunoelectrophoresis. The progesterone binder was selectively absorbed from a 27,000 *g* supernatant of sonified liver by a transcortin specific immunoadsorbant, as demonstrated by the disappearance of the precipitin line and by a large reduction of protein-bound progesterone, relative to a small loss of protein, which could be shown by chromatography on Sephadex G-25. The selective adsorption of bound progesterone from the 27,000 *g* supernatants of sonified liver, uterus, or kidney by a transcortin-specific immunoadsorbant was demonstrated by finding tritiated progesterone on the specific but not on the non-specific immunoadsorbant. The experiments demonstrated that blanched guinea-pig liver and uterus contained transcortin, or transcortin-like molecules, in concentrations exceeding the concentrations that could be obtained from interstitial blood plasma alone.

INTRODUCTION

The existence of cellular macromolecules that bind cortisol and/or progesterone has been documented for various tissues [1–10]. Whenever a further identification of the binding macromolecules has been made, they were shown to be proteins. Similarities to transcortin were noted for a cortisol binding macromolecule in rat liver cytosol [11, 12] and for a progesterone binding macromolecule in the cytosol of uterus and liver of rat and rabbit [3, 4, 13].

Bound radioactive progesterone in the cytoplasm of rat uterus was shown to be precipitable by rabbit IgG anti-rat serum[4]. A cortisol-binding protein in rat liver cytosol and serum transcortin were shown to coprecipitate with antibodies to whole rat serum [12].

In this paper we have demonstrated, by a variety of techniques that make use of transcortin-specific antisera, the existence of cellular macromolecules antigenically related to transcortin. While our work was in progress, it was reported, in abstract form, that the presence of transcortin-like antigen in lymphocytes has been demonstrated with transcortin-specific antibodies [14].

MATERIALS AND METHODS

[7-^{3}H]-Progesterone (10 μCi/181 ng) and [1,2-^{3}H]-cortisol (10 μCi/78 ng) from New England Nuclear were stored at −15°C in ethanol at a concentration of 10 μCi/ml. Tris–KCl buffer consisted of 0·01 M Tris–HCl and 0·04 M KCl, pH 7·8. Borate buffer consisted of 0·17 M sodium borate and 0·14 M NaCl pH 8·0. Tris–sucrose buffer consisted of 0·01 M Tris–HCl, 0·04 M KCl and 0·25 M sucrose, pH 7·8. Incubation medium RPMI 1640 [15] was purchased from Grand Island Biological Co.

Tritium was counted at 35% efficiency in 10 ml of aquasol–toluene scintillation fluid (2 vol. Aquasol from New England Nuclear to 1 vol. toluene) in a Packard Liquid Scintillation Counter. Insoluble tritium samples were oxidized in a Packard Tri-Carb Sample Oxidizer prior to counting. The sample was placed on cot-

ton in a piece of dialysis casing for oxidation. A well-type dual channel γ-spectrometer (Beckman, Model 3320) was used to count ^{125}I with an efficiency of 40% and a background of 85 c.p.m.

Guinea-pig transcortin was isolated by the method of Schneider *et al.* [15]. Rabbit antisera to transcortin and to bovine serum albumin (crystallized, Pentex) were obtained by intramuscular and footpad injections of male Sprague–Dawley rabbits with 1–2 mg of protein in Freund's complete adjuvant, followed 3 and 5 weeks later by a booster of about 0·5 mg protein in the same complete adjuvant. The animals were bled from an ear vein, and the sera stored at −15° C until use.

Guinea-pigs in late pregnancy and non-pregnant guinea-pigs were sacrificed by a blow on the head. Blood was collected by heart puncture before the removal of organs. Blanched tissues were homogenized in Tris–sucrose buffer (3 g wet wt./1 ml) in a Pyrex tissue grinder with Teflon pestle. Homogenates were stored frozen.

Sonification of blanched tissues in Tris–sucrose buffer (3 g wet wt./1 ml) or of homogenates was carried out with a cell disrupter (Heat Systems, Inc.) for 1 min at 60 W or 3–4 min at 40–50 W in a methanol dry ice mixture or ice bath. The sonified tissues were centrifuged for 1 h at 27,000 ***g*** in an International Centrifuge (Model B-20 No. 870 rotor, 12,000 rev./min) and the supernatants were stored frozen.

Ten ml of sonified tissue was equilibrated with 2 μCi of tritiated progesterone (in 200 μl ethanol) under gentle shaking overnight at 5° C.

A Diaflow filter, UM20E, with a model 52 filtration chamber (Amicon Co.) under 50 psi pressure was used for the filtration of low mol wt. substances and the concentration of macromolecules of over 25,000 mol wt. The sample was first concentrated to 0·25 of its vol. and then twice diluted to twice its original vol. and reconcentrated each time. Chromatography through Sephadex G-25, medium grade, from Pharmacia was carried out at 5° C on 0·7 cm dia columns, varying between 31 and 35 cm in length. Sample size was 0·5 or 1 ml, the eluting buffer Tris–KCl buffer, the flow rate 0·2 to 0·8 ml/min, and fractions of 1·1 to 2 ml were collected. Optical density of the effluant was measured continuously by an ISCO model UA2 u.v.-analyzer and 200 μl aliquots were counted for tritium.

A 6–21% sucrose density gradient was produced in a 12 ml tube by a Beckman density gradient former. A 0·2 or 0·4 ml sample was overlayered and centrifugation was performed for 22 h at 41,000 rev./min in a Beckman ultracentrifuge model L2-65B with the SW41–661 rotor. Fractions of 0·1, 0·5 or 1 ml were collected from the bottom. Human IgG, 7S (A grade, Calbiochem) and bovine serum albumin were used for sedimentation standards. Protein concentrations were determined by the method of Lowry [17], with bovine serum albumin as standard.

Counterimmunoelectrophoresis was performed on glass microscope slides (76 × 25 mm) covered with a solution of 1% agarose (Fisher Scientific) in 0·025 M veronal buffer, pH 8·6. Wells holding 10 μl were placed 0·5 cm apart. The wells closer to the cathode were filled with the test solution and those closer to the anode with antisera [18]. Shandon Company equipment was used for electrophoresis of 10–15 min duration of 50 V and about 2 mA/slide.

Radioimmunoassay was performed by two methods. In the first method, guinea-pig transcortin rabbit antiserum was polymerized with ethyl chloroformate [19], hand homogenized and used as a suspension at about 0·25 of the concentration of the original antiserum. A 100 μl sample was mixed with 100 μl of polymerized antiserum suspension in a 6 × 55 mm test tube and shaken gently for 3 days at 5° C [20]. Ten μl of a solution of ^{125}I-guinea-pig transcortin, prepared by a modified chloramine-T method of Greenwood and Hunter [21, 22], was added and the mixture was shaken at 5° C for 2 h. The sediment was washed three times before it was counted. To wash the sediment, borated saline was added, the mixture was agitated for 15 s on a vortex mixer, centrifuged at 2000 rev./min (1000 ***g***) in an International Centrifuge and the supernatant was removed.

The second method is a double precipitation method; it was later superceded by the first method. In brief, sample and antiserum were incubated overnight at room temp, iodinated guinea pig transcortin was then added, followed 1 h later by antirabbit γ-globulin goat serum. The precipitate was washed as before, except that the precipitate was allowed to settle for 5 h between agitation and centrifugation.

Some additional details are noted in the experimental section.

EXPERIMENTAL

Differential centrifugation and immunological tests on sediments and supernatants

Liver and kidney from a pregnant guinea-pig had been stored frozen. The thawed tissues were blanched in three changes of 300 ml each of Tris–sucrose buffer over 3 h at 5° C. Tissue pieces of about equal size were homogenized with a loose fitting Potter hand homogenizer with five times their vol. in buffer. Centrifugations were performed consecutively at about 700, 35,000 and 200,000 ***g***, and the pellets obtained after each centrifugation were washed once with Tris–sucrose buffer. The three pellets and the supernatant of the

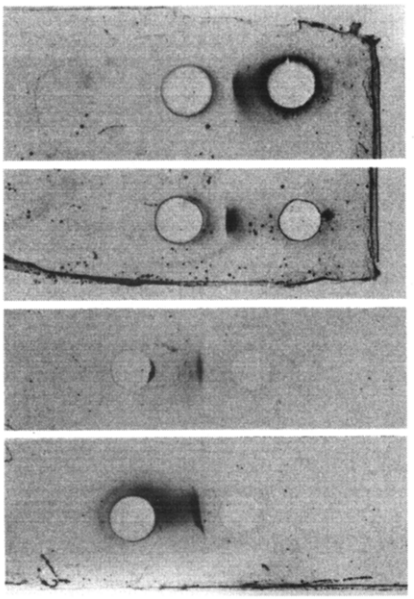
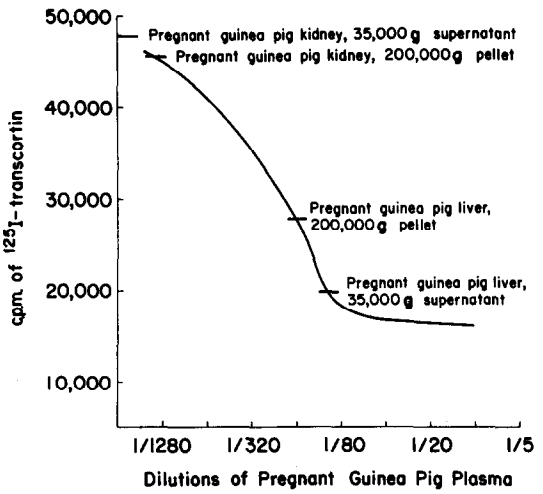


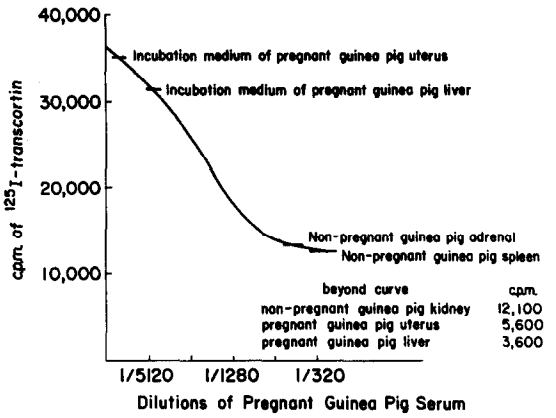
Fig. 1. Counterimmunoelectrophoresis of fractions of differentially centrifuged homogenate of pregnant guinea-pig liver against transcortin-specific rabbit antiserum. From top to bottom: 35,000 *g* pellet, 200,000 *g* pellet, 700 *g* pellet and 200,000 *g* supernatant.

200,000 *g* centrifuged pellet of the liver homogenate were tested by counterimmunoelectrophoresis (Fig. 1).

The supernatants of the 35,000 *g* centrifugation and the pellets obtained at 200,000 *g* of liver and kidney homogenates were compared by the radioimmunoassay of method 2 (Graph 1).



Graph 1. Radioimmunoassay (double precipitation method) of 35,000 *g* supernatants and 200,000 *g* pellets of pregnant guinea pig liver and kidney homogenates. The assay shows transcortin in liver samples, but hardly any in kidney samples.



Graph 2. Radioimmunoassay of 27,000 *g* supernatants of sonified tissues and incubation media. Highest concentrations of transcortin were in liver and uterus.

Immunological tests on sonified tissues

Tissue slices with a total wet wt. of 0·9 g of liver and uterus from a pregnant guinea-pig, and 0·8 g of kidney and 0·4 g of spleen and adrenal from a non-pregnant female guinea-pig were incubated in RPMI 1640 (~1 ml/g) at 37°C for 3·5 h. The tissues were separated from the incubation medium by centrifugation at 1000 *g*. They were blanched further by four successive incubations in small volumes of Tris–sucrose buffer at 5°C over a period of more than 12 h and centrifugation at 1000 *g* after each incubation. The blanched tissues were stored frozen, then thawed, sonified and centrifuged at 27,000 *g*. Only supernatants of the pregnant guinea pig samples were stored frozen again, until they were tested by the radioimmunoassay of method 1, together with the samples from the non-pregnant guinea-pig (Graph 2). Additionally, Graph 2 shows the results of the radioimmunoassay of the RPMI 1640 incubation media of uterus and liver.

Immunoelectrophoresis tests were performed on aliquots of the supernatants of the 27,000 *g* centrifuged, sonified tissues obtained by the method described in the previous paragraph. The experimental procedure was similar to that of counterimmunoelectrophoresis. The wells were filled with 10 μl samples and electrophoresis took place for 50 min. The centertrough was filled with anti-guinea-pig transcortin rabbit serum. The results of tests with samples of uterus and liver of the pregnant animal and of the kidney, uterus and liver of the non-pregnant animal are shown in Fig. 2. In Fig. 3 are shown the results of tests of sonified adrenal and spleen of the pregnant guinea-pig. These two samples were obtained by a slightly different procedure. The whole organs were blanched at 5°C in four changes of 500 ml of Tris–sucrose buffer over a period of 48 h.

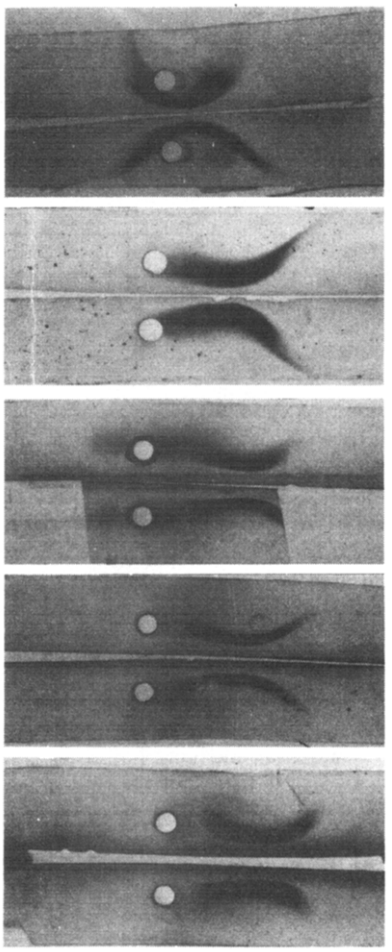


Fig. 2. Immunoelectrophoresis of 27.000 *g* supernatants of sonified tissues. From top to bottom: pregnant guinea-pig liver and uterus, female guinea-pig liver, kidney and uterus. Electrophoresis of the first slide was 35 min, of the other slides 50 min. Transcortin-specific antiserum in troughs.

They were homogenized, sonified and centrifuged at 27.000 *g*.

Coincidence of a progesterone binding macromolecule and antigenic transcortin in sonified blanched tissue

Uterus and liver of a pregnant animal were blanched at 5°C in four changes of 500 ml of Tris-sucrose buffer over a period of 48 h. They were homogenized and stored frozen for about a month prior to sonification. The sonified tissues were incubated with tritiated progesterone, centrifuged at 27.000 *g* and the supernatants were stored frozen. Thawed samples were recentrifuged at 27,000 *g* just prior to further experimentation. They will be referred to, henceforth, as recentrifuged supernatants.

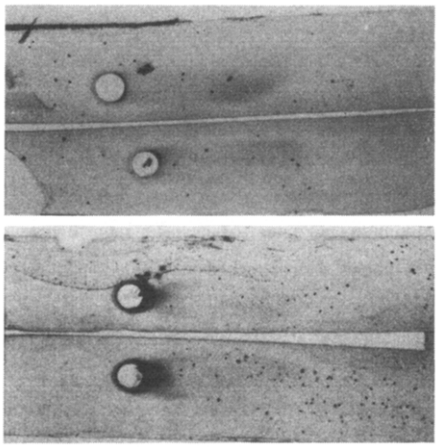
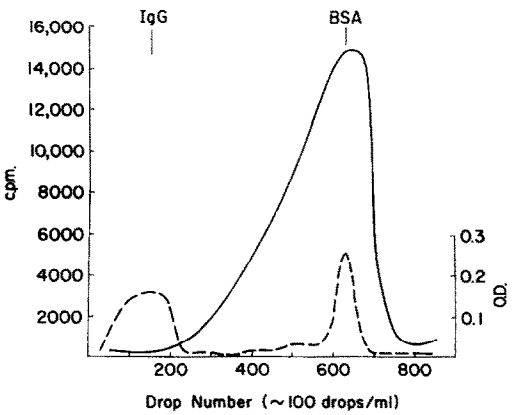


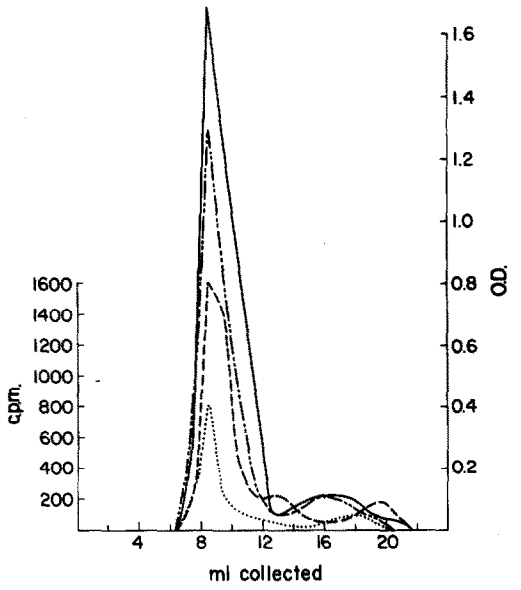
Fig. 3. Immunoelectrophoresis of 27.000 *g* supernatants of sonified adrenal (top) and spleen (bottom) of a pregnant guinea-pig. Transcortin-specific antiserum in trough. Precipitin lines are barely visible.

Aliquots of the recentrifuged supernatants from uterus and liver were chromatographed on Sephadex G-25. The tritium peaks, which followed immediately after the void vol., were rechromatographed on sucrose density gradient (shown for liver in Graph 3). All fractions were tested for transcortin by counterimmunoelectrophoresis.

A sample of the recentrifuged supernatant from liver was concentrated and dialyzed on a Diaflow filter UM20E. Aliquots were incubated with a transcortin-specific or a non-specific immunoadsorbant, the latter prepared by polymerization of the serum of a non-immunized rabbit [19]. The mixtures were shaken for 4 days at 5 C, centrifuged at 1000 *g*, and the supernatants were chromatographed on G-25 (Graph 4). Optical density at 280 nm was monitored and samples of



Graph 3. Sucrose density gradient of a 27.000 *g* recentrifuged supernatant of sonified pregnant guinea-pig liver: $O.D._{600\,nm}^{1\,cm}$ at a 3 in 10 dilution. —c.p.m./100 µl.



Graph 4. Chromatography on Sephadex G-25 of a 27.000 *g* recentrifuged supernatant of sonified pregnant guinea-pig liver. (——) $O.D._{280}^{1cm}$ and (– – –) c.p.m. after incubation with a non-specific immunoadsorbant, (— · · — · · —) $O.D._{280}^{1cm}$ and (·····) c.p.m. after incubation with a transcortin-specific immunoadsorbant.

the peak areas of O.D. and tritium following the void vol. were tested by counterimmunoelectrophoresis.

In another experiment, 75 μl aliquots of the recentrifuged supernatant from liver were incubated at 5°C for 12 h under shaking with 100 μl of a transcortin-specific or a bovine serum albumin-specific immunoadsorbant [19] suspension. The sediments were thoroughly washed as described under methods for radioimmunoassay and quantitatively transferred onto a piece of cotton, oxidized and counted for tritium.

RESULTS AND DISCUSSION

By the use of rabbit antisera produced to isolated guinea-pig transcortin [15], we were able to demonstrate the presence of transcortin in guinea-pig uterus and liver and to some extent in kidney. The antisera used were highly specific, showing only a minute trace of impurity in the α_2- or β-globulin region when tested against pregnant guinea-pig serum by two-dimensional immunoelectrophoresis (Fig. 4). A progesterone binding globulin of plasma has been reported to have an α_2- or β-globulin mobility [23]. Figure 5 shows lines of identity by the method of Ouchterlony [24] between purified transcortin, 105.000 *g* supernatant of sonified, blanched uterus, and pregnant guinea-pig plasma after immunodiffusion against guinea-pig transcortin rabbit antiserum.

We fractionated blanched, homogenized pregnant guinea-pig liver by centrifugation at different *g* forces and tested the washed pellets in addition to the cytosol in order to minimize the possibility of transcortin contamination from circulating blood. Figure 1 shows that all three washed pellets and the cytosol gave precipitin lines with anti-guinea-pig transcortin rabbit serum. A comparison of liver and kidney by radioimmunoassay (Graph 1) showed that transcortin was either absent in these kidney samples or they contained approx. 0·1 of

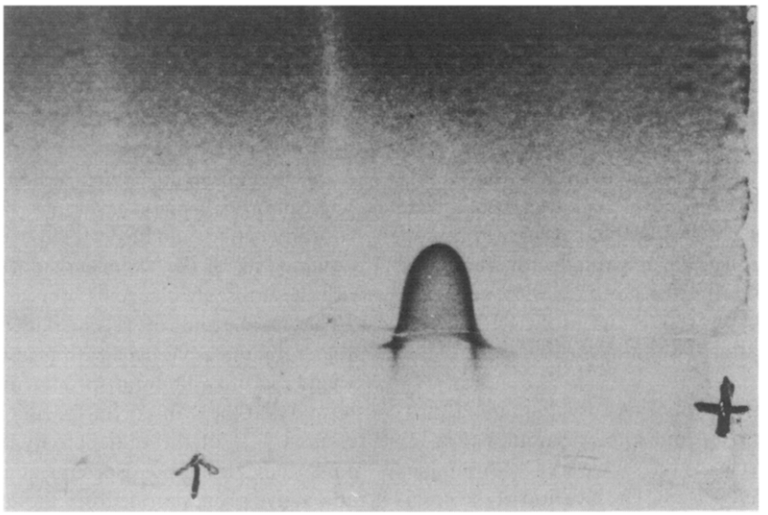


Fig. 4. Two-dimensional immunoelectrophoresis. Electrophoresis of pregnant guinea-pig serun with the anode to the right and the sample applied to the well above the arrow, followed by electrophc esis into a gel containing guinea-pig transcortin rabbit antiserum with the anode at the top of the picture.

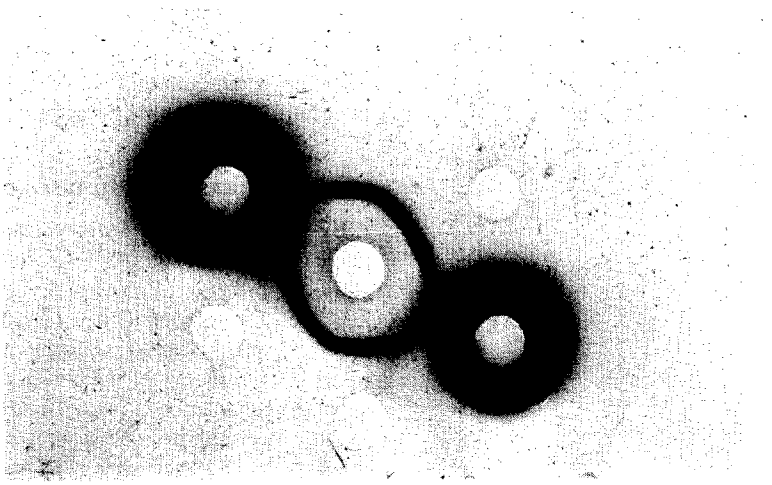


Fig. 5. Immunodiffusion of guinea-pig transcortin rabbit antiserum in the center well against clockwise from top guinea-pig transcortin (0·2 mg/ml), pregnant guinea-pig plasma (20%), 105,000 *g* supernatant on sonified, blanched uterus of pregnant guinea-pig, and duplicates of transcortin, plasma and uterus.

the quantity of transcortin found in liver samples. Radioimmunoassay was chosen for the detection of transcortin in these samples because of the high sensitivity of the assay rather than for quantitation; precise aliquots of tissue fractions were not applied in this assay.

The radioimmunoassay on blanched, sonified tissues (Graph 2) showed some transcortin in kidney, spleen and adrenal of a female non-pregnant guinea-pig. If we compare the quantities found in these tissues with the concentration of transcortin in guinea-pig serum [25], we can estimate that the blanched tissues would have had to retain about 6% of their own wt. in whole blood to give the results shown. Thus, it seems that part of the transcortin found may be cellular. In samples of uterus and liver of a pregnant guinea-pig, we measured transcortin levels far in excess of the accompanying standards (Graph 2) and far higher levels than found in spleen, a blood-rich organ. That the tissues were thoroughly blanched, and that the measured transcortin resided either in the cells or strongly adhered to the cells, is also indicated by the fact that only negligible amounts of transcortin were found in the RPMI 1640 incubation media after the initial incubations of liver or uterus.

Precipitin lines were obtained by immunoelectrophoresis of uterus, liver and kidney samples (Fig. 2), while almost no precipitin lines were seen with samples of adrenal and spleen (Fig. 3). The location of the precipitin line is the same as that for transcortin.

A progesterone-binding macromolecule in liver and uterus appeared on sucrose density gradient just ahead of BSA (Graph 3). We did not find a progesterone binder with a peak in the 6·7 S region as reported by Milgrom *et al.* [8], possibly because, unlike other investigators [3, 8], we sonified the tissue homogenates. We found a very broad peak for the progesterone binder(s), with the fractions in the peak area giving the strongest precipitin reaction against transcortin-specific antiserum.

A more direct proof for the identity of a progesterone-binding macromolecule with transcortin (or an antigenically related molecule) in liver is demonstrated by Graph 4. Chromatography of liver samples after incubation with a transcortin-specific immunoadsorbant shows a loss of about 50% progesterone in the protein peak as compared to a sample that was incubated with a non-specific immunoadsorbant. The latter sample still contained transcortin, as could be shown by a strong precipitin line produced by counterimmunoelectrophoresis against transcortin-specific antiserum. No transcortin could be detected by this method in the protein peak of the sample that had been incubated with the transcortin-specific immunoadsorbant.

The incubation of recentrifuged supernatants of pregnant guinea-pig liver with transcortin-specific and bovine serum albumin-specific immunoadsorbants showed that the transcortin-specific immunoadsorbant retained 32% of the radioactivity, whereas the albumin-specific immunoadsorbant did not adsorb any radioactive progesterone from the sample.

In a similar experiment conducted on uterus, liver and kidney of a non-pregnant female guinea-pig, we could show qualitatively, by direct counting of the im-

munoadsorbant, that progesterone was bound to the transcortin-specific immunoadsorbant, but not to an albumin-specific immunoadsorbant.

A strong adherence of progesterone to transcortin bound to an immunoadsorbant does not preclude the existence of a weaker association of progesterone to transcortin in plasma, since the transcortin bound to antibodies has probably lost some freedom of intrastructural mobility. If guinea-pig serum is preincubated with tritiated progesterone and a precipitate with antiserum is subsequently developed in agarose gel at 5°C, the progesterone label will remain in the precipitate and is not removed by extensive washings. In contrast, radioactive progesterone will not bind to an antigen-antibody precipitate already formed in agarose gel prior to the incubation with the progesterone. Cortisol behaves slightly different; not only will cortisol remain in a precipitate formed subsequently to the incubation of guinea-pig serum with tritiated cortisol, but it also binds to a precipitate formed prior to the addition of cortisol.

The origin of the tissue transcortin remains unknown. It may have been synthesized by the tissues in which we detected it, notably liver and uterus, or it may have diffused into the cell from the circulation. The latter possibility had been postulated and verified by a computer analysis [26]. Some blood proteins are known to enter cells [27, 28]. The penetration of α-globulins and albumin into the cellular space had been observed and measured for rat uterus [29]. Transcortin may also have been adsorbed on the cell surface from blood plasma of interstitial spaces, with some organs showing a preferential adsorption over other organs [30]. Since the interstitial plasma space remaining after blanching was not measured, the transcortin found could be in part plasma transcortin.

The transcortin detected by our antiserum may not be identical to plasma transcortin, but may also be only antigenically related to it. This work presents only a beginning in the comparison of cellular and plasma progesterone and/or cortisol-binding proteins by immunological techniques. A closer inspection of identities and differences of these antigenically related compounds awaits future investigation.

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