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BINDING OF STEROIDS BY PLASMA OF A TELEOST: THE RAINBOW TROUT, *SALMO GAIRDNERII*

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SUMMARY

Steroid binding in female trout plasma has been studied. Two thermolabile binding systems are detected by saturation analysis and competitive assays. One is a "sex binding protein" with high affinity for testosterone ($K_{4°C} = 3{\cdot}8 \times 10^8$ M^{-1}) and oestradiol-17β ($K_{4°C} = 1{\cdot}8 \times 10^8$ M^{-1}). The second is a "transcortin type" system binding testosterone ($K_{4°C} = 3{\cdot}7 \times 10^7$ M^{-1}), progesterone ($K_{4°C} = 2{\cdot}2 \times 10^7$ M^{-1}), oestradiol-17β ($K_{4°C} = 1{\cdot}4 \times 10^7$ M^{-1}) and corticosteroids. Association constants and capacities determined by gel filtration or by equilibrium dialysis are compared. As in other fish, measured capacities are higher than for mammalian binding systems.

Whether the two kinds of sites are on a single or two proteins is discussed. Competitive assays performed with fractions of a DEAE-cellulose chromatography of plasma show a partial separation. But only one peak of radioactivity sediments in the 5–6 S zone when plasma is ultracentrifuged with tritiated steroids in a sucrose gradient.

To test the steroid inactivation by binding, the induction of maturation trout oocytes *in vitro* by 17α-hydroxy-20β-dihydroprogesterone has been used as biological assay. 17α-Hydroxy-20β-dihydroprogesterone activity is suppressed by plasma binding.

Biochemical and biological results are compared and discussed.

INTRODUCTION

Different systems of protein steroid interactions have been recognized in the plasma of several mammalian vertebrates: albumin without specificity [1], α_1-acid glycoprotein or orosomucoid binding progesterone [2], transcortin or corticosteroid binding globulin [3, 4], sex binding protein [5], progesterone binding protein from pregnant guinea-pig [6] and foetal protein [7, 34].

Plasma binding of corticosteroids (cortisol or cortisone) has been demonstrated in all non-mammalian vertebrate classes which have been investigated by Seal and Doe[8]. Martin *et al.*, have found in the sera of four amphibian species a sex binding protein and a corticosteroid binding protein [9–12]. Using an electrophoretic technique, Corvol and Bardin detected testosterone binding in amphibia and fishes, but not in birds [12].

In fish, corticosteroids are always bound by blood serum [8, 11, 13–15]. Testosterone binding occurs in the blood of the carp [12], atlantic salmon, atlantic cod [13], halibut and bowfin [16]. However, whether there are one or several systems remains unknown since no competition studies have been done. More detailed studies have been undertaken in only three species: skate, *Raja radiata* [14, 17, 18], dogfish, *Scyliorhinus canicula* [15] and lamprey, *Petromyzon marinus* [9, 11]. In the elasmobranch sera C_{18}, C_{19} and C_{21} steroids are bound by one protein fraction, which has been partially purified in skate by Idler and Freeman[7, 18].

In the cyclostome two electrophoretic fractions bind both protesterone and oestradiol-17β.

The present work deals with protein steroid interactions in the plasma of the trout, *Salmo gairdnerii*.

EXPERIMENTAL

Animals

Three- or four-year-old ripe female trout were purchased from a private fish farm during the spawning period. Blood was collected from the heart in heparinized tubes and centrifuged. After pooling plasma, endogenous steroids were removed with charcoal [20]. Samples were stored at −20°C, a temperature at which Idler and Freeman did not find degradation of corticosteroid binding in salmon plasma [21].

Steroids

[1,2,6,7-^{3}H]-Progesterone (110 Ci/mM), [2,4,6,7-^{3}H]-oestradiol-17β (100 Ci/mM), [1,2,6,7-^{3}H]-testosterone (87 Ci/mM) have been provided by the Radiochemical centre (England). [1,2-^{3}H]-Cortisone (9 Ci/mM) was obtained from the Commissariat à l'Energie Atomique (France). They were chromatographically pure. Unlabelled steroids were purchased from Steraloids (U.S.A.) and Merck (Germany), and stored in ethanol solutions at a concentration of 100 μg/ml. Before use, an aliquot was diluted in buffer, 0·1 M phosphate–0·05 M NaCl, pH 7·8, to give a final concentration of ethanol less than 1‰.

Sephadex filtration

Micro-columns of Sephadex (G-25 fine) were used to separate bound and unbound steroids [22, 23].

Glass columns 0·9 × 15 cm. are packed with swelled Sephadex (0·5 g dry) to give a 3·5 cm. length bed of gel. The 0·5 ml incubate was placed on the top of the column, the eluate was discarded, and then the bound fraction was eluted with 1·5 ml phosphate buffer and counted for radioactivity in a liquid scintillation spectrometer (Packard) with an efficiency of 30%.

Equilibrium dialysis

One ml diluted plasma (1/40) was dialyzed for 48 h at 4°C (dialysis tubing 18/100, Union Carbide Corporation, U.S.A.) against 15 ml phosphate buffer containing tritiated steroid (10^{-10} M) and various amounts of the same non-radioactive steroid (10^{-10} M to 10^{-8} M). After dialysis, 1 ml aliquots taken from inside and outside the bag were counted for radioactivity. A Scatchard plot was used to determine association constants [24].

Competitive binding

Plasma was diluted in phosphate buffer, and incubated with tritiated and/or unlabelled steroids in a plastic tube at 4°C for at least 4 h. A plasma dilution was chosen to obtain 50% binding of the tritiated steroid (5×10^{-10} M) without competitive unlabelled steroid. Tritiated steroid (10^{-10} M) and non-radioactive steroid (10^{-9} M to 5×10^{-8} M) were simultaneously incubated at 4°C with 0·5 ml of diluted plasma. All binding assays were conducted in triplicate. As an index of binding specificity we used the "Relative Potency Estimate" described by Pichon and Milgrom[25].

DEAE cellulose chromatography

Trout plasma was dialyzed for 16 h at 4°C against Tris 0·01 M–NaCl 0·05 M, pH 8, and then fractionated on DEAE cellulose Whatman DE23 with a discontinuous gradient of Tris–NaCl as described by Boffa *et al.*[11]. Each protein peak was dialyzed against phosphate buffer, concentrated against 50% glycerol and then used for competitive assays.

Protein concentration was measured by means of the technique of Lowry *et al.* with bovine serum albumin as standard protein [26].

Polyacrylamide gel electrophoresis

Electrophoresis was performed at 4°C in the polyanalyst apparatus (Buchler Instrument Inc., U.S.A.) with anionic system (pH 9·3), Tris–glycine (pH 8·9) and Tris–HCl (pH 8·07) respectively used for upper and lower buffer.

Sucrose gradient ultracentrifugation

Diluted plasma (1/10) was incubated with tritiated steroid (10^{-10} M) and 0·2 ml was centrifuged in a 5 to 20% sucrose gradient in phosphate buffer. Sedimentation coefficients were evaluated by means of serum albumin simultaneously run as a reference ($S_{20,w} = 4{\cdot}6$) according to Martin and Ames[27].

Biological assay

As described previously by Jalabert *et al.*[28, 29], progestagens and principally 17α-hydroxy-20β-dihydroprogesterone are able to induce the meiotic maturation of trout oocytes *in vitro*. In order to test the possible inactivation of bound steroid, we measured the 17α-hydroxy-20β-dihydroprogesterone efficiency in inducing oocyte maturation when trout plasma was used instead of physiological salt solution as incubation medium.

RESULTS

Binding characteristics of steroids

Tritiated progesterone, testosterone, oestradiol-17β and cortisone (10^{-10} M) are bound by trout plasma, and association constants and capacities have been measured for the first three steroids by two separate techniques (Table 1). Results are similar for association constants. Equilibrium dialysis gives higher values for capacities than gel filtration. Testosterone and oestradiol-17β are each bound by two systems (S-I, S-II). For these steroids the association constants of the second system are about ten times less than association constants of the first system, but capacities are four to five times greater. Progesterone is bound by only one system whose affinity is similar to affinities of testosterone and oestradiol S-II systems. Relative affinities calculated with competitive studies corroborate the presence of two binding systems whose decreasing order of affinity is: testosterone, oestradiol-17β, for S-I, and testosterone, progesterone, oestradiol-17β, corticosteroids for S-II (Table 2). A strong testosterone affinity is always observed. There is an increasing affinity with hydroxylated progesterone in the 17α or 20β position. But hydroxylation of deoxycorticosterone in the 17α position does not

Table 1. Sex steroid binding by female trout plasma

		Testosterone		Oestradiol-17β		Progesterone
		SI	SII	SI	SII	SII
Association constant (l./mol)	Gel filtration	$2{\cdot}2 \times 10^{8}$	$5{\cdot}9 \times 10^{7}$	$1{\cdot}5 \times 10^{8}$	$5{\cdot}6 \times 10^{7}$	$5{\cdot}9 \times 10^{7}$
	Equilibrium dialysis	$3{\cdot}8 \times 10^{8}$	$3{\cdot}7 \times 10^{7}$	$1{\cdot}8 \times 10^{8}$	$1{\cdot}4 \times 10^{7}$	$2{\cdot}2 \times 10^{7}$
Binding capacity (mol/l.)	Gel filtration	$1{\cdot}3 \times 10^{-6}$	$1{\cdot}8 \times 10^{-6}$	$1{\cdot}5 \times 10^{-6}$	$1{\cdot}8 \times 10^{-6}$	$1{\cdot}5 \times 10^{-6}$
	Equilibrium dialysis	$2{\cdot}4 \times 10^{-6}$	8×10^{-6}	2×10^{-6}	$11{\cdot}6 \times 10^{-6}$	10×10^{-6}

Table 2. Relative steroid affinities for protein binding in trout plasma

Competing unlabelled steroids	Tritiated steroids P-^{3}H	E2-^{3}H	T-^{3}H
Testosterone	135	108	100
11-Ketotestosterone	35		15
Oestradiol-17β	39	100	23
Oestrone	10	10	
Oestriol	<1	<1	
Progesterone	100	10	19
17α-Hydroxyprogesterone	117	<1	
20α-Dihydroprogesterone	120		
20β-Dihydroprogesterone	111		
17α-Hydroxy-20β-dihydroprogesterone	98	<1	
11-Deoxycorticosterone	62	<1	<4
11-Deoxycortisol	62		
Cortisone	10		
Cortisol	<1		
20β-Dihydrocortisone	<1		

P = Progesterone, E2 = oestradiol-17β, T = testosterone.

Affinity indices are "Relative Potency Estimate" in % calculated according to Pichon and Milgrom[25]. RPE of steroid *b* relative to steroid *a* was obtained by dividing the mass of unlabelled steroid *a* necessary to displace 50% of the same radioactive steroid (10^{-10} M) by the mass of the unlabelled steroid *b* necessary to obtain the same effect.

increase affinity. Cortisone and cortisol are poor competitors, but elimination of the 11-hydroxyl group induces better competition. Similarly, addition of an 11-keto group to testosterone depresses its strong affinity.

Little competition occurs between oestradiol-17β and progesterone. Oestradiol-17β is a better competitor against progesterone than progesterone against oestradiol-17β, but oestrone and oestriol always have weak affinities.

Scatchard plots (Fig. 1) and competitive studies show two binding systems, which are both destroyed by heating the diluted plasma (1/40) at 60°C for 30 minutes (Table 3). Whether there are two proteins or two sites on one protein has been investigated.

Separation of protein binding systems

DEAE cellulose chromatography eluted with a discontinuous NaCl gradient gives six protein peaks (Fig. 2). Every one of them were tested for steroid binding (Table 4). Only the last three fractions (F4, F5, F6) are active. Progesterone competition against oestradiol-17β decreases from F4 to F6. In the same way oestradiol competes against progesterone more in F6 than in F4, and F6 has a greater specific activity for oestradiol. It appears that there are more progesterone sites in F6 and more oestradiol sites in F4. Specific activity of testosterone is higher in the fractions F4, F5, F6. DEAE cellulose chromatography does not give a clear separation between binding systems, but the more electronegative F6 fraction contains more S-II sites than F4.

Table 3. Thermolability of steroid protein-binding in trout plasma

Bound steroid	B/T before heating	B/T after heating (60°C × 30 mn)
Progesterone	73·0	4·1
Oestradiol-17β	56·0	2·7
Testosterone	73·0	5·2
Cortisone	28·1	0

Diluted plasma (1/40) was heated at 60°C during 30 minutes.

B = Bound steroid, T = total steroid (5×10^{-10} M).

B/T is given in per cent.

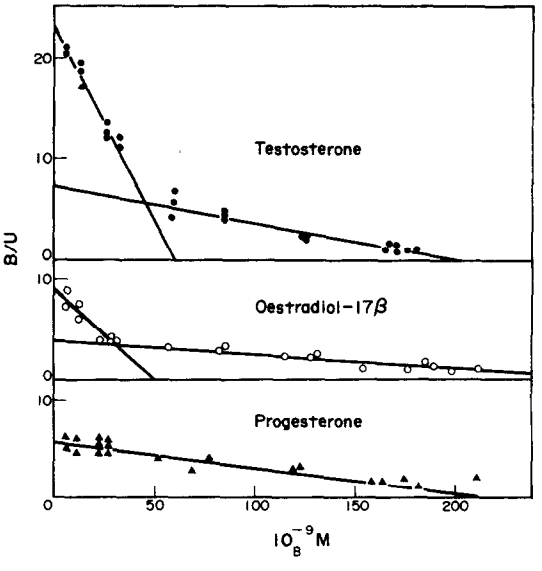

Fig. 1. Scatchard plot for equilibrium dialysis of plasma female trout. Diluted plasma (1/40) was dialysed against testosterone (●—●—●), oestradiol (○—○—○) or progesterone (▲—▲—▲). B/U = Bound/Unbound. B = Bound.

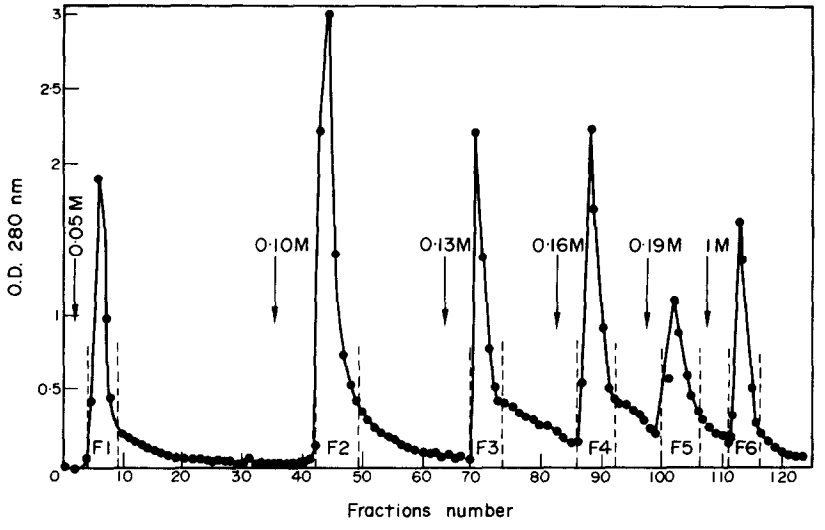

Fig. 2. Trout plasma was fractionated on DEAE-cellulose Whatman DE23 ($\phi = 9$ mn, $1 = 320$ mn, flow rate = 20 ml/h). The elution is performed with a discontinuous gradient of NaCl (0·05 M, 0·10 M, 0·13 M, 0·16 M, 0·19 M, ·1 M in Tris 0·01 M–HCl pH 8 buffer as described by Boffa *et al.* (1972). After concentration against 50% glycerol each protein peak was used for competitive assay.

Plasma electrophoresis with tritiated steroids gives only one peak of radioactivity with progesterone or cortisone, but two peaks with testosterone or oestradiol-17β (Fig. 3). One of these two peaks is four to five times smaller than the other. The peaks of ^{3}H-progesterone and ^{3}H-cortisone and the largest peak of ^{3}H-testosterone and ^{3}H-oestradiol have the same R_F (0·63). But a single peak of radioactivity always sediments in the 5–6 S zone in sucrose gradient ultracentrifugation (Fig. 4).

Therefore the more electronegative protein band can bind testosterone and oestradiol-17β but not progesterone and cortisone like S-I. The other protein band can bind all steroids like S-II. Electrophoresis was run also with male plasma. We obtained identical results for male and female plasma.

Inactivation of steroid by plasma binding in an in vitro *biological assay*

17α-Hydroxy-20β-dihydroprogesterone induces *in vitro* meiotic maturation of trout oocytes. Vitellus migration and germinal vesicle breakdown are

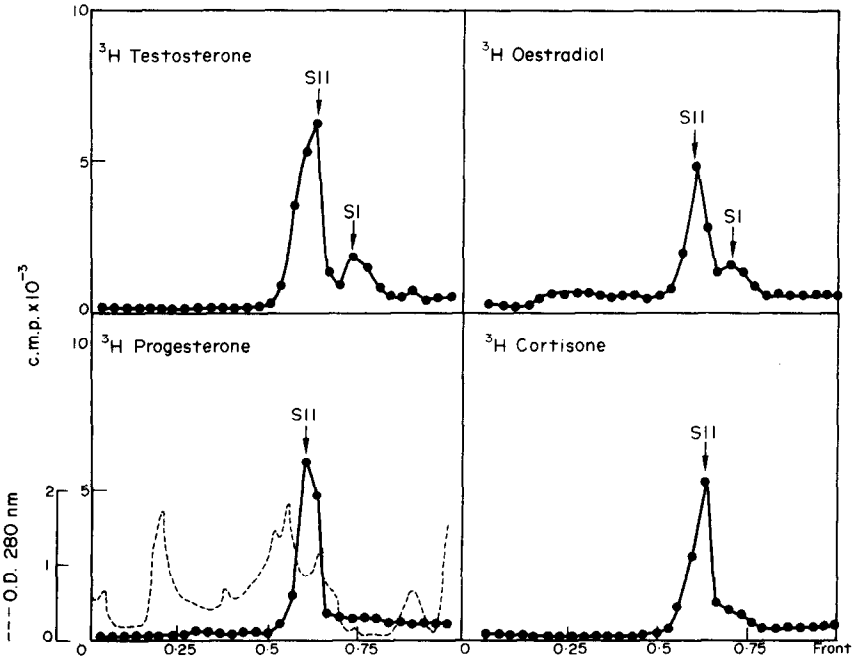

Fig. 3. Tritiated steroids were incorporated both in gel and plasma (about 10^{-9} M). After electrophoresis the migration gels were frozen at −20°C and cut into 2 mm slices which were agitated during the night in 10 ml scintillation fluid and then counted.

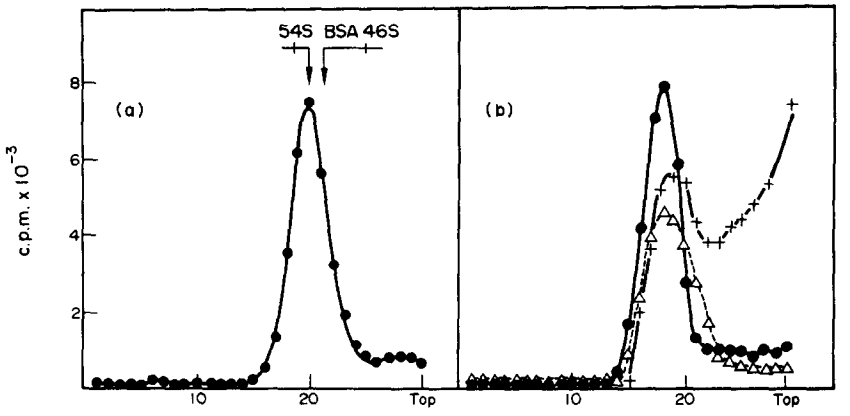

Fig. 4. Radioactivity pattern after sucrose gradient ultracentrifugation of trout plasma incubated with tritiated steroids. 0·2 ml Diluted plasma (1/10) incubated with 10^{-10} M ^{3}H-testosterone (A), ^{3}H-progesterone (B, ●—●—●), ^{3}H-oestradiol-17 (B, O—O—O) or ^{3}H-cortisone (+ – + – +) are layered on the top of a 5 to 20% sucrose gradient and centrifuged at 4°C for 18 h at 37,000 rev./min in the SW 30 rotor of a Spinco L50 ultracentrifuge. About 30 fractions were collected by perforating the bottom of the cellulose nitrate tubes and counted. Bovine serum albumin (BSA) was used as reference.

observed within 72 h after the start of oocyte incubation. When incubation is performed in a physiological salt solution a steroid concentration of 5 to 30 ng/ml is sufficient to induce 50% maturation, but use of plasma as an incubation medium multiplies by at least ten the 17α-hydroxy-20β-dihydroprogesterone dose inducing 50% maturation (ED50). The measurement of the unbound steroid fraction gives values of the same magnitude of ED50 as in physiological salt solution (Table 5).

DISCUSSION

There is good evidence for the presence of at least two binding systems occurring in trout plasma. One binds oestradiol-17β and testosterone with high affinity and specificity. The other with lower affinity and specificity binds testosterone, progesterone, oestradiol-17β and corticosteroids. The former system S-I could be a sex binding protein (SBP) and the latter S-II a "transcortin type" binding.

The good agreement of association constants determined by dialysis and gel filtration can be explained by the use of microcolumns in which dissociations are low [23]. This low dissociation is indicated by the detection of 50% bound progesterone after gel filtration of a 4% albumin solution incubated with 10^{-9} M tritiated progesterone.

Testosterone association constants in trout plasma are higher than the values reported by Freeman and

Table 4. Steroid binding with DEAE chromatography fractions

DEAE fractions	Protein concentration (mg/ml)	Progesterone ^{3}H B_1	Progesterone ^{3}H + oestradiol B_2	B_1/B_2	Oestradiol ^{3}H B'_1	Oestradiol ^{3}H + progesterone B'_2	B'_1/B'_2	Testosterone ^{3}H
F1	0·52	0	—	—	0	—	—	0·7
F2	0·61	1	—	—	0	—	—	1·3
F3	0·68	2·9	—	—	2·3	—	—	8·4
F4	0·74	42	26	1·6	45	18	2·5	80·6
F5	0·49	77·5	36	2·1	59	28	2·1	130
F6	0·49	30·6	15	2·1	36	26	1·4	62·4

Aliquots of DEAE fractions (0·3 ml) were incubated with tritiated steroids (10^{-10} M) or with tritiated (10^{-10} M) and unlabelled steroids (5×10^{-8} M). Binding indices (B) are calculated by dividing bound radioactivity used in the incubation. Indices are expressed for 1 mg/ml of protein. Competition index is obtained by dividing bound radioactivity without competition (B_1) by bound radioactivity with competition (B_2).

Table 5. Suppression of 17α-hydroxy-20β-dihydroprogesterone activity on oocyte maturation by plasma binding

Fish	ED50 in balanced salt medium (ng/ml)	ED50 in undiluted plasma (ng/ml)	Unbound steroid in undiluted plasma (ng/ml)	ED50 in 1/10 diluted plasma (ng/ml)	Unbound steroid in diluted plasma (ng/ml)
1	25·1	214·5	23	—	—
2	12·2	122	5	21·3	2
3	7·9	120	—	—	—

Idler for atlantic salmon [13]. The trout sex-steroid binding system is different from the skate and dogfish "Sex Hormone Binding Protein" ("SHBP") which bind C_{18}, C_{19} and C_{21} steroids with a higher affinity for [15, 17, 18] oestradiol than for testosterone. It resembles more closely the human SBP [31, 32] but with higher capacity. The trout "transcortin type" system binds progesterone and corticosteroids but also oestradiol and testosterone with a strong affinity. In this respect it resembles skate and dogfish "SHBP" but with weaker affinity and larger capacity. Mammalian corticosteroid binding globulin possesses 10 to 100 times higher association constants [33]. These high capacities have been also reported in the other fish species studied.

On DEAE-cellulose the binding fractions of trout plasma are eluted later than those of lamprey and dogfish [11, 15]. The last DEAE fraction which especially binds oestradiol may be associated with the faster electrophoresis peak (R_F: 0·73) obtained with oestradiol and testosterone and should be the sex binding protein.

As sucrose gradient ultracentrifugation of plasma gives only one radioactive peak with ^{3}H-oestradiol or ^{3}H-testosterone, the separation of S-I and S-II sites may be an experimental artefact. Dissociation by the experimental procedure has been postulated by Nunez *et al.*[34] for oestradiol-17β and corticosterone sites in the plasma of newborn rats.

Heat lability of steroid-binding in trout plasma is similar to human corticosteroid binding globulin [35, 36], human testosterone binding globulin [31] and salmon cortisol binding protein [13], whereas skate sex hormone binding protein is heat stable [17].

In trout, as in salmon plasma [14], cortisol has a lower affinity than cortisone. The 20β-dihydrocortisone, an original corticosteroid isolated in salmon [41] does not compete with progesterone. It is of interest to note that 11-ketotestosterone, which is an efficient physiological androgen in salmonids [18, 39, 40], competes poorly with testosterone. In the same way 17α-hydroxy-20β-dihydroprogesterone, which is the more active progestagen in inducing maturation of trout oocytes [30], has the weaker affinity of all the progestagens studied.

Pathological situations in man [42], metabolic clearance rate observation [43] and *in vivo* and *in vitro* biological assays in the rat [44, 45] corroborate the hypothesis of biological inactivation of steroids by plasma protein binding. Our biological assay agrees with this hypothesis. Plasma binding increases the 17α-hydroxy-20β-dihydroprogesterone ED50 for oocyte maturation *in vitro*.

When quantities of unbound steroid incubated with plasma are compared with the ED50 for incubation with physiological salt solution, it appears that only unbound steroid is active in inducing maturation. This fact might partly explain the great difference between the effective dose of 17α-hydroxy-20β-dihydroprogesterone for *in vitro* incubation conducted with a physiological saline medium and plasma hormone concentration reported *in vivo* by Schmidt and Idler[46]. From our competitive studies and the biological assay we have come to the conclusion that bound steroids are inactive, but physiological active steroid is less bound.

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