# Trout steroidogenic testicular cells in primary culture: 1. Changes in free and conjugated androgen and progestagen secretions: effects of gonadotropin serum and lipoproteins

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GENERAL AND COMPARATIVE ENDOCRINOLOGY 78, 374–387 (1990)

# Trout Steroidogenic Testicular Cells in Primary Culture

## I. Changes in Free and Conjugated Androgen and Progestagen Secretions: Effects of Gonadotropin, Serum, and Lipoproteins

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Accepted July 19, 1989

Isolated trout steroidogenic testicular cells were cultured for 10–15 days, either mixed with other round cells or after enrichment in interstitial cells. Free and conjugated progestagen and androgen secretions were assayed using specific radioimmunoassays (RIA). Free progesterone, 17α-hydroxyprogesterone (17α-OH-P), 17α-hydroxy,20β-dihydroprogesterone (17α,20β-OH-P), androstenedione, testosterone (T), and 11-ketotestosterone (11KT) were produced by testicular cells prepared from testes in spermatogenesis and mature testes. Discrete amounts of dehydroepiandrosterone (DHA) and of estradiol were secreted by mixed testicular cells prepared from mature testes, but no estradiol was detected in interstitial cell media. Conjugated androgens were produced by interstitial cells. While the production of progestagens by cells from spermatogenetic and mature testes either remained constant or increased throughout culture duration, those of free and conjugated androgens progressively decreased to low values whatever the components added to the medium. When salmon gonadotropin (s-GtH) was present permanently, androgen (free and conjugated) and progestagen secretions were stimulated for 3 to 4 days. When GtH was present discontinuously (1 day in every 3 days), the sensitivity of the cells was maintained for at least 7 days. While the GtH-stimulated/basal ratio was high for androgens, it was rather low for 17α20β-OH-P as compared to the values obtained with testis fragments. Trout serum (5%) stimulated the secretion of free and conjugated T and 11KT when testes were mature, but not when they were in spermatogenesis, while it stimulated 17α20β-OH-P secretion at the two stages. Total trout lipoproteins (125–500 μg/ml) stimulated 17α20β-OH-P secretion by cells from spermatogenetic testes, but not 11KT secretion. 

Annual variation of free and conjugated steroids in plasma of seasonal breeders such as salmonids have been well documented (Baynes and Scott, 1985; Kime and Manning, 1982). It appeared that (1) androgens and 17α-hydroxy,20β-dihydroprogesterone (17α20β-OH-P)[1] levels were low at the beginning of spermatogenesis, while increasing thereafter; (2) changes in 11-ketotestosterone (11KT) levels are preceded by similar changes in testosterone (T) levels; (3) at the time of milt production the fall in T levels is concomitant with an increase in 17α20β-OH-P; and (4) the ratio of conjugated:free androgens rises during the same period, the major glucuronide being that of T.

It is now clearly established that free and conjugated androgens are both major products of the salmonid testis (Idler *et al.*, 1971; Hews and Kime, 1978; Saad and Depêche, 1987; Schulz and Blüm, 1987a). However, if there is now evidence that the Leydig cells are the main source of testic-

[1] Steroid nomenclature: 17α-OH-P, 17-hydroxyprogesterone, 17-hydroxy-4-pregnene-3,20-dione; 17α20β-OH-P, 17α-hydroxy,20β-dihydroprogesterone, 17,20β-dihydroxy-4-pregnen-3-one; DHA, dehydroepiandrosterone; androstenedione, 4-androstene-3,17-dione; T, testosterone, 17β-hydroxy-4-androsten-3-one; 11KT, 11-ketotestosterone, 17β-hydroxy-4-androstene-3,11-dione; $E_2$, estradiol, 1,3,5(10)-oestratriene-3,17β-diol; 5-ene pathway, $\Delta^5$ pathway; 4-ene pathway, $\Delta^4$ pathway.



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ular steroids in fish, it is unknown if the glucuronyltransferase activity is associated with these cells. In any case, extragonadal steroid sources could also participate in the regulation of plasma androgens, such as interrenals (Schulz and Blüm, 1987b), liver, and blood cells (Schulz, 1986). 17α20β-OH-P has also been demonstrated to be a major testicular steroid (Ueda *et al.*, 1984; Saad and Depéche, 1987). Estradiol could be produced by the teleost testis (Fostier and Le Gac, 1987).

Until recently, a single carbohydrate-rich, "maturational" gonadotropic hormone (GtH) was assumed to control the salmonid testis function and was demonstrated to enhance androgen and progestagen production by testicular tissue *in vitro* (Ueda *et al.*, 1984; Le Gac and Fostier, 1987; Saad and Depéche, 1987; Schulz and Blüm, 1987a; Le Gac and Loir, 1988). Two biochemically distinct GtHs have been recently purified from salmon (Suzuki *et al.*, 1988). Their respective possible functions in the changes in the steroidogenic pathway are unknown. Furthermore, it cannot be excluded at the present time that nongonadotropin pituitary hormones, such as growth hormone for instance, may influence testicular steroidogenesis (Singh *et al.*, 1987).

Various mammalian steroid-secreting cells use preferentially lipoprotein-bound cholesterol for steroid synthesis rather than newly synthesized cholesterol and there is a species-dependent preference for the class of lipoproteins utilized (high-density lipoproteins (HDL) or low-density lipoproteins (LDL); Andersen and Dietschy, 1978; O'Shaughnessy and Wathes, 1985). In trout, annual qualitative and quantitative changes of the plasmatic lipoproteins have been demonstrated (Fremont and Marion, 1982) but it is unknown if they are related to changes in the gonadal steroidogenic activity.

A method has been recently proposed to maintain viable isolated trout somatic testicular cells in culture for 1–2 weeks, either mixed together or separated in at least two populations (Loir, 1988). In this paper, we have checked the possibilities, limits, and usefulness to develop more advanced research on the regulation of steroidogenesis, of this *in vitro* method. To do this, we have (1) followed the changes of either free or conjugated androgen and progestagen secretions *in vitro* for 10–15 days, (2) looked at the effects of gonadotropic stimulation, and (3) evaluated the ability of the cultured cells to respond to components added to the culture medium, such as trout serum and lipoproteins. We discussed and compared our results to data obtained either *in vivo* or *in vitro* with testis fragments. At the same time, we have obtained some original data dealing with the steroidogenic activity of the trout testicular cells.

## MATERIALS AND METHODS

### *Animals*

One- to 3-year-old males of summer-spawning, autumn-spawning, and spring-spawning strains of rainbow trout were used. They were kept in recycled freshwater at 12–15° under a natural photoperiod.

The maturation stage of the testes was determined according to Billard and Escaffre (1975). Briefly, stage III corresponds to the beginning of spermatogenesis, stages IV and V to spermatogenesis, stages VII and VIIIa to mature spermiating testes, stage VIIIb to testes approaching the end of spermiation, and stage IX to regressed testes. A total of 23 experiments were run for this study with two fishes at maturation stage III, four fishes at stage V, two at stage VI, two at stage VII, three at stage VII–VIIIa, six at stage VIIIa, and four at stage VIIIb–IX.

### *Preparation of Testicular Cell Populations and Culture*

All operations were carried out in sterile conditions at 12°. Modified L-15 Leibovitz medium (Loir, 1988) was used routinely except for testis dissociation. The technique used to dissociate trout testes at various maturation stages and to prepare testicular cell populations has been described in detail elsewhere (Loir, 1988). It is summarized in Fig. 1. Briefly, for every

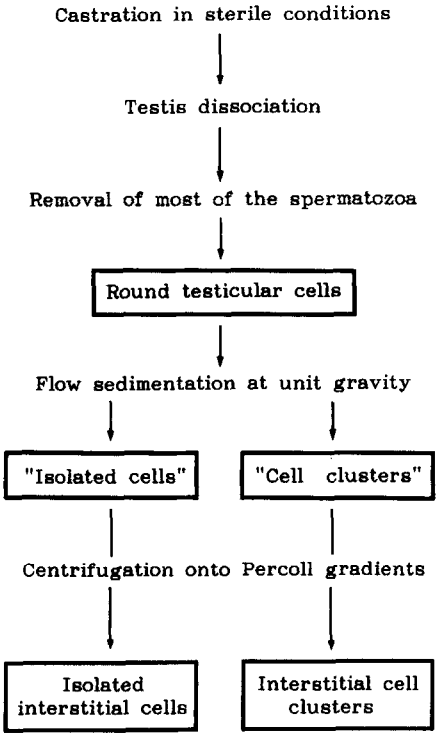


FIG. 1. Scheme of preparation of testicular cell populations (in squares). An average of 32 to 34 hr separated castration from seeding of the cells (Loir, 1988).

experiment, one of both testes of a fish was dissociated by perfusion for 5 to 6 hr with 0.8 mg collagenase/ml in Swim S77 medium plus 1 mg pronase/ml during the first hour. The cells were washed overnight in 1% bovine serum albumin (BSA). Then, most of the spermatozoa were removed by centrifugation after mixing with an isotonic Percoll solution. The resulting cell suspension containing somatic cells and variable numbers of germ cells and spermatozoa was fractionated into an "isolated cell" population and a "cell cluster" population. These two populations were fractionated by Percoll density gradient centrifugation and two interstitial cell populations (isolated cells and cell clusters) were collected. In terms of somatic cell composition, the interstitial cell (Leydig cells and fibroblast/myoid cells) populations were usually at least 95% pure, i.e., they contained less than 5% of Sertoli cells. Round germ cells and spermatozoa were also present in variable numbers according to the testes (Loir, 1988).

Routinely, cells were cultured in triplicates in plastic multiwell plates coated with fibronectin, Usually, 1.3 to 35 $\times$ $10^6$ round testicular cells/ml were seeded, depending on the maturation stage. Routinely, the culture medium was supplemented with 2% Ultroser (steroid-free serum substitute, IBF, France). Highly purified salmon gonadotropin (s-GtH; Breton *et al.*, 1978) was either added or omitted in the range of 0–180 ng/ml.

## *Preparation of Trout Serum and Lipoproteins*

Trout serum was prepared in the classical way from juvenile animals (50 to 120 g) because plasmatic steroid and GtH concentrations are very low at this age. Total trout lipoproteins were prepared from males at the same maturation stage as those used to prepare the testis cells (stages V or VI). They were prepared according to Fremont and Marion (1982) (centrifugation at 150,000*g* during 40 hr at 10° after the density of serum was raised to 1.21 g/ml). They were exhaustively dialyzed against 0.15 *M* NaCl and then against culture medium and finally stored at 4° being used less than 3 weeks after preparation. Antibiotics were present throughout preparation and storage. Protein was determined according to Fremont and Marion (1982). Total cholesterol was determined by the enzymatic method (Biochemica test combination Boehringer). After electrophoretic analyses, around 50% of the obtained lipoproteins were HDL. They contained negligible amounts of steroids and GtH. The concentrations of (lipo)proteins used were 125, 250, and 500 μg/ml culture medium (equivalent to about 1.25, 2.5, and 5% serum) and corresponded respectively to 17, 34, and 68 μg total cholesterol/ml.

## *Steroid Radioimmunoassays*

Steroids were assayed in the culture medium after extraction with ethylacetate:cyclohexane (50:50), except progesterone which was extracted with hexane. No chromatography was carried out.

Progesterone (P) was assayed according to Lecouteux *et al.* (1985). The main cross-reactivities, expressed as the ratio of the mass of the assayed steroid to the mass of steroid required to decrease bound levels of the tracer of half its value without competitor, were under 5% with the other progestagens.

*17α-Hydroxyprogesterone (17α-OH-P)*: The radioimmunoassay (RIA) was performed as for 11KT (Fostier *et al.*, 1982). The anti-17α-OH-P-3-CMO BSA was purchased from Steranti. The main cross-reactivities with progestagens, androgens, and estrogens were under 3%.

*17α-Hydroxy-20β-dihydroprogesterone*. RIA was performed according to Fostier and Jalabert (1986). Cross-reactivities with progestagens and other steroids were equal to or under 2%.

*Dehydroepiandrosterone (DHA)*. RIA was performed according to Garnier *et al.* (1978). The anti-

body used was against DHA-15α-$CH_2$-CO BSA. Cross-reactivities with related steroids were equal to or under 3.5%.

*Androstenedione*. The antibody against 4-7-carboxyethylthioether ovalbumen was purchased from Steranti. The main cross-reactivities were with $\Delta^5$ androstenedione (60%) and with 5α-androstanedione (67%). With androsterone, DHA, T, estrone, and P, cross-reactivities were under 2%. The RIA was performed as for 11KT.

*11-Ketotestosterone*. RIA was performed as described by Fostier *et al.* (1982). The main cross-reactivities with the other androgens were under 2%.

*Testosterone*. RIA was performed according to Fostier and Jalabert (1986). The main cross-reactivities were with 5α-dihydrotestosterone (41%), 11KT (31%), androstenedione (14%), and 5α-androstane-3α,17βdiol (6.2%). With the other androgens the cross-reactivities were under 3%.

*Estradiol-17β ($E_2$)*. The procedure described by Terqui *et al.* (1973) was used. The main cross-reactivities were with estrone (29%) and epiestriol (16%).

*Conjugated steroids*. The aqueous residues remaining from the extraction by ethylacetate:cyclohexane were treated for 16 hr at 37° with 1.7% *Helix pomatia* enzyme solution (SHP, IBF, France; 1700 Fishman units of β-glucuronidase and 17,000 Roy units of sulfatase) followed by ethylacetate:cyclohexane extraction and RIA for 17α20β-OH-P, "total androgens," testosterone, and 11KT. In two experiments, the enzymatic treatment was carried out on half of the samples before organic extraction (total steroids) while the second half was processed as above (free steroids and conjugated steroids). The total androgens RIA was performed as for 11KT (Fostier *et al.*, 1982) using an antibody against testosterone-3-CMO BSA. The main cross-reactivities were with 5α-dihydrotestosterone (84%), 11KT (61%), 5α-androstane-3α,17β-diol (89%), 5α-androstane-3β,17β-diol (26%), and androsterone (18%).

The sensitivity was 5 pg/ml for the androgens, 17α-OH-P and 17α20β-OH-P, 2 pg/ml for $E_2$, and 10 pg/ml for P. The intraassay coefficients of variation were under 10% for all the steroids. To avoid interassay (CV ≤ 15%) variations, all samples from the same experiment were usually measured in the same series. Throughout, three wells were used during culture for each treatment and control and, usually, steroids were assayed in triplicate. Two group comparisons were made by Student's *t* test. However, because there was only 400 or 1800 μl medium per well and the concentration of some steroids was low, when three or four or more steroids were assayed, one or two were assayed in triplicate wells while the others were assayed in the pool of the three remaining culture media. In this case, no standard error (SE) could be calculated. When SE was calculated, the coefficient of variation was usually equal to or lower than 12%.

## RESULTS

### *Free Steroid Secretion in the Presence of Ultroser (Figs. 2–4)*

Free steroids were assayed in the culture medium from round testicular cells, isolated testicular cells, testicular cell clusters, and sometimes from interstitial cell populations (Fig. 1) obtained from testes at various maturation stages. For all the assayed steroids and for maturation stages III to VIIIa, production was far higher than the cell contents at the time of seeding. On the contrary, at stage IX, steroid production was very low.

At maturation stages VII and VIIIa (8 experiments), whatever the cell population, P production usually increased slowly or at least remained unchanged until Day 7.5 or more.

At stages III to VIIIa (11 experiments), 17α-OH-P either remained constant or increased at least until Days 7.5–8.

At stage III (1 experiment), 17α20β-OH-P was not secreted during the first 2.5 days but it was secreted later with a peak at Day 8. At stages V and VI (4 experiments), 17α20β-OH-P secretion either remained constant or increased slightly throughout culture duration, depending on the testicular cell populations and on the animals. At stages VII and VIIIa (10 experiments), it usually increased up until Days 7.5–8. From stage VI to stage VIIIa, the ratio of the GtH-stimulated production of 17α20β-OH-P and 11KT (17α20β-OH-P/11KT), after 1, 2, or 3 days in culture, was always lower than 0.4. With testes at stage IX (regressed after spermiation) and testes still spermiating but assumed to be at stage VIIIb, 17α20β-OH-P production became predominant over androgen production. The ratio was then higher than 1 and rose to 16 in one fish.

Testicular cells from testes at stages VI and VII secreted low amounts (5 to 60 pg/ml/day) of DHA after 1 and 2 days in cul-

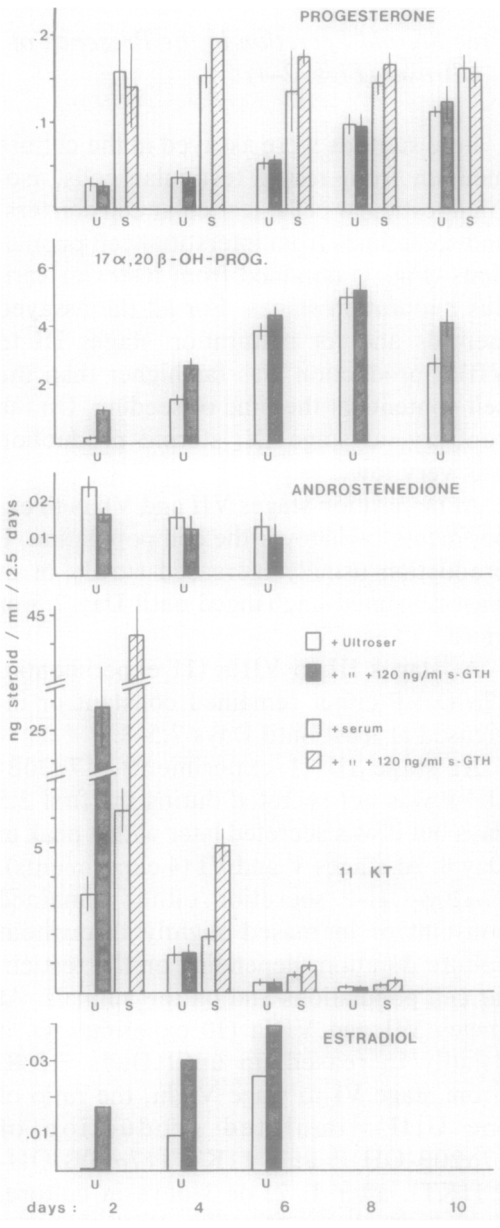


FIG. 2. Changes in the basal and s-GtH-stimulated (100 ng/ml) secretions of five steroids in the free form by a round testicular cell population cultured for 10 days in the presence of either 2% Ultroser (U) or 5% trout serum (S). Stage VIIIa. Two similar representative experiments: Progesterone, 17α20β-OH-P, and 11KT: $4.7 \times 10^6$ round cells/ml; androstenedione and estradiol: $3.1 \times 10^6$ round cells/ml. Means of three wells ± SE or pools of three wells (no SE, estradiol).

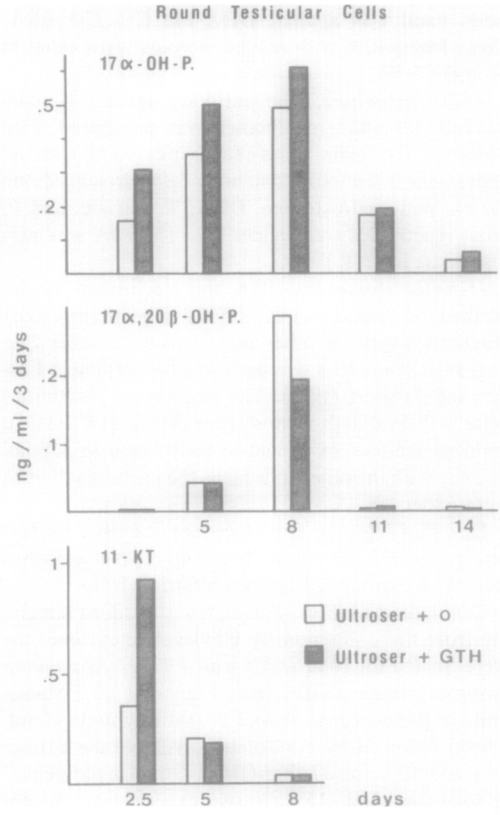


FIG. 3. Changes in the basal and s-GtH-stimulated (100 ng/ml) secretions of three steroids in the free form by a round testicular cell population ($6 \times 10^6$ round cells/ml) cultured for 14 days in the presence of 2% Ultroser. One representative testis at maturation stage III. Pools of three wells.

ture. At stages VII and VIIIa (3 experiments), androstenedione concentrations were always under 100 pg/ml/day, a short time after seeding, then they decreased slowly.

At stages III to VIIIb, 11KT (18 experiments) and T (9 experiments) secretion varied in a similar manner: basal and GtH-stimulated secretion always decreased regularly, more or less rapidly, so that after 5 to 12 days they were nearly undetectable (T unshown). This was true independently of the maturation stage and of the various components added or not into the culture medium. Basal and GtH-stimulated T concentrations were always lower than those of 11KT. The ratio of the s-GtH-stimulated

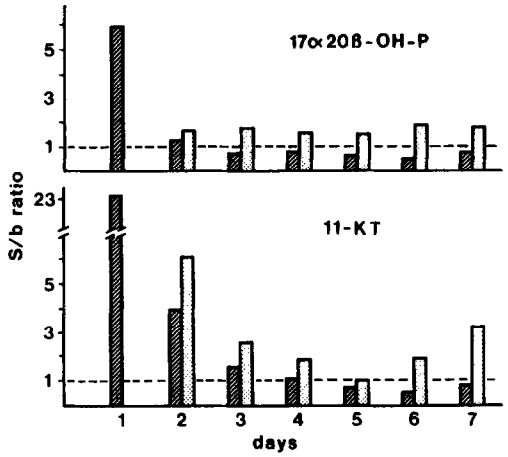


FIG. 4. Changes in the s-GtH (120 ng/ml)-stimulated/basal ratio for the secretions of free 17α20β-OH-progesterone and free 11-ketotestosterone by a round testicular cell population ($1.3 \times 10^6$ round cells/ml) cultured for 7 days in the presence of 2% Ultroser. s-GtH was present either permanently (hatched squares) or only during 1 day before sampling (and every 3 days; spotted squares). Stages VII–VIIIa. Ratio: mean of three "stimulated" wells/mean of three "basal" wells.

production of 11KT and T (11KT/T), after 1, 2, or 2.5 days in culture, showed a tendency to increase with the maturation stage: stage V, 5.3; stage VI, 5.4 ± 2.4 (2 experiments); stage VII, 7 ± 4.2 (2 experiments); stages VII–VIIIa, 9.8 ± 4.2 (2 experiments); stage VIIIa, 11.2 ± 3.6 (2 experiments). At stage IX a negligible amount of 11KT was produced.

We looked for $E_2$ in two fishes at stages VII–VIIIa. Low but significant concentrations were secreted in the culture medium by round testicular cells, especially in the presence of s-GtH, and they increased with culture duration. No $E_2$ was detected in the interstitial cell population.

When 100 ng s-GtH/ml was present permanently, apparent stimulation of the free steroid secretion depended on the considered steroid. For P, significant stimulation was never observed. For 17α-OH-P, either low stimulation (×1.2 to 1.8) was observed 2.5 days or more after seeding or no significant effect was detected, whatever the maturation stage and the considered cell population. For 17α20β-OH-P, the effect of s-GtH after 2–2.5 days was also independent of stages and cell populations: either low stimulation (×1.1 to 3) or no effect, followed sometimes by slight inhibition was observed throughout culture. However, during the first day after seeding, s-GtH stimulated 17α20β-OH-P secretion more efficiently than later (by six times; stage VIIIa, one experiment). After 2 days in culture, 100 ng s-GtH/ml slightly stimulated (×1.4) androstenedione secretion, but had no significant effect later.

In the presence of Ultroser, the GtH-stimulated/basal ratio (S/b ratio) of 11KT secretion by the round testicular cells, after 2 or 2.5 days in culture, was equal to 3 at stage III, to 4 to 8 at stages V–VIIIa, and to 1 to 3.5 at stages VIIIb and IX. In some experiments, the S/b ratio of 11KT secretion by the interstitial cells was far higher (up to 16) than that observed with the corresponding round testicular cells. At stages V–VIIIa, the S/b ratio was equal to 3 to 5 for T. Whatever the maturation stage and the cell population, constantly present s-GtH had, usually, no effect on 11KT and testosterone secretion after 4 or 5 days in culture.

At stage VIIIa (1 experiment), when s-GtH was present during only 24 hr before medium collection and every 72 hr thereafter, the stimulation of 11KT secretion was effective during 1 additional day and then it was stimulated again at a later time, and for 17α20β-OH-P, stimulation was maintained for at least 7 days.

### *Conjugated Steroid Secretion in the Presence of Ultroser (Fig. 5)*

Androgens, T, 11KT, 17α20β-OH-P, and DHA were found to be conjugated in the culture medium of round testicular cells prepared from testes at maturation stages VI to VIIIa (five experiments; these conjugated steroids were not assayed at other

stages). Percentages of these steroids in the conjugated form (conjugation rate, CR) varied according to the maturation stage and to the presence or absence of GtH and trout serum in the culture medium. The CRs observed for T and 17α20β-OH-P in the absence of GtH and serum are shown in Table 1. At stage VI, the CR for DHA was between 68 and 73% in the absence of serum.

GtH stimulated the production of conjugated T, 11KT, and androgens for the first 3 or 4 days in culture. In fact, 1 day and sometimes 2 days after seeding (Table 2),

TABLE 1

CONJUGATION RATES (PERCENTAGE OF STEROID IN THE CONJUGATED FORM) FOR TESTOSTERONE (T) AND 17α,20β-OH-P (17,20P) AS A FUNCTION OF THE MATURATION STAGE AND OF CULTURE DURATION

| Maturation stage | | Day 0 | 2 Days | 6 Days |
|---|---|---|---|---|
| VI | T | 36 | 60 | 68 |
| (one experiment) | 17,20P | | | |
| VII | T | 32 | 81 | 80 |
| (one experiment) | 17,20P | | 2.7 | 3.3 |
| VIIIa | T | 36 | 92–98 | 90–96 |
| (three experiments) | 17,20P | | 20–22 | 19–22 |

*Note.* Round testicular cells cultured in the presence of Ultroser and in the absence of s-GtH. Day 0: aliquots of cells to be seeded were extracted and assayed.

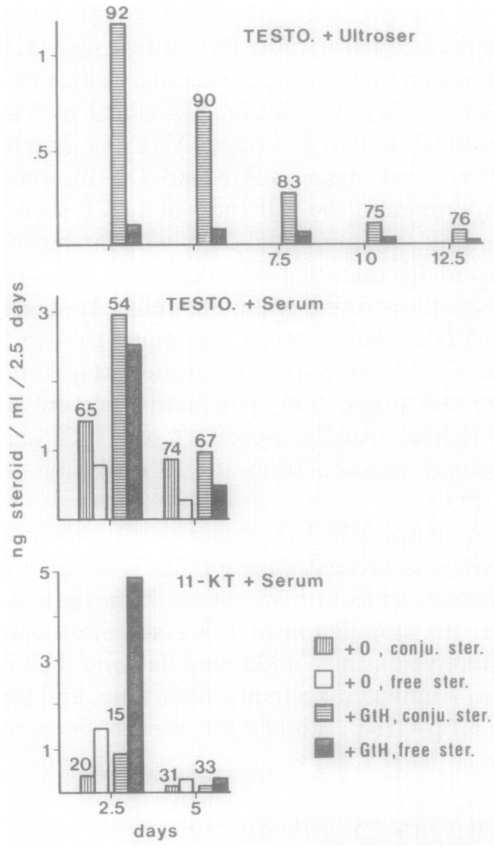


FIG. 5. Changes in the basal and s-GtH-stimulated (100 ng/ml) secretions of free and conjugated testosterone and 11-ketotestosterone by an interstitial cell cluster population ($0.7 \times 10^6$ interstitial cells + $0.025 \times 10^6$ Sertoli cells/ml) cultured for 12.5 days in the presence of either 2% Ultroser or 5% trout serum. One testis at stage VIIIa. Pools of three wells. Numbers at the top of the squares: conjugation rates in percentage of the total secreted steroids.

the increase of the absolute amounts of these conjugated steroids was concomitant with a decrease of the CRs. For 17α20β-OH-P there was rather a slight decrease of the amount of the conjugated form accompanied by a decrease of the CR (Table 2).

Conjugated T was measured in the culture medium of an interstitial cell population (stage VIIIa; 1 Sertoli cell per 28 Leydig cells). The CR varied between 92 and 76% during the first 12.5 days in culture.

In the seminal plasma of spermiating trouts (stages VII–VIIIa; 10 males; not shown), 95% of the total T (M = 70 ng/ml), 70% of the total 11KT (M = 15 ng/ml), and 19.9% of the total 17α20β-OH-P (M = 2 ng/ml) were in the conjugated form.

### *Effect of Trout Serum and Lipoproteins on Steroid Secretion (Figs. 2, 5–7)*

The effect of 5% young trout serum was tested at stages V to VIIIa (five experiments). Whatever the stage was, trout serum enhanced the basal and s-GtH stimulated secretion of P, 17α-OH-P, and free 17α20β-OH-P for at least 10 days. While the secretion of free 11KT was conspicuously enhanced at stages VII and VIIIa, it was either unchanged or partly inhibited at stage V. On the other hand, at stages VI to VIIIa, s-GtH stimulated free 11KT and free

TABLE 2

EVOLUTION DURING THE FIRST 7 DAYS IN CULTURE OF THE CONJUGATION RATE (EXPRESSED AS PERCENTAGE OF TOTAL STEROID-FREE FORM + CONJUGATED FORM) FOR TOTAL ANDROGENS (Andr.), TESTOSTERONE (T), 11-KETOTESTOSTERONE (11KT), AND 17α,20β-OH-PROGESTERONE (17,20P)

| | | Days | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Andr. | 0 | 32 | 83 | | 80 | | 86 | | 86 |
| | G | | 40 | | 81 | | 83 | | 90 |
| T | 0 | | 92 | 93 | | | | | |
| | G | | 80 | 91 | 94 | 91 | 88 | 90 | 91 |
| 11KT | 0 | | 51 | 56 | | | | | |
| | G | | 11 | 44 | 54 | 54 | 58 | 56 | 61 |
| 17,20P | 0 | | 24 | 21 | | | | | |
| | G | | 4 | 12 | 16 | 18 | 20 | 16 | 20 |

*Note.* Maturation stage VIIIa. Round testicular cell population; one representative experiment. Culture in the presence of 2% Ultroser and of either 100 ng s-GtH/ml (G) or without GtH (0)

T secretion, in the presence of trout serum, for at least 2 additional days, as compared with Ultroser only.

At stages VI to VIIIa (three experiments), trout serum also slightly increased the secreted amounts of conjugated T, 11KT, and DHA. However, because the secretion of these free steroids was simultaneously enhanced, the CRs were lowered, especially for T. At stage VI after 1 day in the presence of s-GtH (round testicular cells), the CR for T was 17% with serum vs 56% with Ultroser. After 2 days it was 29% vs 60%. At stage VIIIa after 2.5 days (interstitial cell population), it was 54% vs 92%. The decreasing effect of GtH on the CRs, during the first 1 or 2 days in culture, was also observed in the presence of serum.

The effect of total trout lipoproteins on steroidogenesis was checked at stage V (round testicular cells; two experiments). Lipoproteins prepared from animals at this maturation stage (125 to 500 μg/ml) enhanced significantly but slightly (×1.4 to 1.8) the basal and s-GtH-stimulated 17α20β-OH-P secretion during the first 2.5 days. After 5 and 7.5 days, only the highest lipoprotein concentration significantly enhanced (×1.5 to 1.6) these steroid secretions. On the contrary, lipoproteins never enhanced either the basal or the s-GtH-stimulated 11KT secretions. These lipoprotein concentrations had no obvious influence on the fine ultrastructure (mitochondria, smooth ergastoplasmic reticulum) of the Leydig cells (unshown).

### *Changes in Maximal Steroidogenic Capacity during Culture (Maturation Stage V; Fig. 8)*

The addition of 100 μ*M* 22-hydroxycholesterol enhanced steroidogenesis in all the various testicular cell populations prepared from two fishes. For every assayed steroid (17α20β-OH-P, T, and 11KT), basal and s-GtH-stimulated production reached the same level, irrespective of whether or not s-GtH was present. After the first 2.5 days, in the presence of 100 ng s-GtH/ml, the mean enhancement as compared to the steroid production measured without 22-OH-cholesterol was 64 ± 19 ($n$ = 6 cell

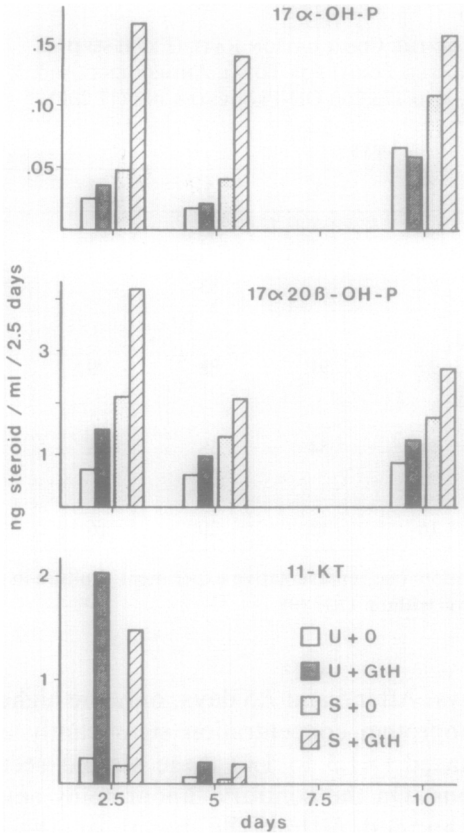


FIG. 6. Changes in the basal and s-GtH-stimulated (100 ng/ml) secretions of three steroids in the free form by an interstitial cell cluster population (contaminated by closed cysts; $4 \times 10^6$ round cells; $0.72 \times 10^6$ Leydig cells) cultured for 10 days in the presence of either 2% Ultroser (U) or 5% trout serum (S). One testis at maturation stage V. Pools of three wells.

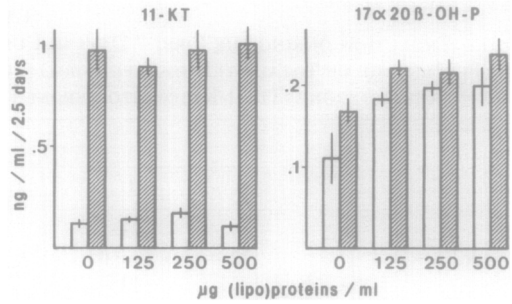


FIG. 7. Influence of three concentrations of trout (stage V) total lipoproteins on the basal (white squares) and s-GtH-stimulated (100 ng/ml; hatched squares) secretions of free 11-ketotestosterone and free 17α20β-OH-progesterone by a round testicular cell population ($2.4 \times 10^6$ round cells/ml) cultured 2.5 days in the presence of 2% Ultroser. One representative testis at stage V. Means of three wells ± SE.

populations) for 17α20β-OH-P, 17 ± 9 times ($n$ = 7 cell populations) for T, and 1.74 times ($n$ = 1) for 11KT. With the interstitial cell cluster population, 10 times more 17α20β-OH-P than 11KT was formed from 22-OH-cholesterol after 2.5 days. Later, for all cell populations, except for the interstitial cell cluster population, the amounts of steroids formed from 22-OH-cholesterol decreased progressively. The amounts of 17α20β-OH-P were 10, 54, and 86 times lower, as a mean, after respectively 5, 7.5, and 10 days than after 2.5 days. Those of T were 16, 96, and 188 times lower. On the contrary, in the case of the interstitial cell cluster population, the amounts of 17α20β-OH-P increased by 1.17 and 1.26 times after respectively 5 and 7.5 days, then they decreased (2.9 times after 10 days).

## DISCUSSION

### *Steroidogenic Activity and Effect of s-GtH*

A short time after seeding, populations of mixed testicular cells produced all the androgens and progestagens that we assayed, except 17α20β-OH-P which was not detected in significant amounts at stage III during the first 2 days in culture. Free T, 11KT, and 17α20β-OH-P are present at rather high concentrations in blood plasma of *Salmo gairdneri* (Baynes and Scott, 1985). These steroids were predominantly secreted *in vitro* by testicular cells. The 11KT/T ratio increased with the maturation stage and the 17α20β-OH-/11KT ratio became higher than 1 during stage VIII. These *in vitro* observations are in agreement with the changes of these steroids known to occur *in vivo* throughout the trout maturation cycle. The inversion of the 17α20β-OH-P/11KT ratio during stage VIII espe-

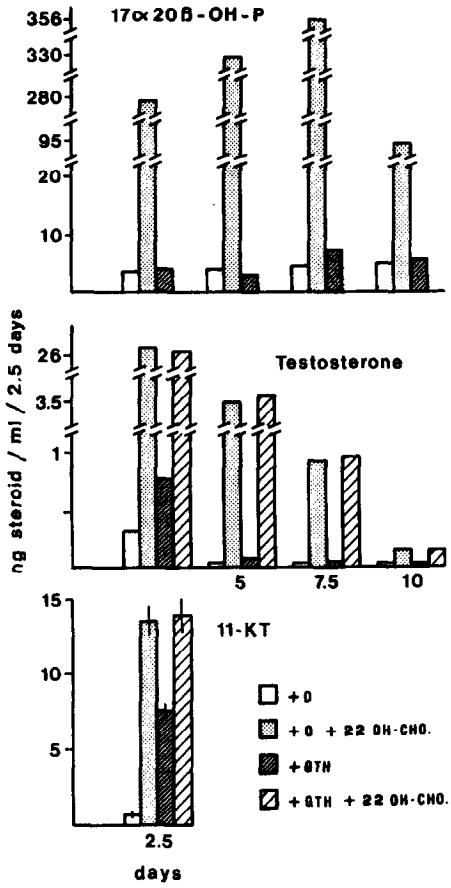


FIG. 8. Changes in the basal and s-GtH-stimulated (100 ng/ml) secretions of three steroids in the free form by an interstitial cell cluster population (contaminated by closed cysts; 29 × $10^6$ round cells/ml; around 2.1 × $10^6$ Leydig cells) cultured for 10 days in the presence of 2% Ultroser and either 100 μ*M* 22-OH-cholesterol or not. One representative testis at stage V. Means of three wells ± SE or pools of three wells (17α20β-OH-P and T).

cially reflects the fall in androgen levels observed at the time of milt production while 17α20β-OH-P plasmatic levels increased (Baynes and Scott, 1985). Although large interexperiment variations occurred, maximal rates of stimulation of 11KT production by s-GtH were observed at stages V to VIIIa, while they were currently low at stages VIIIb–III. Stimulation rates for 17α20β-OH-P did not decrease at stages VIIIb–IX. These similarities between *in vivo* and *in vitro* changes in steroids levels and sensitivity to GTH (Le Gac and Fostier, 1987; Le Gac and Loir, 1988) demonstrate that culture of trout testicular cells constitutes a pertinent tool to elucidate the regulation of steroidogenesis during the perispermiation period.

When s-GtH was permanently present, T, 11KT, and 17α20β-OH-P secretions were stimulated for 3 to 4 days only. This loss of sensitivity to GtH may be induced by *in vitro* conditions. The beneficial effect of trout serum on the sensitivity of 11KT secretion to GtH suggests that some unknown factors could help the cells to retain their sensitivity. However, because GtH was permanently present, it could promote some desensitization of the target cells as already shown in mammals in the case of prolonged stimulation by LH (Nozu *et al.*, 1981). This hypothesis is partly supported by the observation that 24-hr stimulations seemed to be able to maintain the sensitivity of the cells for at least 7 days (Fig. 4). Shorter GtH pulses (1 to 4 hr?) and physiological GtH concentrations (around 10–15 ng/ml) would possibly still enhance and lengthen the cell sensitivity to GtH.

After 2 days in culture, the S/b ratios of 17α20β-OH-P secretion by isolated testicular cells were always low (less than 3). On the contrary, when mature testis fragments were incubated for 20–24 hr (Saad and Depêche, 1987; F. Le Gac, unpublished), the S/b ratios were between 11 to 26. We have no explanation for this discrepancy, although the fact that the S/b ratio, when measured only 1 day after seeding, was equal to 6 suggests that the testicular cells producing 17α20β-OH-P under GtH control (Leydig cells) could rapidly lose their sensitivity to gonadotropin after seeding.

$E_2$ secretion was stimulated by s-GtH for at least 6 days. However, according to Fostier and Le Gac (1987) and to Le Gac and Loir (1988), GtH might not have a direct effect on aromatase activity but may have an indirect effect through increased secretion of T. Effectively, in the round testicu-

lar cell population in which $E_2$ was assayed, T secretion was stimulated during at least 6 days (24-hr pulses of GtH).

The detection in the medium of low concentrations of DHA indicates that, at least at the end of spermatogenesis, the 5-ene pathway of steroid synthesis operated *in vitro*—likely less active than the 4-ene pathway—as already demonstrated *in vivo* (Saad and Depéche, 1987; for review, see Fostier *et al.*, 1983).

*Changes in the Steroidogenic Pathway*

The basal and GtH-stimulated secretions of free and conjugated 11KT and T always irremediably decreased to very low concentrations after 10–12 days. This fall was often concomitant with an increase in progestagen production. So, it can be excluded that it is due to an impairment in cell viability. Some mechanisms possibly involved in the androgen decrease/progestagen increase have been already discussed elsewhere (Le Gac and Loir, 1988).

Ultroser does not contain cholesterol and in the absence of exogenous cholesterol supply, depletion of endogenous cholesterol could lead to a progressive decrease of the steroidogenic activity. When trout serum (supply of lipoprotein-bound cholesterol) was added, progestagen secretion (Figs. 2 and 6) and, at least at the stage VIIIa, 11KT secretion (Fig. 1), were conspicuously stimulated. Nevertheless, the androgen fall was not counteracted but was only slowed down.

In mammals, increased $E_2$ production has been proposed as one factor responsible for the diminished capacity for testosterone production by desensitized Leydig cells (Nozu *et al.*, 1981). Increasing, although low, E2 concentrations were effectively present in trout round testicular cell population media. But, because no $E_2$ was detected in interstitial cell culture media, this excluded the fact that this steroid was responsible for the decrease in androgens observed in these media as well as in the others.

Optimal 22-OH-cholesterol concentrations (100 $\mu M$; Risbridger *et al.*, 1986) allow measurement of the maximal rates of steroidogenesis, providing an estimation of P450scc activity but also of the various enzyme activities involved in the two pathways leading to 17α20β-OH-P on the one hand and to T and 11KT on the other hand. It appeared that these activities, considered as a whole, decreased throughout culture duration in all the cell populations, except in the interstitial cell cluster population where they increased slightly at the beginning. Elucidation of the changes in enzyme activity responsible for the *in vitro* shift from androgens to progestagens will need studies of steroid metabolism using labeled precursors.

*Glucuronated Steroid Synthesis*

The conjugated steroids were hydrolysed with sulfatase and glucuronidase. It has been pointed out that in trout as in other teleosts, inversely to mammals, the conjugated steroids are in the glucuronated form (Hews and Kime, 1978). Idler *et al.* (1971) and Hews and Kime (1978) have shown that conjugates are produced in the testis. Our data agree with this conclusion and in addition, they demonstrate that steroid conjugation occurs at least in the interstitial cells (Fig. 5).

Taking into account that glucuronides concentrations are higher in the testicular than peripheral plasma (Idler *et al.*, 1971), the CRs of 32–36% that we measured for T in the newly dissociated cells are in agreement with the data on the relative amounts of free and conjugated androgens in the peripheral blood obtained by Kime and Manning (1982) in mature and spermiating trout.

A conspicuous effect of the *in vitro* conditions was to enhance rapidly (1 day) the CRs which later remained roughly constant. Numerous factors may affect the glu-

curonyl transferase enzyme (Kime and Manning, 1982). Static culture conditions lead to the accumulation of various molecules and in particular of steroids which may stimulate the enzyme activity to limit the concentrations of free active steroids in the cell environment. Values for T and 11KT CRs are somewhat higher than those obtained by Schulz and Blüm (1987a) with trout testis fragments incubated for 20 hr.

Under our experimental conditions, 100 ng GtH/ml stimulated the conjugated androgen production during the first 3 or 4 days. Stimulation of the secretion of conjugated T, 11KT, and 11β-OH-testosterone by increasing GtH concentrations was also observed by Schulz and Blüm (1987a). During the first 1 or 2 days, in the presence of either Ultroser or serum, the CRs were slightly diminished, especially for T. This could reflect that the enzyme activity would be limiting in these conditions of the production of large amounts of substrate. Saad and Depeche (1987) observed no change of the CR for total steroids with less than 300 ng GtH/ml. *In vivo,* the androgen CRs were higher in mature males when GtH levels were high (Kime and Manning, 1982; Truscott *et al.,* 1986).

*Serum and Lipoproteins*

Juvenile trout serum enhanced both basal and GtH-stimulated progestagen and androgen secretions at stages VII and VIII. The effect of trout total lipoproteins has not been checked at this stage. At stage V, lipoproteins, as serum, enhanced progestagen secretions, but after 2.5 days, the enhancement was about three times lower with lipoproteins than with serum. This suggests that juvenile trout serum enhances steroid secretion by the supply of lipoprotein-bound cholesterol but that some other serum factors are also active on steroidogenesis. Steroids and GtH were in concentrations that were too low at the final serum dilution to be efficient. Various molecules regulating testicular steroidogenesis, such as growth factors and namely insulin-like growth factors, are likely to be in great concentrations in juvenile trout serum. The presence of steroid-binding proteins in serum could explain the increase of steroid concentrations in the culture media. Nevertheless, the different effect of serum on androgens at stages V and VIII suggests the intervention of a more specific mechanism, although this differential effect may be partly due to lipoproteins acting in a stage-dependent manner as already shown in the female trout (Babin, 1986). Our data point out that at the time of intense gametogenesis (stage V), high amounts of lipoproteins present in the blood plasma (Fremont and Marion, 1982) have an influence on testicular steroidogenesis, but on one hand, it is restricted to only progestagens and on the other, they amplify rather moderately their production. At the same time, the lipoproteins would play a crucial role in the synthesis of membranes of the germ cells (Loir, 1989).

In conclusion, the method used to dissociate trout testes and to culture testicular cells enables the maintaining of the differentiated activity of steroidogenic cells, which was modulable by s-GtH, serum, and lipoproteins for several days. It appeared that (1) the stimulation by GtH of the 17α20β-OH-P production by the isolated cells differed from that obtained with testis fragments and this remains to be explained, and (2) throughout culture there was a marked increase in the ratio 17α20β-OH-P/T + 11KT. Because this *in vitro* change mimics that observed *in vivo* during the perispermiation period, it is especially interesting to try to elucidate the mechanisms which underly it. Although the measurement of net steroid productions provides preliminary informations, it will be necessary to analyze the changes in enzyme activity which occur during culture. The data

we have obtained suggest that it may be possible to develop such a study using a culture of dissociated testicular cells.

## ACKNOWLEDGMENTS

This work was supported in part by a grant from the "Institut National de la Recherche Agronomique." We thank Dr. B. Breton for the kind gift of s-GtH, and Drs. D. H. Garnier, A. Fostier, and F. Le Gac for helpful discussions. We are grateful to Mrs. M. Bougoussa and B. Mourot for technical assistance and to J. Hall for help with the English.

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