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***In vitro* metabolism of progesterone
and 17 α -hydroxyprogesterone in the testis
of the rainbow trout, *Salmo gairdneri* Rich.,
at different stages of spermatogenesis**

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Summary. Rainbow trout testicular fragments were incubated at 8 different stages of the spermatogenetic cycle in the presence of tritiated progesterone or 17 α -hydroxyprogesterone. This paper describes the potential enzymatic activities involved in the Δ_4 route leading to the production of « major » derivatives.

- 1) A 5-reductase converted progesterone into 5 α (β)-pregnane-3,20-dione.
 - 2) A 20 β -oxydoreductase converted 17 α -hydroxyprogesterone into 17 α -hydroxy-20 β -dihydroprogesterone. At some stages this synthesis reached or exceeded androgen production.
 - 3) 20 β -oxydoreductase, 17-20 desmolase and 17 β -oxydoreductase are proposed as putative enzymatic steps in the regulation and modulation of steroidogenic metabolism in the trout testis.
 - 4) The physiological role of the different major steroids synthesized by the testis *in vitro* is discussed, and the hypothesis of the involvement of 17 α -hydroxy-20 β -dihydroprogesterone in the control of germ cell mitotic activity and/or feedback on the hypothalamo-pituitary system is examined.
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Introduction.

Steroid biosynthesis in the testis varies in different vertebrate species (Ozon, 1972 ; Kime, 1980). This diversity is expressed by differences in metabolic routes, types of steroids produced, and probably also in modalities of regulation, depending on the particular reproductive strategy. It is thus primordial to know the steroidogenic metabolism of a given species before trying to analyze the role of the steroids.

The steroidogenic function of the testis plays a complex role in controlling the initiation and progress of spermatogenesis. In fact, the various steroids produced act either locally or at many extra-gonadal sites, such as the hypothalamo-pituitary system, where they contribute to the modulation of gonadotropic secretion.

The testis of teleost fish with an annual reproductive cycle seems a good model for analyzing the relationships between steroidogenic metabolism and the spermatogenetic process, including spermiogenesis and spermiation. The proliferation and differentiation of male germ cells spread over several months of the year (Billard *et al.*, 1972 ; Drance *et al.*, 1976 ; van den Hurk *et al.*, 1978). Stages, corresponding to the progression of the front of the spermatogenetic wave, can be defined by the synchronous evolution of germ cells within the testicular cysts (Billard and Escaffre, 1975).

Potential enzymatic activities in the gonad can be identified by studying the *in vitro* metabolism of radioactive steroids, but none of the studies in teleosts (see review by Kime, 1980) has traced qualitative or quantitative modifications in metabolism during the cycle.

Due to the numerous metabolic routes operating in the gonad, the complete pattern of enzymatic activities cannot be determined when a single precursor is used. This paper reports results obtained with progesterone and 17 α -hydroxyprogesterone, two C21 steroids of the Δ_4 synthesis route.

Material and methods.

Fish. — Three-year old male rainbow trout (*Salmo gairdneri* Rich.) in their second reproductive cycle were used. They were anesthetized by immersion in a bath of 0.05 p. 100 phenoxy-2-ethanol. Whole transversal sections, of about

TABLE 1

Stages of spermatogenesis in the rainbow trout

Stage	Correspondance with <i>S. fario</i> stages	Definition (most advanced stage of spermatogenesis)	GSI (1) and no. of fish	Month
I	I ₁	A spermatogonia (and residual spermatozoa)	0.3-0.9 (6)	3-5
II	II	A and B spermatogonia	0.3-0.9 (5)	4-6
III a	III	Meiotic prophase does not exceed the zygotene stage	0.3-0.9 (5)	5-6
III b		Spermatids	0.3-0.9 (3)	6-7
IV	IV	Onset of spermiogenesis (few spermatozoa)	0.3-1 (7)	6-7
V	V	End of spermatogenesis (many spermatozoa)	1.5-2.5 (6)	7-8
VI-VII	VI VII	Onset of spermiation (some spermatocytes still present)	2.5-3.5 (7)	9-11
VIII	VIII	Advanced spermiation	3-4 (3)	12-1

(1) GSI : testicular weight/fresh weight of intact fish \times 100.

100 mg each from the middle of the testis, were cut in small pieces and washed in three successive baths of Eagle's medium (Eagle, 1959) before incubation with the radioactive precursor.

The stage of spermatogenesis was determined from the most advanced stage observed in several histological sections. We used a slightly modified version of the classification proposed by Billard and Escaffre (1975) for the *fario* trout (table 1).

Steroids. — $1,2,6,7\text{-}^3\text{H}$ progesterone (specific activity : 96 Ci/mmol) and 17α -hydroxy-7 (n)- ^3H progesterone (specific activity : 10.7 Ci/mmol) were furnished by the Radiochemical Centre (Amersham, UK). The non-radioactive steroid standards were provided by Sigma Chemical Co. 1.10^6 dpm of radioactive precursor was mixed with the non-radioactive steroid to obtain a final concentration of 5.10^{-7} M (specific activity : 0.3 Ci/mmol).

Incubations. — The fragments of testis were incubated for 3 h at 14 °C in 3 ml of Eagle's medium in 15-ml vials which were shaken constantly. The medium was equilibrated with a gaseous mixture of 50 p. 100 nitrogen, 49 p. 100 oxygen and 1 p. 100 CO_2 . In these conditions, the pH was about 7.9 which is the value of the coelomic medium (Jalabert, personal communication). The radioactive precursor was introduced at the beginning of the incubation which was carried out at a set time (11 h) to take into account the effect of possible circadian variations in metabolism. At the end of incubation, the fragments were blotted, then homogenized in the incubation medium. Into the homogenate, we introduced 50 μg of various steroids used as carriers and 10 000 dpm of 4 tracers labelled with ^{14}C (testosterone, 5α -dihydrotestosterone, estrone and 17β -estradiol).

Extraction. — The free steroids were extracted by ether (3×10 ml), and the homogenate was treated with 1 300 Fishman units of β -glucuronidase at 37 °C for 48 h. The glucuroconjugated steroids were then extracted by adding 2×10 ml of ether.

Separation and identification of steroids. — The steroids were separated by thin-layer chromatography on Merck silica gel (60 F 254) in the presence of steroid standards. The extracts were first chromatographed in the chloroform/ethanol system (95/5 ; v/v) ; the steroids were identified in the presence of authentic steroids after chromatography in different systems : (i) chloroform/ethyl acetate 5/1, (ii) chloroform/ethanol 95/5, (iii) chloroform/acetone 9/1, (iv) chloroform/methanol/water 188/12/1, (v) chloroform/ethyl acetate/methanol 45/45/10, (vi) ethyl acetate/hexane/acetic acid/ethanol 72/13.5/10/4.5, (vii) benzene/ethanol 90/10, (viii) chloroform/ethyl acetate 1/1, (ix) chloroform/methanol 96/4, (x) cyclohexane/isopropanol 70/30, (xi) hexane/ethyl acetate 75/25, (xii)

Common designation and systematic nomenclature of the steroids cited : progesterone : 4-pregnene-3,20-dione ; $5\alpha(\beta)$ -pregnane dione : $5\alpha(\beta)$ -pregnane-3,20-dione ; 17α -hydroxyprogesterone : 17α -hydroxy-4-pregnene-3,20 dione ; 17α -hydroxy-20 β -dihydroprogesterone : $17\alpha,20\beta$ -dihydroxy-4-pregnen-3-one ; androstenedione : 4-androstene-3,17-dione ; 11β -hydroxy-androstenedione : 11β -hydroxy-4-androstene-3,17-dione ; testosterone : 17β -hydroxy-4-androsten-3-one ; 11β -hydroxytestosterone : $11\beta,17\beta$ -dihydroxy-4-androsten-3-one ; 11-ketotestosterone : 4-androstene-3,11,17-trione ; cortisol : $11\beta,17\alpha,21$ -trihydroxy-4-pregnene-3,20-dione.

chloroform/ethanol 90/10. A steroid was identified by its isopolarity with the authentic steroid in at least two of the following chromatographic systems :

Steroid :	Successive systems used :
5 α (β)-pregnane-3,20-dione	ii xi
17 α -hydroxyprogesterone	ii vii
17 α -hydroxy-20 β -dihydroprogesterone	ii v iv ix x
androstenedione	i (2 times) viii
11 β -hydroxyandrostenedione	i (2 times) xii
testosterone	i (2 times) iv iii
11 β -hydroxytestosterone	i (2 times) iv iii
adrenosterone	i (2 times) iv
11-ketotestosterone	i (2 times) iv
cortisol	ii vi x

The usual reactions of oxidation, reduction and acetylation were initiated by methods described elsewhere (Sire and Dépêche, 1981). In particular, 17 - hydroxy-20 β -dihydroprogesterone was acetylated by a mixture acetic anhydride-pyridine (12 h at 20 °C) and the acetates were subsequently hydrolyzed by 5 p. 100 KOH (1 h at 60 °C). The 17-hydroxy group was oxidized with chromic acid in the dark for 1 h.

The derivatives were analyzed by chromatography in the presence of authentic derivatives.

The chromatograms were analyzed with a scanning radiochromatograph (Panax xy) at a speed of 120 or 600 mm/h.

Measurement of radioactivity. — After each steroid was eluted, an aliquot fraction (1/10 : 100 μ l) was put into 15 ml of a PPO/POPOP/toluene mixture. Radioactivity was measured by double labelling in a liquid scintillation spectrometer equipped with an external standard (Packard Tricarb 3320). Extraction percentages were calculated from aliquot fractions (20 μ l) of the homogenate whose radioactivity was counted in a mixture of PPO/POPOP/toluene/Triton X 100.

Expression of results. — The amount of radioactivity found in each derivative formed was compared to the total radioactivity of the synthesized derivatives, using the formula :

$$\text{steroid S (p. 100)} = \frac{\text{dpm of steroid S}}{\text{total dpm recovered} - \text{precursor dpm}} \times 100 .$$

The amount of precursor metabolization by the testis was expressed by the « percentage of metabolization » :

$$\frac{\text{dpm of the whole of the derivatives}}{\text{total dpm recovered} \times \text{mg of incubated fragment fresh weight}} .$$

We used only those steroids (« major » metabolites) representing more than 1 p. 100 of the total radioactivity.

Results.

1) *Metabolization rate.* — The percentage of metabolization of both precursors was based on three incubations at different stages of the cycle (table 2). Progesterone metabolization ranged between 11 and 23 p. 100, whatever the stage of the cycle, except at stage VIII when it rose sharply to 70 p. 100. The percentage of 17 α -hydroxyprogesterone metabolization was higher (40-50 p. 100) than that of progesterone. It exceeded 50 p. 100 at stages III a, III b and IV.

TABLE 2

Percentage of precursor metabolization at different stages of the cycle (means of 3 incubations)

Precursor	Stage							
	I	II	III a	III b	IV	V	VI-VII	VIII
Progesterone	12	23	11	11	15	11	20	68
17 α -Hydroxyprogesterone	45	30	53	50	59	28	30	—

2) *Steroid glucuroconjugation.* — The total estimation of the percentage of glucuroconjugated steroids was based on three incubations at different stages of the cycle (table 3). In our experimental conditions, it seemed to be low, never exceeding 3 p. 100.

TABLE 3

Percentage of total glucuroconjugated steroids (means of 3 incubations)

Precursor	Stage						
	I	II	III a	III b	IV	V	VI-VII
Progesterone	0.9	< 1	0.4	0.5	0.5	3.0	2.0
17 α -Hydroxyprogesterone	< 1	< 1	< 1	0.4	1.0	1.0	0.2

3) *In vitro metabolism of progesterone* (table 4).

a) *C21 steroids.* — After chromatography in systems (ii) and (xi), we identified a derivative as being 5 α (β)-pregnane-3,20-dione ; this derivative represented 14-23 p. 100 of the radioactivity recovered in the derivatives at stages I to III a and 5-10 p. 100 recovered at more advanced stages (IV or more). At stage VIII, the percentage recovered was less than the detection threshold.

TABLE 4

In vitro metabolism of progesterone (in p. 100 of the whole of the derivatives)

Derivative	Stage							
	I	II	III a	III b	V	V	VI-VII	VIII
5 β (β) Pregnane dione	23	14	16	17	7	10	5	< 1
17 α -Hydroxyprogesterone	14	18	13	20	24	27	31	2
17 α -Hydroxy-20 β -dihydroprogesterone	11	4	7	9	10	30	20	23
Androstenedione	26	20	31	16	20	5	3	1
11 β -Hydroxyandrostenedione	5	4	< 1	< 1	< 1	< 1	< 1	< 1
Testosterone	9	14	5	4	8	4	6	8
11 β -Hydroxytestosterone	< 1	< 1	< 1	< 1	< 1	< 1	< 1	22
Adrenosterone	8	2	< 1	< 1	< 1	< 1	3	< 1
11-Ketotestosterone	< 1	< 1	< 1	< 1	< 1	< 1	< 1	39

17 α -Hydroxyprogesterone was found at all stages ; between 14 and 31 p. 100 were recovered during the cycle, except at stage VIII when only 2 p. 100 was recovered.

17 α -Hydroxy-20 β -dihydroprogesterone was identified in several chromatographic systems (see above). The percentages of this steroid tended to increase from the early stages of the cycle up to stage V, reaching a maximum of 30 p. 100. Afterwards, the percentage decreased only slightly (23 p. 100 at stage VIII).

b) *C19 steroids*. — Androstenedione and testosterone were formed at all stages. The androstenedione/testosterone ratio decreased constantly from stage III a to stage VIII. This decrease corresponded to an apparent stimulation of 17 β -oxydoreductase activity favourizing testosterone production (reduction) (table 5).

TABLE 5

Androstenedione/testosterone ratio at different stages of the cycle (precursor : progesterone)

Stage							
I	II	III a	III b	IV	V	VI-VII	VIII
2.89	1.43	6.20	4.00	2.50	1.25	0.50	0.25

11 β -Hydroxyandrostenedione was synthesized at stages I and II, while 11 β -hydroxytestosterone was not formed until stage VIII. Adrenosterone production was not stable in our experimental conditions, only appearing at stages I, II and VI-VIII. At stage VIII, most of the radioactivity (30 p. 100) was recovered in a derivative identified as 11-ketotestosterone ; its synthesis was not detected at earlier stages.

4) *In vitro* metabolism of 17 α -hydroxyprogesterone (table 6).

a) *C21 steroids*. — More 17 α -hydroxy-20 β -dihydroprogesterone was produced from 17 α -hydroxyprogesterone rather than from progesterone. The percentage of radioactivity recovered in this derivative varied considerably with the stage. The highest percentages were observed at stage I (34 p. 100), stage III a (49 p. 100), and especially at stage V (82 p. 100) when the steroidogenesis was almost completely devoted to the synthesis of this derivative (less than 14 p. 100 of C19 steroids were formed). Traces of cortisol were found at stages VI-VII.

TABLE 6

In vitro metabolism of 17 α -hydroxyprogesterone (in p. 100 of the whole of the derivatives)

Derivative	Stage						
	I	II	III a	III b	IV	V	VI-VII
17 α -Hydroxy-20 β -dihydroprogesterone	34	16	49	13	22	82	48
Androstenedione	10	6	7	5	10	< 1	1
11 β -Hydroxyandrostenedione	20	32	22	32	34	3	6
Testosterone	6	8	4	6	2,5	2	11
11 β -Hydroxytestosterone	2	13	4	7	7	2,5	13
Adrenosterone	5	11	< 1	< 1	1	< 1	< 1
11-Ketotestosterone	< 1	< 1	6	24	17	6	17

b) *C19 steroids*. — Due to the fact that 17 α -hydroxyprogesterone is situated at the « crossroad » of steroidogenesis, the total amount of C19 derivatives recovered was inversely proportional to the amount of C21 steroids. The synthesis of one or the other of these steroid types thus predominated at a given stage.

At all stages, the « androgen » fraction included androstenedione (only traces at stage V), testosterone, 11 β -hydroxyandrostenedione and 11 β -hydroxytestosterone. Adrenosterone was produced at stages I, II and IV. 11-Ketotestosterone was not formed at stages I and II but it represented 6 to 24 p. 100 of the radioactivity at stages III to VI-VII.

Overall C19 steroid production predominated over (stages II, III b, IV) or was equivalent to (stages I, III a, VI-VII) the synthesis of 17 α -hydroxy-20 β -dihydroprogesterone, except at stage V when there was a massive production of this C21 steroid.

Discussion and conclusions.

Several works have been carried out on the *in vitro* steroidogenic metabolism of rainbow trout testis (Arai and Tamaoki, 1967 ; Suzuki and Tamaoki, 1972 ; Kime, 1979). The only data on changes in steroid production during the cycle have been obtained from plasma assays, and those assays included only a small

number of steroids (Billard *et al.*, 1978 ; Scott *et al.*, 1980). Considerable changes in the gonadosomatic index during the cycle complicate assessment of the physiological significance of the observed variations. Moreover, it is difficult to extract from these data any exact information on the nature of the steroids acting locally in the gonad. If study of the relative percentages of the different metabolites synthesized from a precursor *in vitro* cannot reflect the global production by the whole testis, it gives information about the orientation of steroidogenic metabolism in the gonad.

Pregnane 5-reduction activity has been reported in the ovary (Reinboth *et al.*, 1966), hermaphroditic gonad (Reinboth, 1978) and testis (Tesone and Charreau, 1980) of some teleosts. In our incubations, more $5\alpha(\beta)$ -pregnane-3,20 dione was synthesized at early stages (I to III b) than later. However, this decrease in apparent progesterone reduction coincided with an increase in 17α -hydroxylase activity and C19 steroid synthesis. As in mammals (Lerner and Eckstein, 1976) progesterone 5-reduction could be one of the mechanisms regulating testicular androgen production.

The formation of 17α -hydroxy-20 β -dihydroprogesterone has already been reported in incubations of trout testis (Arai and Tamaoki, 1967). The assays carried out by Campbell *et al.* (1980), on the plasma of spermiating animals, do not show very high concentrations (less than 9 ng/ml) contrary to the values found in females (480 ng/ml). *In vitro*, great amounts of 17α -hydroxy-20 β -dihydroprogesterone were synthesized at four stages (I, II, V, VI-VII) of the cycle and, at stage V, the synthesis of this steroid predominated over C19 steroid synthesis. This may be due either to increased 20 β -oxydoreductase reductive activity or to lowered 17-20 desmolase activity. In fact, an inhibition of this latter activity alone cannot explain this high synthesis since both activities can be increased simultaneously at stages of high androgenic production.

A control of 20 β -oxydoreductase and 17-20 desmolase activities may be hypothesized. During the cycle, this control could favourize the production of 17α -hydroxy-20 β -dihydroprogesterone or that of C19 steroids by orienting the metabolism towards one or the other. At high doses in the rat, HCG inhibits 17-20 desmolase activity (Cigorrage *et al.*, 1978).

17 β -Oxydoreductase activity, insuring androstenedione-testosterone inter-conversion, may be another site of the regulation of steroidogenic metabolism. In incubations carried out in the presence of progesterone, the androstenedione/testosterone ratio decreased regularly from stage III a to the end of the cycle, a period when androgenic production as well as plasma gonadotropin concentrations increase (Billard *et al.*, 1978). In a study on the female rainbow trout, we showed that salmon gonadotropin stimulated 17 β -oxydoreductase activity in favour of testosterone production in the incubated perfused ovary (Sire and Dépêche, 1981).

Taking into account all these data, we propose 4 putative sites which may be involved in the control of steroidogenic metabolism in the rainbow trout testis during the annual cycle (fig. 1).

Gonadotropic control of the enzymatic activities responsible for the cleavage of the lateral cholesterol chain, a well-known process in mammals (Hall, 1970), has

not yet been studied in fish where the mechanisms of pregnenolone formation have not been elucidated.

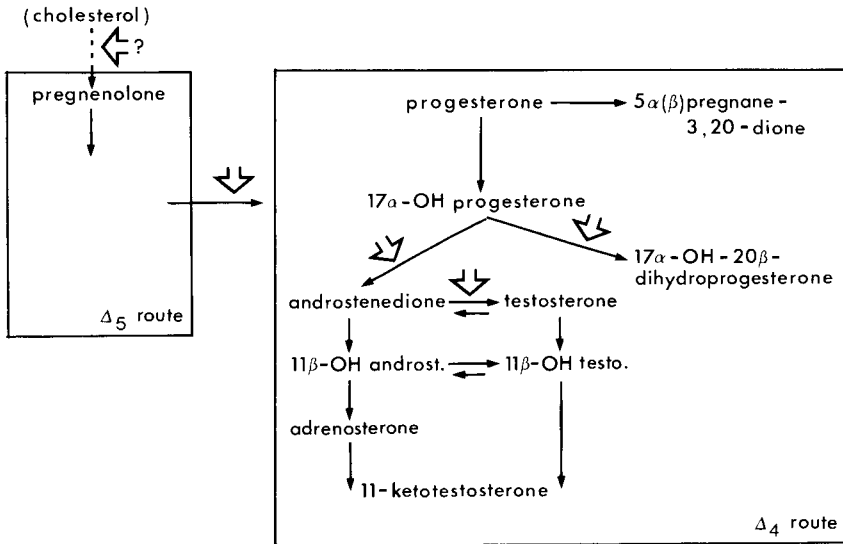


FIG. 1. — Simplified diagram of the routes of major steroid biosynthesis in the rainbow trout testis. Open arrows show the putative sites of metabolic regulation.

The regulation of enzymatic activities involved in the passage from the Δ_5 route of synthesis to that of Δ_4 is suggested by work on the rainbow trout showing that Δ_5 -3 β -hydroxysteroid dehydrogenase activity is stimulated during the cycle (van den Hurk *et al.*, 1978) or by studies on other species using gonadotropic stimulation of intact (Satyanarayana Rao and Nadkarni, 1978) or hypophysectomized (Pickford *et al.*, 1972) animals.

From our results, it seems that three other steps are modulated: 17-20 desmolase activity, 20 β -oxydoreductase activity and 17 β -oxydoreductase activity.

In our incubation, the synthesis of 17 α -hydroxy-20 β -dihydroprogesterone predominated at stages I and III a, when gonial mitotic activity is observed, and at stage V, corresponding to the period at the end of spermatogenesis, that is the time when the spermatocytes in a large number of cysts resume meiosis and divide.

17 α -Hydroxy-20 β -dihydroprogesterone is considered as the inductor of oocyte maturation in several teleost species (Fostier *et al.*, 1973; Jalabert, 1976; Duffey and Goetz, 1980). This steroid, produced by the ovarian follicle (Fostier *et al.*, 1981), would act on post-transcriptional processes controlling the mechanisms of germinal vesicle breakdown and the resumption of meiosis (Jalabert, 1976). Spermatogenesis does not include phenomena strictly identical to oocyte maturation. However some cellular mechanisms common to both germinal lines are involved in the mitotic activity of the spermatogonia and the meiotic evolution

of the spermatocytes. As a working hypothesis, we suggest that 17α -hydroxy- 20β -dihydroprogesterone in the male may act upon some step of the mitotic cycle and meiotic processes of the male germ cells.

In the female, a possible role for 17α -hydroxy- 20β -dihydroprogesterone in feedback on the hypothalamo-pituitary system has been suggested. Weil (1981) indicates that LH-RH sensitivity could depend on the plasma progesterone level or on a « certain oestradiol/ 17α -hydroxy- 20β -dihydroprogesterone ratio ». The plasmatic content of this steroid is low in males during spermiation (Campbell *et al.*, 1980), a time when the testicular potential for synthesis is relatively high. This would be a point in favour of an intratesticular site of steroid action. More research is needed to test the various hypotheses proposed.

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Résumé. Des fragments de testicule de truite arc-en-ciel ont été prélevés aux 8 stades de la spermatogenèse et incubés en présence de progestérone ou de 17α -hydroxyprogestérone tritiée. Le spectre des activités enzymatiques potentielles de la voie Δ_4 de synthèse conduisant à des métabolites majeurs a été établi pour chaque stade.

- 1) Une 5-réductase convertit la progestérone en $5\alpha(\beta)$ -prégnane 3,20-dione.
- 2) Une 20β -oxydoréductase convertit la 17α -hydroxyprogestérone en 17α -hydroxy- 20β -dihydroprogestérone. Son activité est élevée et, à certains stades, la production de ce dérivé égale ou excède celle des androgènes.
- 3) Certaines activités enzymatiques semblent impliquées dans le contrôle et la modulation du métabolisme stéroïdogène au cours du cycle : 20 -oxydoréductase, 17 - 20 desmolase, 17β -oxydoréductase.
- 4) Le rôle physiologique possible des différents stéroïdes majeurs est discuté : l'hypothèse d'une participation de la 17α -hydroxy- 20β -dihydroprogestérone au contrôle de l'activité mitotique des gonies et des spermatocytes et (ou) au rétrocontrôle de l'axe hypothalamo-hypophysaire est examinée.

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