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IN VITRO STUDIES ON STEROID METABOLISM OF TESTICULAR TISSUE IN AMBISEXUAL TELEOST FISH

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SUMMARY

Ambisexual teleost fish are the only vertebrates among which either simultaneous hermaphroditism or spontaneous sex inversion normally occur. In some cases both heterologous germinal tissues coexist during a certain period of the animal's life cycle and can be separated mechanically from each other. Under these conditions metabolic changes during the reproductive cycle and eventual alterations related to sex inversion can be investigated. From samples of this kind sliced tissues have been incubated with C^{14}-labeled steroid precursors (progesterone, testosterone) for 2 h at 26°C without adding cofactors. Testicular tissue from the simultaneous hermaphrodite *Serranus cabrilla*, the protandric *Pagellus acarne* and the diandric protogynous *Coris julis* have been used in the experiments. The metabolites were separated by paper- and thin layer-chromatography and identified by derivative formation and crystallization to constant specific activity. Besides the formation of various saturated 5β-reduced C_{19}- and C_{21}-steroids the occurrence of Δ_4-steroids with a hydroxyl- or oxo-group at C-11 including 11-hydroxyprogesterone is remarkable. Differences in metabolic patterns are more important comparing different species than tissues of the same animal at various physiological stages.

INTRODUCTION

The growing interest in comparative endocrinology has initiated a considerable number of studies on teleost fish which represent by far the largest group of vertebrates. But in the field of steroid biochemistry the teleosts must be considered as nearly unexplored.

At present a general picture of steroid biosynthesis and metabolism in these animals does not exist and the physiological role of steroid hormones which are familiar to us from other vertebrates is largely unknown in this group [1]. The peculiar phenomenon of migration between fresh and salt water in certain species has stimulated a relatively larger amount of research work devoted to corticosteroids but our knowledge of sex hormones remains very unsatisfactory. The review by Ozon[2] on androgens gives a striking illustration of this state of affairs. Although the tables which the author provides should not be considered as being complete, it remains nevertheless remarkable when he reports the isolation of 4 steroids only from altogether 7 species.

In a few scattered experiments on C_{19}-steroid biosynthesis in testicular tissue 6 different labeled precursors have been used and a total of 10 steroid metabolites was found in which the characterization procedure merits the rating as "tentative" or better.

An evaluation of these data is hardly possible. Obvious difficulties in making reasonable correlations between the reported results and the respective physiological conditions of the biological samples account for this situation. Moreover, it is hardly realized so far that a tremendous variety in the reproductive biology of teleosts has to be taken into consideration.

The occurrence of various types of natural ambisexuality in a fairly large number of teleost species [3], has been the starting point for this report.

Teleosts are the only vertebrates among which the deviation from the usual gonochoristic condition can be observed as a natural phenomenon [3, 4] and from a comparative point of view it seems to be worthwhile asking the question whether differences in the sexual organization are reflected in some way in the biosynthesis and metabolism of steroid hormones.

A preliminary *in vitro*-experiment on steroid metabolism in the protogynous sea bass *Centropristes striatus* [5] served as a pilot study only. A paper by Chan & Phillips[6] suggested for the first time some evidence that the process of protogynous sex-inversion might be correlated with a change in the pattern of gondadal steroid metabolism. Colombo *et al.*[7] claimed a reduction of enzymatic activities in regressing gonadal tissue from a protandric species. Our experience with a larger number of ambisexual species [3, 8] provided the basis to start a comparative study of metabolic activities of gonadal tissue at various stages of the reproductive cycle in a functional simultaneous hermaphrodite (*Serranus cabrilla* (L.)—Serranidae) and species with spontaneous sex-inversion (the diandric protogynous *Coris julis* (L.)—Labridae and the protandric *Pagellus acarne* (Risso)—Sparidae).

EXPERIMENTAL

(a) *Collection of biological samples*

A large number of ambisexual fishes offer the advantage of having the heterologous germinal tissues clearly separated from each other within the gonad [3, 8]. In those cases ovarian and testicular components were dissected. All preparations were made

with the gonad immersed in the same medium that was used for later incubation. In *Pagellus acarne* transforming gonads can be identified in late winter and during spring only [8]. During this period of the year the following tissues can be used:

(1) Ovarian and testicular parts from functional males,

(2) enlarging ovarian and regressing testicular tissue from transforming specimens,

(3) ovaries from functional females.

In *Coris julis* primary and secondary males are distinguished throughout the whole year by their color pattern. Inverting specimens are available only during a relatively short period after the spawning season. They are recognized by their intermediate color pattern [8]. Mostly the gonadal material was taken from animals which were killed by decapitation. In cases when trawler catches sometimes provided dead fish only (*Pagellus*) such animals were selected in which the heart was still beating.

(b) *Incubation*

After preparation the gonadal tissue was cut into very small pieces with a pair of scissors and suspended in 50 ml Erlenmeyer flasks in 10 ml of the incubation medium (after Young for *Uranoscopus*—cf. Lockwood[9]) to which 1 μCi of labeled precursor (4-^{14}C-testosterone or -progesterone dissolved in 0·1 ml 1,2-propanediol) had been added shortly before. The pH was adjusted to 7·4. Besides 2 g glucose/1000 ml no cofactors were added. The amount of tissue usually was kept below 0·5 g/10 ml. The time between preparation of the fish and beginning of the incubation rarely exceeded 30 min. The incubation was carried out in a shaking apparatus (Braun–Melsungen) at 26°C under normal atmosphere. The incubation was stopped (mostly after 2 h) by freezing medium and tissue in small glass vials put on dry ice. The material was kept below −25°C until extraction.

(c) *Extraction*

After thawing medium and tissue were homogenized and separated into neutral and phenolic fractions by a combination of the methods after Short[10] and Baggett *et al.*[11].

(d) *Separation and characterization of metabolites*

In all cases the neutral and phenolic fractions were spotted on a paper chromatogram and developed either in the Bush A System (progesterone as precursor) or Bush B_3 and B_1 (testosterone as precursor). After sufficient purification the various metabolites were characterized by current procedures: comparison with the mobility of standard compounds in various chromatographic systems (paper, thin layer silica-gel), by observing the results after oxidation (CrO_3 in pyridine overnight), reduction (with sodium borohydride), acetylation and saponification. When sufficient evidence for the probable identity of certain metabolites had been obtained crystallization to constant specific activity [12] was performed using a Beckman spectrophotometer and a BF-liquid scintillation counter. Technical details will be reported elsewhere.

RESULTS

(a) *Metabolites*

In the neutral extracts of incubations with progesterone it was possible to discover up to 15 clear cut radioactive peaks after using a generalized separation scheme which was published elsewhere [13] and which has to be modified according to particular requirements. The number of metabolites with testosterone as a precursor was smaller than 10. I have not yet been able to characterize all of them. Particular difficulties are raised by the saturated polar C_{19}- and C_{21}- di- and trihydroxy-steroids because of the many isomers which have to be taken into account and which are hard to separate by the conventional methods described before. Oxidation of an aliquot of the unknown substance and separation of the reaction products by various TLC systems with multiple developments indicates that 5β-reduction seems to be the predominant metabolic pathway in all species that have been investigated so far.

There is no doubt that 5β-androstane-3α, 17β-diol and 5β-androstane-3β, 17β-diol are among the metabolites of testosterone. In the less polar group of steroids from progesterone large amounts of 5β-pregan-3α-ol-20-one and lesser quantities of 5β-pregnan-3β-ol-20-one are regularly observed. 5β-pregnan-3,20-dione was isolated from all incubations with progesterone. But the question remains to be answered whether 5α-reduced steroids do occur also in my material.

In the more polar group of steroids from incubations with progesterone the occurrence of 17α-hydroxyprogesterone, 11β-hydroxyprogesterone and 5β-pregnan-17α-ol-3,20-dione merit particular attention. To the best of my knowledge 11β-hydroxyprogesterone is reported here for the first time as a gonadal metabolite in vertebrates. Its finding disproves once again the still widespread but erroneous opinion that under normal conditions 11β-hydroxylase activity in vertebrates is restricted to adrenal tissue only (cf. [14] p. 64). Testosterone and 4-androstenedione could be observed sometimes, but the amount is extremely small and I cannot exclude that failure to discover them in some of the extracts might be attributed to inadequate techniques at the beginning of my work. It should be mentioned that the largest part of radioactivity in the polar fraction of all extracts had in various chromatographic systems a mobility similar to 11-keto-testosterone (androst-4-ene-17β-ol-3, 11-dione). But upon acetylation and running in suitable TLC-systems (e.g. chloroform/acetone 95:5) it splits into 2–3 different compounds none of which corresponding to 11-ketotestosterone. Despite many efforts to characterize at least the main peak of

radioactivity I have not reached a firm conclusion about its nature other than that it must have two acetylable hydroxy-groups. There is some reason to assume that the unknown compound is an unusual steroid which merits much more thorough investigation.

After incubations with testosterone, 11β-hydroxytestosterone (androst-4-ene-11β, 17β-diol-3-one) is the most prominent polar steroid. It occurs in all species irrespective of the physiological stage of the tissue that was used. Androstenedione, the formation of which *in vitro* has been reported repeatedly [2] could be found in some incubations only and then its amount was invariably very low (less than 5%).

(b) *Comparison between metabolic activities from various tissues of different species*

At the onset of our studies it was expected that in a given species physiological differences between the same germinal tissues (e.g. in the protandric *Pagellus acarne* testicular tissue from functional males and testicular tissue from animals in the process of transformation from functional males to functional females) might be clearly reflected in different metabolic activities. This hypothetical assumption was backed up by a study of Chan & Phillips[6] on the protogynous ricefield eel *Monopterus albus* in which the authors described conspicuous changes in the ratio of neutral and phenolic steroids. Colombo *et al.*[15] provided similar arguments when they claimed that in the protandric *Sparus auratus* regressing testicular tissue should have a much lesser enzymatic activity than testicular tissue of functional males of the same species. But the data of these authors should be evaluated with great caution since their comparison was made between tissue samples that have been taken from entirely different stages of the annual reproductive cycle. In the closely related *Pagellus acarne* which is protandric also [8] I have not been able to observe similar differences when I compared testicular tissue from functional males and specimens undergoing sex-inversion when they had been taken from the same trawl-net catch. It is true that after incubation of regressing testicular tissue, with progesterone as a precursor, very small amounts of the substrate have been rediscovered whereas after incubation of functional male tissue practically all progesterone had been used up after the 2 h incubation period. But this difference seems to be well within the range of normal biological variation. According to my observations intraspecific variations are considerable even when comparing tissues that appear to be physiologically identical from a biological point of view (same stage of the reproductive cycle and conditions of incubation of the same kind). As long as we lack sufficient data about the degree of variability that can be encountered when tissues of several specimens are used, any conclusion with regard to the parallelism between histological conditions (and this is the only reliable biological parameter which we have at present) and enzymatic activities seem to be premature. A study by one of my students (Krone, unpublished) on metabolic activities *in vitro* by testicular tissue from the gonochoristic Cichlid fish *Haplochromis burtoni* (Guenther) corroborates this opinion.

At the present stage of our work interspecific differences seem to be much more prominent. The most striking example is the observation that in *P. acarne* —in spite of extensive search—not a trace of 11β-hydroxyprogesterone could be found in any type of gonadal tissue (ovarian material included) after incubation with progesterone. But it was always present in the neutral fraction of incubations with testicular and ovarian tissues of *Coris*. The hermaphroditic *Serranus* is different again since neither 11β-hydroxyprogesterone nor 17α-hydroxyprogesterone could be discovered so far. Interspecific differences seem to be important also after using testosterone as a precursor although the variations are less conspicuous than in incubates from progesterone. From a general comparison between the metabolic conversion of progesterone and testosterone as labeled precursors by tissues of the same physiological type it is obvious that progesterone is metabolized more actively than testosterone.

DISCUSSION

In spite of the limitations inherent in the use of *in vitro*-techniques for the study of steroid production and steroid metabolism in the respective endocrine tissues our investigations demonstrate clearly that this type of work is worthwhile to be undertaken. This is particularly true in our case where it is sometimes difficult to obtain sufficient biological samples and where heterologous germinal tissues which are considered to be somewhat antagonistic in other vertebrates coexist in the same individual. Our studies add further evidence that every effort should be undertaken to characterize the various metabolites since steroid patterns in teleosts may be quite different from other vertebrates as was shown for the first time so convincingly by Idler and his coworkers [16–18].

It is puzzling that in our investigation and in other studies [15] both 11β-hydroxytestosterone and 11-ketotestosterone could not be found as metabolites when progesterone is used as a precursor although both compounds are so prominent as metabolic products from testosterone. This observation might be explained by the fact that the yield of testosterone and androstenedione from progesterone was always very low provided that both steroids were even found. Incubations made with labeled DHA and pregnenolone await extraction and further analysis in order to obtain some ideas about the possible significance in the use of the Δ_4- and Δ_5-pathway by the gonadal tissue of ambisexual teleosts. The final purpose of our studies, however, should be to obtain some ideas for future biological experimentation with steroids other than testosterone and androstenedione in teleost fish. Sufficient evidence has accumulated in the meantime that biologically active androgens of higher vertebrates might not be the same in lower forms.

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