Corticosteroid Biosynthesis in the Interrenal Cells of the Teleost Fish, Oreochromis mossambicus

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Applying high-performance liquid chromatography and thin-layer chromatography to separate corticosteroids, we studied the biosynthesis of steroids by the interrenal cells of the head kidneys (the adrenocortical homolog) of Oreochromis mossambicus. Intact head kidneys converted exogenous 17α-hydroxyprogesterone into mainly cortisol, but 11-deoxycortisol, cortisone, and androstenedione were also recovered from the medium. Incubation of intact tissue with pregnenolone in addition resulted in the formation of large amounts of an unidentified product, which was absent in incubations of tissue homogenates with pregnenolone. © 1989 Academic Press, Inc.

There is substantial literature on the biosynthesis of steroids by the teleost interrenal cells, the adrenocortical homolog of fish. In vitro, these cells seem capable of synthesizing every corticosteroid identified in the vertebrates (Butler, 1973). Despite this apparent in vitro versatility of the teleost interrenal cells, to date cortisol is considered the most important corticosteroid in fish, partly because of the quantities produced and the range of biological activities reported, but also because searches for biological activities of some of the other vertebrate corticosteroids (corticosterone, aldosterone) in teleosts have been unfruitful (Henderson and Garland, 1981; Henderson and Kime, 1987; Mayer-Gostan et al., 1987).

Information on steroid biosynthetic routes in teleosts generally has been obtained from incubations of head kidney homogenates with labeled precursors (Sangaang et al., 1972; Colombo et al., 1972). A problem associated with much of the earlier work is that it "should be examined with the understanding that identifications are at best tentative" (Butler, 1973) with the methods available at that time. We therefore have reevaluated the biosynthesis of steroids by the interrenal cells (the adrenocortical homolog of fish) of Oreochromis mossambicus (tilapia) by applying high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). This report is part of a study on the control of synthesis and release of corticosteroids by the interrenal cells of teleost fish.

MATERIALS AND METHODS

Animals

Sexually mature female Oreochromis mossambicus (formerly Tilapia mossambica and Sarotherodon mossambicus, Cichlidae) were used. They were kept isolated from males at least 2 months prior to the experiments to reduce reproductive activity (Silverman, 1978). When sacrificed, the animals usually were 7 months old and sexually mature, with an average body weight of 20 g, unless indicated otherwise.

Fish were kept in 120-liter freshwater aquaria under constant aeration and filtration (Eheim aquarium pumps). The water (for composition, see Wendelaar and van der Meij, 1981) was renewed regularly. The temperature was maintained at 28°C. The fish were kept under a 12L/12D regimen (light on: 7:00 AM) and were fed Tetramin tropical fish food.

Animals received their last meal 24 hr before sacri-
lice. Fish were netted rapidly and killed by spinal dissection. In view of the reported daily rhythmicities in plasma cortisol levels in fish (Spieler, 1979; Spieler and Noeske, 1981; Rance et al., 1982; Pickering and Pottinger, 1983; Nichols and Weisbart, 1984) care was taken to sacrifice all animals at 9:00 AM. Head kidneys were excised and processed for incubation.

**Incubations**

Homogenates of head kidneys were prepared in a sucrose buffer (0.25 M sucrose, 0.1 M sodium phosphate buffer, pH 7.4; 1/2 w/v; 0°) with an all-glass homogenizer. The homogenate was centrifuged (10 min, 800 g, 2°, Beckman TJ-6) to prepare a cell-free system. The supernatant (1 ml) was transferred to a glass vial containing [3H]pregnenolone (3 μCi in 0.5 ml propylene glycol), 2 mM NAD, 2 mM NADPH, and sodium phosphate buffer (0.1 M, pH 7.4). Incubation volume was 4.5 ml. During the incubations, which lasted 3 hr, samples were taken and dichloromethane was added to terminate steroid bioconversion. Tissue incubations, which lasted 4 hr, were carried out with various amounts of tissue (25—400 mg; see legends) in 2 ml Dulbecco's modified Eagle medium (MDM), containing 4 μCi [3H]pregnenolone or 4 μCi [3H]-17α-hydroxyprogesterone, both dissolved in 0.2 ml propylene glycol. No cofactors were added. All incubations were carried out at 28° in an air atmosphere under continuous shaking, and were terminated by adding dichloromethane.

**High-Performance Liquid Chromatography**

Samples of medium or homogenate (1—2 ml) were extracted three times with dichloromethane (5 ml). The organic phase was evaporated, redissolved in 0.5 ml dichloromethane, and analyzed by HPLC (Spectra Physics SP 8000, Eindhoven, The Netherlands), which was equipped with a LiChroSorb Si-60-5 column (250 × 4.6 mm, Chrompack Middelburg, The Netherlands). Steroids were eluted with dichloromethane—ethyl acetate-water (94/5/1) at a flow rate of 0.9 ml/min. One hundred fractions (0.2 min) were collected with an LKB Redirac fraction collector. Time between runs was 15 min. Four milliliters of scintillation fluid was added to the fractions and radioactivity was determined with an LKB Wallac 1216 Rackbeta liquid scintillation counter. Elution times for several reference corticosteroids were analyzed using commercially available tritiated compounds. Results are expressed as the percentage of total radioactivity for every fraction (% 3H).

**Thin-Layer Chromatography**

Before sample extraction, 25 μg of each of the following carriers was added: pregnenolone (P5), progesterone (P4), 17α-hydroxypregnenolone (17α-P5), 17α-hydroxyprogesterone (17α-P4), 11-deoxy cortisol, corticosterone, 11-deoxycortisol ("compound S"), cortisol, and cortisone. The steroids were extracted from the sample of medium or homogenate with dichloromethane (3 × 10 ml). To remove the excess nonsteroidal lipids, residues were extracted with 0.5 ml hexane and then with acetone (6 × 1 ml). The extracts were stored overnight (−20°) to precipitate apolar lipids. These lipids were centrifuged (10 min, 800 g, 0°), washed twice with acetone (−20°), and then discarded. The combined supernatants were evaporated and the residue (dissolved in a few drops of dichloromethane—methanol 9/1) was subjected to TLC. Thin-layer chromatography was carried out on precoated plates with silica gel F 254 (Merck) in saturated tanks. Three systems were used. In system 1, plates were developed in toluene—cyclohexane (1/1; 3 ×) to separate apolar compounds (triglycerides) from steroids. In system 2, steroids were separated in benzene—ethyl acetate (3/1; 4 ×); and in system 3, in chloroform—methanol (95/5; 2 ×). Carrier and reference steroids were detected by UV absorption (in the case of 3-keto-4-steroids) or by spraying with primuline (Wright, 1971) (other steroids). Radioactive areas on TLC plates were located by means of a Berthold thin-layer chromatogram scanner.

**Reagents**

The tritiated compounds were purchased from Amersham U.K.; the specific activity of pregnenolone was 12.5 Ci/mmol and of 17α-hydroxyprogesterone, 11 Ci/mmol. Reference steroids were from Steraloids or Makor. NADPH and NAD were obtained from Boehringer-Mannheim. Chemicals and solvents (Baker) were of analytical grade.

**RESULTS**

The HPLC elution profiles of the steroid standards (Fig. 1) show that under the conditions employed the method is suitable for separation of corticosteroids derived from 17α-hydroxyprogesterone: deoxycorticisol, cortisol, and cortisone. When CH2Cl2 extraction and HPLC were used for steroid separation, total recovery (radioactivities in the water phase + HPLC fractions) was calculated as 92 ± 3% (mean ± SEM for six samples).
Steroid HPLC profiles of incubation of head kidney homogenate with tritiated pregnenolone for 10, 90, and 180 min are given in Fig. 2. The peak eluting at 7 min likely consists of a mixture of steroids (pregnenolone, 17α-hydroxypregnenolone; see Fig. 1) and is termed precursor peak. It should be noted here that products eluting after 9 min tended to shift to shorter elution times with consecutive runs. To identify cortisol, and especially cortisone, standards were run between sample runs. It is clear from Fig. 2 that homogenates of tilapia head kidneys converted pregnenolone into one major end product which coeluted with cortisol. In addition to the two unidentified minor peaks eluting at 17 and 19 min, the product eluting from the column at 10 min might also qualify as an end product. Cortisone serves as a likely candidate for this peak and the rate at which it was produced seems to decline after 90 min. The unidentified compounds eluting at 9.2 and 11.8 min apparently represent intermediates of steroidogenesis.

**Intact Tissue Supplied with [3H]Pregnenolone**

Incubation of intact head kidney tissue with tritiated pregnenolone resulted in a strikingly different picture (Fig. 3a). Pregnenolone again was converted into cortisol (16.1 min) and cortisone (10.3 min) but the larger part of the radioactivity was recov-
Fig. 2. Head kidney homogenate incubation with [3H]pregnenolone. Head kidney tissue from 4 FW females (average weight 55 g) was homogenized and prepared for incubation as outlined under Materials and Methods. Weight of the head kidney tissue: 284 mg. Incubation volume: 4.5 ml; [3H]pregnenolone: 3 μCi. At 180 min, 6.6% of the total radioactivity could be recovered from the water phase. Arrows indicate elution positions of cortisone and cortisol standards.

ered from a peak eluting at 11.5 min. We subjected the sample of medium to TLC and the resulting radiochromatogram is shown in Fig. 3b. Apparently the HPLC “precursor peak” (8.7 min; Fig. 3a) consisted mainly of pregnenolone. No progesterone was present. Chromatography of the peak marked with an asterisk in chloroform-ethanol 95/5 (system 3; 1×) resulted in separation of 17α-hydroxyprogrenolone (80% of the total peak dpm) and 17α-hydroxyprogesterone (20%). The compound with the same $R_f$ as deoxycortisol (Fig. 3b) still behaved as deoxycortisol in system 3 (not shown). The peak containing cortisol and cortisone was also redeveloped in system 3 (2×) and the radiochromatogram is in Fig. 3c. We conclude on the basis of the relative peak areas that the larger peak from Fig. 3c probably is identical to the HPLC peak eluting at 11.5 min (Fig. 3a).

After the incubation the remaining tissue was homogenized and extracted; subsequent HPLC analysis (not shown) revealed that very small amounts (2%) of the newly synthesized cortisol (medium + tissue) were tissue bound, indicating that storage was negligible, the more since this value was not corrected for extracellular spaces. Neither were conjugated steroids stored. The thin-layer chromatogram of the homogenate (not shown) illustrated that pregnenolone was the principal intracellular steroid, while 17α-hydroxyprogrenolone and 17α-hydroxyprogesterone were present in much smaller amounts. No progesterone could be demonstrated in the tissue.

**Intact Tissue Supplied with 17α-[3H]Hydroxyprogesterone**

Figure 4 depicts the HPLC profile and radiochromatograms of the medium after incubation of head kidney tissue with tritiated 17α-hydroxyprogesterone. Judging from the HPLC profile it seems that *tilapia* interrenal cells converted 17α-hydroxypro-
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Fig. 3. Head kidney incubation with [3H]pregnenolone (240 min). (a) HPLC elution profile of the medium. (b) TLC radiochromatogram of the medium. TLC was carried out in systems 1 (3×) and in 2 (4×). *See Text. (c) TLC radiochromatogram of the shaded area from (b) after chromatography in system 3 (2×). At 240 min, 8.5% of the radioactive products were water soluble and 2.1% of the newly synthesized cortisol was in the tissue. The tissue did not contain any conjugated steroids; 25 mg head kidney tissue was incubated (material from four females, weighing 10 g on average). F, cortisol; E, cortisone; S, 11-deoxycortisol; T, testosterone; 17αP₄, 17α-hydroxyprogesterone; P₅, pregnenolone.

gestosterone almost exclusively into cortisol, whereas only small amounts of the steroid were processed to cortisone (10.5 min). Thin-layer chromatography (systems 2 and 3), however, revealed the presence of substantial amounts of deoxycortisol and androstenedione in the medium (Fig. 4b). Testosterone could not be identified in both medium and tissue. Important in this respect is that the tissue incubated with pregnenolone did not release androstenedione to the medium as could be evaluated from TLC of the pregnenolone peak (Fig. 3b) in system 3 (data not shown). The peak containing cortisol and cortisone could be shown to contain mainly cortisol (Fig. 4c). The tissue again contained minimal amounts of newly synthesized cortisol (not shown). Head kidneys incubated with pregnenolone as exogenous substrate released
Fig. 4. Head kidney tissue incubation with 17α-[3H]hydroxyprogesterone (240 min). (a) HPLC elution profile of the medium. (b) TLC radiochromatogram of the medium. TLC was carried out in systems 1 (3x) and 2 (4x). (c) TLC radiochromatogram of the shaded area from (b) after chromatography in system 3 (2x). At 240 min, 1.0% of the radioactive products were water soluble and 1.0% of the newly synthesized cortisol was tissue bound. The tissue did not contain any conjugated steroids; 26 mg head kidney tissue was incubated (material from four females, weighing 10 g each). F, E, S, T, 17αP₄, and P₃, as in Fig. 3; A₄, androstenedione.

markedly more conjugated steroids (8.5% of the total medium radioactivity) than tissue supplied with 17α-hydroxyprogesterone (1.0%).

DISCUSSION

Steroid Biosynthesis

Several authors who incubated teleost head kidney tissue or homogenates with or without added substrates in vitro have demonstrated the capacity of the interrenal cells to synthesize corticosteroids. Reviewing the older literature (Butler, 1973) it appears that, although species differences exist, the teleost interrenal cells synthesize many of the corticosteroids found in mammals. Because of the arrangement of the interrenal
cells in the head kidneys of most fish it is not possible to incubate the cell without renal elements (glomeruli and tubules), pigment cells, chromaffin tissue, and hemopoietic elements. In tilapia the picture is relatively simple due to the absence of renal elements and the predominantly myeloid nature of the hemopoietic tissue. Nandi and Bern (1965) incubated tilapia head kidneys with or without ACTH and reported the presence of cortisol, cortisone, 17α-hydroxy-, 11-deoxycorticosterone (= 11-deoxycortisol), and 11-dehydrocorticosterone. Seven years later Colombo et al. (1972) investigated corticosteroidogenesis by tilapia head kidneys from [14C]progesterone and identified 17α-hydroxyprogesterone, cortisol, cortisone, 11-deoxycorticosterone, corticosterone, 11-dehydrocorticosterone, 11β-hydroxyprogesterone, and 21-deoxycortisol. In this latter study Colombo et al. used radioactive progesterone as precursor because their preliminary results, utilizing [14C]acetate, had indicated that progesterone might be an intermediate in tilapia head kidney corticosteroidogenesis. Since the data from these preliminary experiments were unconvincing (they obtained only 0.002% conversion of [14C]acetate to progesterone), we decided to use pregnenolone rather than progesterone as radioactive substrate. Pregnenolone is an obligatory intermediate in corticosteroidogenesis, whereas progesterone is not.

High-performance liquid chromatography has been used to separate and analyze steroids from the mammalian adrenal cortex (Kessler, 1982; Cavina et al., 1979; Rose and Juskov, 1979) and teleost plasma and testis (Huang et al., 1983; MacFarlane, 1984). The results of our incubation of Tilapia head kidney homogenate with tritiated pregnenolone illustrated the rapid conversion of pregnenolone into cortisol and cortisone. After 3 hr, 26 and 13% of the radioactivity were associated with cortisol and cortisone, respectively. Other species appear less effective in this respect: a comparable incubation of rainbow trout head kidney homogenate (using approximately ten times as much tissue as in the present study) with pregnenolone yielded only 2.5% cortisol after 150 min (Arai et al., 1969). Sandor et al. (1967) also observed only 4% conversion of pregnenolone into cortisol after a 3-hr incubation of eel head kidney homogenate.

Intact tilapia head kidney tissue converted pregnenolone not only into cortisol and cortisone but also into a product eluting between these two steroids. Thin layer chromatography of the incubation medium confirmed the presence of this product with a polarity between those of cortisol and cortisone. It was present in the tissue as well and therefore probably does not represent a modification of cortisol or cortisone associated with secretion. Subsequent TLC in other systems did not reveal the nature of this product. In relation to this, Patiño et al. (1987) observed that head kidneys of coho salmon, when supplied with added substrate in vitro, secrete large amounts of an unidentified steroid. The authors further reported that this peak was absent in medium when head kidney tissue of coho salmon was incubated without added substrate. Goodman (1980), perfusing the head kidneys of the eel with tritiated pregnenolone in situ, also observed an unknown steroid in the perfusate. Whether or not these products are related to our unidentified steroid remains to be answered. The fact that this product was not present in media of incubations with 17α-hydroxyprogesterone indicates that it must have been derived from either pregnenolone or 17α-hydroxy-pregnenolone. We suggest that its formation might be due to the large amounts of pregnenolone (100 nM) supplied to the tissue. Since in vivo the conversion of cholesterol into pregnenolone is the rate-limiting step in corticosteroidogenesis, the interrenal cells normally do not experience high pregnenolone concentrations. This sugges-
tion is corroborated by the data on coho salmon cited earlier (Patino et al., 1987).

In our study it was established that the principal pathway to cortisol and cortisone in tilapia proceeds via pregnenolone, 17α-hydroxypregnenolone, and 17α-hydroxyprogesterone rather than via progesterone since this steroid was shown to be virtually absent in both tissue and medium. Preference for this route has also been established for the herring and the Atlantic salmon (Sangalang et al., 1972). The formation of tritiated cortisol, cortisone, and 11-deoxycortisol by tilapia head kidneys supplied with labeled 17α-hydroxyprogesterone confirms results obtained by Colombo et al. (1972). Our rate of cortisol production, however, was higher than that reported by these authors. Using 26 mg head kidney tissue we observed some 30% conversion after 4 hr, whereas Colombo et al., incubating 600 mg tissue with approximately the concentration of 17α-hydroxyprogesterone we used, obtained a 17% yield of cortisol after 6 hr. They did not report the formation of any steroids other than cortisol, cortisone, and 11-deoxycortisol from 17α-hydroxyprogesterone. No subsequent androgen formation (testosterone) could be demonstrated. Arai et al. (1969) identified small amounts of androstenedione (1% after 30 min) in incubations of trout head kidney homogenates. Adding androstenedione as a substrate to their incubations yielded 22% testosterone after 150 min. The biological significance of androgen formation by tilapia interrenal cells apparently are able to convert 17α-hydroxyprogesterone into androstenedione by cleaving the C-20 and C-21 side chains of 17α-hydroxyprogesterone. Although Colombo et al. (1972) identified 17-deoxycorticosteroids in incubation media, we suggest that this is due to their choice of large amounts of progesterone as exogenous substrate, a steroid we have shown to be bypassed when pregnenolone was provided as a substrate and it might therefore be related to the high concentration of 17α-hydroxyprogesterone in the in vitro preparation. In line with this, Huang et al. (1983) did not observe androstenedione in carp head kidneys which had not been supplied with exogenous substrates.

Storage of Steroids

The results presented indicate that Tilapia interrenal cells in vitro do not store newly synthesized cortisol, either free or conjugated. Huang et al. (1983) analyzed plasma and unincubated head kidneys from carp, and it can be deduced from their data that in vivo the head kidneys contain enough cortisol to raise the plasma concentration by some 50% upon instantaneous total release. The occurrence and significance of cortisol storage in teleosts in vivo remain controversial. Singley and Chavin (1975) argued that the rise in plasma cortisol they observed 15 sec after subjecting fish to a saline stress [although 15 sec probably must be interpreted as 60 sec; see personal communication in Spieler (1974)] must have originated from the release of stored hormone. Ashcom (1979) also concluded that during the first 15 min of acid stress, brook trout increased their plasma cortisol levels without appreciable de novo synthesis of cortisol. It seems unlikely that the rapid stress-induced rise in plasma cortisol can be attributed exclusively to the release of stored cortisol.

In summary, we conclude that cortisol and cortisone probably are the only two corticosteroids produced in significant amounts by tilapia interrenal cells in vivo. Although Colombo et al. (1972) identified 17-deoxycorticosteroids in incubation media, we suggest that this is due to their choice of large amounts of progesterone as exogenous substrate, a steroid we have shown to be bypassed when pregnenolone was converted by tilapia head kidneys. Nevertheless, species differences may exist and some teleosts might synthesize aldosterone in vivo (Whitehouse and Vinson, 1975; Reinking, 1983). Taking into account the
low sensitivity of the gill and gut mucosal corticosteroid receptor of fish to aldosterone and cortisone (DiBattista et al., 1984), we conclude that cortisol quantitatively and qualitatively is the most important cortico-

steroid in *tilapia*. Suggestions have been put forward that cortisone, although considered biologically inactive itself, may facilitate cortisol action at the target level (Leloup-Hatey, 1974, 1979).

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