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25OHD, A CIRCULATING VITAMIN D METABOLITE IN FISH

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Summary: Serum and hepatic 25-hydroxyvitamin D (25OHD), and serum calcium, phosphate, $25OHD_3$ binding capacity and binding affinity were measured in male and female trout. Both serum and hepatic 25OHD levels are decreased in female trout with elevations in protein bound calcium and phosphate. Whereas the apparent dissociation constant (Kd) for serum binding of $25OHD_3$ of 1.0-2.0 x 10^{-9}M is similar in males and females, the $25OHD_3$ binding capacity of hypercalcemic spawning trout (1.39 x 10^{-7}M) is significantly less than that of male fish (1.88 x 10^{-7}M). At circulating serum concentrations of 25OHD which average 9.5 x 10^{-9}M only 5-7% of trout serum 25OHD binding sites are occupied.

INTRODUCTION

Although fish represent an exceedingly rich repository for antirachitic substances, considerable controversy exists with regard to the chemical nature of the antirachitic factors (1-6) and the metabolism of vitamin D in this vertebrate (4-7). In 1975, Henry and Norman detected renal 25-hydroxycholecalciferol-hydroxylase activity in both fresh and salt water fish (7) although previous attempts to demonstrate the existance of more polar metabolites of vitamin D and 25-hydroxyvitamin D (25OHD) *in vivo* in goldfish were unsuccessful (5). In 1976, Hay and Watson, isolated a circulating 25OHD binding globulin from bony fish; these authors also cited unpublished work of Fraser that 25OHD circulates in marine and fresh water bony fish species in concentrations of

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3.9 - 29.7 ng/ml (6). Subsequent to these reports, Hollis et al, using a calciferol binding globulin from bovine plasma in a competitive binding assay, were unable to detect vitamin D metabolites in rainbow trout plasma (4). Since fish have no parathyroid glands (8) and demonstrate striking elevations in circulating calcium and calcitonin levels during spawning (9), they may serve as a unique model to evaluate the role of vitamin D metabolites in the mobilization of skeletal and scale-calcium depots (10). We were therefore prompted to measure 25OHD in male and female trout and to further evaluate the effect of spawning thereon.

METHODS

Fresh male and female trout one year of age were purchased from a nearby fishery, and blood samples obtained by cardiac puncture without anesthesia. Following centrifugation at 2000 rpm for 20 minutes at 4C, the serum was separated; aliquots were subjected total and ionized calcium measurements utilizing the Turner Fluorometer Model III and the Orion Electrode respectively; phosphate (as inorganic phosphorus) was determined colormetrically (11), and 25OHD measured according to the method of Haddad and Chyu (12) employing [^{3}H-26,27] 25OHD$_3$ (specific activity 12.2 Ci/mmole). Livers were also removed in toto, flushed with isotonic saline and homogenized in 3 x W/V distilled water. Aliquots of the homogenate were subjected to protein analysis (13), and prepared for 25OHD assay utilizing extraction and chromatographic precedures described earlier (12).

In a separate series of experiments, serum was obtained from trout with blood calcium values ranging from 11.8-12.6 mg/dl and spawning trout with calcium levels of 19.4-21.0 mg/dl; 0.5-1.25 µl aliquots of sera were diluted in cold 0.05 M sodium phosphate buffer at pH 7.4. Saturation analyses were performed by adding increasing amounts of ^{3}H-25OHD$_3$ in 50 µl of ethanol to tubes containing 1 µl of unlabeled 25OHD$_3$ in 50 µl of ethanol. Each experiment was performed in triplicate. Control tubes contained 50 µl of ethanol without 25OHD$_3$. (^{3}H-26,27) 25-hydroxycholecalciferol (specific activity 94 Ci/mmole) was purchased from Amersham-Searle and purified on 55 cm columns of Sephadex LH-20

slurried in chloroform: n-hexane (65:35 vol/vol) before use (14). Crystalline reference $25OHD_3$ was kindly supplied by M. Uskokovic of Hoffman LaRoche. The sterol was diluted in absolute ethanol with further dilution based on the molar extinction coefficient: λ263nm (ε18,550). For the binding assay, one ml of diluted trout serum was added to tubes containing labeled and unlabeled (0.25, 0.50, 0.75, 1.0, 4.0, 10,.and 1000 ng) $25OHD_3$, vortexed and then incubated in the dark for 50 minutes at 4C. Each incubation was performed in triplicate. The unbound $25OHD_3$ was absorbed for 30 minutes onto Dextran-20 coated charcoal suspensior as previously described (15). Following centrifugation at 2000 rpm for 20 minutes at 4C, 500λ of the supernatant was added to liquid scintillation vials containing 10 ml of Formula-963. Non-specific or low affinity binding was determined by measuring the supernatant dpm in the presence of a large molar excess (1 μg) of unlabeled $25OHD_3$. Specific binding was determined graphically as described by Rosenthal (16). In these experiments aliquots of trout serum were also subjected to 25OHD assay.

RESULTS AND DISCUSSION

The data in Table 1 confirms earlier reports of elevated total serum calcium in spawning fish (8,9), without alterations in the ionized fraction. These changes were attended by higher serum phosphate levels and lower 25OHD values. As noted previously (8) a significant correlation was found between circulating total calcium and phosphate in male ($r=0.54$, $p<0.01$) non-spawning female ($r=0.68$, $p<0.001$) and spawning female ($r=0.82$, $p<0.01$) trout. Such was not the case when serum total calcium and 25OHD values were compared. Serum and hepatic 25OHD content, similar in both spawning and non-spawning females,were significantly lower in both groups than values obtained in male fish (Table 1).

^3H-$25OHD_3$ was displaced from trout serum by $25OHD_3$ and only one class of high-affinity binding sites observed. There was no significant correlation between individual serum 25OHD values and $25OHD_3$ binding capacities. However, the $25OHD_3$ binding capacity was decreased in hypercalcemic spawning females (Table 2). Preliminary studies with sera from non-spawning female trout reveal

Table 1

SERUM TOTAL CALCIUM (Ca_T), IONIZED CALCIUM (Ca_I) PHOSPHATE (Pi) AND 25OHD IN MALE AND FEMALE TROUT

SEX	n	Ca_T	Ca_I	Pi	25OHD (serum)	25OHD (liver)
		mg/dl			ng/ml	ng/g protein
Male	10	11.1 ± 0.2	3.7 ± 0.4	10.3 ± 0.6	4.2 ± 0.4	31.1 ± 3.1
Female	18	9.8 ± 0.5	4.0 ± 0.2	9.4 ± 0.3	2.9 ± 0.3*	21.2 ± 1.0*[t]
Spawning Females	29	18.2 ± 0.3*	4.3 ± 0.2	12.6 ± 0.4*	2.8 ± 0.2*	

Data expressed as mean ± S.E.

*$p < 0.005$ compared with male

[t]Combined female and spawning females

Table 2

SERUM BINDING CAPACITY AND AFFINITY FOR 25OHD$_3$ IN TROUT

SEX	n	25OHD ng/ml	Binding Capacity x 10^{-7}M	Binding Affinity (Kd) x 10^{-9}M
Male*	10	5.6 ± 0.3	1.88 ± 0.10	1.35 ± 0.23
Spawning Females**	12	3.5 ± 0.4^{t}	1.39 ± 0.04***	1.97 ± 0.52

*Serum total calcium, 11.8-12.6 mg/dl
**Serum total calcium, 19.4-21.0 mg/dl
***$p<0.025$ compared to male
$^{t}p<0.005$ compared to male

25OHD$_3$ binding capacities which are indistinguishable from those of male fish, although as noted earlier, 25OHD levels of non-spawning females are also lower than males of similar ages and similar to those of the spawning female (Table 1). If one assumes that the decreased circulating 25OHD binding capacity reflects a decrease in protein production, and if the fish 25OHD binding globulin (6) is of hepatic origin, estrogen-induced alterations in hepatic protein production which characterize the spawning period in fish (8) may conceivably affect the production of 25OHD binding protein and/or hepatic 25-hydroxylase, the latter resulting in decreased hepatic 25OHD stores (Table 1). In this regard, it is worth noting that trout can metabolize vitamin D$_3$ to 25OHD$_3$ with a metabolic efficiency of 30% in 6 hrs (6). Although the site of metabolic conversion of vitamin D$_3$ is presently unknown, the hepatic concentration of 25OHD in fish is appreciable (Table 1).

Since the average 25OHD level in trout sera approximates 9.5 x 10^{-9}M (Tables 1 and 2) and the serum binding capacity is 1-2 x 10^{-7} (Table 2), as much as 93-95% of the serum 25OHD binding sites are unoccupied in bony fish. The reason for this large reserve supply of circulating 25OHD binding

sites in fish, an observation previously demonstrated in humans (15) and rats (17) is still obscure. Moreover, the fact that the circulating 25OHD levels in female trout are lower than those of male trout (Table 1), and do not change during spawning suggests that this vitamin D metabolite plays an insignificant role in the mobilization of calcium depots in fish.

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