Japan. J. Ichthyol.
42(1): 7–10, 1995

魚類学雑誌
42(1): 7–10, 1995

Hydroxyl-apatite Crystals in the Urine of Stanniectomized Freshwater North American Eels (*Anguilla rostrata* LeSueur)

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(*Received July 29, 1994; in revised form February 6, 1995; accepted February 7, 1995*)

Abstract A white micro-crystalline precipitate was observed in the urine of freshwater North American eels from 7–16 days after Stanniectomy. Scanning electron microscopy and energy dispersive x-ray microanalysis showed that the amorphous crystals contained calcium and phosphorous but no magnesium or other elements. Subsequent x-ray diffraction analysis showed that the crystals were hydroxyl-apatite. These crystals are formed in urine as a consequence of the increased urinary excretion of calcium that always follows removal of the corpuscles of Stannius.

Precipitates are found in fish urine, within normal ranges of pH, when there is a high urinary concentration of divalent cations. Elevated urinary magnesium and calcium concentrations are typical of marine teleost fishes which drink seawater and secrete magnesium and calcium through the wall of the proximal segments of the renal tubules, into the urine. As a consequence, sediments or precipitates are observed not only in the urine but also in the lumina of the urinary tubules (Guitel, 1906; Grafflin, 1936; Pitts, 1934; Lahlou, 1967; Hickman, 1968) of marine teleost fishes. No urinary precipitates or sediments have been previously observed in freshwater fishes in which the urine is very dilute and the concentration of ions is extremely low, particularly divalent ions (see Hickman and Trump, 1969 for review). However, we recently observed urinary precipitates in freshwater North American eels from 7–14 days after the removal of the corpuscles of Stannius, a time when urinary magnesium and calcium concentrations increase significantly (Butler, 1969, 1994). It was of interest to learn about the form and composition of the precipitate in the course of learning how the corpuscles of Stannius regulate renal function in freshwater eels.

Materials and Methods

Animals.—Female freshwater North American eels (*Anguilla rostrata* LeSueur) weighing approximately one kg were collected from the St. Lawrence River near Quebec City, Canada in June 1992. They were held in 100 gallon tanks supplied with flowing, aerated dechlorinated tap water (Na^+, 0.45; Cl^-, 0.95; K^+, 0.02; Ca, 0.98; Mg, 1.59 mmol. l^{-1}) at 13°C for about three months before they were used for our experiments. They were fed earthworms and chopped beef heart, *ad libitum* twice weekly.

Experimental groups.—Daily urine samples were collected from 6 sham-operated eels and 6 stanniectomized (STX) eels on the 16th day after surgery.

Observation tanks.—Experimental eels were selected at random, acclimated to 13°C for two weeks, and starved for the final week before they were used for experiments. During urine collection the tanks were covered with black plastic so that the eels would not be disturbed.

Removal of the corpuscles of Stannius.—Eels were anesthetized in an aqueous solution (1 gram. l^{-1}) of tricaine methane sulphonate (Sigma Chemical Co., St. Louis, MO, USA) and placed on a wet cotton towel. The corpuscles of Stannius were removed through a 8.0 cm lateral incision at the level of the posterior kidney using a method described in detail by Butler (1994). The wound was sutured tightly with 3-0 stainless steel wire and the eel was given an i.m. injection of 50 mg of Ampicillin (Penbritin-500, Ayherst Labs, Montreal, Canada) in 1.0 m*l* of 0.9% saline. Sham-operated eels were treated similarly but the corpuscles of Stannius were not removed.

Plasma and urine collection.—14 days after surgery each eel was anesthetized fully and a semicircular incision through the body wall near the anus exposed the uropore which drains the urinary bladder. A urine drainage catheter (Tygon tubing, i.d.

1.0 mm, o.d. 1.8 mm, Norton Co., OH, USA) was inserted through the urogenital papilla into the urinary bladder. It was tied in place with a 3-0 silk ligature around the neck of the bladder drainage duct. The body wall incision was closed with 5-0 stainless steel wire. The eel was then given an i.m. injection of 50 mg of Ampicillin in 1.0 m*l* of 0.9% sterile NaCl solution. The eel rested for 48 hours, then urine was collected for 6 hrs. Urine samples from 6 STX eels were pooled and centrifuged at 2°C. Urine was decanted and the urinary precipitate was dried in air at 0°C then stored at the same temperature. Precipitates from six eel urine samples were pooled, mixed, and analysed using scanning electron microscopy and energy dispersive x-ray micro-analysis/spectroscopy (EDS) followed by x-ray diffraction.

Blood collection.—Vacutainer tubes containing ammonium heparin (Becton Dickinson, Rutherford, NJ) fitted with 22G needles were used to collect caudal venous blood from each eel at the end of the urine collection. Blood was centrifuged and plasma was stored at −60°C until it was analyzed; Na^+ and K^+ by flamephotometry (IL Model 943); Cl^- by a coulimetric method (Buchler digital chloridometer); Ca and Mg by atomic absorption spectrometry (IL Model 351); and osmolality by freezing point depression (Advanced micro-osmometer Model 3M0).

Scanning electron microscopy and energy dispersive x-ray microanalysis.—A small sample of the white amorphous material was mounted on aluminum stubs with double-stick tape and coated with carbon in an Edwards High Vacuum Sputter-coater (E12E). The material was examined in a scanning electron microscope (Hitachi Model S2500) attached to an energy dispersive x-ray microanalyis system (Link eXL). The secondary electrons emitted by the specimen were detected by a photomultiplier attached to a cathode screen. The image formed by the secondary electrons was projected on a television screen and captured on polaroid P55 film. X-rays were collected at a fixed angle of 45 degrees. X-ray spectra were recorded using a count-time of 40 seconds and a 20 kV acceleration voltage. X-rays were first measured by a detector equipped with a berylium window and a lithium drift-drift silicon crystal. The first signal emitted from the detector went to an amplifier and then to an analyser which displayed the signal in the form of an x-ray spectrum which was printed by a computer. Each element has a characteristic x-ray spectrum so it was possible to complete an elemental analysis of the white amorphous precipitate prior to the x-ray diffraction analysis.

X-ray diffraction analysis.—A capillary tube was sealed at one end with plasticine and a small amount of white amorphous precipitate was placed in the tube. The tube was mounted on a Philips Model PW 1720 x-ray defractometer (Holland). The sample was bombarded with x-rays so that electrons hit the crystals and were diffracted in a pattern of rings specific to their molecular structure which were recorded on photographic film. The spacing and intensity of the concentric rings were matched to those of known reference standard to determine the chemical structure of the unknown crystals.

Results and Discussion

A white precipitate was present in the urine of Stanniectomized eels but not in the urine of sham-operated eels. Scanning electron microscopy showed that these crystals were amorphous (Fig. 1). X-ray micro-analysis showed they contain only calcium and phosphorus (Fig. 2). There was no evidence of magnesium or any other elements including sodium, chloride, or potassium (Fig. 2). Subsequent x-ray diffraction analysis (Fig. 3) showed that the unknown precipitate is hydroxyl-apatite 1/2 $[Ca(OH)_2 \cdot 3Ca_3(PO_4)2]$ since it has the same diffraction pattern as the standard reference hydroxyl-apatite (see Mineral Powder Diffraction File, 1980).

Sediments or precipitates have been observed in the renal tubules and/or urinary bladder of a number of marine species. Munz and McFarland (1964) identified solid material in the turbid urine of Pacific hagfish (*Eptatretus stouti*) but found no evidence of magnesium or calcium. The acid soluble material was thought to represent organic phosphates. Youson (1982a, b) has observed precipitates of "chalky" material in the distal segments of juvenile lampreys (*Petromyzon marinus*) adapted to 80% and 100% seawater. This material was thought to be $MgHPO_4$ as a consequence of magnesium and sulphate secretion by the proximal tubules during the marine phase when lampreys drink seawater (Logan et al., 1980; Rankin et al., 1980). Precipitates are also found in the urine of the higher bony fishes, the teleosts. For example, urine from longhorn sculpins (*Myoxocephalus octodecimspinosus*) was turbid and contained a sediment both before and after it was drained from the urinary bladder. After this sedi-

Fig. 1. Hydroxyl-apatite crystals recovered from urine of freshwater eels 16 days after removal of the corpuscles of Stannius (scanning electron micrograph; 2000×).

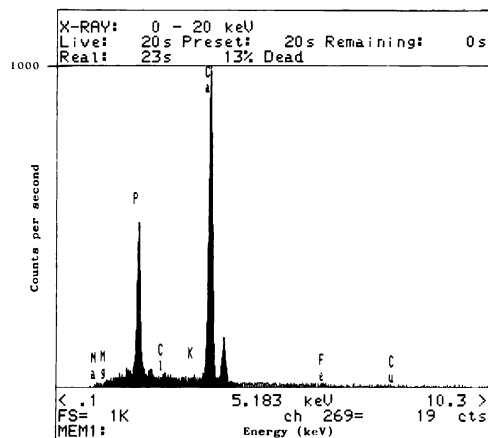

Fig. 2. X-ray spectra of amorphous precipitate in the urine of freshwater eels 16 days after removal of the corpuscles of Stannius. Note that the only significant peaks are those of phosphorus and calcium.

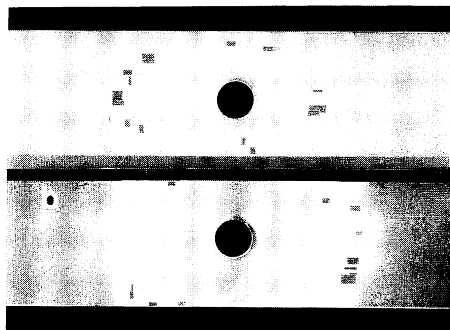

Fig. 3. X-ray diffraction pattern of amorphous precipitate found in the urine of freshwater eels 16 days after removal of the corpuscles of Stannius. Note the pattern for (a) standard reference hydroxyl-apatite and (b) the urine precipitate.

ment was centrifuged, collected and washed with distilled water it was analyzed and found to be $MgHPO_4 \cdot 3H_2O$. This bladder urine was a supersaturated solution of magnesium phosphate and would remain so unless water was reabsorbed by the bladder (Pitts, 1934; Grafflin, 1936) whereupon a precipitate would form. Moreover, Hickman (1968) reported that freshly collected urine from the southern flounder (*Paralicthyes lethostigma*) was turbid due to the presence of a calcium precipitate which was heavier in alkaline urine than in acidic urine although the amount of precipitate was influenced more by phosphate than by pH.

Precipitates are found in the urine of marine or euryhaline fishes adapted to seawater because seawater is ingested and the divalent cations are excreted renally, especially the large quantities of ingested magnesium and sulphate (Hickman and Trump, 1969). However, precipitates are not found in the

Table 1. Plasma and urinary electrolyte concentrations (mmol. l^{-1}) and osmolality (mosm. kg^{-1}) in freshwater North American eels (*Anguilla rostrata*) 16 days after removal of the corpuscles of Stannius

	Group	Na^+	K^+	Cl^-	Mg	Ca	PO_4^{2-}	osmolality
PLASMA	SHAM	147.2±2.9	1.99±0.03	99.5 ±2.0	1.06±0.07	2.61±0.06	4.60±0.29	281 ±3.1
	STX	138.1±1.0*	2.83±0.18*	80.8 ±3.3*	0.69±0.04*	5.53±0.39*	2.34±0.61*	269 ±3.6
URINE	SHAM	13.9±1.5	1.72±0.21	2.03±0.47	0.11±0.03	1.38±0.13	4.98±0.99	33.7±2.4
	STX	22.3±3.7*	1.63±0.40	2.43±0.53	0.34±0.07*	4.06±0.28*	3.43±0.20	36.7±5.3

SHAM = sham-operated control; STX = corpuscles of Stannius removed. $n = 6$ for each experimental group. Values are means±S.E.M. *$p < 0.05$ compared with SHAM using Students t test.

urine of freshwater fishes where, as a rule, ions are conserved and urine is dilute and copious. Hypercalcemia, hypomagnesia, hyponatremia and hypophosphatemia all followed Stanniectomy (Table 1) conforming to changes which have been observed already in freshwater North American eels (Butler, 1969, 1994; Fenwick, 1974) and European eels (Chan et al., 1969). In our experiments, the pronounced hypercalcemia led to a substantial increase in urinary calcium and magnesium concentrations followed, in turn, by the precipitation of pure hydroxyl apatite but not of magnesium salts.

Acknowledgments

This research was supported by grant A2359 from the National Sciences and Engineering Research Council to D. G. B. The authors gratefully acknowledge the technical assistance of Mrs. Rossana Soo and D. Holmyard.

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スタニウス小体を除去された北米産淡水ウナギ尿中のハイドロキシアパタイト結晶

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スタニウス小体除去 7-14 日後に，北米産淡水ウナギの尿中に白色の微細沈澱物が観察された．走査電顕およびエネルギー分散型 X 線微量分析の結果，この無定形結晶は，カルシウムとリンを含有するが，マグネシウムや他の元素は含んでいなかった．X 線回折によりこの結晶はハイドロキシアパタイトであることが示された．この結晶は，スタニウス小体除去に伴い，常に尿中へのカルシウムの排出が増加する結果形成される．