Production of 11-Ketotestosterone and Other Steroids by the Sliced Ovaries of Crab, *Portunus trituberculatus*

Shin-ichi TESHIMA and Akio KANAZAWA*

(Received December 23, 1969)

The presence of steroid-hormonelike materials in marine invertebrates has been suggested by many workers.¹⁻⁶⁾ Recently, the chemical identifications of these effects have been carried out with the eggs of lobster,⁷⁻⁹⁾ the ovaries of starfish and sea urchin.^{10,11)} On the other hand, it has been demonstrated by tracer experiments that the gonads of sea urchin,¹²⁾ oyster,¹²⁾ lobster,¹³⁾ scallop,¹⁴⁾ and blue crab¹⁵⁾ contain the enzyme systems for steroid hormone biosynthesis.

The purpose of the present study is to obtain further information on steroid metabolism in marine invertebrates.

Experimental and Results

The sliced ovaries (1000 mg) of crab, *Portunus trituberculatus*, were incubated in a mixture of 8 ml incubation medium with progesterone (1.0 mg), cofactors^{14,16)} (2.5 mg of glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase, and 1.0 mg of nicotinamide-adenine dinucleotide phosphate) and antibiotics (9000 units of penicillin G and 240 μ g of aureomycin). The incubation medium contains the following salts (g/1000 ml): NaCl, 23.0; KCl, 0.964; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 5.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 7.27; tris, 3.0; CaCl_2 , 1.119; $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 0.138; pH, 7.4. After oxygenating for 2 minutes, the flasks were incubated with shaking at 20°C for 1 hour, and then the medium was changed with the fresh medium containing cofactors, antibiotics and progesterone (1.0 mg). After incubating for 4 hours successively, the tissues and two changes of medium were extracted twice with 7 volumes of dichloromethane, and the extracts were evaporated under nitrogen below 40°C. In total, 8.0 g of ovaries was used for incubation.

For the purpose of investigating the steroids produced from endogenous precursors, 8.0 g of the ovaries was incubated without addition of progesterone under the same conditions. In addition, the analysis of steroid hormones in the fresh ovaries was carried out as control.

The dichloromethane extracts were chromatographed initially on a thin-layer plate (Kieselgel G - Kieselgel GF₂₅₄ (5:1)) using chloroform-methanol (97:3) as a solvent. The UV-absorbing substances were eluted from the plate with dichloromethane-methanol

* Faculty of Fisheries, Kagoshima University. Shimoarata-cho, Kagoshima City (手島新一・金沢昭夫: 鹿児島大学水産学部)

(9: 1),¹⁹⁾ evaporated, and rechromatographed on a thin-layer using three or more solvent systems, successively. The solvent systems^{18,19)} used for thin-layer chromatography (TLC) are shown in Table 1. The chromatographic homogenities and mobilities of eluted fractions were investigated by TLC along with standard steroids. The steroids on thin-layer plates were located under UV-light (at 253 m μ) and by blue tetrazolium reagent. Moreover, the final eluates from TLC were analyzed by gas-liquid chromatography (GLC) using two different columns.

Table 1. Solvent systems in TLC.

Solvent systems	Compositions
S1	Chloroform-methanol (97: 3)
S2	Benzene-acetone (8: 2)
S3	Benzene-chloroform-methanol-ether (2: 2: 1: 1)
S4	Benzene-acetone (3: 1)
S5	Benzene-methanol (9 : 1)
S6	Heptane-ethyl acetate (8: 2)

Table 2. Rf values in TLC and relative retention times in GLC of the steroid hormones detected from the ovaries incubated with progesterone.

Steroids identified	Rf in TLC					Relative Rt in GLC*	
	Solvent systems					Column packings**	
	S1	S2	S3	S4	S5	QF-1	OV-17
11-Ketotestosterone	0.21	0.17	0.50	0.17	0.12	7.86	0.72
11 β -Hydroxy-androst-4-ene-3,17-dione	0.38	0.29	0.60	0.35	0.17	11.47	0.86
Testosterone	0.45	0.30	0.56	—	0.20	3.88	—
17 α -Hydroxy-progesterone	0.55	0.35	0.67	0.44	—	17.65	1.20
Progesterone (precursor)	0.67	0.53	0.73	0.53	0.50	8.59	0.93

* Relative to cholestane

** QF-1: Column 2.0 m $\times$ 4 mm I.D., 1.0% QF-1 on 60-80 mesh Chromosorb W; Column temp. 210°C; N₂ 1.0 kg/cm²

OV-17: Column 3.0 m $\times$ 4 mm I.D., 1.5% OV-17 on 80-100 mesh Shimalite W; column temp. 210°C; N₂ 1.0 kg/cm²

From the ovaries incubated with progesterone, 17 α -hydroxy-progesterone, testosterone, 11-ketotestosterone, and 11 β -hydroxy-androst-4-ene-3,17-dione were tentatively identified by TLC and GLC as shown in Table 2. Under the conditions of this experiment, 17 α -hydroxy-progesterone was present in relatively high concentration in comparison with other steroids.

Two additional UV-absorbing substances were detected in initial TLC of the extract from the ovaries incubated with progesterone. One (Rf, 0.60), blue tetrazolium-positive, revealed the same mobilities as deoxycorticosterone in TLC using the system S1, S2,

and S5, however it was left un-analyzed by GLC. The other (Rf, 0.15), blue tetrazolium-negative, was observed to be more polar than both cortisol and 11-ketotestosterone in TLC using the system S1, S2, and S3. The gas-chromatographic retention time of this substance was not identical with that of the steroid hormones usually found in the ovaries.

From the ovaries incubated without addition of progesterone, the three UV-absorbing substances were detected in initial TLC, although these substances were in minute quantity. One of them (Rf, 0.38) was identical with authentic 11 β -hydroxy-androst-4-ene-3,17-dione in TLC using the system S1, S3, and S5. However, no further identification could be done due to insufficient material. The other substances (Rf, 0.15 and 0.75) were conceivable to be different with steroid hormones from the mobilities in TLC and the retention time in GLC.

From the fresh ovaries, the two UV-absorbing substances were detected in initial TLC. From the results of sequential TLC and GLC, these substances were assumed to be different with steroid hormones. However, the insufficient materials did not allow to carried out further investigation. Table 3 summarized the steroids identified from the ovaries incubated and non-incubated.

Table 3. Steroid hormones identified from the incubated and non-incubated ovaries of crab, *Portunus trituberculatus*.

Steroids	Non-incubated ovaries	Incubated ovaries	
		Without progesterone	With progesterone
11-Ketotestosterone	—	—	+
11 β -Hydroxy-androst-4-ene-3,17-dione	—	$\pm$	+
Testosterone	—	—	+
17 α -Hydroxy-progesterone	—	—	++
Progesterone (precursor)	—	—	+++

Discussion

11-Ketotestosterone first isolated from the blood of sockeye salmon²⁰⁾ has also been obtained from the blood of Atlantic salmon²¹⁾ and the ovaries of common mullet,²²⁾ revealing strongly androgenic activities for sockeye salmon²³⁾ and for medaka, *Oryzias latipes*.²⁴⁾ The *in vitro* production of this substance has also been demonstrated by the Atlantic salmon tissues,²⁵⁾ the testes of rainbow trout^{26,27)} and the ovaries of mollusc.²⁸⁾ However, it has not yet been isolated from a marine invertebrate such as the crab. This report is the first case showing the production of 11-ketotestosterone in a marine invertebrate.

From the present data, it may be suggested that crab, *Portunus trituberculatus*, possesses the enzyme systems at least for the synthesis of 11-ketotestosterone, 11 β -hydroxy-

androst-4-ene-3,17-dione, testosterone, and 17 α -hydroxy-progesterone.

Acknowledgement

The authors wish to express their thanks to Prof. K. KASHIWADA, University of Kagoshima, for his kind advice.

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