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In Vitro Biosynthesis of Steroids from Progesterone by the Ovaries and Pyloric Ceca of the Starfish *Asterias rubens*

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In vitro biosynthesis of steroids from progesterone in ovaries and pyloric ceca of *Asterias rubens* has been investigated. The biosynthesis of 17α-hydroxyprogesterone, androstenedione, testosterone, 20α-dihydroprogesterone, 11-desoxycorticosterone, and 5α-pregnane-3,20-dione could be demonstrated to take place in the tissues of both organs by using [1,2-^{3}H]progesterone as a precursor. The yields of intermediates of the Δ^4-pathway and of 11-desoxycorticosterone are small, being higher in the ovaries than in the pyloric ceca. The yields of 20α-dihydroprogesterone are low, those of 5α-pregnane-3,20-dione are high. In both cases the yields in the pyloric ceca exceed those in the ovaries. The results indicate the presence of the following biosynthetic enzyme systems in ovaries and pyloric ceca of *Asterias rubens:* 17α-hydroxylase, C_{17}–C_{20}-lyase, 17β-HSD, 20β-HSD, 21-hydroxylase, and 5α-reductase. The importance of these enzymes for the metabolism of progesterone, i.e., the biosynthesis of C_{19}-steroids, 20α-dihydroprogesterone, and corticosteroids, will be discussed.

Steroidogenesis in invertebrates is less well known than in vertebrates. Only a few reports are available on the presence of steroids and steroid biosynthesis in arthropods (Teshima and Kanazawa, 1971a, b; Tcholakian and Eik-Nes, 1971) and in mollusks (Gottfried and Dorfman, 1970; De Longcamp *et al.*, 1974; Lupo di Prisco *et al.*, 1973; Lupo di Prisco and Dessi'Fulgheri, 1975; Carreau and Drosdowsky, 1977). For Echinodermata some data suggest a steroid-synthesizing capacity. Colombo and Belvedere (1976) reported steroid biosynthesis in the gonads of the echinoid *Paracentrotus lividus*, the ophiuroid *Ophiothrix fragilis*, the holothuroid *Cucumaria planci*, and the asteroid *Astropecten irregularis pentacanthus*.

Some indications for the steroid-synthesizing capacity in the gonads of *Asterias rubens* have been obtained by demonstrating the presence of the 3β-hydroxysteroid dehydrogenase (3β-HSD) and the 17β-hydroxysteroid dehydrogenase (17β-HSD), two enzymes essential for steroid biosynthesis (Schoenmakers *et al.*, 1976). In addition, ultrastructural evidence has been obtained for the presence of steroid-synthesizing cells in the ovaries of *Asterias rubens* (Schoenmakers *et al.*, 1977). Cholesterol[1] can be converted into pregnenolone and progesterone in the ovaries and pyloric ceca of *Asterias rubens* (Schoenmakers, 1977, 1979). Therefore, the purpose of this study is to examine the conversion of progesterone into other C_{21}-steroids and the biosynthesis of C_{19}-steroids from progesterone. Since the pyloric ceca are assumed to be involved in the growth of the ovaries, the steroid biosynthesis of the ovaries will be compared to that of the pyloric ceca.

Progesterone was reported to be present in the ovaries of the starfish *Pisaster ochraceus* (Botticelli *et al.*, 1960) and *Asterias*

[1] Steroid nomenclature: cholesterol = 3β-hydroxycholest-5-ene; pregnenolone = 3β-hydroxypregn-5-ene-20-one; progesterone = pregn-4-ene-3,20-dione; 17α-hydroxyprogesterone = 17α-hydroxypregn-4-ene-3,20-dione; 20α-dihydroprogesterone = 20α-hydroxypregn-4-ene-3-one; 11-desoxycorticosterone = 21-hydroxypregn-4-ene-3,20-dione; androstenedione = androst-4-ene-3,17-dione; testosterone = 17β-hydroxyandrost-4-ene-3-one.

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amurensis (Ikegami *et al.*, 1971) and in the sea urchin *Strongylocentrotus franciscanus* (Botticelli *et al.*, 1961). In studying the *in vivo* metabolism of progesterone in *Asterias rubens* and *Marthasterias glacialis* Gaffney and Goad (1974) found the conversion of progesterone into some intermediates, which are supposed to be possibly involved in the biosynthesis of asterosaponins, unique steroid-like compounds present in Asteroidea (Rio *et al.*, 1965; Yasumoto *et al.*, 1966; Grossert, 1972).

The present paper reports the results of experiments in which ovaries and pyloric ceca of female specimens of *Asterias rubens* were incubated *in vitro* with tritiated progesterone.

MATERIALS AND METHODS

Animals. Specimens of *Asterias rubens* were collected in the Wadden Sea, east of the island of Texel (The Netherlands) on March 14, 1975. The animals were kept in aerated seawater at 6° for 3 days before being processed.

Chemicals. All chemicals were of analytical grade; organic solvents were redistilled once, just before use. NAD, NADH, NADP, NADPH, and ATP were obtained from Boehringer (Mannheim, West Germany). [1,2-^{3}H]Progesterone (sp act 47.8 Ci/mmol), purchased from New England Nuclear (Boston), was purified by thin-layer chromatography (tlc), according to the method advised by manufacturer.

Dissection of the animals. Dissection and sexing of starfishes, and checking parasitism, were carried out as described elsewhere (Schoenmakers, 1979). The ovaries and pyloric ceca of one not-parasitized female animal were used for this study. The animal was histologically examined to determine the stage of the annual reproductive cycle (Schoenmakers and Goedhart, submitted for publication).

Incubation. For incubation 2 g ovarian and 2 g pyloric ceca tissue were homogenized separately with a Potter–Elvehjem homogenizer at 0° in 0.25 *M* sucrose (w/v, 1:3).

The incubation mixture consisted of 0.5 ml propyleneglycol containing about 5 μCi [1,2-^{3}H]progesterone, 3 ml of a solution of NAD, NADH, NADP, NADPH, and ATP in phosphate buffer, and 12 ml phosphate buffer (0.1 *M*, pH 7.4; final concentration of each cofactor 1 m*M*), to which 4 ml homogenate (corresponding to 1.0 g tissue) was added.

The incubations were carried out under continuous shaking at 25° in an air atmosphere. After 30, 60, and 120 min, 6-ml aliquots were pipetted from the incubation mixture and the enzyme reactions were brought to stop by addition of 10 ml dichloromethane; three fractions were thus obtained for each tissue.

Extraction and processing. To detect the steroids on tlc and to measure recovery, we added before extraction to each fraction 50 μg of each of the following steroids: progesterone, 17α-hydroxyprogesterone, androstenedione, testosterone, 20α-dihydroprogesterone, 11-desoxycorticosterone, and 5α-pregnane-3, 20-dione. The extraction, tlc, detection of steroids, derivative formation, recrystallizations, recovery measurements, measurement of radioactivity, and calculation of steroid conversion were carried out as described elsewhere (Schoenmakers, 1979). For tlc the following solvent systems were applied:

System 1—toluene–cyclohexane (1:1, v/v) to remove most of the lipid materials. In this system the plates were developed three times;

System 2—benzene–ethylacetate (3:1, v/v), three times developed;

System 3—dichloromethane–methanol (97:3, v/v), once developed.

For recovery measurements with gas–liquid chromatography (GLC), *o*-methyloximes were prepared of the steroids 17α-hydroxyprogesterone and 11-desoxycorticosterone (Mambara and Iwata, 1973).

RESULTS

The fresh weight of the ovaries is 52.00 g, that of the pyloric ceca 30.50 g. This corresponds to 20.39 and 11.96% of the total fresh weight of the animal (255.0 g), respectively. The stage of the annual reproductive cycle of the ovaries was determined to be stage 4, the maturation stage.

After extraction of the different incubation mixtures triglycerides were removed from the extracts of pyloric ceca tissues by precipitation with 8 ml aqueous methanol (70%). The organic phases were evaporated *in vacuo* and the residues subjected to tlc following systems 1 and 2. Radioscans proved that [1,2-^{3}H]progesterone was converted into at least eight compounds by both ovarian and pyloric ceca tissues. Figure 1 shows radioscans of the steroids isolated from ovarian and pyloric ceca tissue, respectively, after an incubation of 120 min.

The radioactive areas corresponding to the added carriers were eluted. However, using tlc system 2, 17α-hydroxyprogesterone, testosterone, 20α-dihydroprogester-

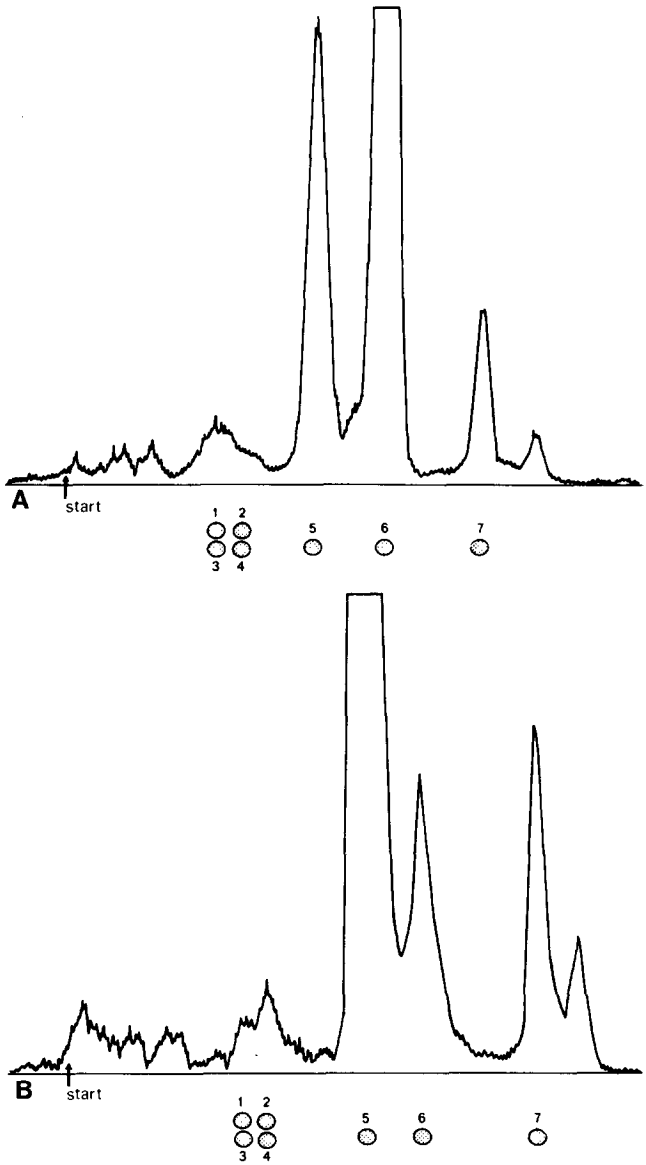

FIG. 1. Thin-layer radioscans of steroids extracted from ovarian (A) and from pyloric ceca tissue (B) of *Asterias rubens* after 120 min incubation [1,2-^{3}H]progesterone. References are: (1) testosterone; (2) 17α-hydroxyprogesterone; (3) 11-desoxycorticosterone; (4) 20α-dihydroprogesterone; (5) androstenedione; (6) progesterone; (7) 5α-pregnane-3,20-dione.

one, and 11-desoxycorticosterone were inadequately separated. Therefore, these compounds were eluted as one fraction and resubjected to tlc, using system 3. This resulted into an effective separation of these steroids.

The radioactive areas corresponding with authentic steroids were eluted and further purified by tlc following system 3, formylation, and recrystallization.

17α-Hydroxyprogesterone

The 17α-hydroxyprogesterone areas were eluted and formylated. The radioac-

tive compound of each fraction remained unaffected and showed the same mobility in tlc in system 3 as authentic 17α-hydroxyprogesterone. Furthermore, a constant specific radioactivity was measured in most fractions after repeated recrystallization.

Androstenedione

The androstenedione areas were eluted. Rechromatography in tlc system 3 showed that these areas are composed of a number of radioactive compounds, one of which—accounting for only a relatively small part of the total metabolites—corresponds with authentic androstenedione. These androstenedione areas were eluted and the radioactive substance of each fraction resisted formylation. The individual fractions showed a constant specific radioactivity after repeated recrystallization.

Testosterone

The testosterone areas were eluted and formylated. Each fraction contained a radioactive compound with the same R_f value as authentic testosterone-formate. Repeated recrystallization of the individual fractions to constant specific radioactivity further confirmed the identity of the labeled substance.

The results of recrystallization and the conversion percentages after correction for recovery are summarized in Table 1.

20α-Dihydroprogesterone

The 20α-dihydroprogesterone areas were eluted. Formylation of each fraction pro-

TABLE 1

SPECIFIC ACTIVITIES (dpm/mg) DURING THE LAST THREE RECRYSTALLIZATIONS OF 17α-HYDROXYPROGESTERONE, ANDROSTENEDIONE, AND TESTOSTERONE-FORMATE AND THE CONVERSION PERCENTAGES (AFTER CORRECTION FOR RECOVERY) OF THESE STEROIDS ISOLATED FROM OVARIAN AND PYLORIC CECA TISSUES OF *Asterias rubens* AFTER INCUBATION WITH [1,2-^{3}H]PROGESTERONE

Tissue	Incubation time (min)	17α-Hydroxyprogesterone		Androstenedione		Testosterone-formate	
		dpm/mg	Percentage conversion	dpm/mg	Percentage conversion	dpm/mg	Percentage conversion
Ovaries	30	23		799		2541	
		24		789		2517	
		23	0.042 ± 0.003	801	0.77 ± 0.03	2532	1.34 ± 0.04
Ovaries	60	19		586		22	
		19		584		21	
		18	0.022 ± 0.003	587	0.78 ± 0.03	22	0.026 ± 0.02
Ovaries	120	—		4495		687	
		—		4452		713	
		—	<0.01	4382	2.6 ± 0.1	707	0.49 ± 0.02
Pyloric ceca	30	13		616		30	
		14		605		29	
		13	0.022 ± 0.003	613	0.32 ± 0.01	30	0.029 ± 0.002
Pyloric ceca	60	26		174		21	
		26		178		22	
		27	0.032 ± 0.005	175	0.13 ± 0.01	20	0.019 ± 0.002
Pyloric ceca	120	—		196		11	
		—		187		10	
		—	<0.01	194	0.09 ± 0.01	9	0.004 ± 0.002

TABLE 2

SPECIFIC ACTIVITIES (dpm/mg) DURING THE LAST THREE RECRYSTALLIZATIONS OF 20α-DIHYDROPROGESTERONE-FORMATE, 11-DESOXYCORTICOSTERONE-FORMATE, AND 5α-PREGNANE-3,20-DIONE AND THE CONVERSION PERCENTAGES (AFTER CORRECTION FOR RECOVERY) OF THESE STEROIDS ISOLATED FROM OVARIAN AND PYLORIC CECA TISSUES OF *Asterias rubens* AFTER INCUBATION WITH [1,2-^{3}H]PROGESTERONE

Tissue	Incubation time (min)	20α-Dihydroprogesterone-formate		11-Desoxycorticosterone-formate		5α-Pregnane-3,20-dione	
		dpm/mg	Percentage conversion	dpm/mg	Percentage conversion	dpm/mg	Percentage conversion
		47		417		3,607	
Ovaries	30	46		396		3,013	
		48	0.039 ± 0.002	395	0.314 ± 0.010	3,017	2.9 ± 0.1
		76		168		4,642	
Ovaries	60	75		164		4,229	
		75	0.075 ± 0.004	166	0.168 ± 0.007	4,236	2.7 ± 0.1
		53		41		10,810	
Ovaries	120	43		14		10,276	
		40	0.024 ± 0.002	15	0.008 ± 0.002	9,672	6.4 ± 0.2
		393		145		12,020	
Pyloric ceca	30	409		148		11,345	
		411	0.249 ± 0.008	146	0.073 ± 0.002	11,377	10.6 ± 0.3
		236		460		3,819	
Pyloric ceca	60	241		413		3,832	
		239	0.145 ± 0.005	409	0.184 ± 0.007	3,822	3.2 ± 0.2
		254		29		10,008	
Pyloric ceca	120	258		19		9,489	
		254	0.090 ± 0.003	20	0.008 ± 0.001	9,720	4.3 ± 0.1

duced a compound with the same mobility in tlc in system 3 as authentic 20α-dihydroprogesterone-formate. A constant specific radioactivity was obtained after repeated recrystallization of the individual fractions.

11-Desoxycorticosterone

The 11-desoxycorticosterone areas were eluted and formylated. The formylated product of each fraction showed the same mobility in tlc in system 3 as authentic 11-desoxycorticosterone-formate. Finally, a constant specific radioactivity was reached after repeated recrystallization of the individual fractions.

5α-Pregnane-3,20-dione

The 5α-pregnane-3,20-dione areas were eluted and subjected to formylation. The radioactive compound of each fraction remained unaffected and showed the same R_f value as authentic 5α-pregnane-3,20-dione. The individual fractions showed a constant specific radioactivity after repeated recrystallization.

The results of recrystallization and the conversion percentages after correction for recovery are summarized in Table 2.

After 30, 60, and 120 min incubation of ovarian tissue a large amount of progesterone had not been converted: 72, 69, and 54%, respectively. The total amount of compounds which were not further identified increased to 19, 25, and 26%, respectively.

On the contrary, after 30, 60, and 120 min incubation with pyloric ceca tissue a minor amount of unchanged progesterone remained: 23, 13, and 10%, respectively; the percentages conversion into nonidentified steroids were: 66, 82, and 83%, respectively.

DISCUSSION

After 30, 60, and 120 min incubation of ovarian tissue and pyloric ceca tissue, the conversion *in vitro* of progesterone into 17α-hydroxyprogesterone, androstenedione, testosterone, 20α-dihydroprogesterone, 5α-pregnane-3,20-dione, and 11-desoxycorticosterone has been proved on the basis of the following evidence:

—The tritiated free steroids have a chromatographic mobility, similar to that of the authentic materials.

—The radioactive compounds, in case of testosterone, 20α-dihydroprogesterone, and 11-desoxycorticosterone, can be formylated and their radioactive derivatives have the same mobility in tlc as the authentic steroid formates. The radioactive material, in case of 17α-hydroxyprogesterone, androstenedione, and 5α-pregnane-3,20-dione, resisted formylation and therefore identification of these steroids can only be considered as tentatively.

—After addition of carriers, recrystallization of the steroids and steroid formates can be carried out up to constant specific radioactivities (Tables 1 and 2).

The *in vitro* incubation experiments thus demonstrate that the ovaries and the pyloric ceca of female *Asterias rubens* are able to synthesize from progesterone the steroids just mentioned. This means that in both organs the following biosynthetic enzymes are present: 17α-hydroxylase, $C_{17}-C_{20}$-lyase, 17β-HSD, 20α-hydroxysteroid dehydrogenase (20α-HSD), 5α-reductase, and 21-hydroxylase. These enzymes will be briefly discussed.

17α-Hydroxylase

From Table 1 it appears that the percentage conversion from progesterone into 17α-hydroxyprogesterone is low (ovaries: 0.04 to 0.02%; pyloric ceca: 0.03 to <0.01%). The 17α-hydroxyprogesterone yield in time decreases in the ovaries; the decrease is irregular and less evident in the pyloric ceca.

$C_{17}-C_{20}$-Lyase and 17β-HSD

Table 1 shows that the conversion values of androstenedione are higher in the ovaries (0.8–2.6%) than in the pyloric ceca

(0.3−0.1%). Since the androstenedione yield in time increases in the ovaries and decreases in the pyloric ceca, the metabolism of androstenedione in the ovaries may be different from that in the pyloric caeca.

The yields of C_{19}-steroids are higher than those of 17α-hydroxyprogesterone (Table 1). Thus, the C_{19}-steroids show a greater tendency to be accumulated. In the ovaries the yield of 17α-hydroxyprogesterone decreases in time, whereas that of androstenedione increases. Thus, likely the biosynthesis of 17α-hydroxyprogesterone preceded that of androstenedione, which is in accordance with the biosynthetic sequence in vertebrates. The testosterone yield in the ovaries is irregular and allows no conclusions as to the biosynthetic sequence. In the pyloric ceca the yield of 17α-hydroxyprogesterone is difficult to explain, but most likely it decreases in time. The yields of androstenedione and testosterone distinctly decrease, which points to at least some quantitative differences in C_{19}-steroid metabolism in ovaries and pyloric ceca. In conclusion, though it has not been proved, it is likely that also in echinoderms the biosynthetic sequence will be: progesterone → 17α-hydroxyprogesterone → androstenedione.

As follows from Table 1, the yields of testosterone in the ovaries (1.3−0.03%) are larger than those in the pyloric ceca (0.03−0.01%). The presence of 17β-HSD is in agreement with and confirms the earlier observations of Schoenmakers *et al.* (1976). Colombo and Belvedere (1976) demonstrated this enzyme in the ovaries of *Astropecten irregularis pentacanthus* and in the gonads of *Paracentrotus lividus*.

20α-HSD

The yields of 20α-dihydroprogesterone are small in the ovaries (0.02−0.08%) and higher in the pyloric ceca (0.09−0.25%) (Table 2). Thus the enzyme seems more active in the pyloric ceca than in the ovaries. The 20α-dihydroprogesterone yield in time is irregular, without large fluctuations, in the ovaries; the yield slightly decreases in the pyloric ceca. The enzyme was demonstrated before by Colombo and Belvedere (1976) in the gonads of *Astropecten irregularis pentacanthus*, *Paracentrotus lividus* and *Ophiothrix fragilis*.

21-Hydroxylase

Table 2 shows that the yields of 11-desoxycorticosterone are small (ovaries: 0.31−0.01%; pyloric ceca: 0.18−0.01%). The enzyme seems to be more active in the ovaries than in the pyloric ceca. The ovaries show decreasing 11-desoxycorticosterone production in time; in the pyloric ceca this decrease is less evident. Therefore, 11-desoxycorticosterone is probably not an end product. This enzyme was found before in *Astropecten irregularis pentacanthus* (Colombo and Belvedere, 1976).

5α-Reductase (5α-R)

As follows from Table 2, the percentage conversion of progesterone into 5α-pregnane-3,20-dione is high in both organs, but the yields are higher in the pyloric ceca than in the ovaries (ovaries 2.7−6.4%; pyloric ceca 10.6−3.2%). The accumulation of 5α-pregnane-3,20-dione and probably its formation are faster in the pyloric ceca than in the ovaries, since the yields are decreasing in the former and increasing in the latter. A 5α-reductase was also demonstrated by Smith *et al.* (1972) for the conversion of cholesterol into 5α-cholestan-3β-ol in *Asterias rubens*.

From the percentage unchanged progesterone it appears that the metabolic capacity of the ovaries is different from that of the pyloric ceca. It may be possible that in this stage of the annual reproductive cycle, i.e., the maturation stage, the capacity to metabolize progesterone in the ovaries is reduced, or that this low capacity is inherent to the nature of the organ. Both organs, however, are able to convert progesterone

via different pathways. First, progesterone can be metabolized into intermediates of the Δ^4-pathways. In this experiment the yields of these intermediates are in general low but they are higher in the ovaries than in the pyloric ceca. The organs show decreasing yields of these steroids in time with the exception of an increasing yield of androstenedione in the ovaries. Therefore, the identified steroids probably do not represent end products.

These results can be compared with previously obtained data on the ability of steroid biosynthesis of the starfish *Asterias rubens*, i.e., the presence of side-chain cleaving enzyme complex, Δ^5-Δ^4-isomerase, and 3β-HSD (Schoenmakers, 1979) and of steroid-synthesizing cells in the ovaries (Schoenmakers *et al.*, 1977). It may then be concluded that *Asterias rubens* is able to synthesize in the ovaries and the pyloric ceca C_{19}-steroids from cholesterol. Formation of these steroids via the Δ^4-pathway, just as in vertebrates, seems to be not impossible. Moreover, the ovaries appear to be more active in the biosynthesis of these steroids than the pyloric ceca. No reports are available on the biosynthesis of C_{19}-steroids in Echinodermata.

A second metabolic route of progesterone is the conversion into 20α-dihydroprogesterone, also a common pathway in vertebrates. The formation of 11-desoxycorticosterone from progesterone indicates a progesterone metabolism leading to corticosteroids.

However, the metabolic pathways of progesterone in *Asterias rubens* mentioned so far and comparable with those found in other animals are not intensively used when compared with the conversion of progesterone into 5α-pregnane-3,20-dione. In the present experiments the highest yields are found for 5α-pregnane-3,20-dione in ovaries and pyloric ceca. Moreover, in both organs, especially in the pyloric ceca, the production of unidentified steroids is important. Therefore, a unique route leading from progesterone to asterosaponins is supposed to be present in *Asterias rubens*. Gaffney and Goad (1974) and Teshima *et al.* (1977), studying the metabolism of progesterone in *Asterias rubens*, found some intermediates for the biosynthesis of asterosaponins, namely 3β-hydroxy-5α-pregnane-20-one and 3β,6α-dihydroxy-5α-pregnane-20-one. Structurally it does not seem impossible that 5α-pregnane-3,20-dione is another intermediate for the formation of asterosaponins. The 5α-pregnane-3,20-dione yield in time decreases in the pyloric ceca and increases in the ovaries; the amount unchanged progesterone is much smaller in the pyloric ceca than in the ovaries; the yield of nonidentified steroids which might be intermediates in the synthesis of asterosaponins is much higher in the pyloric ceca than in the ovaries. Therefore, it may be supposed that the pyloric ceca are more active in the biosynthesis of the asterosaponins than the ovaries.

No information is available on the function of progesterone and its metabolites presently identified in *Asterias rubens*. Studies on the biosynthesis and functions of steroids with regard to the reproduction of *Asterias rubens* will be continued.

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