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Radioimmunoassays to Determine the Presence of Progesterone and Estrone in the Starfish *Asterias rubens*

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RIA's have been made for progesterone and estrone with the antisera S74B7 and 7604-7 40, respectively, which will be described. The characteristics of the RIA for progesterone resemble those of other reported RIA's. The antiserum for the RIA of estrone is highly specific, with main cross-reactions of 0.32%, 0.27% and 0.13% for estradiol-17β, estradiol-17α and oestriol, respectively. The RIA for estrone is sensitive (affinity constant: 28.2×10^9 l/mol), with a standard curve in the range from 1 to 10 pg. Accuracy and precision were reliable, as proved by the significant correlation between steroid added and steroid estimated.

With the use of these RIA's, progesterone and estrone were demonstrated in the ovaries and pyloric ceca of single specimens of *Asterias rubens*. Progesterone was also present in the perivisceral fluid, but estrone appeared to be absent. The progesterone level was lower and the estrone level higher in the ovaries than in the pyloric ceca.

The significance of the presence of these steroids in the ovaries and pyloric ceca of *Asterias rubens*, and of the differences in the steroid levels between both organs will be discussed.

Radioimmunoassays were developed for nearly every steroid hormone in biological fluids of many species of vertebrates. Antisera are often used with a limited specificity requiring laborious purification, *e.g.*, column chromatography, so that larger samples are necessary to compensate procedural losses. Lately antisera with a high specificity were described for the estimation of steroids (Cameron *et al.*, 1975).

In this study the analysis with radioimmunoassay (RIA) has been carried out of hardly or not purified biological extracts. With such a RIA a reliable quantification depends largely on the specificity of the applied antiserum. The type of steroid–protein conjugate used for immunization determines this characteristic, and especially the site of the steroid, where it is linked to the coupling bridge for the attachment to the carrier protein, is of great importance (Kohen *et al.*, 1975). Using BSA conjugates of 11α-hydroxyprogesterone-hemisuccinate and 6-ketooxime-estrone as immunogens, highly specific RIAs for progesterone and estrone have been developed. With this type of RIAs steroidogenesis and steroid levels can be examined in organs of specimens of vertebrates. No detailed information is available, however, on the application of RIA on endogenous steroids in invertebrates.

The starfish *Asterias rubens* is known to synthesize steroids in ovaries and pyloric ceca (Schoenmakers, 1977, 1979a,b,c) and steroid synthesizing cells have been observed in the ovaries (Schoenmakers *et al.*, 1977); determining the presence of endogenous steroids would confirm the results obtained hitherto and might provide conclusive evidence for steroidogenesis in *Asterias rubens*.

Evidence for the presence of proges-

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terone and estrogens in echinoderms has remained scarce and was mainly obtained by physicochemical and bioassay techniques applied to the extracts of pooled gonads from a number of animals (Steidle, 1930; Donahue, 1940; Hagerman *et al.*, 1957; Botticelli *et al.*, 1960, 1961; Ikegami *et al.*, 1971). No information was available on the presence of steroids in ovaries, pyloric ceca, and perivisceral fluid of *Asterias rubens*.

The purpose of this study was to develop highly specific and sensitive RIAs with antisera, raised against BSA conjugates of 11α-hydroxyprogesterone-hemisuccinate and 6-keto-oxime-estrone and to apply these to the estimation of progesterone[1] and estrone in ovaries, pyloric ceca, and perivisceral fluid.

MATERIAL AND METHODS

Animals. Specimens of *Asterias rubens* were collected in the Wadden Sea, east of the island of Texel (The Netherlands), in September 1976. The animals were kept in aerated seawater at 6° for 3 days before being processed.

Chemicals. *n*-Hexane, 99.5% (Merck AG, Darmstadt, W. Germany) and twice distilled benzene (Uvasol, Merck AG) were used for the extraction of progesterone and estrone, respectively. Steroids were purchased from Steraloids Inc. (Wilton, N.H.) and Sigma Chemical Company (St. Louis, Mo.); [1,2,6,7(*n*)-^{3}H]-progesterone (sp act 84 Ci/mmol) and [2,4,6,7(*n*)-^{3}H]-estrone (sp act 86 Ci/mmol) were obtained from the Radiochemical Centre Amersham (England). The tracers were subjected to routine checks for purity by thin-layer chromatography and, if necessary, purified by gel filtration on Sephadex LH-20, according to Mikhail *et al.* (1970). All other chemicals were of analytical reagent grade.

Samples for radioactivity measurements were dissolved in a mixture of Instafluor (Packard Instruments Co., Downers Grove, Ill.,) and Triton X-100 (Serva, Heidelberg, W. Germany) (2:1, v/v). Buffer systems were as follows:

PBS	0.01 *M* phosphate buffer (pH 7.4) in physiological saline containing 0.01% (w/v) merthiolate
PBS^{++}	PBS (pH 7.4) containing 0.29% (w/v) human immunoglobin (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and 0.36% (w/v) bovine serum albumin (BSA; Povite, Organon Oss, The Netherlands)
PBS-G	PBS (pH 7.0) containing 0.1% (w/v) gelatin (BDH Chemical Ltd., England)
PBS-G^{++}	PBS-G (pH 7.0) with the same addition as for the PBS^{++} buffer

Dissection of animals. Dissecting, sexing, and checking parasitism were carried out as described by Schoenmakers (1979a). Parasitized animals were discarded. A histological examination was made to determine for each animal the stage of the annual reproductive cycle according to Schoenmakers and Goedhart (1979). The ovaries, pyloric ceca, and perivisceral fluid of 16 females were stored at −25°, before homogenization.

Homogenization. After thawing for 15 min at 37°, at least 1 ml PBS in a weight:volume ratio 1:1 was added to the ovaries and the pyloric ceca. Subsequently, the organs were separately homogenized in a Potter–Elvehjem homogenizer at 0° for 2 min, followed by sonification for 30 sec (Sonifier B-10, Branson Sonic Power Co., Danbury, Conn.). The homogenates were stored at −25° before determination of total protein in quadruplicate according to Lowry *et al.* (1951) and RIA for progesterone and estrone.

Estimation of progesterone. Progesterone levels were estimated in total homogenates by RIA similar to the method described by De Jong *et al.* (1974). Instead of 10,000 dpm of the tracer, 5000 dpm (8.3 pg tritiated

[1] Steroid nomenclature: cholesterol, 3β-hydroxycholest-5-ene; pregnenolone, 3β-hydroxypregn-5-ene-20-one; progesterone, pregn-4-ene-3,20-dione; 6β-hydroxyprogesterone, 6β-hydroxypregn-4-ene-3,20-dione; 11α-hydroxyprogesterone, 11α-hydroxypregn-4-ene-3,20-dione; 11β-hydroxyprogesterone, 11β-hydroxypregn-4-ene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxypregn-4-ene-3,20-dione; 20α-hydroxyprogesterone, 20α-hydroxypregn-4-ene-3-one; 20β-hydroxyprogesterone, 20β-hydroxypregn-4-ene-3-one; corticosterone, 11β,21-dihydroxypregn-4-ene-3,20-dione; cortisol, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; cortisone, 17α,21-dihydroxypregn-4-ene-3,11,20-trione; dehydroepiandrosterone, 3β-hydroxyandrost-5-ene-17-one; androstenedione, androst-4-ene-3,17-dione; testosterone, 17β-hydroxyandrost-4-ene-3-one; 11-keto-testosterone, 17β-hydroxyandrost-4-ene-3,11-dione; androsterone, 3α-hydroxy-5α-androstane-17-one; estradiol-17α, 3,17α-dihydroxyestra-1,3,5(10)-triene; estradiol-17β, 3,17β-dihydroxyestra-1,3,5(10)-triene; 6-keto-estradiol-17β, 3,17β-dihydroxyestra-1,3,5(10)-triene-6-one; estriol, 3,16α,17β-trihydroxyestra-1,3,5(10)-triene; estrone, 3-hydroxyestra-1,3,5(10)-triene-17-one; 6-keto-estrone, 3-hydroxyestra-1,3,5(10)-triene-6,17-dione.

progesterone) was added before extraction to obtain a higher sensitivity. Triplicate samples of the homogenized ovaries (0.1–0.2 ml), duplicate samples of the homogenized pyloric ceca (10 and 20 μl), and duplicate samples of the perivisceral fluid (0.5 ml) were twice extracted with 2 ml *n*-hexane. The solvent layer was collected by decantation after freezing of the extraction mixture for 3.5 min at −65°. After evaporation of the solvent under a nitrogen stream at 45°, the residues were dissolved in 100 μl PBS^{++}. One hundred microliters of the antiserum (S74B7), diluted 1:5000 in PBS^{++}, was added to the samples and standards (in triplicate in the range 10–400 pg progesterone in 100 μl PBS^{++} with 5000 dpm tritiated progesterone). After incubation for 15 min at 37° and subsequently overnight at 0°, 50-μl aliquots of samples and standards were used to correct for procedural losses and for mass of tritiated progesterone (mean percentage recovery: ovaries 82.03 ± 3.7 (SD)%, n = 58; pyloric ceca 81.61 ± 5.0 (SD)%, n = 36; perivisceral fluid 83.24 ± 4.2 (SD)%, n = 41).

Bound and free fractions were separated with Dextran-T70 coated charcoal according to De Jong *et al.* (1974). After dilution to 1 ml with double-distilled water the 50-μl aliquots and aliquots of the bound fractions each were dispersed in 10 ml scintillation mixture and counted in a liquid scintillation counter (LKB-Wallac 81000, Sweden) until a statistical error of 1.6% is reached, and corrected for quenching.

The antiserum was raised in 3-year-old ovariectomized Texel ewes, similar to the procedure of Scaramuzzi *et al.* (1975). The 11α-hydroxyprogesterone-hemisuccinate BSA conjugate (Steraloids Inc., Q-3253) was dissolved in sterile physiological saline and emulsified with an equal volume of complete Freund's adjuvant; 5 ml of the stable emulsion containing 5 mg of the conjugate was immediately injected at 10 subcutaneous sites in the abdomen.

The primary immunization was followed by bleedings at regular intervals. Upon reaching a constant level of the titer of the antisera samples, a booster injection of 5 mg of the conjugate was administered 12.5 weeks after the primary immunization and blood was collected 2 weeks after the booster injection for the above-mentioned RIA.

Estimation of estrone. Estrone levels were estimated in total homogenates by RIA partly similar to the procedure for progesterone. One hundred microliters of PBS-G^{++} and 5000 dpm of the tritiated estrone (7.0 pg) were added to 20-μl duplicate samples of the ovaries and pyloric ceca. The tracer, 5000 dpm, was added to duplicate samples (0.2–0.5 ml) of the perivisceral fluid and to the standards (in triplicate: 0, 1, 2, 3, 4, 5, 6, 7, 8, and 10 pg estrone in benzene). The samples were twice extracted with 2.5 ml benzene. After collecting with a Pasteur pipet, the solvent layer and the standards were evaporated under a nitrogen stream at 45°. Next 200 μl of the antiserum (7604-7 40) diluted 1:500,000 in PBS-G was added. After incubation for 15 min at 37° and overnight at 0°, 50-μl aliquots of samples and standards were used to correct for procedural losses and for mass of tritiated estrone (mean recovery: ovaries 84.5 ± 7.2 (SD)%, n = 40; pyloric ceca 80.7 ± 6.4 (SD)%, n = 45; perivisceral fluid 82.3 ± 5.2 (SD)%, n = 21). Samples and standards were then processed as described for progesterone.

The antiserum was raised in 4-month-old female white New Zealand rabbits, similar to the procedure of Nieschlag *et al.* (1975). The 6-keto-oxime-estrone BSA conjugate (Steraloids Inc., E-2056) was dissolved in sterile physiological saline and emulsified with an equal volume of complete Freund's adjuvant; 2 ml of the stable emulsion containing 50 μg of the conjugate was immediately injected at 10 intradermal sites along the spine.

The primary immunization was followed by bleedings at regular intervals. Upon reaching a constant level of the titer of the antisera samples, a booster injection of 50 μg of the conjugate was administered 8 weeks after the primary immunization and blood was collected 2 weeks after the booster injection for the above-mentioned RIA.

Calculations. Progesterone and estrone values were corrected for solvent blanks (3.4 ± 2.3 (SD) pg, n = 22, and 0.20 ± 0.48 (SD) pg, n = 49, respectively), and for procedural losses, mass of the tritiated steroid, and sample volume. Since the a-specific binding, *i.e.*, the fraction of tracer not adsorbed by the charcoal in absence of antiserum, was 4 and 3% for the whole standard range of the RIA of progesterone and estrone, respectively, no correction has been made.

Progesterone values were calculated by computer from a standard curve of percentage bound plotted versus log of mass of steroid. Estrone values were calculated from a standard curve of percentage bound plotted versus mass of steroid. The statistical error due to errors in pipetting and radioactivity measurements was 8%. The standard deviation was calculated as:

$$SD = \left(\frac{\Sigma (x_i - \bar{x})^2}{n - 1} \right)^{1/2};$$

the coefficient of variation CV = SD × 100/$\bar{x}$; the *mean* SEM = SD/$(n)^{1/2}$(n = number of estimations) (Dixon and Massey, 1969).

RESULTS

Radioimmunoassays

The specificity of the two antisera was tested against several steroids; the cross-reaction is expressed as the proportional ratio of the mass of steroid and interfering compound, at 50% displacement of the tracer (Abraham, 1969). Table 1 shows the

results for the progesterone antiserum, Table 2 for the estrone antiserum. The remarkably high specificity of the estrone antiserum is even better illustrated in Fig. 1.

The affinity constants calculated as the negative slope from a Scatchard plot (Goodfriend and Sehon, 1958) were 1.88×10^9 and 28.2×10^9 liters/mol at 0° for the antisera against progesterone and estrone, respectively. Figure 2 is an example of a standard curve in the range 0–10 pg for the RIA of estrone.

Data on accuracy and precision of both methods are presented in Table 3 for the estimation of progesterone and in Table 4 for the estimation of estrone. The relation between steroid added and steroid estimated tested by linear regression, produces the regression equations: $y = 3.69 + 0.97\,x$ and $y = -0.22 + 1.06\,x$, with correlation coefficients 1.00 and 0.99 for progesterone and estrone assays, respectively.

Since the estimations of progesterone and estrone levels in ovaries, pyloric ceca, and perivisceral fluid of *Asterias rubens* were made with a limited number of RIAs no interassay coefficients of variation can be calculated for the results. However, in other experiments the interassay coefficient of variation for the progesterone RIA as determined on the basis of two pools of plasma was 12.2% ($n = 11$) (Colenbrander *et al.*, 1979). For the estrone RIA, the interassay coefficient of variation as determined on the basis of two pools of plasma with estrone added is 8.8% ($n = 12$).

TABLE 1
SPECIFICITY OF THE ANTISERUM S74B7 RAISED IN SHEEP (TEXEL) AGAINST 11α-HYDROXYPROGESTERONE-HEMISUCCINATE BSA CONJUGATES, EXPRESSED AS PERCENTAGE CROSS-REACTION

Steroid	Percentage cross-reaction
Progesterone	100
17α-Hydroxyprogesterone	1.35
20α-Hydroxyprogesterone	<0.5
20β-Hydroxyprogesterone	1.9
11α-Hydroxyprogesterone	77.5
11β-Hydroxyprogesterone	23.8
6β-Hydroxyprogesterone	<0.5
Pregnenolone	0.52
5α-Pregnane-3,20-dione	12.89
5β-Pregnane-3,20-dione	6.82
3β-Hydroxy-5α-pregnane-20-one	2.27
20α-Hydroxy-5α-pregnane-3-one	<0.5
3β,20β-Dihydroxy-5α-pregnane	<0.5
Estrone	<0.5
Estradiol-17α	<0.5
Estradiol-17β	<0.5
Androstenedione	<0.5
Dehydroepiandrosterone	<0.5
Testosterone	<0.5
Androsterone	<0.5
11-Keto-testosterone	<0.5
Cortisol	<0.5
Cortisone	<0.5
Corticosterone	0.81

TABLE 2

SPECIFICITY OF THE ANTISERUM 7604-7 40 RAISED IN RABBITS (WHITE NEW ZEALAND) AGAINST 6-KETO-OXIME-ESTRONE BSA CONJUGATES, EXPRESSED AS PERCENTAGE CROSS-REACTION

Steroid	Percentage cross-reaction
Estrone	100
6-Keto-estrone	64.9
6-Keto-estradiol-17β	3.16
Estradiol-17β	0.32
Estradiol-17α	0.27
Estriol	0.13
Androstenedione	0.08
Testosterone	0.08
Dehydroepiandrosterone	0.04
Progesterone	<0.02
Pregnenolone	<0.02

Progesterone and Estrone Levels

The mean (± SEM) gonad index of the examined female specimens is 1.74 ± 0.27; the mean (± SEM) pyloric ceca index is 13.77 ± 0.94. The stage of the annual reproductive cycle of the ovaries was histologically determined (Schoenmakers and Goedhart, 1979) and appeared to be the resting stage (stage 1) or the early growth stage (stage 2).

In estimating progesterone in the pyloric ceca, some difficulties due to the high lipid content were encountered. When taking samples of 0.1–0.5 ml homogenate, the percentage a-specific binding ranged from 24 to 85%, for samples of 10 and 20 μl homogenate the percentage was 4%; the bound fraction is then clear after the charcoal separation.

The steroid level in the ovaries and pyloric ceca is expressed as nanograms per gram fresh weight and as picograms per milligram protein, that in the perivisceral fluid as nanograms per milliliter. The mean (± SEM) levels of progesterone and estrone in the ovaries, pyloric ceca, and perivisceral fluid are presented in Table 5.

The progesterone level is lower and the

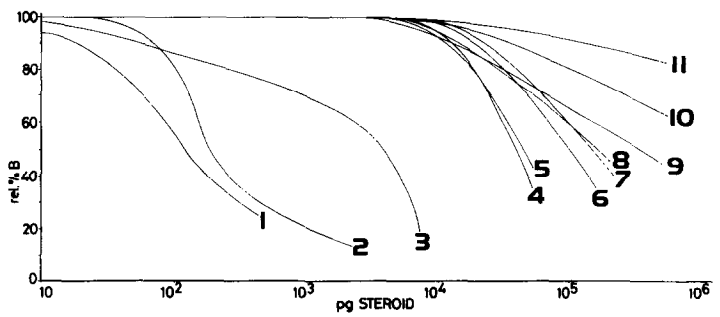

FIG. 1. Specificity of the antiserum (7604-7 40) raised in female white New Zealand rabbits against 6-keto-oxime-estrone BSA conjugates. Relative percentage bound (rel. % B) plotted versus pg mass of tested steroid. 1, Estrone; 2, 6-keto-estrone; 3, 6-keto-estradiol-17β; 4, estradiol-17β; 5, estradiol-17α; 6, estriol; 7, androstenedione; 8, testosterone; 9, dehydroepiandrosterone; 10, progesterone; 11, pregnenolone.

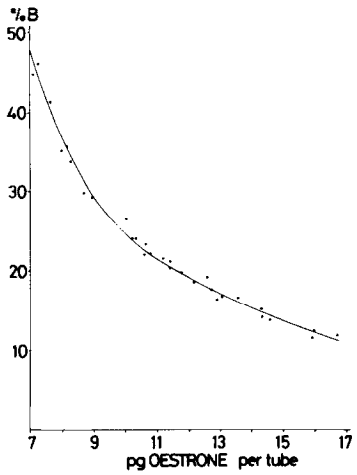

FIG. 2. Standard curve for the radioimmunoassay for estrone. Percentage bound (%B) plotted versus pg mass of estrone (sum of the mass of nonlabeled (0–10 pg) and labeled (7 pg) estrone, corrected for the percentage recovery). Antiserum (7604-7 40) in a dilution 1:500,000.

estrone level higher in the ovaries than in the pyloric ceca. Progesterone is scarcely present in the perivisceral fluid, and estrone appears to be totally absent.

DISCUSSION

Radioimmunoassays

The characteristics of the RIA for progesterone are in agreement with those described by De Jong *et al.* (1974) and Scaramuzzi *et al.* (1975). The specificity of the presented RIA depends not only on the specificity of the antiserum, which is high, but also on the specificity of the extraction solvent. *n*-Hexane is a highly a-polar and saturated aliphatic organic solvent; therefore, polar and aromatic steroids in general are hardly extracted.

Previous *in vitro* studies on the ovaries and pyloric ceca of *Asterias rubens* have revealed the synthesis of 17α-hydroxyprogesterone, 20α-hydroxyprogesterone, pregnenolone, 5α-pregnane-3,20-dione, androstenedione, and testosterone (Schoenmakers, 1977, 1979a, b). Of these steroids, only 5α-pregnane-3,20-dione shows a cross-reaction of some importance (12.89%) with the applied antiserum. Therefore it is possible that the presented progesterone levels have been overestimated. After injecting ^{14}C-labeled progesterone into *Asterias rubens*, Gaffney and Goad (1974) found ^{14}C-labeled 3β-hydroxy-5α-pregnane-20-one, which shows a slight cross-reaction (2.27%).

For estrone RIA, various antisera have been reported. Compared to antisera raised against estradiol-17β-hemisuccinate BSA conjugates (*e.g.*, Robertson *et al.*, 1972), the antiserum (7604-7 40) presented in this paper can be used for RIA without the laborious preceding process for separating estrone from other estrogens. Doerr (1976) reported an antiserum raised against estrone-3-hemisuccinate BSA conjugates with a specificity and affinity similar to the antiserum (7604-7 40), but the titer, as de-

TABLE 3
ACCURACY AND PRECISION OF THE RADIOIMMUNOASSAY FOR PROGESTERONE

Progesterone added to PBS^{++} (pg)	n^a	Progesterone obtained (mean) (pg)	SD (pg)	CV	Recovery (%)
25	12	23.34	6.14	26.3	93.4
50	12	43.85	8.42	19.2	87.7
100	12	101.53	12.15	11.9	101.5
150	10	156.87	19.47	12.4	104.6
200	10	203.51	20.40	10.0	101.8
300	12	303.30	12.69	4.2	101.1
400	11	378.36	37.18	9.8	94.6

[a] Number of estimations.

TABLE 4
ACCURACY AND PRECISION IN THE ESTIMATION OF ESTRONE[a]

Estrone assayed (pg)	n	Estrone obtained (mean) (pg)	SD (pg)	CV	Recovery (%)
2	6	2.202	0.190	8.62	110.1
3	9	2.761	0.330	11.95	92.0
4	6	3.886	0.217	5.59	97.2
5	3	5.217	0.548	10.50	104.3
6	9	5.812	0.441	7.59	96.9
8	6	8.497	1.601	18.84	106.2

[a] Known amounts of estrone were added to PBS-G^{++} and processed as described for the samples (n = number of estimations).

fined by Abraham (1975), of the latter antiserum is higher (1:2,000,000). In comparison with other antisera (Rowe *et al.*, 1973; Dobson and Dean, 1974; McNatty *et al.*, 1976) raised against similar conjugates also linked to BSA via a 6-keto-oxime, the antiserum (7604-7 40) has a specificity in the same order of magnitude or better.

Accuracy and precision of the standard curves and of the samples were satisfactory for both RIAs. The high affinity constant of the estrone antiserum (28.2×10^9 liters/mol) permits a high sensitivity of detection to be obtained.

Progesterone and Estrone Levels

Since the antisera against progesterone and estrone are highly specific, it can be concluded from Table 5 that the presence of progesterone and estrone has been proved in ovaries and pyloric ceca of female specimens of *Asterias rubens*. Progesterone is probably present in the perivisceral fluid to a neglectable degree, whereas estrone cannot be detected.

The presence of progesterone confirms the *in vitro* synthesis of progesterone (Schoenmakers, 1977, 1979a). The presence of estrone indicates that in *Asterias rubens* estrogens may be synthesized, but biosynthesis of these steroids could not yet be proved (Schoenmakers, 1979c).

Evidence is available for the presence of progesterone in the ovaries of the starfish *Pisaster ochraceus* (Botticelli *et al.*, 1960), the sea urchin *Strongylocentrotus franciscanus* (Botticelli *et al.*, 1961), and the starfish *Asterias amurensis* (Ikegami *et al.*, 1971). Indications were reported for the presence of estrogens in echinoderms. Steidle (1930), using a mouse bioassay, reported traces of estrogen-like activity in the sea urchin *Psammechinus miliaris*. Donahue (1940) found small amounts of

TABLE 5
MEAN (± SEM) PROGESTERONE AND ESTRONE LEVELS IN 16 FEMALE SPECIMENS OF *Asterias rubens*[a]

	Progesterone	Estrone	Expressed as
Ovaries	2.08 ± 0.37	1.33 ± 0.21	ng/g
	23.4 ± 3.5	20.8 ± 6.0	pg/mg
Pyloric ceca	35.8 ± 1.5	0.73 ± 0.03	ng/g
	204.7 ± 11.9	4.36 ± 0.33	pg/mg
Perivisceral fluid	0.04 ± 0.01	nd	ng/ml

[a] Progesterone and estrone levels expressed as ng/g fresh wt (ng/g), pg/mg protein (pg/mg), and ng/ml. ND, not detectable, *i.e.*, less than 2 pg/ml.

material with estrogen-like activity in extracts of the Bermuda urchin *Lytechinus variegatus*, the reef urchin *Echinometria sp.*, and a holothurian *Stichopus mobii*. Hagerman *et al.* (1957), using an enzymatic assay, reported that the ovaries of the starfish *Asterias forbesi* and of the sea urchins *Arbacia punctulata* and *Strongylocentrotus droebachiensis* contain minor amounts of estrogens. Botticelli *et al.* (1960, 1961) identified estradiol-17β in the ovaries of *Pisaster ochraceus* and *Strongylocentrotus franciscanus* after extracting pooled ovaries of a large number of animals followed by countercurrent distribution, paper chromatography, and bioassay. The presence of estrone was not demonstrated. Also in other phyla of invertebrates, material with estrogen-like activity was reported by several authors with similar methods. Preparations with estrogen-like activity were obtained from Coelenterata (Schwerdtfeger, 1932), Annelida (Steidle, 1930), Arthropoda (Steidle, 1930; Donahue, 1940, 1948; Hagerman *et al.*, 1957; Lisk, 1961; Gottfried *et al.*, 1967). De Longcamp *et al.* (1974), using RIA, found indications for the presence of estrogens in the gonad of *Mytilus edulis*. The estrone content was about the same in male and female mussels, but 10-fold higher than the estradiol-17β content. Carreau and Drosdowsky (1977), however, reported the absence of estrogens in the gonad of the cuttlefish *Sepia officinalis* by means of RIA. As far as known, no information is available on the presence of progesterone and estrone in the pyloric ceca of echinoderms.

The progesterone level in *Asterias rubens* is about 10-fold lower in the ovaries than in the pyloric ceca, whereas the estrone level is about 3-fold higher in the ovaries than in the pyloric ceca. Consequently, the ratio estrone/progesterone is about 30-fold higher in the ovaries than in the pyloric ceca. These differences in steroid levels between the two organs have an important bearing on a possible function of these steroids. Studies are being continued on the significance of the presence of progesterone and estrone in ovaries and pyloric ceca and on the meaning of the different steroid levels in the two organs for the reproduction of *Asterias rubens*.

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REFERENCES

Abraham, G. E. (1969). Solid-phase radioimmunoassay of estradiol-17β. *J. Clin. Endocrinol. Metabol.* **29**, 866–870.

Abraham, G. E. (1975). Characterization of antisteroid antisera. *In* "Steroid Immunoassay (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), pp. 67–78. Alpha-Omega, Cardiff, Wales, U.K.

Botticelli, C. R., Hisaw, F. L. Jr., and Wotiz, H. H. (1960). Estradiol-17β and progesterone in ovaries of starfish *(Pisaster ochraceous)*. *Proc. Soc. Exp. Biol. Med.* **103**, 875–877.

Botticelli, C. R., Hisaw, F. L. Jr., and Wotiz, H. H. (1961). Estrogens and progesterone in the sea urchin (*Strongylocentrotus franciscanus*) and pecten *(Pecten hericius)*. *Proc. Soc. Exp. Biol. Med.* **106**, 887–889.

Cameron, E. H. D., Hillier, S. G., Griffiths, K. (1975). "Steroid Immunoassay." Alpha-Omega, Cardiff, Wales, U.K.

Carreau, S., and Drosdowsky, M. (1977). The *in vitro* biosynthesis of steroids by the gonad of the cuttlefish (*Sepia officinalis* L.). *Gen. Comp. Endocrinol.* **33**, 554–565.

Colenbrander, B., Dieleman, S. J., Kruip, Th. A. M., Willemse, A. H., and Wensing, C. J. G. (1979). Effect of estradiol benzoate on LH secretion in pigs. Submitted.

De Jong, F. H., Baird, D. T., and Van der Molen, H. J. (1974). Ovarian secretion rates of oestrogens, androgens and progesterone in normal women and in women with persistent ovarian follicles. *Acta Endocrinol. (Copenhagen)* **77**, 575–587.

De Longcamp, D., Lubet, P., and Drosdowsky, M. (1974). The *in vitro* biosynthesis of steroids by the gonad of the mussel *(Mytilus edulis)*. *Gen. Comp. Endocrinol.* **22**, 116–127.

Dixon, W. J., and Massay, F. J. (1969). "Introduction to Statistical Analysis." McGraw–Hill, New York.

Dobson, H., and Dean, P. D. G. (1974). radioimmunoassay of oestrone, oestradiol-17α and -17β in bovine plasma during the oestrous cycle and last stages of pregnancy. *J. Endocrinol.* **61**, 479–486.

Doerr, P. (1976). Radioimmunoassay of oestrone in plasma. *Acta Endocrinol. (Copenhagen)* **81**, 655–667.

Donahue, J. K. (1940). Occurrence of estrogens in the ovaries of certain marine invertebrates. *Endocrinology* **27**, 149–152.

Donahue, J. K. (1948). Fluorimetric and biological determination of estrogens in the eggs of the American lobster *(Homarus americanus). Proc. Soc. Exp. Biol. Med.* **69**, 179–181.

Gaffney, J., and Goad, L. J. (1974). Progesterone metabolism by the echinoderms *Asterias rubens* and *Marthasterias glacialis. Biochem. J.* **138**, 309–311.

Goodfriend, L., and Sehon, A. H. (1958). Preparation of an estrone–protein conjugate. *Canad. J. Biochem. Physiol.* **36**, 1177–1187.

Gottfried, H., Dorfman, R. I., and Wall, P. E. (1967). Steroids of invertebrates: Production of estrogens by an accessory reproductive tissue of the slug *Arion ater rufus. Nature (London)* **215**, 409–410.

Hagerman, D. D., Wellington, F. H., and Villee, C. A. (1957). Estrogens in marine invertebrates. *Biol. Bull.* **112**, 180–183.

Ikegami, S., Shirai, H., and Kanatani, H. (1971). On the occurrence of progesterone in ovary of the starfish, *Asterias amurensis. Dobutsugaku Zasshi* **80**, 26–28.

Kohen, F., Bauminger, S., and Lindner, H. R. (1975). Preparation of antigenic steroid–protein conjugates. *In* "Steroid Immunoassay" (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), pp. 11–32. Alpha-Omega, Cardiff, Wales, U.K.

Lisk, R. D. (1961). Estradiol-17β in the eggs of the American lobster, *Homarus americanus. Canad. J. Biochem. Physiol.* **39**, 659–662.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

McNatty, K. P., Baird, D. T., Bolton, A., Chambers, P., Corker, C. S., and McLean, H. (1976). Concentration of oestrogens and androgens in human ovarian venous plasma and follicular fluid throughout the menstrual cycle. *J. Endocrinol.* **71**, 77–85.

Mikhail, G., Wu, C. H., Ferin, M., and Vande Wiele, R. L. (1970). Radioimmunoassay of plasma estrone and estradiol. *Steroids* **15**, 333–352.

Nieschlag, E., Kley, H. K., and Usadel, K. H. (1975). Production of steroid antisera in rabbits. *In* "Steroid Immunoassay" (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), pp. 87–96. Alpha–Omega, Cardiff, Wales, U.K.

Robertson, H. A., Smeaton, T. C., and Durnford, R. (1972). A method for the extraction, separation and estimation of unconjugated oestrone, oestradiol-17α and oestradiol-17β in plasma. *Steroids* **20**, 651–667.

Rowe, P. H., Cook, I. F., and Dean, P. D. G. (1973). Oestrone specific antisera raised against oestrone-6-(O-carboxymethyl)oxime-bovine serum albumin. *Steroids and Lipids Res.* **4**, 24–29.

Scaramuzzi, R. J., Corker, C. S., Young, G., and Baird, D. T. (1975). Production of antisera to steroid hormones in sheep. *In* "Steroid Immunoassay" (E. H. D. Cameron, S. G. Hillier, and K. Griffiths, eds.), pp. 111–122. Alpha–Omega, Cardiff, Wales, U.K.

Schoenmakers, H. J. N. (1977). Steroid synthesis of *Asterias rubens. Proc. Int. Union Physiol. Sci.* **13**, 673.

Schoenmakers, H. J. N. (1979a). *In vitro* biosynthesis of steroids from cholesterol by the ovaries and pyloric caeca of the starfish *Asterias rubens. Comp. Biochem. Physiol.* **63B**, 179–184.

Schoenmakers, H. J. N. (1979b). *In vitro* biosynthesis of steroids from progesterone by the ovaries and pyloric ceca of the starfish *Asterias rubens.* Submitted.

Schoenmakers, H. J. N. (1979c). *In vitro* biosynthesis of steroids from androstenedione by the ovaries and pyloric ceca of the starfish *Asterias rubens.* Submitted.

Schoenmakers, H. J. N., and Goedhart, M. J. (1979). The annual reproductive cycle of the ovaries of *Asterias rubens* (Echinodermata). Submitted.

Schoenmakers, H. J. N., Colenbrander, P. H. J. M., and Peute, J. (1977). Ultrastructural evidence for the existence of steroid synthesizing cells in the ovary of the starfish *Asterias rubens* (Echinodermata). *Cell Tissue Res.* **182**, 275–279.

Schwerdtfeger, H. (1932). Beiträge zum Vorkommen und zur Wirkung der weiblichen Sexualhormone. *Arch. Exp. Pathol. Pharmakol.* **163**, 487–492.

Steidle, H. (1930). Über die Verbreitung des weiblichen Sexualhormones. *Arch. Exp. Pathol. Pharmakol.* **157**, 89.