Estradiol-17β and Progesterone in Ovaries of Starfish (*Pisaster ochraceous*).* (25704)

CHARLES R. BOTTICELLI, FREDERICK L. HISAW, JR.,† AND HERBERT H. WOTIZ‡
(Introduced by Frederick L. Hisaw)
Biological Labs, Harvard University, Mass.; Dept of Biochemistry, Boston University School of Medicine; and University of Washington, Friday Harbor Labs, Seattle, Wash.

Extracts that produce estrogenic reactions in mammals have been obtained from representatives of several Phyla of invertebrates (1) but the chemical identity of substances responsible for these effects is unknown. Estrogenic preparations have been obtained from gonads of starfish by Steidle(2), Donahue and Jennings(3), and more recently by Hagerman *et al.*(4). That such active agents are chemically related to steroid hormones of vertebrates is, of course, a possibility and their presence may be of some evolutionary significance. The present report presents results of effort to isolate estrogens and progesterone from ovarian tissue of starfish (*Pisaster ochraceous*) by methods commonly employed for obtaining these hormones from mammalian material.

Procedures. Starfish ovaries (6552 g) were collected and stored in approximately twice their volume of acetone. At time of extraction the acetone was poured off, ovarian tissue homogenized and reextracted with acetone. The combined acetone extracts were taken to dryness by gentle stream of warm air passing over the solution in large evaporating dishes. The oily residue was dissolved in sufficient 70% ethanol and extracted 3 times with ligroin (bp. 30-60°C). The ethanolic fraction was diluted with water containing 35% alcohol and subsequently washed with ligroin (3x). The alcoholic solution, bioassayed by the Astwood method(5), showed presence of estrogenic material in very small amount. The ethanolic fraction was then dried *in vacuo* and the residue partitioned countercurrently between 70% methanol and a 50-50 mixture of chloroform and carbon tetrachloride with 29 transfers. Five fractions were collected by combining tubes 0-2, 3-9, 10-19, 20-25, and 26-29, and dried *in vacuo*. The residue for each group of tubes was dissolved in 10 ml of methanol and 1 ml aliquot was taken from each for bioassay of estrogenic potency. Propylene glycol (1 cc) was added to each aliquot and the alcohol evaporated. Rats from inbred strain were 24 or 25 days old and weighed 54 to 60 g. Two animals were used to test material from each set of tubes. They were given 0.4 cc of propylene glycol solution in single subcutaneous dose and uteri removed 6 hours later and weighed.

The *results* (Table I), indicated estrogenic activity only in material obtained from tubes 3-9, and 10-19. Therefore, these 2 fractions were recombined and again partitioned in the same system with 29 transfers. The tubes of this second run were combined in 3 groups, 0-8, 9-20, and 21-29 and were processed as before. Results of the bioassay (Table II) show activity in fractions 0-8, and 9-20. It seems quite probable that estrogenic activity in tubes 3-9 of the first run and subsequently in 0-8 of the second run was caused by the tailing of estradiol, a supposition subsequently borne out by chromatographic evidence.

Fractions 0-8 and 10-19 were each divided into 3 aliquot portions and subjected to the following 3 paper chromatographic systems, at room temperature: (1) Benzene-formamide (1:1) for 22 to 24 hours; (2) toluene-propy-

TABLE I. Results of Bioassay from First Countercurrent Separation.

Fraction	Uterine wet wt, mg	% increase over control
Controls*	22.4	
0- 2	21.9	.0
3- 9	26.2	15.9
10-19	28.8	28.5
20-25	22.4	.0
25-29	22.0	.0

* 30 animals.

* Aided by grants from National Science Fn. and U.S.P.H.S.

† Department of Zoology, Oregon State College, Corvallis, Oregon.

‡ Senior Research Fellow, U.S.P.H.S.

TABLE II. Bioassays for Second Countercurrent Distribution.

Fraction	Uterine wet wt, mg	% increase over control
Controls*	22.4	
0- 8	23.4	4.5
9-20	25.7	14.7
21-29	22.6	.0

* 30 animals.

lene glycol (1:1) for 6 hours, and (3) ligroin-propylene glycol (1:1) for 64 hours. The unknown fraction in each of these systems was applied to one of 4 strips, estrone to another, estradiol to a third and a mixture of estrone and estradiol to the fourth. The 4 strips in each system were run simultaneously. The 3 strips treated with authentic estrogens were stained with ferric chloride-ferricyanide stain. Areas on experimental strips corresponding to the standards were cut and eluted 5 times with 10 ml portions of absolute ethanol. Similar elutions also were made of the remaining paper from experimental strip. Alcoholic eluates were evaporated in part, transferred to small vials, and reduced to dryness. Propylene glycol (0.5 cc) was added to each vial and 0.2 cc was injected in single subcutaneous dose into each of two 24-25 day old rats. Results of these bioassays (Table III) indicate presence of estradiol-17β, while test fractions corresponding to authentic estrone gave uniformly negative results. Also, the eluates of the paper outside the test areas gave negative responses.

While these results strongly indicate presence of estradiol-17β in ovarian tissue of the starfish, they also emphasize its small amount. In view of this, and assuming that estradiol can be converted to estrone in the starfish, it seems quite probable that estrone also might have been present but in a quantity insufficient for detection by our methods. The bioassay used has been very carefully standardized on a strain of inbred rats, in which minimal dose of estradiol that induces a 33% increase in uterine weight in 6 hours is 0.025 μg and estrone 0.45 μg(5,6). It has been estimated, for comparison, that ripe ovaries of the starfish contain between 0.04 μg and 0.1 μg of free estradiol-17β/kg of fresh tissue.

The brown oily residue obtained on evaporation of the ligroin fractions was used in procedures for isolation of progesterone. This material was first applied in benzene and ligroin (30 ml) to an alumina (Merck) column (24 mm x 170 mm) and eluted with petroleum ether, benzene and ethyl acetate, primarily for cleaning the preparation. Seven eluates were obtained, of which the third (petroleum ether, 100 ml) and the seventh (ethyl acetate, 300 ml) gave positive reactions when tested by Hooker-Forbes method(7).

Eluates 1-4 and 5-7 were combined, evaporated to dryness and applied in benzene to 2 separate aluminum (100 g Woelm, neutral activity grade II) columns (24 mm x 170 mm). A similar column was also treated with authentic progesterone. The columns were eluted with 100 ml of ligroin plus graded amounts of benzene, benzene plus chloroform, and chloroform plus methanol. Eluates were tested by Hooker-Forbes method, and positive reactions were obtained in fractions from both experimental columns corresponding to those containing authentic progesterone from the control column. The only difference was

TABLE III. Bioassay of Eluted Paper Strips from 3 Different Chromatographic Systems.

	Countercurrent fractions 0-8 Chromatography*				Countercurrent fractions 9-20 Chromatography*			
	Estrone		Estradiol		Estrone		Estradiol	
Chromatographic systems	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase	Uterine wet wt, mg	% increase
Benzene-formamide	22.4	0	25.5	13.8	21.0	0	26.0	16.1
Toluene-propylene glycol	22.5	0	24.2	8.1	21.4	0	24.3	8.5
Ligroin-propylene	21.1	0	24.1	6.7	22.7	0	26.6	18.7

* Areas of test strips corresponding to authentic estrone and estradiol-17β. Uterine weights are avg for 2 animals in each experimental test. Avg uterine wet wt for 30 untreated control animals was 22.4 + 0.3 mg.

a somewhat wider spread of activity in fractions from experimental columns than in those of the control. However, the active substance was present in such small amount that further identification was not attempted.

Our results strongly indicate that ovaries of starfish (*Pisaster ochraceous*) at time of spawning contain estradiol-17β. Estrone was not found. However, as concentration of estradiol was estimated to be approximately 0.04 to 0.1 μg/kg of fresh tissue, the amount of estrone that could have been derived as a metabolite from this source would probably be too small for detection by methods employed.

Discussion. Evidence for presence of progesterone, though not as convincing as that for estradiol, is, nevertheless, significant. The chromatographic agreement between authentic progesterone and the substance capable of evoking Hooker-Forbes response may not be conclusive proof of their identity but it is a definite possibility. The amount of active material obtained was too small for a more thorough characterization so absolute identification will require further study.

The significance of these observations lies in the possibility that ovarian, steroid hormones of vertebrates may also be present in gonads of invertebrates and raises questions regarding physiological and evolutionary importance.

Summary. Ovaries of starfish (*Pisaster ochraceous*) were examined for sex steroids. A low level of estrogenic activity, approximately 0.1 μg or less/kg of ovarian tissue, was determined by the Astwood 6 hour assay method. The biologically active material was identified as estradiol 17β by countercurrent distribution, paper chromatography and bioassay. Presence of progesterone was tentatively identified by methods of column chromatography and Hooker-Forbes assay.

1. Hanström, B., *Hormones in Invertebrates,* Oxford Univ. Press, 1939.

2. Steidle, H., *Arch. f. Exp. Path. Pharm.,* 1930, v157, 89.

3. Donahue, J. K., Jennings, E. R., *Endocrinol.,* 1937, v21, 690.

4. Hagerman, D. D., Wellington, F. M., Villee, C. A., *Biol. Bul.,* 1957, v112, 180.

5. Astwood, E. B., *Endocrinol.,* 1938, v23, 25.

6. Hisaw, F. L., Jr., *ibid.,* 1959, v64, 276.

7. Hooker, C. W., Forbes, T. R., *ibid.,* 1947, v41, 158.

Received February 23, 1960. P.S.E.B.M., 1960, v103.