Sulphation as a Metabolic Pathway for Oestradiol in the Sea Urchin *Strongylocentrotus franciscanus*

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1. Aerobic incubation of [^{14}C]oestradiol, in the presence of surviving gut tissue of the sea urchin *Strongylocentrotus franciscanus*, or a soluble enzyme system prepared therefrom, resulted in rapid formation of a water-soluble metabolite, identified as oestradiol 3-sulphate. 2. No evidence was obtained for the formation of other metabolic derivatives by the urchin-gut enzymes, or for the presence of the sulphating capacity in any other tissues of the organism under the conditions used. 3. The data are consistent with the possibility that oestradiol, previously detected in the gonads, is synthesized therein and excreted in a conjugated, highly water-soluble form via the gut.

Great gaps appear in the literature on comparative biochemistry of oestrogenic hormones that render difficult or impossible interpretation of the significance of the widespread distribution (Butenandt & Jacobi, 1933; Doisy, 1939; Dorfman & Ungar, 1953; Steidle, 1930; Zondek, 1941) of these phylogenetically ancient molecules. Only recently has the chemical nature of the oestrogenic activity been investigated, and evidence been obtained for the occurrence of the common mammalian oestrogens, oestrone, oestradiol and oestriol in many lower vertebrates (see Gottfried, 1964). Among invertebrates, oestradiol has been identified in ovaries of *Pisaster ochraceus* (Botticelli, Hisaw & Wotiz, 1960) and *Strongylocentrotus franciscanus* (Botticelli, Hisaw & Wotiz, 1961; see also Creange, 1964); both oestrone and oestradiol were found in ovaries of *Pecten hericinus* (Botticelli *et al.* 1961). Pathways of synthesis and metabolism of these steroids, as well as their regulatory functions, if any, in the aforementioned organisms are not known.

The present experiments were undertaken as an initial step in the attempt to elucidate predominant metabolic pathways for oestrogens in selected invertebrates. This paper concerns the identification of oestradiol 3-sulphate as the principal metabolite formed after incubation of oestradiol in the presence of surviving gut tissues from *Strongylocentrotus franciscanus*, or soluble enzymes prepared from that tissue. A preliminary account of some of this work has been presented (Creange & Szego, 1962). The data constitute a portion of

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MATERIALS AND METHODS

[16- ^{14}C]Oestradiol (2.7 $\mu\text{C}/\text{mg.}$) was purchased from C. A. Frosst Co., Montreal, Quebec, Canada; [4- ^{14}C]oestradiol (37.3 $\mu\text{C}/\text{mg.}$) from New England Nuclear Corp., Boston, Mass., U.S.A. Reference standards oestradiol 17-sulphate, oestradiol 3-sulphate, oestradiol 3,17-disulphate, oestriol 3-sulphate and oestriol 3,16,17-trisulphate were generous gifts from Dipl. Eng. H. Fex, A. B. Leo, Hälsingborg, Sweden. Oestrone [^{35}S]sulphate and carrier-free inorganic [^{35}S]sulphate were purchased from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. These compounds behaved as homogeneous substances during chromatographic and electrophoretic analyses and were not further purified.

All reagents used were analytical grade with the exception of chloroform, which was of spectrophotometric quality. Benzene, ethyl acetate, ethylene dichloride, hexane, light petroleum (b.p. 80–100°), pyridine and toluene were purified according to methods described by Bush (1961). Diisopropyl ether was distilled over FeSO_4 . The anti-oxidant Topanol (6-*tert.*-butyl-2,4-dimethylphenol), made available through the courtesy of Imperial Chemical Industries Ltd., Durham, was added in small amounts (0.01%, v/v) to the freshly distilled diisopropyl ether (see Bush, 1961). Diethyl ether was freshly distilled over FeSO_4 each day. Butan-1-ol and 2-methylpropan-2-ol were redistilled before use; methanol (J. T. Baker, Phillipsburg, N.J., U.S.A.), ethanol and 95% (v/v) ethanol (both from U.S. Industrial Chemicals, New York, N.Y., U.S.A.) were used without further purification.

Incubation of surviving tissues. Tissues from the ampullae, gonads (from animals collected without regard to

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sex) or the gut were excised, rinsed in ice-cold sea water and blotted. Portions (50–60 mg.) were rapidly weighed into 20 ml. beakers containing, in a total volume of 1.5 ml. of filtered autoclaved sea water, 42–44 μ moles of [14 C]-oestradiol (about 0.15 μ C in 0.014 ml. of 95% ethanol), 17 μ moles of glucose and 45 μ moles of tris. The final pH was 7.6–7.9. Incubation was carried out for various times at 15° in an oxygen atmosphere. Freshly prepared incubation media were always filtered through Whatman no. 50 filter paper immediately before use to remove undissolved steroid, if any. In all experiments reported, the amount of steroid indicated as originally present was the amount recovered by actual extraction and analysis of control samples without tissue, not necessarily the calculated amount added.

After incubation, the samples were rapidly chilled and centrifuged at 0°. Portions of the supernatant medium were removed as required for paper-electrophoretic analysis. The paper electrophoresis was carried out by the horizontal open-strip method of Szego (1957), except that a constant current of 0.45 mA/cm. was used. The dried electrophoretograms were scanned for radioactivity with a windowless digital-output strip-scanner (Electro-Fab Co., Van Nuys, Calif., U.S.A.). Radioactivity is expressed as counts/min. corrected for background and for absorption of radioactivity by the paper (Szego & Roberts, 1956). Generally, tissues and the remainder of the incubation medium together were macerated in 5 ml. of acetone at 0° and centrifuged. The residue was extracted three times with 5 ml. of acetone-methanol (1:1, v/v), then three times with 5 ml. of diethyl ether-acetone (1:1, v/v). The combined organic extracts were evaporated to dryness; the residue was dissolved in 10 ml. of water and extracted three times with 10 ml. of diethyl ether. The combined ether extracts, containing free steroid, were evaporated to dryness, plated on 1 in. aluminium planchets and analysed for radioactivity in a windowless gas-flow automatic sample counter. All results were corrected, if necessary, to infinite thinness.

The aqueous phase from the above extraction procedure was made to 1 N with NaOH and extracted three times with equal volumes of butan-1-ol. Portions of the organic extracts containing the steroid metabolites were analysed for radioactivity as described above. Recovery of total radioactivity in both ether and butan-1-ol extracts by this fractionation procedure averaged $85 \pm 5\%$.

Incubation of acetone-dried powder preparations. Acetone-dried powders of the *S. franciscanus* gut were prepared by homogenization of frozen tissues in a Waring Blendor with 10 vol. of acetone at -10° to -15° . The resultant suspension was rapidly filtered; the residue was resuspended in cold acetone, filtered and dried *in vacuo*. The enzyme preparation obtained was stable and could be stored several weeks at -30° . Just before use, the acetone-dried powder was extracted for 1 hr. in ice-cold 5 mM-phosphate buffer, pH 7.0 (50 mg. of powder/ml. of buffer). The mixture was centrifuged 10 min. at 0°. Portions (1 ml.) of the supernatant solution, containing 19–22 mg. of protein, as determined by semimicro-Kjeldahl analysis, were added to 2 ml. of incubation medium containing various amounts of [14 C]oestradiol, 45 μ moles of K_2SO_4 , 15 μ moles of $MgCl_2$, 15 μ moles of ATP and 100 μ moles of tris, final pH 8.1–8.2. Incubation was carried out in air in a Dubnoff metabolic incubator at 25°. Temperature and pH optima, the influence of substrate and enzyme concentrations, substrate

specificity and other properties of the enzyme system were established in previous investigations (Creange, 1964).

After incubation, samples were extracted three times with acetone-methanol (1:1, v/v). The combined organic extracts were dried *in vacuo*. Sulphated metabolites were separated from free steroids in the extracts by means of silica-gel column chromatography, by using a modification of the method of Conrad, Mahesh & Herrmann (1961). The columns (1 cm. diam.) were packed with 4–5 g. of silica gel (100–325 mesh, grade 922; Davison Chemical Division, W. R. Grace and Co., Baltimore, Md., U.S.A.) in benzene. The samples were transferred to the columns with minimum volumes of the acetone-methanol mixture. Eluting solvents, volumes and the elution sequence are shown in Table 1. Fractions II and IV together contained 95–100% of the total radioactivity present in the incubation mixture. This procedure was more suitable for preparation of large quantities of the oestrogen metabolites than was the solvent partition method described above.

RESULTS

Fig. 1 illustrates the distribution of radioactivity in the medium after a typical aerobic incubation of surviving sea-urchin gut tissue with

Table 1. *Elution sequence for the silica-gel column partition method used to separate free steroids from steroid sulphates*

Columns were eluted at a rate of about 1 drop every 5 sec. Fraction II contained free steroids; fraction IV contained steroid sulphates.

Eluting solvent	Vol. (ml.)	Fraction
Benzene	10	I
Benzene-ethyl acetate (1:1, v/v)	10	II
Ethyl acetate	10	
Ethyl acetate-ethanol (9:1, v/v)	10	III
Ethyl acetate-ethanol (1:1, v/v)	25	IV

Fig. 1. Presence of polar [14 C]oestradiol metabolite in the medium after 2 hr. incubation of 50 mg. of *S. franciscanus* gut tissue in the presence of [14 C]oestradiol. Incubation conditions were as described in the text. Portions (0.02 ml.) of the preincubation medium (b) and of media after incubation (a) were subjected to electrophoretic analysis as described in the text.

[^{14}C]oestradiol. Fig. 1(b), representing electrophoretic analysis of the preincubation medium, shows one radioactive peak near the origin. This

peak comprises the free oestrogen. Fig. 1(a) shows that radioactivity attributable to the original free steroid was no longer present in the medium after 1 hr. of incubation. Instead, there was a smaller radioactive peak, about 12 cm. from the origin, which represents the [^{14}C]oestradiol metabolite formed in the presence of the urchin tissue and released into the medium.

The rapidity of the conversion of oestradiol is illustrated in Fig. 2. Even in the unincubated controls electrophoretic analysis showed a decrease of almost 50% in the radioactivity in the medium. A 15 min. incubation resulted in a further decline in free steroid. At this time an appreciable amount of radioactivity was present in the area of the electrophoretogram occupied by the polar oestradiol metabolite. During incubation for longer periods, successively increasing amounts of [^{14}C]oestradiol metabolite were formed until about 60 min., at which time negligible amounts of free steroid remained in the medium and maximal production of the metabolite appeared to have taken place. These phenomena were strongly inhibited by anaerobic incubation.

In the present investigations, the electrophoretic mobility of the radioactive metabolite is reminiscent of the relative mobility of 'oestropoetin', a metabolite formed after incubation of the parent steroid in the presence of surviving rat liver and homologous serum protein (Szego, 1953, 1955, 1957; Szego & Roberts, 1956). However, incubation of labelled oestrogen with surviving sea-urchin gut tissue failed to reveal association of the steroid with protein, even when the medium was fortified with homologous protein. On the contrary, the oestradiol metabolite moved faster than any of the urchin-tissue proteins on the paper electrophoretograms. Nor was it possible to demonstrate an association between steroid and protein by cold acetone treatment of the incubation medium.

Fig. 2. Distribution of radioactive products in medium before and after a typical incubation of surviving sea-urchin gut tissue with [^{14}C]oestradiol were carried out for various times. Incubation conditions and electrophoretic analysis were as described in the text. Unincubated controls, represented by '0 min.' on the abscissa, were samples in which the tissue was added to ice-cold medium, the mixture immediately centrifuged at 0° for 10 min. and a sample of the supernatant medium removed at once for analysis. $\circ$, Radioactivity attributable to free [^{14}C]oestradiol; $\bullet$, radioactivity due to accumulation of the polar oestradiol metabolite. $\triangle$ and $\blacktriangle$, Radioactivity present in the preincubation medium without tissue added, which, after electrophoresis, occupied the region of the electrophoretogram normally containing free steroid and metabolite respectively.

Table 2. *Distribution of radioactivity originating from [^{14}C]oestradiol after various periods of incubation in the presence of *S. franciscanus* gut tissue in vitro*

Between 50 and 60 mg. fresh wt. of tissue was used. The medium and conditions of incubation were as described in the text. The fractionation procedure was similar to that described in the text, except that the tissue and medium were fractionated separately. In this particular instance the tissue was digested briefly in boiling 0.1 N-NaOH, then made to pH 6 before ether extraction.

Incubation time (min.) Fraction.....	Tissue (counts/min.)			Medium (counts/min.)			Recovery* (%)
	Total	Ether-soluble	Water-soluble	Total	Ether-soluble	Water-soluble	
0	11558	6246	5312	8749	8325	434	82
30	19355	1365	17990	5957	2716	3241	102
60	18026	436	17590	5958	584	5374	97
120	18799	279	18520	6255	129	6216	101

* 24760 counts/min. present originally, equivalent to 44 μmoles of steroid.

In additional experiments (Table 2) it was found that radioactivity not present in the medium after incubation could be accounted for in the tissues, and that even in unincubated controls about 45% of the radioactivity in the tissue had been rendered water-soluble. Table 2 also demonstrates that after only 30 min. incubation over 85% of the total steroid was converted into a water-soluble form; by 60 min., virtually no ether-soluble radioactivity remained. Thus the formation of the polar oestrogen metabolite in the medium paralleled the metabolic conversion of oestradiol into water-soluble products.

When incubation of gonad tissue or ampullae of the sea urchin was carried out, analysis of the resultant media revealed no polar oestradiol metabolites. Gut tissue alone, of the three tissues tested, appeared to have the ability to transform the free steroid into a water-soluble metabolite, which was subsequently identified as oestradiol 3-sulphate (see below). That the process was a property of an enzyme system in the gut itself and not of the associated flora was demonstrated by failure of washings from equivalent amounts of gut tissue to exhibit any steroid-transforming activity *in vitro* after 60 min. incubation.

Identification of the metabolite. Preliminary evidence, based on solvent partition, stability to alkali, sensitivity to Mylase-P (phenol sulphatase) and resistance to β -glucuronidase, indicated that the predominant metabolite formed from oestradiol

by surviving gut tissues of the sea urchin might be a steroid sulphate (Creange & Szego, 1962). To test this possibility directly, gut tissue was incubated for 90 min. in the presence of unlabelled oestradiol and 150 μ C of carrier-free inorganic [35 S]sulphate. A parallel incubation was carried out with [14 C]oestradiol as substrate. Analysis of the resultant media, by paper electrophoresis under conditions that removed all traces of inorganic 35 S, revealed that the electrophoretic mobilities of the 35 S- and the 14 C-labelled metabolites were identical (Creange, 1964). Thus presumptive evidence was obtained for the formation of oestradiol sulphate by surviving gut tissues of *S. franciscanus*.

Butan-1-ol extracts of media and tissues, pooled from the [14 C]oestradiol and the [35 S]sulphate incubations respectively, were subjected to mixed chromatography with reference standard oestrogen sulphates in seven different solvent systems. The results are summarized in Table 3. It is clear from these data that, in all chromatographic systems used, both the 14 C- and the 35 S-labelled metabolites had mobilities identical with that of authentic oestradiol 3-sulphate. The oestradiol metabolite formed by the acetone-dried powder preparations was also subjected to mixed paper chromatography in solvents 1, 3, 6 and 7 (Table 3). The R_F values obtained (not shown in Table 3) were identical with those of authentic oestradiol 3-sulphate.

In an effort to characterize the steroid moiety of the oestrogen metabolite, pooled butan-1-ol extracts

Table 3. *Chromatographic mobility of 14 C- and 35 S-labelled metabolites formed after incubation of surviving *S. franciscanus* gut with [14 C]oestradiol or unlabelled oestradiol together with inorganic [35 S]sulphate*

The solvent systems used were as follows: 1, di-isopropyl ether-2-methylpropan-2-ol-acetic acid-water (7:3:3:7, by vol.); 2, ethyl acetate-butan-1-ol-aq. ammonia (sp.gr. 0.90)-water (9:1:1:9, by vol.); 3, ethyl acetate-butan-1-ol-aq. 0.2% ammonia (8:7:1 by vol.); 4, ethylene dichloride-2-methylpropan-2-ol-aq. 2N-ammonia (3:1:4, by vol.); 5, propan-2-ol-aq. ammonia (sp.gr. 0.90)-water (7:1:2, by vol.); 6, di-isopropyl ether-2-methylpropan-2-ol-aq. 2N-ammonia (2:1:3, by vol.); 7, butan-1-ol-toluene-aq. ammonia (sp.gr. 0.90)-water (5:5:1:9, by vol.).

Solvent system	Method	14 C- and 35 S-labelled metabolites	R_F Authentic reference compounds		
			Oestradiol 3-sulphate	Oestradiol 17-sulphate	Oestrone sulphate
1	Present investigation	0.30	0.29	0.38	0.56
2	Schneider & Lewbart (1959)	0.72	0.72		
3	Schneider & Lewbart (1956)	0.46	0.46	0.54	
4	Bush (1957)	0.80	0.80		
5	cf. Smith (1960)	0.86	0.86		0.86
6	Bush & Gale (1960)	0.17	0.17	0.21	
7*	Schneider & Lewbart (1959)	0.65	0.68	0.56	

* In this system, the R_F value obtained for oestriol 3-sulphate was 0.09; for oestradiol 3,17-disulphate and oestriol 3,16,17-trisulphate, the R_F was 0.00.

containing the [^{14}C]oestradiol metabolite were subjected to solvolysis according to the method of Burstein & Lieberman (1958). The free steroid obtained was subjected to mixed chromatography with authentic oestrone, oestradiol and oestriol in the Bush B1 system (Bush, 1952). After development, the chromatograms were dried, analysed for radioactivity (see the legend to Fig. 1) and stained by the method of Barton, Evans & Gardner (1952).

Fig. 3. Distribution of [^{14}C]oestradiol metabolite (O) mixed with authentic carrier oestradiol 3-sulphate (●) in two 24-transfer countercurrent distribution systems. (a) Distribution obtained in the system ethyl acetate-0.1% NaOH (1:1, v/v; Diczfalussy, Cassmer, Alonso & de Miquel, 1961); (b) distribution in ethyl acetate-butan-1-ol-aq. 0.2% ammonia (1:1:2, by vol.; Menini & Diczfalussy, 1960). [^{14}C]Oestradiol radioactivity was measured in a windowless gas-flow automatic sample changer as described in the text. Carrier oestradiol 3-sulphate was determined by the Kober reaction utilizing the modification of Brown (1955).

Radioactivity on the chromatograms coincided only with oestradiol; none was associated with oestriol or oestrone (Creange, 1964).

When the [^{14}C]oestradiol metabolite, pooled from several incubations utilizing the soluble enzyme preparation, was subjected to countercurrent distribution along with carrier oestradiol 3-sulphate, both substances exhibited identical distribution (Fig. 3). The correspondence between the partition coefficients of the acetylated derivatives of both the unknown and the standard oestradiol 3-sulphate is shown in Table 4. Further, K values for their subsequently solvolysed products were similar to each other, and corresponded to the partition coefficient observed for authentic oestradiol 17 β -acetate (Table 4). Thus, on the basis of the above investigations, it is apparent that the metabolite formed by enzymes from *S. franciscanus* gut tissue was oestradiol 3-sulphate.

DISCUSSION

On the basis of the work by Botticelli, Hisaw & Wotiz (1961) it can be assumed that the organism is normally exposed to oestradiol, the endogenous origin of which is yet to be determined. In the present experiments, it has been conclusively demonstrated that the major product of the metabolism of oestradiol by incubation *in vitro* in the presence of surviving sea-urchin gut tissue, or enzymes prepared therefrom, is oestradiol 3-sulphate. The enzyme system involved in the reaction is highly active, as indicated in greater detail elsewhere (Creange, 1964), and capable of rapid transformation of amounts of oestradiol very far in excess of those found in the urchin tissues. In fact, under optimum incubation conditions (Creange, 1964) rates of oestradiol 3-sulphate formation by the soluble enzymes of 6-7 $\mu\text{moles/g.}$ of protein/hr. could be obtained. Such a rate of sulphate ester formation is comparable with the

Table 4. Partition coefficients of derivatives of [^{14}C]oestradiol metabolite and of authentic oestradiol 3-sulphate

Solvent systems were those described by Diczfalussy *et al.* (1961): A, ethyl acetate-aq. 0.1% ammonia (1:1, v/v); B, benzene-hexane-methanol-water (1:9:6:4, by vol.). Partition coefficients (K) were calculated according to the method of Way & Bennet (1951). Acetylation was carried out according to the method of Dominguez, Seely & Gorski (1963), with a 3:1 ratio of pyridine to acetic anhydride.

Acetylation product	Solvent system	Partition coefficient (K)	
		[^{14}C]Oestradiol metabolite	Authentic oestradiol 3-sulphate
Acetylation product	A (12 transfers)	0.499	0.499
Acetylation product after solvolysis	B* (24 transfers)	1.404	1.500

* K for authentic oestradiol 17 β -acetate in this system was 1.500.

rates reported for extracts of adult human adrenal glands (Boström, Franksson & Wengle, 1964).

These observations do not, of course, rule out the possible exogenous origin of the free oestradiol found in the urchin gonad tissues, inasmuch as the steroid might well be present in the diet of the organism. It is considered unlikely, however, that ingested oestradiol would escape sulphation and reach the gonads in quantities sufficient to account for the occurrence of free oestradiol therein, without the participation of a gonadal sulphatase. The extreme water-solubility of the steroid sulphate, and thus its ready excretion into the sea water environment, would render still more unlikely the exogenous origin of gonadal steroid in this organism. Hence the likelihood is that gonads of *S. franciscanus* synthesize oestradiol, and that the turnover of this steroid is far greater than would be inferred merely on the basis of the absolute amounts present in the urchin tissue.

Comparative studies carried out with soluble enzymes prepared from gut tissue of the sea urchins *S. franciscanus* and *S. purpuratus*, as well as from tissues taken from invertebrates representing four other phyla, revealed that under the incubation conditions used *S. franciscanus* yielded the most active preparation. It was also the only preparation that catalysed the synthesis of oestradiol 3-sulphate alone (Creange, 1964). However, the physiological significance of this powerful oestradiol-sulphation system is obscure.

Considerable interest has been centred recently on the role of steroid sulphates as intermediates in the biological synthesis of steroid hormones. However, throughout the present work, there was no sign of any alteration of the steroid molecule other than sulphation at C-3. Thus it seems most likely that the oestradiol 3-sulphate is not an intermediate, but an end product, of oestrogen metabolism in *S. franciscanus*. It is, of course, possible that alterations of the steroid structure involve the participation of organs other than the gut, or that the present conditions of incubation lacked some factors essential for the further metabolism of oestradiol 3-sulphate or its precursor steroid. Thus the conversion of oestradiol into oestrone by sperm of sea urchins and other invertebrates requires NAD or NADP (Hathaway, 1965).

It is conceivable that sulphation may be involved in the transport of oestradiol across the gut wall of the sea urchin, as part of an excretory or 'detoxication' process. Such an intestinal transport system might be analogous to that described for steroid glucosiduronates in the rat (Kreek, Guggenheim, Ross & Tapley, 1963; Smith, Tapley & Ross, 1963). Finally, the possibility cannot be ignored that production of large quantities of a

highly water-soluble compound such as oestradiol 3-sulphate may play some significant pheromonal role in the regulation of reproductive activity in populations of *S. franciscanus*. These and additional postulates remain to be tested.

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