Proceedings
of the
Society
for
Experimental Biology and Medicine

VOL. 111 DECEMBER 1962 No. 3

SECTION MEETINGS

MARYLAND
Carnegie Institution of Washington October 25, 1962

MINNESOTA
Mayo Foundation October 5, 1962

Conversion of 6,7-H^3-Estradiol-17β into Estrone and Estradiol-17α in the Mature Male Dog.*‡ (27845)

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The estrogens and related compounds in dog urine have not been studied carefully. Some early reports indicated the presence of estrogens(1-4) while others reported negative results even in the urine from pregnant bitches(5,6). More recently Kristoffersen (7), using the method of Brown(8), reported trace amounts of estrogens in pregnancy urine. The present study involved administration of 6,7-H^3-estradiol-17β to a mature male dog and the study of the metabolites in urine. It was hoped that by this means metabolites of estradiol-17β could be established so that an acceptable analytical method, useful for biochemical studies of estrogen metabolism in dogs, could be developed.

Experimental. Paper chromatographic systems.

A = Bush A(9); Heptane : methanol : water (200 : 160 : 40)

B = Iso-octane : toluene : methanol : water (90 : 110 : 150 : 50)

C = Bush C(9); Toluene : ethyl acetate : methanol : water (180 : 20 : 100 : 100)

D = n-Heptane : ethyl acetate : methanol : water (170 : 30 : 140 : 60)

E = Ligroin (90-120°C) : toluene : methanol : water (100 : 100 :130 : 70)

F = Toluene/propylene glycol(10)

Purification of 6,7-H^3-estradiol-17β. The

* This investigation was carried out during the tenure of Postdoctoral Research Fellowship from Nat. Cancer Inst. and grant of U. S. Public Health Service.

‡ This work is part of a thesis to be submitted by the senior author in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Physiology at Jefferson Medical College.

6,7-H^3-estradiol-17β (150 mC/mg) was successively purified in chromatography systems B and E. After dilution with non-radioactive estradiol-17β the labelled material was subjected to additional purification by rechromatography in two additional systems, D and F.

For the estrogen metabolism study a 1½-year-old 15-lb male mongrel dog was catheterized. Ten ml of blood were drawn, and 15 μc of 6,7-H^3-estradiol-17β in a mixture of 0.5 ml of ethanol and 10 ml of saline was injected intravenously. Additional blood samples were taken at 6, 25, 60 and 180 minutes. All samples were centrifuged at 3600 rpm for 30 minutes and the plasma immediately frozen. Twenty-four-hour urine samples, saturated with chloroform during collection, were obtained for the first 2 post-injection days. These were stored in the frozen state.

Plasma studies. The plasma samples were thawed at 5°C and homogenized to break up clotted material present, and 4-C^{14}-testosterone added as an internal standard to control the recoveries during the extraction procedure. This was justified on the basis that 97% of the 4-C^{14}-testosterone could easily be recovered in preliminary extractions. Plasma samples amounting to 0.1 ml were diluted to 1 ml with water and extracted once with 5 ml of ethyl acetate. The ethyl acetate extracts were transferred to scintillation vials and the solvent was evaporated with heat under a stream of nitrogen. Ten ml of POPOP/DPO scintillation counting solvent was added to each vial and counting was done in an automatic liquid scintillation counter (Tri-Carb-Packard) for 30 minutes with the apparatus adjusted to count both H^3 and C^{14}. Calculation for each isotope was done using simultaneous equations, and all samples were checked for quenching with H^3-toluene.

Urine. The urine samples were thawed and filtered in a cold room (5°C). Counting of crude urines was accomplished by solvolizing 0.2 ml of the urine in the scintillation fluid with ethanol, corrections for quenching being made.

Each of the urine samples was extracted once with twice their volume of ethyl acetate. The ethyl acetate was washed twice with water, and the water reextracted once with ethyl acetate. The ethyl acetate extracts were dried overnight with sodium sulfate, then evaporated to dryness *in vacuo*. The dried material was designated "free fraction." The extracted urine plus the water washings were adjusted to pH 6.4 and phosphate buffer (pH 6.4) added at the rate of 1/20 that of the urine volume. To this mixture 100 units/ml of bacterial β-glucuronidase (Sigma) were added and the final solution incubated at 37° ± 1°C for 48 hours. The liberated steroids were extracted with ethyl acetate and designated as "glucuronide fraction."

The aqueous portion was again adjusted to pH 6.4 and treated by the solvolysis procedure of Burstein and Lieberman(11) somewhat modified. After making the urine up to 20% with NaCl, extraction was carried out with ethyl acetate as described for preparation of the free fraction, and the extract dried with sodium sulfate, filtered, and concentrated H_2SO_4 added to a final concentration of 0.001 M. This mixture was incubated at 37° ± 1°C for 24 hours, washed with $NaHCO_3$, and with water until neutral, dried overnight with sodium sulfate, and finally evaporated to dryness. This material was designated as "solvolysis fractions."

Finally, the urine remaining after the solvolysis procedure was brought to boiling, and concentrated HCl was added at the rate of 15 ml of concentrated HCl to 100 ml of urine as recommended by Marrian(12). After refluxing for one hour the urines were cooled, and the dry ethyl acetate extract prepared as described previously. This material was designated as the "acid hydrolysis fraction."

Results. At 6, 25, 60, and 180 minutes after administration of the tritiated estradiol-17β 2400, 233, 70, and $<$ 1 dpm per ml of plasma was found. From these data a biological half-life of 10.5 minutes was calculated for the early phase, and 25.0 minutes for the later time. 180 minutes after injection no significant radioactivity could be detected. Of the injected dose 29% was excreted into the first day's urine, and 2% in the second day's urine. The greatest amount of H^3-steroids was found in the glucuronide fractions (Fig. 1).

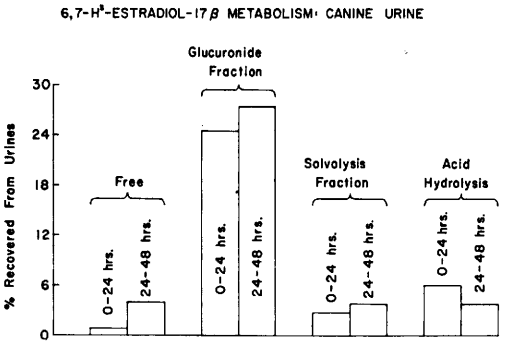

FIG. 1

The phenols from the glucuronide fraction (1st day) were chromatographed in system B along with parallel standards of estrone, estradiol-17β, and estriol. The areas corresponding to the respective standards were eluted with chloroform/methanol and 5 mg of estrone, 5 mg of estradiol-17β, and 10 mg of estriol were added to the corresponding fractions. Three successive crystallizations were done for each estrogen (Table I). The estriol fraction had a minimum amount of radioactivity but there was no change in specific activity between the second and third crystallization. Estradiol-17β and estrone did show a constant specific activity (within experimental error). The neutral fraction of the 1st day urine contained no significant radioactivity.

To study the metabolites of estradiol-17β further a pool consisting of the free fractions from both urines, the glucuronide fraction from day 2, and the solvolysis fractions from both urines were chromatographed in system B. The chromatogram was divided into 6 zones. Zone I (11,900 cpm) included compounds with the mobility of estriol and the more polar estrogens, including the origin to just behind the estradiol-17β area. Zone II (20,200 cpm) corresponded to the estradiol-17β area; Zone III (8,840 cpm), the area between estradiol-17β and estrone; Zone IV (7,910 cpm), estrone; and Zones V (2,260 cpm) and VI (4,240 cpm), the less polar area which ran in front of estrone.

TABLE I. Specific Activities of Estrogens Isolated from the Glucuronide Fraction of Day 1.

Compound	Specific activities, cpm/mg Crystallization 1st	2nd	3rd
Estradiol-17β	5,577	5,274	5,025
Estrone	3,960	4,794	4,014
Estriol	899	376	392

The material in Zone I was rechromatographed in system C, along with the standards estriol and 16-epiestriol. The chromatogram was scanned in a strip-counter (Atomic Accessories, Inc. – Scanogram II) at very slow speed. Two peaks of radioactivity were found, one which corresponded to 16-epiestriol (2,812 c/m) and another less polar material (2,804 c/m) with an R_F of .90. The estriol area contained 1,628 c/m. Five mg of estriol were added to the material eluted from the estriol area and the mixture crystallized from aqueous ethanol. After the second crystallization the count dropped to 14 cpm/mg, and it was concluded the concentration of estriol was insignificant. The other zones were not further investigated.

Zone II was rechromatographed in system D, and a large well-defined peak, containing 14,700 cpm, was found which corresponded exactly to estradiol-17β. This zone was rechromatographed in system E which separates the estradiol-17 epimers. Two peaks were found which had the mobilities corresponding to estradiol-17β and estradiol-17α. The estradiol-17α area had a count of 2,590 cpm and the estradiol-17β area had a count of 6,080 cpm. Five mg of estradiol-17β were added to the corresponding radioactive zone and radiochemical purity was attained by 3 successive crystallizations of the free compound followed by 3 recrystallizations of the monoethyl ether(8) (Table II).

Zone III was rechromatographed in system D, and also showed a single peak which corresponded to the estradiol epimers. A portion of this zone, containing 7,016 cpm was combined with the 2,592 cpm eluted from the estradiol-17α area of Zone II, and after addition of 5 mg of reference estradiol-17α, recrystallization to constant specific activity was attained (Table II). The formation of the monomethyl ether(8) helped identify the compound. Estradiol-17α appeared to be the major radioactive constituent of Zone III.

Zone IV was rechromatographed in system

TABLE II. Specific Activities of Free and Monomethyl Ethers of Estradiol-17α, Estradiol-17β, and Estrone.

	Specific activities, cpm/mg						
	Crystallization			Crystallization of monomethyl ethers of the rediluted compounds			
Compound	1st	2nd	3rd	Theoretical specific activity	1st	2nd	3rd
Estradiol-17α	900	874	864*	124	118	116	118
Estradiol-17β	1060	1012	1006†	120	114	110	110
Estrone	918	912	911‡	200	196	192	190
Estriol	71	14	—	—	—	—	—

* .7 mg diluted with 4 mg of non-radioactive estradiol-17α.
† .54 mg diluted with 4 mg of non-radioactive estradiol-17β.
‡ 1.2 mg diluted with 4 mg of non-radioactive estrone.

D, and a single peak corresponding exactly to estrone was observed. 6,036 cpm was eluted, and this was crystallized after addition of 5.3 mg of estrone. By recrystallization and derivative formation followed by additional recrystallization, radiochemical purity was obtained (Table II).

Zones V and VI did not yield any recognizable metabolites.

Discussion. Earlier studies provided indirect evidence for *in vivo* conversion of estradiol-17β to estrone in the dog(13,14). In another experiment estradiol-17β was isolated from dog bile after subcutaneous injection of estrone acetate(15). We have confirmed the oxidation of estradiol-17β to estrone and further demonstrated that estradiol-17α is also formed. This later compound undoubtedly resulted from the reduction of estrone.

The present study does not add precise information on the 16 substituted estrogens but indicates, as previously suggested by Pearlman *et al.*(15), that estriol is not a significant metabolite in the dog. Other 16 oxygenated estrogens are present in the urine, but additional studies are needed to elucidate their structures.

The bulk of the urinary estrogens appears to be conjugated to glucuronic acid.

Summary. 1. H^3-labelled estrone, estradiol-17β and estradiol-17α were isolated from dog urine after intravenous injection of 6,7-H^3-estradiol-17β. 2. The urinary estrogens were mostly bound to glucuronic acid. 3. Estradiol-17α is a major metabolite in the urine.

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Received July 11, 1962. P.S.E.B.M., 1962, v111.