The author wishes to express his sincere appreciation to The Upjohn Co. for the support which greatly aided this study. The efforts of Dr. Albert Notation in preparation of sulfated steroids are also acknowledged.

1. Rugh, R., Experimental Embryology, Burgess Publishing Co., Minneapolis, 1962, 91.

2. Langan, W. B., 16th Internat. Cong. Zoology, 1963, v2, 304.

3. Chang, C. Y., Witschi, E., Endocrinology, 1955, v61, 514.

4. Wright, P. A., Proc. Soc. Exp. Biol. & Med., 1960, v104, 77.

Received January 4, 1967. P.S.E.B.M., 1967, v124.

Metabolism of Estradiol-17β-4-^{14}C in a Non-Pregnant Rhesus Monkey.* (31994)

GEORGE L. FLICKINGER AND CHUNG-HSIU WU†
(Introduced by Luigi Mastroianni)
Department of Obstetrics and Gynecology, School of Medicine, University of Pennsylvania, Philadelphia

Although the rhesus monkey is being used extensively for investigations in reproductive biology, only a few studies of estrogen metabolism have been carried out in this species. Short and Eckstein identified estrone and estradiol-17β in the urine of pregnant monkeys but were unable to detect estriol(1). Recently Laumas reported estriol to be present in the urine of rhesus monkeys (2).

The present study was carried out to determine the metabolism of estradiol-17β-4-^{14}C in a non-pregnant rhesus monkey during the follicular stage of the menstrual cycle.

Materials and methods. A 6 kg *Macaca mulatta* was injected intravenously with 4.9 μC of estradiol-17β-4-^{14}C (specific activity, 8.18 mC/mM; New England Nuclear Corp.) dissolved in 4.5 ml of saline and 0.5 ml of ethanol. The estradiol-17β-4-^{14}C was purified by paper and thin layer chromatography prior to its use in the present experiment.

Injection was carried out on the eighth day of a 29-day menstrual cycle. The animal was housed in a metabolic cage with the collection flask packed in dry ice so as to insure immediate freezing of voided urine throughout three consecutive 24-hour collections.

Five ml of heparinized blood, drawn at 5, 30, 60 and 180 minutes after injection, was centrifuged for 30 minutes at 3600 rpm. One-tenth ml of plasma was diluted to 1 ml with water and extracted 2 $\times$ with 5 ml of ethyl acetate. The ethyl acetate was evaporated in scintillation vials and counted. Fifty μg each of carrier estrone, estradiol-17β, estradiol-17α, 16 epiestriol and estriol was added to each 24-hour urine. One ml aliquot of each 24-hour volume was assayed for radioactivity. The remaining total volume of each day's urine was extracted 3 $\times$ with 2 volumes of freshly distilled diethyl ether ("free fractions"). The extracted urines were adjusted to pH 6.8 following addition of 1/10 volume of 0.2 M maleate buffer(3). The urine was incubated in the presence of bacterial glucuronidase (300 units/ml; Sigma) for 24 hours at 37°C. Extraction was carried out as before ("glucuronide fractions").

The aqueous phases were adjusted to pH 6.0 with 0.2 M maleic acid and incubated for 24 hours at 50°C in the presence of phenolic sulfatase (10 mg/ml; Nutritional Biochemicals). Following extraction as above ("sulfate fractions"), 15 volumes percent concentrated HCl was added to the extracted urines. They were refluxed for 1 hour, cooled and extracted with ether ("acid fractions").

The ether from each of the above frac-

*This work was supported in part by U.S.P.H.S. Grants HD-01199 and AM,14013 and a grant from the Ford Foundation.

†Ford Foundation Fellow in Reproductive Biology.

TABLE I. Percent of Injected Radioactivity in Urine.

Day	Urine vol (ml)	Steroid fractions				
		Total	Free	Glucuronides	Sulfates	Acid hydrolyzed
1	280	52	.47	44.0	4.2	1.8
2	355	10	.13	7.7	1.3	.41
3	435	3	.02	2.4	.25	.07

tions was evaporated and the residues were partitioned between toluene and 1 N NaOH. The NaOH was neutralized with HCl and extracted 3 × with two volumes of ether. The ether was washed 1 × with 1/10 volume of $NaHCO_3$, 2 × with 1/10 volume of water, dried over anhydrous sodium sulfate and evaporated.

Chromatography was carried out on strips of Whatman #1 paper. Reference estrogens were located on the strips with the ferric chloride-potassium ferricyanide reagent(4). Estrogens were acetylated in 1.0 ml pyridine and 0.5 ml acetic anhydride at room temperature overnight. The acetates were located on thin layer plates (0.5 mm thick, silica gel G) by spraying with 2% phosphomolybdic acid in ethanol followed by heating at 90°C for 5 minutes.

Radioactive zones were detected on paper strips and thin layer chromatograms with a radiochromatogram scanner (Baird Atomic; Model RSC-363). Liquid scintillation was performed on a Packard Automatic Tri Carb Spectrometer. Fifteen ml of scintillation media, consisting of 4.6 g of 2,5 diphenyloxazole (PPO) and 116 mg of 1,4-bis-2-(5-phenyloxazolyl-benzene (POPOP) per liter of toluene was added to each sample. When necessary samples were solubilized in the scintillation media according to the procedure of Flood *et al*(5). Sufficient counting times were alloted to yield a maximum relative standard error of ±2% for each sample. The count rate (cpm) was corrected for quenching by means of an external standard. All count rates were corrected to 100% efficiency and expressed as disintegrations per minute (dpm).

Results. At 5, 30, 60 and 180 minutes after intravenous injection of estradiol 17β-4-^{14}C; 2150, 193, 64 and less than 1 dpm per ml of plasma was found. The calculated biological half lives were 7½ minutes for the first phase and 21 minutes for the later phase.

Of the injected radioactivity 52% was found in the first day's urine, 10% in the second and 3% in the third. The majority of the radioactivity was present in the glucuronide fractions of each 24-hour urine (Table I).

The urinary metabolites of estradiol-17β-4-^{14}C were identified in the glucuronide fraction of the first day's urine, which contained 44% of the injected radioactivity. Paper chromatography of this fraction was carried out initially in the system methylcyclohexane-propylene glycol for 72 hours. Three zones of radioactivity were detected. The least polar zone, which ran parallel to reference estrone, was eluted and chromatographed with toluene-propylene glycol for 4 hours. A single area of radioactivity corresponding to reference estrone was found. The second radioactive zone, corresponding to estradiol-17β and estradiol-17α was chromatographed for 20 hours in the system toluene-propylene glycol. Radioactivity was not detected by the radiochromatogram scanner or liquid scintillation of the eluted area corresponding to estradiol-17α. A radioactive area which ran parallel to reference estradiol-17β was eluted, and chromatography in the system toluene-methanol-H_2O (100:75:25) revealed a single peak with mobility identical to authentic estradiol-17β.

The area eluted from the starting line of the initial methylcyclohexane-propylene glycol chromatogram was run in the toluene-propylene glycol system for 5 days. In this system a zone of radioactivity was found adjacent to reference 16 epiestriol. This zone was eluted and rechromatographed successively in the systems methylene chloride-ethylene glycol (20 hours), isopropyl ether-ethylene glycol (19 hours), and toluene-ethyl acetate-methanol-H_2O (9:1:6:4, 6

TABLE II. Recrystallization of Estrogens in Urine of a Monkey Administered Estradiol-17β-4-^{14}C.

Steroid	Steroid carrier (mg)	Crystallization data (dpm/mg)				
			Free steroids			
			1st		2nd	
		Theoretical*	Crystals	Mother liquor		Acetates†
Estrone	19.67	32,700	31,978	31,157	30,863	31,424
Estradiol-17β	19.74	70,600	68,435	67,891	68,505	67,939
16 Epi-estriol	10.00	7,300	7,013	6,942	6,786	7,005
Estriol	10.52	10,400	10,250	10,104	10,217	10,196

* Calculated from dpm eluted from final paper chromatograms.
† Corrected for difference in molecular wt.

hours). In each system a radioactive area with mobility the same as reference 16 epi-estriol was found. Less than 5% of the initial radioactivity corresponding to 16 epi-estriol was lost through this multiple chromatographic procedure.

A more polar zone corresponding to estriol in the toluene-propylene glycol system (5 days) was rechromatographed successively in the systems methylene chloride-ethylene glycol (42 hours), isopropyl ether-ethylene glycol (25 hours) and toluene-ethyl acetate-methanol-H_2O (9:1:6:4, 10 hours). A radioactive zone corresponding to authentic estriol was detected on each strip and 82% of the original radioactivity associated with estriol was recovered.

Radioactive zones corresponding to estriol, estradiol-17β, estrone, and 16 epi-estriol, eluted from the final paper chromatograms, were crystallized to constant specific activity from aqueous ethanol following the addition of carrier steroids. The specific activities of the acetates formed from the final crystallization of the free estrogens were the same as the corresponding parent steroids (Table II). Aliquots of the estrogen acetates were chromatographed on thin layer plates with methylcyclohexane ethyl acetate (6:4). In every instance the radioactivity was associated with the carrier estrogen acetate.

Discussion. The present study demonstrates that estriol and 16 epi-estriol are urinary metabolites of estradiol-17β in the rhesus monkey during the follicular stage of the menstrual cycle. These 16- oxygenated estrogens represented, however, only a small portion of the total radioactivity recovered in the glucuronide fraction of the first day's urine. It appears that the majority of the injected dose of estradiol-17β-4-^{14}C was excreted without further metabolism or as estrone. A recent study reported estriol levels in non-pregnant and pregnant monkeys to be higher than either estrone or estradiol-17β(2). Large amounts of estriol have also been found in the urine of a pregnant chimpanzee(6). The formation of 16 epi-estriol from estradiol-17β in the rhesus monkey is of interest since this steroid has only been reported in the human(7) and the chicken(8).

The pattern of conjugation of estrogens in the rhesus monkey seems to be like that of the human in that the majority of the radioactivity present in the urine was hydrolyzed by glucuronidase. Much smaller amounts of the radioactivity were detected as the free steroids or as sulfates. This differs from the study in the pregnant chimpanzee where the majority of estrogens in the urine were excreted as free steroids(6).

Summary. The metabolism of estradiol-17β-4-^{14}C was studied in a rhesus monkey during the follicular stage of the menstrual cycle. The major portion of the injected dose was excreted in the urine during the first 24 hours as glucuronosides. Estradiol-17β and estrone were the principal radioactive estrogens found in this fraction of the urine. Smaller amounts of the radioactivity were identified as estriol and 16-epi-estriol.

1. Short, R. V., Eckstein, P., J. Endocrinol., 1961, v22, 15.
2. Laumas, K. R., ibid., 1965, v31, 297.
3. Touchstone, J. C., Murawec, T., Biochemstry, 1965, v4, 1612.

4. Barton, D. H. R., Nature, 1952, v170, 250.
5. Flood, C., Layne, D. S., Ramcharon, S., Rossipal, E., Tait, J. F., Tait, S. A. S., Acta Endocrinol., 1961, v36, 237.
6. Jerku, H., Layne, D. S., Steroids, 1965, v5, 37.
7. Marrian, G. F., Bauld, W. S., Biochem. J., 1955, v59, 136.
8. MacRae, H. F., Common, R. H., Poult. Sci., 1960, v39, 707.

Received January 16, 1967. P.S.E.B.M., 1967, v124.

Organic Acid Movement Out of the Central Canal.* (31995)

DONALD T. FRAZIER, SIDNEY SOLOMON, AND HARALD SONNENBERG
Department of Physiology, University of New Mexico School of Medicine, Albuquerque

The function of ependymal cells has long been under consideration and diverse views have been advanced in ascribing a biological role to the ependyma. Because ependymal cells are ciliated, it has been suggested that these cells can circulate cerebrospinal fluid and indeed such has been reported for *Xenopus* larvae(1). Evidence exists that the choroid plexus is involved in the production of cerebrospinal fluid and, by implication, its ependymal cells(5,13,14,15,21). More recently, a specific function of the ependymal cells of the choroid plexus has received support, *i.e.*, that these cells are involved in the absorption of organic acids and other anions(4,7,14,20).

Although it is technically convenient to study transfer from brain ventricle to blood, interpretation of results is complicated by the fact that the ventricular lining is a structurally mixed cellular layer. Since the central canal of the spinal cord is lined almost exclusively by ependymal cells, investigation of translocation of solute and solvent into and out of the central canal should more clearly define the functional role of ependymal cells. To this end, experiments involving perfusion of the central canal have been carried out.

Methods. In the first developmental experiments, rabbits were chosen as the experimental animals, while later studies were performed exclusively on cats. Both species were anaesthetized with nembutal. Laminectomies were performed at a low thoracic and a lumbar region of the cord. V cuts were made at both levels. A fine polyethylene catheter connected to a Sage infusion pump was inserted into the thoracic canal for delivery of perfusate. In all experiments, delivery was at a rate of 3 lambda/min. A second catheter was inserted at the lumbar region for collection. Sampling of perfusate was done by inserting a glass capillary tube into the collection catheter and allowing it to fill for fixed times. Periods varied from 15 minutes to one hour depending on the rate at which fluid was collected. The perfusate contained radio inulin (C^{14}) and had the following composition in mM/l: NaCl 150; $NaHCO_3$ 2.4; KCl 5.6; $CaCl_2$ 2.2; glucose 2.5. Appropriate test substances were then added to the perfusates.

It was not possible to collect fluid at every insertion of the collection catheter. It is likely that at least two factors were responsible. 1) It was found in some experiments that the catheter had passed out of the canal into the cord and had become plugged with bits of neural tissue. 2) The canal itself was blocked by cellular debris. Under such conditions, the lumbar laminectomy was extended caudally, the cord recut and collection tried again. Such procedure often led to a successful experiment.

The following analytical procedures were used: 1) Volume recovery was estimated by weight difference of the collection capillary tube. 2) Chloride was determined using an electro-titrator (Aminco). 3) Radio inulin was determined with an automated thin-window gas-flow counter (Nuclear, Chicago). 4) Chlorphenol red [CPR] was measured spectrophotometrically in micro cuvettes at

*This work was supported in part by NIH Grant HE-08477.