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7. We thank S. Voerman (Laboratory for Research on Insecticides, Wageningen, Netherlands) for providing test chemicals and D. J. de Jong (Experimental Station for Fruit Growing, Wilhelminadorp, Netherlands) for providing the *C. spectrana* moths.
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Estrogen Formation by the Isolated Perfused Rhesus Monkey Brain

Abstract. *Perfusion of two isolated brains from immature male rhesus monkeys with [^{3}H]androstenedione resulted in the identification of free and conjugated [^{3}H]estrone and free [^{3}H]estradiol from the perfusates. In the dissected cerebral tissues, estrogens were recovered only from the hypothalamus and limbic system. The production of estrogens from androstenedione during the 40-minute perfusions in these two experiments totaled 1.58 and 2.83 nanograms.*

The in vitro conversion of androstenedione to estrone and of testosterone to estradiol has been demonstrated with the use of homogenates of hypothalamic and limbic tissues from immature and adult rhesus monkey brains. In addition, biotransformation of androstenedione to estrone, and in some cases to estradiol, has been observed with the same tissues from human fetuses and from adult rats and rabbits (*1*). The crucial actions that estrogens exert centrally, such as control of gonadotropins, sexual behavior, and perhaps sexual differentiation of the hypothalamus (*2*), make on-site hormone synthesis particularly intriguing. Estrogens formed in the brain at their site of action would not be subject to systemic dilution and metabolic degradation before exerting a local effect. Confirmation in vivo of estrogen formation in the brain was sought to determine the applicability of the above in vitro observations to a physiological system.

The substrate, 7α-[^{3}H]androstenedione (specific activity, 7.7 c/mmole; Amersham/Searle) was purified by paper chromatography (in a ligroin, 96 percent methanol system). The techniques of perfusion and monitoring were as described by White *et al.* (*3*). The experimental subjects were two prepubertal male monkeys weighing 2.5 kg each. In order to minimize the amount of extraneous tissues perfused, the dissection included removal of the lower jaw, tongue, skin, salivary glands, orbital tissues, and muscles of the head. Thus, the experimental preparations consisted only of the isolated cranial vault, brain, and pituitary. After cannulation of the carotid arteries and separation of the head and spinal cord at the foramen magnum, perfusion was established at approximately 20 ml/min with intra-arterial pressures of 85/50 mm-Hg and normal electroencephalogram (EEG) patterns. In the first experiment, 350 μc of [^{3}H]androstenedione in 2 ml of absolute ethanol were slowly injected into the 250-ml perfusion volume. The injection was soon followed by a flat EEG and a rising arterial pressure over the 40-minute perfusion period. The brain, on sectioning, showed edema without hemorrhage. In order to minimize the effects of ethanol in the second study, the [^{3}H]androstenedione was slowly injected in 0.5 ml of 50 percent ethanol. The perfusion pressure and flow remained similar to that in the period before injection, and no edema developed. The EEG lost fast wave components which were regained by the end of the 40-minute perfusion period. On sectioning, no edema or hemorrhage were present. Immediately after perfusion, the hypothalamus, limbic system, the pituitary, and the cortex tissues were dissected and homogenized (*1*), except that, in the case of the second monkey, the pons was also studied.

The perfusate from each monkey was extracted with 20 volumes of a mixture of ethanol and ether (3 : 1). For tissues, the extraction was carried out with three 50-ml portions of the above-mentioned solvents. After evaporation, lipid precipitation was performed by resuspending the residue in 50 ml of 70 percent methanol and storing the samples overnight at −20°C. They were then centrifuged for 15 minutes at 8000*g*; the supernatant was decanted and evaporated to dryness; the residue was reconstituted in 50 ml of water, which was then extracted with three 50-ml portions of ether. The aqueous or conjugated fractions were hydrolyzed with 0.15 ml of Glusulase (glucuronidase, 26,925 units; sulfatase, 7,050 units; Endo Laboratories). The hydrolyzates were adjusted to *p*H 5 by the addition of 5 ml of acetate buffer and stored at 37°C overnight. They were extracted then with three 50-ml portions of ether.

Table 1. Aromatization of perfused [^{3}H]androstenedione (350 μc; specific activity, 7.7 c/mmole) by rhesus monkey brain. Estrogen was identified in the perfusate (250 ml). For crystallization, 10 mg of nonlabeled estrogen was used. Abbreviations: (c), conjugated fraction; dpm, disintegrations per minute; N, final crystallization; N-1, next to final crystallization; ML, mother liquor.

Product	Monkey No.	Total estrogen characterized			Specific activities (dpm/mg)			
					N−1		N	
		(pmole)	(pg)	(dpm)	Crystal	ML	Crystal	ML
[3-methyl-^{3}H]-estrone	1	4.98	1340	84090	8629	8434	8409	8109
	2	4.08	1096	67670	7175	6840	6767	6475
[3-methyl-^{3}H]-estrone (c)	1	0.06	15	950	96	96	95	93
	2	1.21	325	2045	2088	2032	2045	2004
[3-methyl-^{3}H]-estradiol	1	0.08	23	1430	142	146	143	141
	2	0.13	25	2210	223	217	221	233

All subsequent procedures with all tissues and perfusate samples were as described (*4*). Briefly, these consisted of two separations of the phenolic fractions followed by paper chromatography with ligroin and toluene (1 : 1) and 85 percent methanol to separate estrone and estradiol. This was followed by methylation of the estrogen and purification of the methyl ethers by paper chromatography in a ligroin, 96 percent methanol system. The methyl ethers of estrone and estradiol were identified by reverse isotope dilution and crystallization to constant specific activity. Radioactive portions were counted in a Nuclear-Chicago three-channel liquid scintillation spectrometer (Mark II) for sufficient time to assure a counting error no greater than 3 percent. A difference between final crystals and mother liquors of 5 percent or less was achieved in each case.

With the above-described procedures, it was possible to demonstrate aromatization of androstenedione by the perfused rhesus monkey brain. Free estrone, free estradiol, and conjugated estrone were identified in perfusates from both experiments (Table 1). In addition, the hypothalamic and limbic tissues dissected from the perfused brains contained free and conjugated estrogens (Table 2). No estrogens (less than 500 disintegrations per minute) were recovered from the pituitary, cortex, or, in one case, the pons. Isolation of radioactive estrogen from the hypothalamic and limbic tissue suggests that it is formed at these sites and is consonant with localization of enzymatic activity in these areas by studies in vitro. The contribution of uptake of labeled estrogen to the amounts found in these tissues cannot be assessed at this time. The only common metabolite recovered from both perfusates and all extracted tissues was free estrone (Tables 1 and 2). Free estradiol and conjugated estrone were isolated from both perfusates, but estradiol was found only in the tissues from the first preparation and the estrone was found in tissues from the second (Table 2). While estrone is a direct product of aromatization of androstenedione, the estradiol could be formed by reduction of estrone or by conversion of androstenedione to testosterone with subsequent aromatization. Because there is evidence for both estrone-estradiol and androstenedione-testosterone interconversions in brain tissues (*5*), either or both pathways could be operative in our study. The composition of the conjugated estrone recovered has not been determined, but formation of steroid sulfates by the perfused brain of *Macaca mulatta* has been described (*6*).

The perfusion fluids each contained a total of 1.4 ng of labeled estrogens, representing a conversion of approximately 0.01 percent of the starting substrate in 40 minutes. In the first experiment the tissues contained an additional 1.5 ng of estrogens, while in the second study much less was recovered (Table 2). The obvious effects of ethanol (*7*) in the first experiment preclude interpretations of the differences between the two studies.

It has been reported that, after injection of labeled androstenedione, radioactivity crosses the blood-brain barrier and is selectively accumulated in the hypothalamus (*8*). To this may now be added the observation of conversion of injected androstenedione to estrogens in the isolated perfused brain.

Table 2. Estrogen identified in the cerebral tissues of rhesus monkey brain perfused with [^{3}H]-androstenedione (350 μc, specific activity, 7.7 c/mmole). For crystallization, 20 mg of nonlabeled estrone was used for the unconjugated samples from the hypothalamus and limbic system, while 10 mg of the corresponding nonradioactive estrogen were used for the conjugated fractions.

Product	Monkey No.	Total estrogen characterized*			Specific activities (dpm/mg)			
					N−1		N	
		(pmole)	(pg)	(dpm)	Crystal	ML	Crystal	ML
				Hypothalamus†				
[3-methyl-^{3}H]-estrone	1	1.80	485	30580	1556	1513	1529	1558
	2	0.12	33	4300	209	211	215	211
[3-methyl-^{3}H]-estrone (c)	1			< 500				
	2	0.10	26	1750	167	171	175	169
[3-methyl-^{3}H]-estradiol	1	0.06	27	1090	109	105	109	104
	2			< 500				
				Limbic system‡				
[3-methyl-^{3}H]-estrone	1	3.41	916	57880	2927	2999	2894	2913
	2	0.17	46	6060	287	289	303	301
[3-methyl-^{3}H]-estrone (c)	1			< 500				
	2	0.12	33	2090	210	209	209	209
[3-methyl-^{3}H]-estradiol	1	0.11	29	1850	190	184	185	192
	2			< 500				

* The estrogen reported is in terms of the free steroid present after hydrolysis. † The total weight of the tissue sample from monkey No. 1 was 1500 mg, and from monkey No. 2 it was 767 mg. ‡ The total weight of the tissue sample from monkey No. 1 was 2100 mg, and from monkey No. 2 it was 1464 mg.

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