8. O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
9. R. H. Roth, L. C. Murrin, J. R. Walters, *Eur. J. Pharmacol.* **36**, 163 (1976).
10. J. V. Luco and F. Goni, *J. Neurophysiol.* **11**, 497 (1948); M. Vogt, *J. Physiol. (London)* **123**, 451 (1954).
11. T. Nagatsu, M. Levitt, S. Udenfriend, *J. Biol. Chem.* **239**, 2910 (1964).
12. R. J. Miller and P. H. Kelly, *Nature (London)* **255**, 163 (1975); G. Nistico, J. D. Stephenson, P. Preziosi, *Eur. J. Pharmacol.* **36**, 459 (1976).
13. B. K. Schrier and L. Shuster, *J. Neurochem.* **14**, 977 (1967); J. L. Meek, *Anal. Chem.* **48**, 375 (1976).
14. S. Matthysse, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **32**, 200 (1972).

15 September 1976

Participation of a Nonenzymatic Transformation in the Biosynthesis of Estrogens from Androgens

Abstract. *The biosynthesis of estrogens from androgens proceeds via three enzymatic hydroxylations, of which the first two take place on the C-19 methyl group and convert it to aldehyde. The final and rate-determining hydroxylation occurs at the 2β position, and the product rapidly and nonenzymatically collapses to an estrogen.*

All the estrogens in man are formed from androgen precursors by a sequence of enzymatic reactions referred to as aromatization (*1*). Location of this transformation, originally thought to be limited to the gonads and the placenta, has now been extended also to specific brain sites (*2*), as well as to adipose tissue (*3*). The implications of estrogen biosynthesis in the brain for behavior regulation (*4*) and in fat for the induction of postmenopausal carcinoma (*5*) emphasizes further the need for an understanding of the precise nature of the androgen to estrogen conversion as a prerequisite to any rational attempts at its modification or control. Previous studies have shown that aromatization involves the loss of the angular C-19 methyl group and the stereospecific elimination of the 1β and 2β hydrogens (*6*). The process includes the participation of three enzymatic hydroxylations requiring 3 moles of O_2 and NADPH (nicotinamide adenine dinucleotide phosphate reduced) per mole of estrogen formed (*7*). The first two of the hydroxylations take place on the C-19 methyl group giving rise successively to the 19-hydroxy (*8*) and 19-aldehyde structures (*9*). The site of the third hydroxylation and the nature of the final stage of the androgen to estrogen transformation remain unknown. The synthesis of 2β-hydroxy-19-oxoandrost-4-ene-3,17-dione (**1d**) (*10*) and its collapse to estrone in aqueous solution have been described. The extremely rapid aromatization of **1d** under physiological conditions and the coincidence of its structural features with the known requirements of biological aromatization suggested that 2β may be the site of the third and rate-determining hydroxylation in the aromatization process and that **1d** may be the immediate precursor of the female sex hormone. We now present experimental evidence in support of the above hypothesis and describe the complete mechanism of estrogen biosynthesis from androgen precursors including the unusual nonenzymatic transformation as a final step.

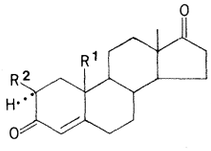
Because of the extremely rapid conversion of **1d** to estrone in neutral or basic aqueous solutions, we were unable to isolate labeled **1d** during the aromatization of labeled androstenedione with human placental aromatase under the usual conditions of *p*H 7.2 (*11*). Since **1d** is distinctly more stable under acidic conditions we repeated the incubation with ^3H- or ^{14}C-labeled androstenedione at *p*H 6, the lowest *p*H still consistent with adequate aromatase activity (*12*), and also added carrier **1d** to the labeled substrate to assist in the trapping of the labeled intermediate. To permit isolation and purification of **1d** without its further decay to estrone, we found it necessary to work up the incubation under acidic conditions and to convert the product to a stable derivative early in the isolation procedure. Reduction with sodium cyanoborohydride [$NaB(CN)H_3$], which is rapid and can be carried out in acid solutions, was the method we selected. This reagent converts the labile aldehyde **1d** to the stable tetrol **2c**, and also reduces both of the other aromatization intermediates **1b** and **1c** to the triol **2b**. The recovered substrate **1a** and the end product estrone are at the same time transformed to the 3β, 17β-diol **2a** and 17β-estradiol, respectively. Reduction of the 3-ketone function with $NaB(CN)H_3$ generated some 3α epimers in addition to the predominant 3β alcohols, but we isolated and analyzed only the 3β alcohol. The 3β stereochemistry in tetrol **2c** was established by means of NMR spectroscopy and conforms to the known orientation of the other main reduction products, **2a** and **2b** (*13*).

Table 1 shows the results obtained from the incubation of [1,2-^3H, 4-^{14}C]androstenedione without addition of carrier **1d** (A), with carrier **1d** added (B), and from the nonenzymatic control (C). The yields of the recovered substrate, intermediates, and product, isolated as their reduced forms, are given as percent of ^{14}C substrate content. Labeled **1d** as the tetrol **2c** derivative could be isolated only in experiment B, in which the car-

Table 1. Products isolated from the incubation of doubly labeled androst-4-ene-3,17-dione ($^3H/^{14}C$ = 5.8) and subsequent reduction. [1,2-^3H,4-^{14}C]Androst-4-ene-3,17-dione (0.1 μ*M*) (with a tritium content of 5.8×10^7 count/min and a ^{14}C content of 1.0×10^7 count/min) was incubated with placental microsomes in the presence of NADPH. The incubation was carried out in 3 ml of phosphate-citrate buffer (*p*H 6) for 30 minutes at 37°C. Simultaneously, another incubation with the same placental preparation was run exactly as above except that 950 μg (3 μ*M*) of the 2β-hydroxy derivative **1d** was added to the substrate before the start of incubation. A third incubation in which the placental microsomes were heated at 100°C for 3 minutes served as the nonenzymatic control. At termination, the incubation mixtures were adjusted to *p*H 3 with 0.1*N* HCl, and 6 ml of ethanol was added. The precipitated protein was removed and washed with ethanol. The combined ethanolic extracts (22 ml) were reacted with 200 mg of $NaB(CN)H_3$. After 3 hours at room temperature, excess reagent was decomposed with acetone, the reaction mixture was neutralized with 0.1*N* NaOH, and the volume was reduced. The remaining aqueous solution was extracted with ethyl acetate–(*n*)-butanol (10:1) and the organic layer was taken to dryness. The residue was purified by successive paper chromatography as follows: (system a) isooctane, toluene, methanol, water (75 : 125 : 160 : 40); (system b) isooctane, toluene, methanol, water (3:1:3:1); (system c) benzene, methanol, ethyl acetate, water (1:1:0.1:1); (system d) benzene, methanol, water (1 : 1 : 1). Radioactive regions corresponding to androst-4-ene-3β,17β-diol (**2a**), androst-4-ene-3β,17β,19-triol (**2b**), androst-4-ene-2β,3β,17β,19-tetrol (**2c**), and estradiol were eluted, diluted with the appropriate carrier steroid, and recrystallized until constant specific activity and isotope ratio were achieved. The tetrol **2c** was acetylated to the tetraacetate before crystallization. Abbreviations: A, substrate only; B, substrate + 950 μg of **1d**; C, inactivated enzyme control.

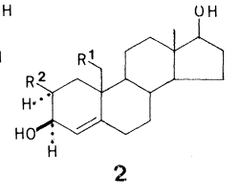
Product	Yield (percent ^{14}C)			$^3H/^{14}C$			Percent ^3H lost		
	A	B	C	A	B	C	A	B	C
Diol **2a**	31	38	65	5.0	5.0	5.0	13.8	13.8	13.8
Triol **2b**	1.7	6.1		5.0	5.0		13.8	13.8	
Tetrol **2c**		0.08			3.9			32.8	
Estradiol	12.0	5.9		1.5	1.5		74.1	74.1	

rier material had been added. The successive ^{14}C specific activities of recrystallized **2c** were 215, 203, and 205 count/min mg; and the total ^{14}C associated with the material was 8000 count/min. Since 5 count/min mg and 200 count/min, respectively, are the lowest limits of detection, this provides strong evidence for the generation of labeled **1d** during the ^{14}C-androstenedione aromatization. The low yield of the isolated material, 0.13 percent when corrected for the recovered substrate, is explained by the following: (i) the short half-life of **1d** even at *p*H 6; (ii) the decreased enzymatic formation of the intermediate **1d** at the less-than-optimum *p*H necessarily used; and (iii) exclusion from the calculation of the 3α epimers of **2c** also formed during the reduction of **1d**. It should be noted that **2c** could also be derived from the reduction of 2β, 19-dihydroxyandrost-4-ene-3,17-dione, also a possible aromatization intermediate. The enedione however has been shown not to be an estrogen precursor (*14*) and therefore could not be the source of the ^{14}C-labeled **2c**.



1

a $R^1 = CH_3$, $R^2 = H$
b $R^1 = CH_2OH$, $R^2 = H$
c $R^1 = CHO$, $R^2 = H$
d $R^1 = CHO$, $R^2 = OH$



2

a $R^1 = R^2 = H$
b $R^1 = OH$, $R^2 = H$
c $R^1 = R^2 = OH$

The ^3H to ^{14}C ratio in the isolated intermediate **1d** serves as additional and convincing proof that it is derived from the dual labeled androstenedione substrate. The isotope ratio ($^3H/^{14}C$) of the end product, estradiol, is 1.5, equivalent to a 74.1 percent loss of the tritium originally present in the substrate. Since the stereochemistry of hydrogen loss in the aromatization process is β, 74.1 percent of the tritium in the androstenedione substrate must be located at 1β and 2β, approximately equally divided between the two locations. Therefore, 37 percent of the tritium may be expected to be lost during 2β-hydroxylation, which, similarly to other steroidal hydroxylations, involves hydrogen displacement rather than inversion (*15*). The actual 33 percent tritium loss found in the isolated derivative

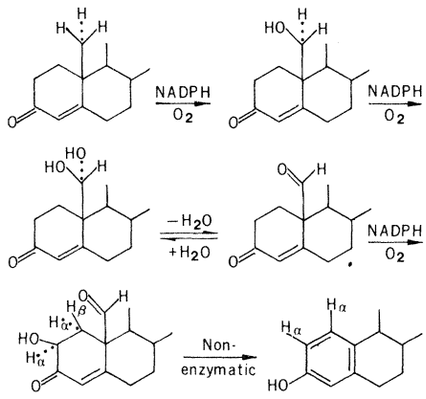


Fig. 1. Sequence for the biological conversion of androgens to estrogens.

of **1d** is close to the calculated value and further substantiates that **1d** is a 2β-hydroxylation product of the substrate. The 13.8 percent loss of tritium found in the other intermediates, represented by the triol **2b**, and in the recovered substrate, as represented by the diol **2a**, is clearly artifactual, since it is also found in the control incubation (C) in which the enzyme was inactivated. The loss results from enolization of the axial 2β tritium under the acidic conditions of incubation and purification (*16*). The substrate for the 2β-hydroxylase which results in the 2β-hydroxyaldehyde **1d** is the 19-aldehyde **1c**. It is significant, therefore, that in the incubation where carrier **1d** was added (B), the yield of the 19-aldehyde **1c**, isolated as the triol derivative **2b**, is almost four times greater than in the incubation where no carrier **1d** was present (A) (6.1 compared to 1.7 percent). This is suggestive of end-product inhibition of the 2β-hydroxylase by **1d** and serves to support further its enzymatic origin.

The evidence presented here permits the construction of a complete mechanism of the conversion of androgen to estrogen by the placental aromatase complex (Fig. 1). The initial enzymatic hydroxylation of C-19 generates the 19-hydroxy intermediate **1b**, which in turn undergoes a second hydroxylation at the 19-pro R position to produce the 19-aldehyde **1c** (*17*). This compound is the substrate for the final and rate determining hydroxylation at 2β to give **1d**, which then rapidly and spontaneously decays to estrone. The three successive hydroxylations may proceed at the same enzyme site without release of intermediates prior to **1d**, or the process may proceed via distinct and separate intermediates resulting from each individual hydroxylation. The weight of the evidence now favors the former path (*7*). It is conceivable that the conversion of **1d** to estrone involves enzymatic participation, but the rate of the chemical conversion is such that the enzyme would be redundant. The lack of end-product inhibition of aromatization by estrogens also indicates that the last step of the process is nonenzymatic.

Available evidence suggests that ovarian estrogen biosynthesis proceeds by the same pathway as that of placental aromatization (*18*), and it is likely that peripheral aromatizations (*19*) including those in the brain and adipose tissue share the same mechanism. The biosynthetic scheme that we describe need not be the only aromatization route. It is, however, the only one for which experimental support is provided, and there is at present no evidence that alternative aromatization sequences do indeed exist.

JUNICHI GOTO, JACK FISHMAN
Institute for Steroid Research, Montefiore Hospital and Medical Center, and *Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10467*

References and Notes

1. L. L. Engel, in *Handbook of Physiology*, R. O. Greep and E. B. Astwood, Eds. (Williams & Wilkins, Baltimore, 1973), sect. 7, vol. 2, part 2, p. 467.
2. F. Naftolin, K. J. Ryan, Z. Petro, *Endocrinology* **90**, 295 (1972).
3. H. M. Bolt and P. Grobel, *Horm. Metab. Res.* **4**, 312 (1972); A. Nimrod and K. J. Ryan, *J. Clin. Endocrinol.* **40**, 367 (1975).
4. H. H. Feder, F. Naftolin, K. J. Ryan, *Endocrinology* **94**, 136 (1974).
5. J. M. Grodin, P. K. Siiteri, P. C. MacDonald, *J. Clin. Endocrinol.* **36**, 207 (1973).
6. J. D. Townsley and H. J. Brodie, *Biochemistry* **1**, 33 (1968); J. Fishman and H. Guzik, *J. Am. Chem. Soc.* **91**, 2805 (1969); _____, D. Dixon, *Biochemistry* **8**, 4304 (1969); H. J. Brodie, K. J. Kripalani, G. Possanza, *J. Am. Chem. Soc.* **91**, 1241 (1969).
7. E. A. Thompson, Jr., and P. K. Siiteri, *J. Biol. Chem.* **249**, 5364 (1974).
8. A. S. Meyer, *Biochim. Biophys. Acta* **17**, 441 (1955); R. B. Wilcox and L. L. Engel, *Steroids* **6** (suppl. 2), 249 (1965).
9. M. Akhtar and S. J. M. Skinner, *Biochem. J.* **109**, 318 (1968).
10. H. Hosoda and J. Fishman, *J. Am. Chem. Soc.* **96**, 7325 (1974).
11. K. J. Ryan, *J. Biol. Chem.* **234**, 268 (1959).
12. K. C. Reed and S. Ohno, *ibid.* **251**, 1625 (1976).
13. S. Julia and C. Moutonnier, *Bull. Soc. Chim. Fr.* (1964), p. 321.
14. H. Hosoda and J. Fishman, *J. Steroid Biochem.* **5**, 315 (1974).
15. S. Bergstrom, S. Lindstedt, B. Samuelsson, E. J. Corey, G. A. Gregoriou, *J. Am. Chem. Soc.* **80**, 2337 (1958); M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek, D. H. Peterson, *ibid.*, p. 2336.
16. J. Fried and J. A. Edwards, *Organic Reactions in Steroid Chemistry* (Van Nostrand Reinhold, New York, 1972), vol. 1, p. 145.
17. Y. Osawa, K. Shibata, D. Rohrer, C. Weeks, W. L. Duax, *J. Am. Chem. Soc.* **97**, 4400 (1975); D. Arigoni, R. Battaglia, M. Akhtar, T. Smith, *J. Chem. Soc. Chem. Commun.* (1975), p. 185.
18. L. R. Axelrod and J. W. Goldzieher, *Biochim. Biophys. Acta* **187**, 450 (1969).
19. W. G. Kelly, *Endocrinology* **95**, 308 (1974).
20. We thank the G. D. Searle and Co. for providing the 19-hydroxy-androstenedione used in these studies. Supported by NCI grant CA 07304. J.G. is a visiting research associate from Tohoku University, Sendai, Japan.

12 July 1976