Biological Aromatization of Steroids*

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The conversion of androgenic steroids to the phenolic estrogens by mammalian tissue has been well documented. The reaction has been reported in relatively low yield, *in vitro* with ovarian and placental slices (1–3), and *in vivo* after the administration of androgens to various species including man (4, 5). The present report describes the conversion of androgens to estrogens in high yield by a system consisting of human placental microsomes, the reduced form of triphosphopyridine nucleotide, and oxygen. Localization of the enzymatic activity has permitted evaluation of enzyme kinetics, cofactor requirements, and substrate specificity. A preliminary note on the conversion of Δ^4-androstene-3,17-dione to estrone by this system has been published (6).

EXPERIMENTAL

Tissue Preparation—Human placentas, obtained immediately after delivery, were dissected free of fetal membranes and processed at 4°. The placental tissue was teased free of large blood vessels, put through a meat grinder, weighed, and homogenized in a Waring Blendor for 1 minute in buffer containing 0.25 M sucrose, 0.05 M phosphate, pH 7, and 0.04 M nicotinamide. 1 volume of buffer to 3 parts of tissue by weight was found to provide preparations of optimal activity. The homogenate was fractionated by the differential centrifugation techniques of Schneider and Hogeboom (7), and subcellular fractions were obtained as listed in Table I. Active preparations could be stored in the Deep Freeze for weeks without loss of activity. The protein content was determined by weighing the dry tissue after trichloroacetic acid precipitation and extraction with organic solvents (8).

Incubation Techniques—The placental preparations containing the steroids dissolved in propylene glycol and cofactors were incubated in 50-ml. Erlenmeyer flasks at 37° in a Dubnoff incubator with air as the usual gas phase. Except where noted, all steroids, cofactors, reagents, and inhibitors were commercial preparations. TPNH was prepared by the method described by Kaplan *et al.* (9).

Extraction and Purification Procedures—Extraction of the incubation mixtures was carried out three times with 6 volumes of chloroform. The pooled chloroform solutions were washed with 10 ml. of distilled water and evaporated. The residues were dissolved in 20 ml. of pentane and extracted three times with 20 ml. of 90 per cent methanol. The pooled methanol extracts were evaporated and the residues dissolved in 50 ml. of toluene. The toluene was extracted four times with 15 ml. of 1 N NaOH and twice with 10 ml. of water. The combined alkali and water extracts were adjusted to pH 8.5 and extracted three times with diethyl ether (10). The ether was evaporated and the residues subjected to an 8-transfer countercurrent distribution in toluene-NaOH (1 N) to purify further the phenolic steroids as described by Baggett *et al.* (1). The phenolic material was next put through a 24-transfer countercurrent distribution in 50 per cent methanol-CCl_4 with the use of techniques previously reported (10). In this manner the estrogens could be isolated and characterized.

Paper chromatography of the extracts was carried out at all stages of the purification procedure according to the techniques of Bush (11) with the solvents described in previous communications (6, 12). A mixture of equal parts of aqueous ferric chloride and potassium ferricyanide, each 1 per cent, was used as a spray to detect the estrogens on paper. Ultraviolet absorption and the Zimmermann reaction adapted for paper were employed to locate other compounds.

Quantitative assays for the estrogens were carried out in duplicate at all stages of purification by the sulfuric acid fluorescence method of Engel *et al.* (10) and the less elaborate technique described by Sweat (13). Kober chromogens were determined according to the modifications of Brown (14). Androgens were measured by the Callow modification of the Zimmermann reaction (15). The radioactive samples were measured at infinite thinness on stainless steel planchets with a windowless gas flow counter operated in the Geiger region. Infrared spectroscopy was carried out with a Perkin-Elmer model No. 21 double beam instrument with sodium chloride optics. Colorimetry was performed with a Bausch and Lomb spectrophotometer and fluorometry with the Farrand fluorometer.

Identification of Estrone and Estradiol[1]—Upon incubation of a complete system such as described in Tables I and II, the estrogen metabolites formed were identified by the following criteria:

1. There was *always* a net formation of fluorogenic and Kober chromogenic metabolites.

2. Solvent partition and isolation of a phenolic fraction demonstrated that the metabolites were phenolic steroids.

3. Comparison of standard samples of estrone and of estradiol with the incubation products by Bush chromatography (11) revealed identical R_F values in two solvent systems.

4. Separation and characterization of the enzymatically formed estrogens by countercurrent distribution revealed good agreement of experimental and theoretical curves, and the partition coefficients obtained in the 24-transfer system with 50 per cent methanol-CCl_4 were identical with known estrone and estradiol.

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[1] Estradiol as used herein refers to estradiol-17-β.

TABLE I

Subcellular distribution of enzymes concerned with ring A aromatization

Placental preparations equivalent to those obtainable from 12.5 gm. (wet weight) of tissue were employed, and the fractions were reconstituted with 0.05 M phosphate buffer, pH 7, to their original concentration in the 800 × *g* supernatant. The following were added: 200 μg. of Δ⁴-androstene-3,17-dione, 10 μmoles of ATP, and 2.5 μmoles of DPN. The mixtures were incubated in air for 1 hour at 37° in a total volume of 5 ml. Extractions and assays were carried out as described in the text. It should be noted that the "Microsomes plus soluble" recombination is equivalent to a 10,000 × *g* supernatant fraction.

Fraction	Estrone formed
	μg.
Homogenate; 800 × *g* supernatant	53
Mitochondria; 10,000 × *g* precipitate	0
Microsomes; 80,000 × *g* precipitate	0.5
Soluble; 80,000 × *g* supernatant	0
Mitochondria plus soluble; recombination	0
Microsomes plus soluble; recombination	52

5. Infrared spectroscopy of the acetates of the estrogen metab olites produced spectra characteristic of estrone and estradiol acetates.[2]

Control Experiments—Extraction of the placental tissue at zero time or after incubation for 1 hour without steroid failed to reveal the presence of estrogens (Table II) as determined by the fluorescence and Kober assays. Since the estrone formed was usually 80 times the amount isolated by Diczfalusy and Lindkvist (16) from comparable weights of placenta, no difficulty was encountered in measuring net formation of estrogens. It was possible to recover 85 ± 4 per cent of added estrone from the system at zero time (10 experiments), and added estrone was stable during the course of a 1-hour incubation. All data provided are typical results based on two or more replications for each experiment. Duplicate experiments agreed within 7 per cent. The placentas tested were from 13- to 40-weeks gestational age and were all active. The substrates were checked by the chromatographic and assay techniques listed and found to be free of contaminating steroids. When allowance is made for the losses associated with the purification procedures, all of the starting material could be accounted for as either estrogen conversion product or unchanged substrate.

RESULTS

Incubation of Δ⁴-androstene-3,17-dione with placental homogenates, ATP, and DPN, resulted in the formation of estrone (Table I). It should be noted that the particular substrate and type of enzyme preparation employed determined whether estrone, estradiol, or a combination of the two were formed (see below).

Localization of Aromatizing Activity—Upon subcellular fractionation of the placenta, the activity of the whole homogenate was found to reside in a preparation containing both the microsomal and supernatant fractions (Table I). These two fractions were inactive when incubated alone, but upon recombination, the system was fully active. By analogy with the adrenal hydroxylation systems previously described (12), it was determined that the soluble component provided the enzymes and substrates for TPN formation and reduction, and the aromatizing activity resided in the microsomal fraction. The supernatant fraction could thus be replaced by a TPNH-generating system or by the reduced coenzyme (Table III).

[2] Infrared spectroscopy was carried out by Mrs. D. Wheeler and Miss M. Collins through the courtesy of Dr. L. L. Engel.

TABLE II

Control experiments: steroid aromatization

A 10,000 × *g* placental supernatant fraction equivalent to 12 gm. (wet weight) of tissue was incubated with 10 μmoles of ATP, 2.5 μmoles of DPN, and 200 μg. of Δ⁴-androstene-3,17-dione in air at 37° for 1 hour in a total volume of 5 ml. Fluorescence and Kober assays were carried out on a purified phenolic extract prepared as described in the text.

Conditions	Estrone formed	
	Kober chromogen assay	Fluorescence assay
	μg.	μg.
Complete system at zero time	0	0
System incubated without Δ⁴-androstene-3,17-dione	0	0
Complete system incubated	40	40

TABLE III

Cofactor requirements for ring A aromatization

Microsomal fraction (2.3 mg. of protein per ml.) in 0.05 M phosphate buffer, pH 7, was incubated in air for one hour at 37° with 200 μg. of Δ⁴-androstene-3,17-dione and the additions noted below in a total volume of 5 ml.

Additions	Estrone formed
	μg.
None	0
TPN (5 μmoles)	0
Glucose 6-phosphate and glucose 6-phosphate dehydrogenase	0
TPN (1 μmole), glucose 6-phosphate, glucose 6-phosphate dehydrogenase	23
TPNH (7 μmoles)	35
DPNH (7 μmoles)	0

Enzyme Kinetics—The relationships of estrone formation to incubation time and to substrate, TPNH and microsomal protein concentrations are indicated in Figs. 1 to 4. Although the aromatization of androgens probably represents a multienzyme system, it is interesting to note that the time curve is linear for the 1st hour. No identifiable intermediates were uniformly noted in the incubations described (see below). Along the ascending portion of the substrate concentration curve (Fig. 2) the estrone yield was 50 to 60 per cent. Translated into a theoretical potential for an average-sized whole placenta of 500 gm., this would be equivalent to at least 1 mg. of estrone per hour.

A pH optimum of 7 was observed, and the reaction was inhibited in the presence of nitrogen. Disodium ethylenediaminetetraacetate, Cu^{++}, Hg^{++}, cyanide, and iodoacetate at 10^{-3} M concentration had slight or no inhibitory effects.

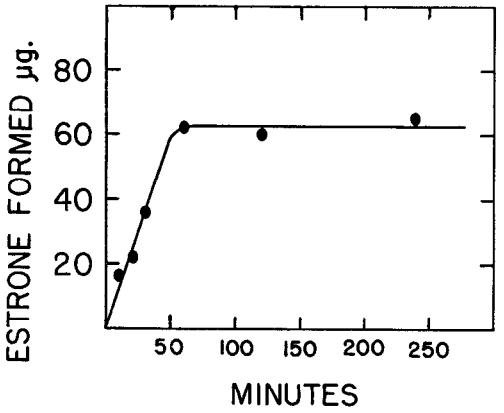

FIG. 1. The conversion of Δ^4-androstene-3,17-dione to estrone by human placenta as a function of time. Microsomal fractions (4.6 mg. of protein per ml.) were incubated with 1 mg. of Δ^4-androstene-3,17-dione, 2 µmoles of TPN, 40 µmoles of glucose-6-P, and 1.2 mg. of glucose-6-P dehydrogenase (Sigma) in a total volume of 5 ml. at 37° for the times indicated.

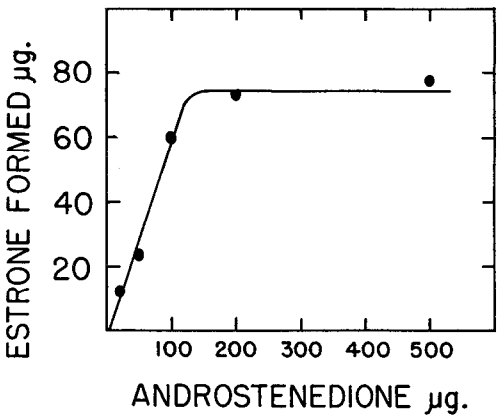

FIG. 2. Formation of estrone as a function of substrate concentration. Placental 10,000 × *g* supernatant fractions prepared from homogenates equivalent to 12 gm. (wet weight) of tissue were incubated with 10 µmoles of ATP, 5 µmoles of DPN, and Δ^4-androstene-3,17-dione in the amounts indicated, for 1 hour at 37° in a total volume of 5 ml.

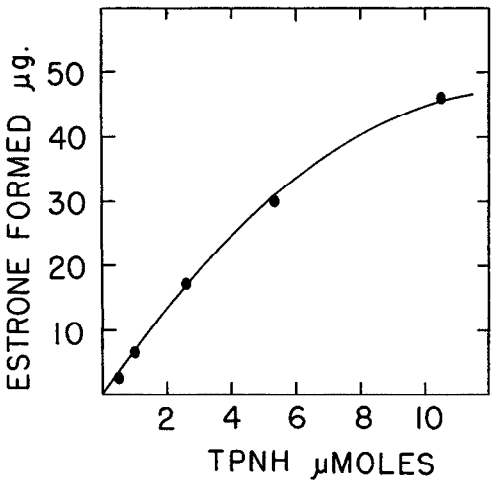

FIG. 3. The formation of estrone by placental microsomes as a function of TPNH concentration. Placental microsome fractions (4.6 mg. of protein per ml.) were incubated with 1 mg. of Δ^4-androstene-3,17-dione and TPNH in the amounts indicated for 1 hour at 37° in a total volume of 5 ml.

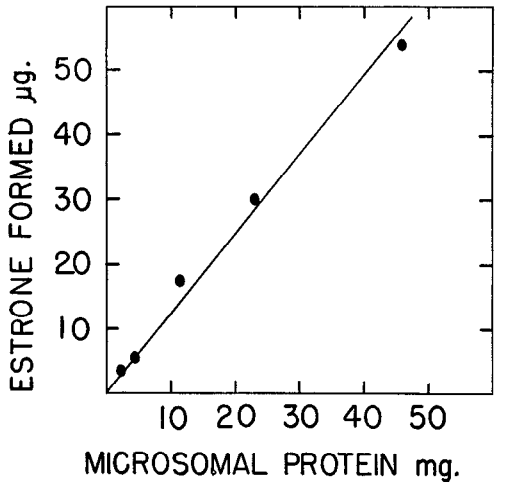

FIG. 4. The formation of estrone by human placental microsomes as a function of microsomal protein concentration. Human placental microsomes in the amounts indicated were incubated with 1 mg. of Δ^4-androstene-3,17-dione, 2 µmoles of TPN, 40 µmoles of glucose-6-P, and 1.2 mg. of glucose-6-P dehydrogenase (Sigma) in a total volume of 5 ml. at 37° for 1 hour.

TABLE IV

Substrate specificity for ring A aromatization

A 10,000 × *g* placental supernatant fraction equivalent to 12 gm. (wet weight) of tissue was incubated in air with 10 µmoles of ATP, 5 µmoles of DPN, and the substrates indicated at 37° for 1 hour. Those substrates which were active were also tested with a system consisting of microsomal fractions (2.3 mg. of protein per ml.) and 7 µmoles of TPNH under similar conditions. The results with the two systems were identical.

Substrate added	Relative activity
	%
Δ^4-Androstene-3,17-dione	100
Testosterone	100
Dehydroepiandrosterone	100
19-Hydroxy-Δ^4-androstene-3,17-dione	100
$\Delta^{1,4}$-Androstadiene-3,17-dione	25
19-Nortestosterone	20
Progesterone	0
17-Hydroxyprogesterone	0
Adrenal steroids*	0

* "Adrenal steroids" refer to the following compounds: adrenosterone, cortisol, corticosterone, deoxycorticosterone, 17,21-dihydroxyprogesterone, pregnenolone, and 17-hydroxypregnenolone.

Substrate Specificity[3]—Of the various steroid substrates which were tested in the aromatization reaction, only certain C_{19} compounds were active (Table IV). From his studies with placental slice incubations, Meyer (3) reported that 19-hydroxy-Δ^4-androstene-3,17-dione was a more active precursor of estrone than was Δ^4-androstene-3,17-dione. The observation here that the 19-hydroxy compound was not more active in this regard sheds no light on its potential role as an intermediate since 19-hydroxylation might not be a limiting factor in these preparations. However, in occasional incubations, with Δ^4-androstene-3,17-dione used as a substrate, an ultraviolet absorbing,

[3] The aromatization of 16α-hydroxylated C_{19} compounds with the formation of estriol is currently under study.

Zimmermann-reacting compound with chromatographic mobility of the 19-hydroxy derivative was noted. Insufficient material was available for identification. The data on 19-nortestosterone and $\Delta^{1,4}$-androstadiene-3,17-dione suggest that these compounds are less likely intermediates in the conversion. The results of the incubations with dehydroepiandrosterone as substrate are not surprising in view of the known presence of the Δ^5-3β-ol dehydrogenase which can convert dehydroepiandrosterone to Δ^4-androstene-3,17-dione (17). Evidence for this conversion was obtained by the accumulation of the latter compound in addition to estrone when dehydroepiandrosterone was the substrate. The Δ^4-androstene-3,17-dione was characterized by its R_F values in the paper chromatographic systems described, but no attempt to isolate the compound in quantities sufficient for rigorous identification was made.

When testosterone was incubated with placental homogenates or the initial microsome fractions, the product was estrone or a mixture of estrone and estradiol. The principal conversion was difficult to establish because of the observed presence of the 17β-ol dehydrogenases which interconvert Δ^4-androstene-3,17-dione and testosterone and also estradiol and estrone. These dehydrogenases have been reported in the supernatant fraction of human placental homogenates (18), but in the present case they were also microsomal contaminants. When the microsomal preparations were washed with buffer and resedimented at 80,000 × *g* at least five times, they could be freed of all dehydrogenase activities, and then Δ^4-androstene-3,17-dione was converted to estrone, and testosterone was converted to estradiol with comparable yields (Table V).

In an attempt to pick up trace intermediates and further check the conversion described, 4-C^{14}-testosterone was incubated under similar conditions. Radioactive estrone was recovered from the experiments with homogenates, and radioactive estradiol from the experiments with washed microsomal preparations. There was sufficient conversion to permit direct measurement of the estrone and estradiol by the assay techniques described *without* the addition of carrier and without the utilization of an isotope dilution procedure (Table VI). No rigorous tests for radiochemical purity were undertaken because this portion of the work was designed as a screening procedure for intermediates. Several trace radioactive compounds were detected by radioautography of paper chromatograms, but none of these substances have as yet been identified. No estrogen or other conversion products were observed in the zero time controls containing 4-C^{14}-testosterone.

DISCUSSION

The aromatization of C_{19} steroids to form phenolic estrogens is here described in a system *in vitro* consisting of human placental microsomes, TPNH, and oxygen.

Mechanisms for the enzymatic conversion of androgens to estrogens have been proposed, based in part on experience with chemical methods for removing the angular methyl group (19) and in part on the biological observations with placental slices and microbial systems (20). Among the possibilities suggested have been 19-hydroxylation, removal of the angular methyl grouping as formaldehyde, formation of the Δ^1-double bond, and rearrangement of the $\Delta^{1,4}$-dieneone system to form the phenolic steroid. Hydroxylation at C-1 or C-2 may play a part (21). Thus far the finer details have not been established, but the counterparts of several of these proposed steps have been clearly

TABLE V

Ring A aromatization with washed microsomal preparations

Microsomal fraction (4.6 mg. of protein per ml.) in 0.05 M phosphate buffer, pH 7, was incubated in air for 1 hour at 37° with 1 mg. of the substrates noted below and 7.2 μmoles of TPNH in a total volume of 5 ml. Estrone and estradiol were assayed as noted in the text. The microsomal fractions were washed and resedimented 5 times at 80,000 × *g*, and were thus freed of 17β-ol dehydrogenases before incubation.

Substrate	Estrogen formed	
	Estradiol	Estrone
	μg.	μg.
Δ^4-Androstene-3,17-dione	0	70
Testosterone	54	0

TABLE VI

Conversion of 4-C^{14}-testosterone to estrone

A placental 10,000 × *g* supernatant fraction equivalent to 12 gm. (wet weight) of tissue was incubated in air at 37° for 1 hour with 121 μg. of 4-C^{14}-testosterone (9.0 × 10^5 c.p.m.), 10 μmoles of ATP, and 5 μmoles of DPN, in a total volume of 5 ml. The incubation mixture was extracted as described in the text, and fluorescence and radioactivity measurements were carried out on the same samples. Radioactive estrone was also characterized by Bush chromatography in two systems as described in the text.

Incubation time	Radioactivity				Fluorescence	
	Total extracted	Percentage of total recovery	Purified phenolic fraction	Conversion to estrone	Estrone	Conversion to estrone
	c.p.m. × 10^{-6}	%	c.p.m. × 10^{-6}	%	μg.	%
Zero	8.7	97	0.03		0	
1 hour	6.4	71	1.92	21	31	26

demonstrated in steroid metabolism with adrenal microsomes (12) and with microorganisms (20–22).

The aromatization reaction described here invites comparison with the conversion of hexahydrobenzoic acid to benzoic acid reported by Beer *et al.* (23) and Mitoma *et al.* (24). This conversion has been shown to occur in liver mitochondria in the presence of ATP, cytochrome *c*, glycine, ethylenediaminetetraacetic acid, $MgSO_4$, and α-ketoglutaric acid. The conditions described are sufficiently dissimilar to the androgen-estrogen conversion to suggest that the mechanisms for the formation of benzoic acid and for steroid aromatization are unrelated.

Steroid aromatization as described here appears to fall into the general category of oxidation reactions which require TPNH and molecular oxygen. Mason (25) has extensively reviewed this type of biological oxidation, but despite the many examples described, no new insight into the mechanisms involved is available.

SUMMARY

A steroid aromatization reaction which reproducibly converts C_{19} steroids to estrogens in high yield has been described. The enzymatic activity resides in the microsomal fraction of the

human placenta and requires the reduced form of triphosphopyridine nucleotide and oxygen. The relationship of this reaction to other aromatization systems has been considered.

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REFERENCES

1. Baggett, B., Engel, L. L., Savard, K., and Dorfman, R. I., *J. Biol. Chem.*, **221**, 931 (1956).
2. Wotiz, H. H., Davis, J. W., Lemon, H. M., and Gut, M., *J. Biol. Chem.*, **222**, 487 (1956).
3. Meyer, A. S., *Biochim. et Biophys. Acta*, **17**, 441 (1955).
4. Heard, R. D. H., Jacobs, R., O'Donnell, V., Peron, F. G., Saffran, J. C., Solomon, S., Thompson, L. M., Willoughby, H., and Yates, C. H., *Recent Progr. in Hormone Research*, **9**, 383 (1954).
5. West, C. D., Damast, B. L., Sarro, S. D., and Pearson, O. H., *J. Biol. Chem.*, **218**, 409 (1956).
6. Ryan, K. J., *Biochim. et Biophys. Acta*, **27**, 658 (1958).
7. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950).
8. Siekevitz, P., *J. Biol. Chem.*, **195**, 549 (1952).
9. Kaplan, N. O., Colowick, S. P., and Neufeld, E. F., *J. Biol. Chem.*, **195**, 107 (1952).
10. Engel, L. L., Slaunwhite, W. R., Jr., Carter, P., and Nathanson, I. T., *J. Biol. Chem.*, **185**, 255 (1950).
11. Bush, I. E., *Biochem. J.*, **50**, 370 (1952).
12. Ryan, K. J., and Engel, L. L., *J. Biol. Chem.*, **225**, 103 (1957).
13. Sweat, M. L., *Anal. Chem.*, **26**, 773 (1954).
14. Brown, J. B., *Biochem. J.*, **60**, 185 (1950).
15. Callow, N. H., Callow, R. K., and Emmens, C. W., *Biochem. J.*, **32**, 1312 (1938).
16. Diczfalusy, E., and Lindkvist, P., *Acta Endocrinol.*, **22**, 203 (1956).
17. Pearlman, W. H., Cerceo, E., and Thomas, M., *J. Biol. Chem.*, **208**, 231 (1954).
18. Langer, L., and Engel, L. L., *Federation Proc.* **15**, 296 (1956).
19. Sneeden, R. P. A., and Turner, R. B., *J. Am. Chem. Soc.*, **77**, 130 (1955).
20. Talalay, P., *Physiol. Revs.*, **37**, 362 (1957).
21. Kushinsky, S., *J. Biol. Chem.*, **230**, 31 (1958).
22. Levey, H. R., and Talalay, P., *J. Am. Chem. Soc.*, **79**, 2658 (1957).
23. Beer, C. T., Dickens, F., and Pearson, J., *Biochem. J.*, **48**, 222 (1951).
24. Mitoma, C., Posner, H. S., and Leonard, F., *Biochim. et Biophys. Acta*, **27**, 156 (1958).
25. Mason, H. S., *Science*, **125**, 1185 (1957).

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