THE INTERMEDIATE STEPS IN THE BIOSYNTHESIS OF ESTROGENS FROM ANDROGENS[1]

Tomás Morato, Mika Hayano, Ralph I. Dorfman and Leonard R. Axelrod

Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts and the Southwest Foundation for Research and Education, San Antonio, Texas

Received November 9, 1961

In the biosynthetic steps in the formation of estrogens from androgens, the intermediate role of 19-hydroxy-androst-4-ene-3,17-dione is well established (cf. Longchampt et al., 1960). The structures, if any, implicated in the biological sequence beyond this point have not been clarified. A preliminary experiment (Hayano et al., 1960) with 19-oxo-androst-4-ene-3,17-dione indicated that this substance was more rapidly transformed to estrone than its 19-hydroxy analogue. Further work has now been completed with placental microsomes and androst-4-ene-3,17-dione, androst-4-en-19-ol-3,17-dione, 19-oxo-androst-4-ene-3,17-dione and 10β-carboxy-estr-4-ene-3,17-dione, which presents evidence that the steroid acid is not an obligatory intermediate in the major synthetic pathway and that the aldo steroid may be.

Human placental microsomes were prepared according to the method of Ryan (1959) and washed three or four times with phosphate buffer pH 7.0, to insure removal of all endogenous TPNH regenerating mechanisms. An amount equivalent to about 7.5 gms. of wet placenta suspended in 1.3 ml. of 0.1 M phosphate buffer, pH 7.0, was used per flask. Fifty μg. of steroid dissolved in about 0.05 ml. of propylene glycol, and cofactors as listed in Table I were included in a final volume of 1.5 ml. Aerobic incubations were carried out in a Dubnoff Metabolic Shaking Incubator for a period of one hour at 38°C. Anaerobic incubations were carried out in Warburg flasks with

[1]Supported in part by U. S. Public Health Service Grants FF-258 and A-2672.

TABLE I

Steroid	Gaseous phase	Cofactor	% Conversion to estrogen
10β-carboxy-estrenedione	N_2	DPN	0
"	N_2	TPN	0
"	N_2	-	0
"	Air	TPNH	<5
19-nor-androstenedione	Air	TPNH	5
19-oxo-androstenedione	N_2	DPN	0
"	N_2	TPN	0
"	N_2	-	0
"	Air	TPNH	100
"	"	DPNH	0
"	"	TPN	0
"	N_2	TPNH	<5
19-hydroxyandrostenedione	Air	TPNH	50-60
"	"	TPN	0
"	"	DPNH	0
"	N_2	TPNH	0
Androstenedione	Air	TPNH	30-40
"	N_2	TPNH	0

DPN, DPNH, or TPN (Sigma) 2.5 μMoles; TPNH regenerating system: TPN 2.5 μMoles, gluc-6-P (Sigma) 4.3 μMoles, gluc-6-P dehydrogenase (Sigma) 0.5 K.U.

alkaline pyrogallol in the center well. In addition, the nitrogen used to flush the system was first passed through a pyrogallol solution. A five minute equilibration period at 38°C was allowed before tipping in the enzyme solution from the side arm. The incubations were continued for an hour and then stopped with the addition of chloroform. The aqueous phase was extracted four times with 10 ml. portions of this solvent and the combined extracts dried and evaporated to a minimal volume for application to paper chromatograms. The total of the material from each flask was transferred to a 1 cm. strip for resolution in the ligroin-propylene glycol system. Standards of known concentrations were run in parallel for visual estimations of recoveries and yields. Steroid recoveries were in the range of 80-90%.

Where estrogens appeared after incubations, both estrone and estradiol-17β, the latter being enzymatically formed because of the presence of TPNH, were observed in a ratio of approximately 1:1. The methods used to locate and identify steroids included the following: Scanning with ultraviolet light for conjugated ketones; the isonicotinic acid hydrazide reaction for conjugated ketones (Smith and Foell, 1959), the Zimmermann test for 17-ketosteroids, and the $FeCl_3$-$K_3Fe(CN)_6$ reagent for phenols.

Our experiments were primarily designed to test for the formation of estrogens from the 10β-carboxy and 19-oxo structures. Under both aerobic and anaerobic conditions the carboxy steroid was transformed almost completely to a material which was concluded to be 19-nor-androstenedione. The trace of estrogen noted under aerobic conditions from the steroid acid undoubtedly arose from the 19-nor-steroid since the latter itself is a substrate, albeit poor, for estrogen formation. No conversion of 19-oxo-androstenedione was observed either aerobically or anaerobically in the presence of DPN, DPNH, or TPN, the steroid being recovered unchanged. With TPNH and in the presence of air a complete transformation to estrogens of this steroid was obtained. Only a trace of estrogen was observed in the anaerobic incubation with this cofactor. Under identical aerobic conditions the conversion of 19-hydroxyandrostenedione and Δ^4-androstenedione was 50-60% and 30-40%, respectively, and that of 19-oxo-androstenedione 100%. At no time was any 19-hydroxyandrostenedione noted following incubations with the 19-oxo structure, while on occasion a trace of material with characteristics of the aldehyde was observed in incubated extracts of the 19-hydroxy steroid. All data are presented in Table I and Figure 1.

It would appear from these findings that the principal sequence of biological estrogen formation should be Δ^4-androstenedione ⟶ 19-hydroxyandrostenedione ⟶ 19-oxo-androstenedione ⟶ ⋯⟶ estrone. In a previous communication (Hayano et al., 1961) from this laboratory, a possible mechanism for the aromatization of 19-hydroxyandrostenedione was

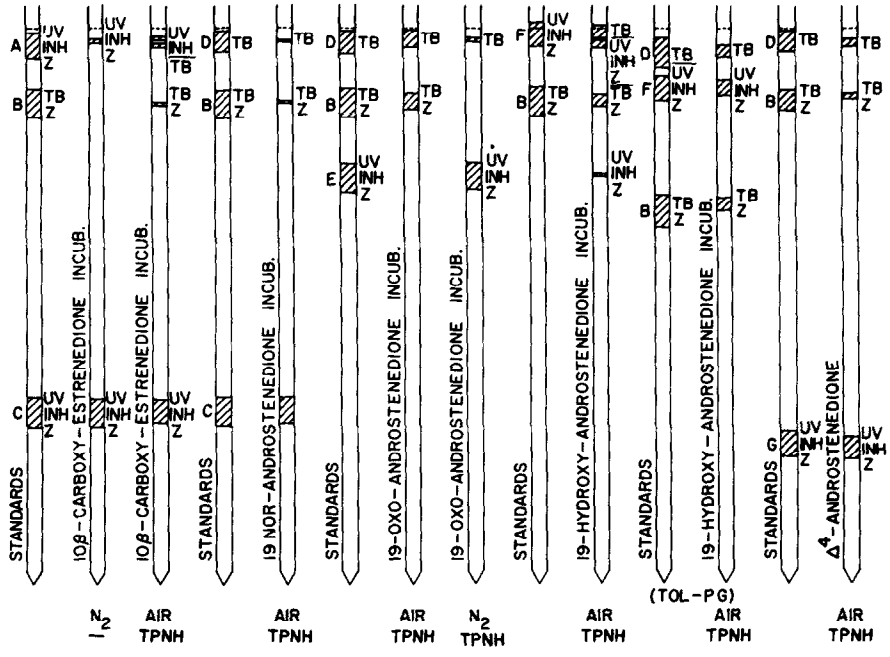

Figure 1. Paper chromatograms of steroid incubations with placental microsomes. Strips: 1 x 44 cm. (starting line to tip). Ligroin-propylene glycol 20 hrs., except where noted for toluene-propylene glycol 4.5 hrs. Atmosphere during incubation and presence of TPNH generating system are noted below each strip. Steroid standards: A = 10β-carboxy-estrenedione; B = estrone; C = 19-nor-androstenedione; D = estradiol-17β; E = 19-oxo-androstenedione; F = 19-hydroxyandrostenedione; G = androstenedione. Positive color reactions and ultraviolet (UV) absorption are noted on the right of each spot: Z = Zimmermann, INH = isonicotinic hydrazide test and TB = Turnbull's blue test.

discussed. This involved an enzymatically catalyzed enolization in ring A followed by a coenzyme abstraction of the C-1α hydride with a concomitant liberation of formaldehyde. This latter substance has recently been identified as appearing in stoichiometric ratio to estrone after placental incubations of Δ^4-androstenedione and 19-hydroxyandrostenedione (Breuer and Grill, 1961). Aromatization of 19-oxo-androstenedione via a similar type of mechanism as outlined above would yield formic acid plus the phenol. The findings in the present study would argue however against the direct entry to the estrogens from the 19-hydroxylated precursor. Further, the strict requirement of TPNH and oxygen for the conversion of both the 19-oxygenated

compounds to estrogens, suggests that the reaction sequence may be more complex than envisioned in the past.

The authors are indebted to the scientists of the Institute for Fermentation, Osaka, Japan, for their gifts of steroids.

References

Breuer, H. and Grill, P., Z. physiol. Chem. 324, 254 (1961).
Hayano, M., Longchampt, J., Kelly, W., Gual, C. and Dorfman, R. I., Abst. First International Congress of Endocrinology, Copenhagen, 1960, pg. 699.
Hayano, M., Ringold, H. J., Stefanovic, V., Gut, M., and Dorfman, R. I., Biochem. Biophys. Research Comm., 4, 454 (1961).
Longchampt, J. E., Gual, C., Ehrenstein, M. and Dorfman, R. I., Endocrinology, 66, 416 (1960).
Ryan, K. J., J. Biol. Chem., 234, 268 (1959).
Smith, L. L. and Foell, T., Anal. Chem., 31, 102 (1959).