POSSIBLE CONVERSION OF ANDROST-4-ENE-3 β , 17 β , 19-TRIOL TO ESTRIOL *IN VITRO* IN RAT TESTIS

MINORU INABA AND TAKESHI NAKAO with technical assistance
of Miss KUNIE KAMATA

*Department of Pharmacology, Jikei University School
of Medicine, Tokyo*

SYNOPSIS

Androst-4-ene-3 β , 17 β , 19-triol (androstetriol) was incubated with the whole testis tissue of a normal rat (Donryu strain). The testis from the right side was incubated with 40 or 50 μ g of androstetriol and the testis of the left side was incubated without substrate to serve as a control. The acidic fraction was extracted from the incubation mixture of tissue and medium. The estrogens in the acidic fraction were separated by the alumina column chromatography method in one experiment, and by the combination of paper chromatography (Bush B₅) and thin-layer chromatography methods in the other two experiments. The fluorescence spectra of estrogen fractions in sulfuric acid and their amounts were determined by spectrophotofluorometry. In all incubations a much larger amount of estriol fraction was determined in the androstetriol incubation than in the control incubation. Incorporation of radioactivity into the acidic fraction was measured in the incubation of testis tissue of one side with dehydroepiandrosterone-¹⁴C and in the other side with testosterone-¹⁴C. The rate of incorporation of ¹⁴C into the acidic fraction from dehydroepiandrosterone incubation was considerably greater than that of the testosterone incubation.

Since it was demonstrated that 4-androstenedione can be hydroxylated enzymatically at the C-19 position by bovine adrenal cortex (Meyer, 1955), 19-hydroxy- or 19-oxo-4-androstenedione and testosterone have been shown as intermediates in the biosynthesis of estrogen by several steroid-producing endocrine tissues.

Recently, several investigators have postulated by means of *in vivo* or *in vitro* experiments with placental tissue, that dehydroepiandrosterone or its sulfate ester would be a more efficient precursor than 4-andro-

stenedione or testosterone, and further that an alternative biosynthetic pathway to estrogen from dehydroepiandrosterone not involving 4-androstenedione or testosterone as an obligatory intermediate would exist in the placenta (Gual *et al.*, 1962; Baulieu and Dray, 1963; Siiteri and McDonald, 1963; Warren and Timberlake, 1964; Bolte *et al.*, 1964; Morato *et al.*, 1965). However, as yet the mechanism by which estrogen is formed from dehydroepiandrosterone has not been fully clarified.

This experiment concerns the *in vitro* for-

Received for publication September 8, 1966.

Trivial names and abbreviations used: 4-androstenedione: androst-4-ene-3,17-dione, dehydroepiandrosterone: 3 β -hydroxyandrost-5-en-17-one, testosterone: 17 β -hydroxyandrost-4-en-3-one, androstenediol: androst-4-ene-3 β , 17 β -

diol, androstetriol: androst-4-ene-3 β , 17 β , 19-triol, estrone or E₁: estra-1, 3, 5(10)-triene-3-ol-17-one, estradiol-17 β or E₂: estra-1, 3, 5(10)-triene-3, 17 β -diol, estriol or E₃: estra-1, 3, 5(10)-triene-3, 16 α , 17 β -triol.

mation of estriol from androst-4-ene-3 β , 17 β , 19-triol (androstenetriol) in rat testis tissue.

MATERIALS AND METHODS

Testis incubation

Whole testis tissue of a normal rat (Donryu) was incubated in 3 ml of Krebs-Ringer bicarbonate buffer solution containing 200 mg glucose per 100 ml (pH 7.4) for 3 hrs. at 35–36°C in a gas phase of 95% O₂ and 5% CO₂. Testis of one side was incubated with 40 or 50 μ g androstenetriol and testis of the other side was incubated without substrate to serve as a control.

Extraction of acidic fraction

After a 3 hrs. incubation period tissue and medium were transferred to a glass homogenizer, homogenized and centrifuged at 3,000 r.p.m. for 15 mins. The aqueous phase was decanted, placed in cold room and subsequently added to the ethanol extract of the tissue residue. The tissue residue was mixed thoroughly with 2ml of 95% ethanol and centrifuged at 3,000 r.p.m. for 15 mins. This extraction of residue was repeated four times and the pooled ethanol extract was combined with the aqueous phase obtained by the first centrifugation. Ethanol was evaporated from the combined aqueous-ethanol extract to an aqueous phase at a temperature below 50°C. Dichloromethane extract of the aqueous fraction was then partitioned between 70% methanol and light petroleum for removal of lipids from the steroid fraction. Methanol was evaporated from the 70% methanol fraction and the steroid fraction in the aqueous phase was extracted with dichloromethane four times. The steroid fraction was finally separated into acidic and neutral fraction by partition between cold toluene and cold 1 *N* NaOH. Acidic fraction was extracted with an equal volume of ethyl ether several times after adjusting the alkaline phase to pH 1 with hydrochloric acid. Average recovery of estrogen at this step was 70%.

Separation of estrogen

Estrone, estradiol-17 β and estriol in the acidic fraction were separated into the individual component by alumina column chromatography in

one experiment. Alumina (Merck, for chromatography) was washed thoroughly with ethanol, dried and activated at 110°C before use. The combination of paper chromatography with thin-layer chromatography was employed for separation of estrogen in the other experiments. For paper chromatography Whatman No. 1 paper washed with benzene-methanol mixture for 4 days was used and the solvent system employed was benzene-methanol-water (2 : 1 : 1 by volume) (Bush, 1954). For thin-layer chromatography silica gel (WAKOGEL B-O for thin-layer chromatography, Wako Chemical Industries Ltd., Tokyo) was used as an absorbent in a thickness of 0.25 mm. Systems employed were chloroform-methanol (1 : 1 by volume) and chloroform-methanol (97 : 3 by volume).

Detection of estrogen

Location of estrogen on the thin-layer plate was detected by exposure to the iodine vapor for several mins. (Aizawa, 1965). Estrogen produced a yellow color which usually disappeared within approximately 30 mins. after the plate was removed from the iodine vapor to the air. Estrogen on the plate was extracted with methanol.

Determination of estrogen

Determination and analysis of the spectrum of fluorescence produced by estrogen in sulfuric acid reagent was performed with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc.).

RESULTS

Incubation A

Testis of the right side (1.05 g) of a normal rat (body weight 250 g) was incubated with 40 μ g of androstenetriol and the testis of the left side (1.1 g) was incubated without substrate for 3 hrs.

It was observed that a much larger amount of compound which produced fluorescence by the addition of the diluted sulfuric acid followed by heating for 10 mins. in the boiling water, was contained in the acidic fraction (one third aliquot) of testis incubated with androstenetriol than in the acidic fraction of the control incubation (Fig. 1)

The remaining aliquots (two thirds) of the acidic fractions of the control and androstenetriol incubation were run in the alumina column chromatography. A glass column of 5 mm internal diameter was filled with 2 g of activated alumina to a height of 9.5 cm. After washing the column with benzene the sample was deposited on top with 1 ml benzene several times and the successive elutions were made with 5 ml of 2%, 3%, 4%, 5%, and 8% methanol in benzene at a flow rate of approximately 15 drops per min. at room temperature. Eluate was added with 1.0 ml of 95% sulfuric acid, heated at 80°C for 10 mins., then diluted with 3.0 ml of 65% sulfuric acid after cooling. Determination was made with the spectrophotofluorometer. Estradiol-17 β was excited by 430 m μ of the activating light and the percent transmission was read at 470 m μ . Estrone and estriol were read at 495 m μ .

Fig. 1. Fluorescence spectra of acidic fractions of control and androstenetriol incubation (Incubation A).

One third aliquot of acidic fraction was directly added to 2.0 ml of 65% H_2SO_4 , heated for 10 mins. in boiling water, and cooled in running water. Fluorescence spectra were read under 463 m μ of activating light.

1. acidic fraction of androstenetriol incubation,
2. acidic fraction of control incubation,
3. standard estriol.

Fig. 2. Separation of estrogens in acidic fractions of control and androstenetriol incubation by alumina column chromatography (Incubation A). Estrogen fraction eluted from alumina column was added to 1.0 ml of 95% H_2SO_4 , heated at 80°C for 10 mins., cooled in running water, and diluted with 3.0 ml of 65% H_2SO_4 for spectrophotofluorometry. Two thirds of the acidic fraction was subjected to column chromatography.

under the excitation by 453 m μ of the activating light, and androstenetriol was determined at 510 m μ under 463 m μ of the activating light.

Although a good separation of estriol from estradiol-17 β or androstenetriol from estriol was not achieved, it was clearly observed that the acidic fraction of the androstenetriol incubation contained more estriol than the acidic fraction of the control incubation (Fig. 2).

Incubation B

Testes from two normal rats (body weight 285 and 288 g) were used for the incubation. Two testes from the right side (2.1 g) were incubated with 50 μ g androstenetriol in one flask and two testes from the left side (2.1 g) were incubated in another flask without substrate as a control. After 3 hrs. of incubation period, the acidic fraction was extracted and run in benzene-methanol-water system of paper chromatography. Rf values of the standard estrogens and androstenetriol

in this system were as follows; estrone 0.88, estradiol-17 β 0.76, estriol 0.06, and androstenediol 0.23, showing a good separation of estriol from androstenediol which was extractable into the acidic fraction. Zone corresponding to the reference estrogen was eluted with methanol and run again in a chloroform-methanol (1 : 1 by volume) system for estrone zone and a chloroform-methanol (97 : 3 by volume) system for estrone and estradiol-17 β zones in the thin-layer chromatography.

Estriol zone of androstenediol incubation was migrated on the plate with Rf 0.62 (location was detected definitely as a yellow spot on the plate by the iodine vapor) which corresponded to the reference estriol. Figure 3 shows the fluorescence spectra of estriol

Fig. 3. Separation of estriol fraction from acidic fractions of control and androstenediol incubation by combination of paper and thin layer chromatography, and their fluorescence spectra (Incubation B).

Standard estriol: 1 μ g, meter multiplier (M.M.) 0.3,

1 - Estriol fraction of androstenediol incubation (1/2 aliquot), M.M. 0.1,

2 - Estriol fraction from control incubation (1/2 aliquot), M.M. 0.03,

Recovery of estriol from thin layer chromatography : 5%.

fractions of the control and androstenediol incubations which corresponded well to the spectrum of the standard estriol. The amounts of estriol determined at this stage were 0.95 μ g/ 2 testes for the control incubation and 7.82 μ g/ 2 testes for the androstenediol incubation with recovery. The average recovery of estrogen at this step was 5 to 6%. It was observed that estrogen was very poorly recovered from the thin-layer chromatography.

Incubation C

Testes from 2 rats (body weight 255 and 254 g) were used for the incubation. Two testes from the right side of each rat (2.0 g) were incubated with 50 μ g of androstenediol and two testes from the left side (2.0 g) were incubated without substrate as a control. The same result was obtained as in Incubation B. The amounts of estriol determined were 0.11 μ g/2 testes for the control and 10.54 μ g/2 testes for androstenediol incubation with recovery.

Incubation D

Three incubations were carried out with 3 normal rats (body weight 198, 208 and 280 g). In each incubation testis of the right side was incubated with testosterone-4-¹⁴C (specific activity 0.05 mC/0.319 mg, New England Nuclear Corp., Boston, Mass.) and the testis of the left side was incubated with dehydroepiandrosterone-4-¹⁴C (specific activity 0.01 mC/0.0709 mg, New England Nuclear Corp., Boston, Mass.). The incorporation of ¹⁴C into the acidic fraction was determined in all incubations. The radioactive substrates were purified by paper chromatography before use. Results are shown in Table 1. In the final column the ratios of the radioactivity in the neutral fraction to that in the acidic fraction are illustrated. The higher incorporation of radioactivity was always observed in the acidic fraction of dehydroepiandrosterone incubation than in that of the testosterone incubation (Table 1).

Table 1. Incorporation of radioactivity into neutral and acidic fraction in the incubation of rat testis tissue with testosterone-4-¹⁴C (T.) or dehydroepiandrosterone-4-¹⁴C (D.)

Incubation No.	Testis weight (g)	Substrate (dpm)	Radioactivity Neutral F.	(dpm) Acidic F.	N. : A.
D1	1.1	T. 1.52×10^4	8,290	3,200	100 : 38
	1.1	D. 6.93×10^4	28,330	24,210	100 : 85
D2	1.07	T. 3.05×10^4	16,100	3,440	100 : 21
	1.08	D. 6.90×10^4	32,770	13,690	100 : 42
D3	0.95	T. 24.60×10^4	198,300	28,440	100 : 14
	0.95	D. 17.80×10^4	130,450	35,330	100 : 27

Specific activities of T. and D. used: T. 0.05mC/0.319mg,
D. 0.01mC/0.0709mg.

Testes for incubation were taken from normal rats. (body weight 198, 208, and 280 g)

Incubation period: 2 hrs. Temperature: 35°–36°C Gas phase: 95% O₂ and 5% CO₂

Fig. 4. Separation of a compound behaving like the reference androstenetriol on paper from acidic fraction of rat testis incubation with dehydroepiandrosterone-4-¹⁴C.

Fourth chromatogram (Bush B5 system) of a fraction corresponding to the reference androstenetriol in the third Bush B₃ system after 18 hrs. development. Paperchromatogram scanner employed was Model JPC-101 with 4π gas flow counters of Nihon Musen Co., Tokyo. Scale 1 K, time constant 3, speed 12.5 mm/min.

Abbreviations: 19OH-4ADL: androst-4-ene-3β, 17β, 19-triol, 19OHT.: 17β, 19-dihydroxy-androst 4-en-3-one, 19OH-4¹⁴ ADN: 19-hydroxyandrost-4-en-3, 17-dione, OR.: origin, S.F.: solvent front.

DISCUSSION

Dehydroepiandrosterone sulfate or free dehydroepiandrosterone has recently been considered an important key precursor in biosynthesis of estrogen in the pregnant woman or in the placental tissue by several investigators (Baulieu and Dray, 1963; Siiteri and McDonald, 1963; Bolte *et al.*, 1964; Warren and Timberlake, 1964; Morato *et al.*, 1965). In the testis as well as the adrenal the role of dehydroepiandrosterone in biosynthesis of estrogen has not been fully clarified.

It was recently demonstrated that the testis of 16-year old boy converted pregneno-

lone to dehydroepiandrosterone as well as to 6α-hydroxyestradiol-17β and 6-ketoestradiol-17β *in vitro* (Axelrod, 1965).

By the incubation of the rat testis tissue with dehydroepiandrosterone-¹⁴C or testosterone-¹⁴C, the authors demonstrated that the incorporation of radioactivity of dehydroepiandrosterone-¹⁴C into the acidic fraction was much greater than that of testosterone-¹⁴C, and that the radioactivity remained in the estrone fraction in a small amount and in the estriol fraction in a much larger amount after the purification by extensive paper chromatography.

The mechanism of estrogen formation from dehydroepiandrosterone or its sulfate

ester is not yet clear. It was suggested that 19-hydroxylation takes place not only in compounds with Δ^4 -3-keto structure but in dehydroepiandrosterone or its sulfate ester by *in vitro* experiment with the human placental preparation (Morato *et al.*, 1965). Our data suggested that 19-hydroxy- Δ^4 -androstene-3 β , 17 β -diol was possibly converted to estriol by the rat testis tissue *in vitro*.

By the rat testis incubation method dehydroepiandrosterone was converted to androstenediol to a considerable extent (Inaba and Nakao, 1966). Although it has not yet been fully identified, a radioactive compound behaving very like androstenetriol in several systems of paper chromatography was separated from the incubation of rat testis with dehydroepiandrosterone- ^{14}C (Fig. 4).

From these data it might be possible to assume that dehydroepiandrosterone converts to androstenediol which is hydroxylated at the C-19 position in succession. In this way androstenetriol might be an intermediate in the formation of estrogen from dehydroepiandrosterone in the rat testis tissue.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Takeda Chemical Industries, Ltd. for the generous supply of androst-4-ene-3 β , 17 β ,

19-triol.

This investigation was supported in part by a Grant-in-Aid for Fundamental Scientific Research from the Ministry of Education of Japan, and reported at the 39th Annual Meeting of the Japan Endocrinological Society (April 1966, Tokyo).

REFERENCES

- Aizawa, Y. (1965). Personal communication
Axelrod, L. R. (1965). *Biochim. Biophys. Acta* **79**, 551.
Baulieu, E. E. and F. Dray (1963). *J. Clin. Endocrinol.* **23**, 1298.
Bolte, E., S. Mancuso, G. Erickson, N. Wijkvist, and E. Diczfalussy (1964). *Acta Endocrinol.* **45**, 535.
Bush, I. E. (1954). *Recent Progr. in Hormone Research* **2**, 111.
Gual, C., T. Morato, M. Hayano, M. Gut, and R. I. Dorfman (1962). *Endocrinology* **71**, 920.
Inaba, M. and T. Nakao (1966). *Endocrinol. Japon.* **13**, 160.
Meyer, A. S. (1955). *Biochim. Biophys. Acta* **17**, 441.
Morato, T., E. Lemus, and C. Gual (1965). *Steroids*, Supplement I, 59.
Siiteri, P. K. and P. C. McDonald (1963) *Ibid.* **2**, 713.
Warren, J. C. and C. E. Timberlake (1964). *Obstet. Gynec.* **23**, 689.