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STUDIES ON 17β-HYDROXYSTEROID DEHYDROGENASE IN IMMATURE MOUSE OVARIAN TISSUE

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SUMMARY

17β-hydroxysteroid dehydrogenase (EC 1.1.1.64) activity was studied in immature mice ovarian tissue. The substrates employed in this investigation were 4-androstene-3,17-dione (androstenedione) and oestradiol-17β. The products of the enzymic reaction, 17β-hydroxy-4-androsten-3-one (testosterone) and oestrone respectively were identified by thin-layer chromatography, gas–liquid chromatography, mass spectrometry and derivative formation. The radiochemical purity was established by recrystallization to constant specific activity. For the reduction of androstenedione to testosterone NADPH was the electron donor whereas the oxidation of oestradiol-17β to oestrone utilized $NADP^+$ as well as NAD^+ as electron acceptor. Triphosphopyridine nucleotide was the preferred cofactor for the latter reaction.

The results of the present experiments demonstrate that the ovaries of immature mice devoid of corpora lutea are capable of converting androstenedione to testosterone and oestradiol-17β to oestrone indicating the presence of 17β-hydroxysteroid dehydrogenase in such tissue.

INTRODUCTION

DURING infancy, the mouse ovary appears not to secrete biologically active sex steroids as evidenced by the fact that the uterus is infantile and the vagina closed. It is of interest to know whether these "non-secreting ovaries" are capable of producing sex hormones or at least of synthesizing these hormones from exogenous precursors.

Previous work has shown that ovarian tissue collected from immature rodents can convert cholesterol to pregnenolone[1], pregnenolone to progesterone and dehydroepiandrostene to androstenedione[2].

The present investigations aim to detect the presence of 17β-hydroxysteroid dehydrogenase in immature mouse ovarian tissue by its ability to convert androstenedione to testosterone and oestradiol-17β to oestrone.

EXPERIMENTAL

Animals

Ovarian tissue was obtained from intact immature female Swiss albino mice. The animals were purchased from Yockna'am (Israel) and varied in age from 21 to 25 days and in weight from 12 to 18 g at the beginning of the experiment. The animals were housed in an air-conditioned room (20°C), fed on Purina lab. chow and given water *ad libidum* up to the time of sacrifice. Only animals with closed vaginas were used in the experiments.

Radioactive steroids

[7α-^{3}H]-androstenedione (3·23 mCi/mmol) was purchased from the Radio-Chemical Center (Amersham, England). [6,7-^{3}H]-oestradiol-17β (40 mCi/mmol)

was obtained from New England Nuclear Corporation (Boston, U.S.A.). The radiochemical purities were verified by thin-layer chromatography on silica gel-G using benzene-ethyl acetate (3:2, v/v) for [7α-^{3}H]-androstenedione and chloroform-ethyl acetate (4:1, v/v) for [6,7-^{3}H]-oestradiol-17β, together with standard reference samples.

Chemicals

Nicotinamide adenine dinucleotide (NAD^+), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate ($NADP^+$) and nicotinamide adenine dinucleotide phosphate reduced (NADPH) were obtained from Serva (Germany). Glucose-6-phosphate (Glc-6-P) and glucose-6-phosphate dehydrogenase (Glc-6-P-dehydrogenase) were purchased from Böehringer (Germany). Standard steroids: androstenedione, testosterone, oestradiol-17β, oestrone, testosterone acetate were acquired from Ikapharm (Israel). Scintillators for liquid scintillating counting were obtained from Packard Instrument Company (U.S.A.). All other chemicals and organic solvents were of reagent grade. The acetone, methanol and carbon disulphide were distilled before use. The rest of the organic solvents were employed without further purification.

Preparation of ovarian tissue

The animals were all killed by dislocation of the cervical spine, and the ovaries were removed by a ventral approach. The uteri were inspected and only ovaries from animals with an infantile uterus were used. Each ovary was rapidly cleaned, relieved of superficial fat and connective tissue, and kept on ice until the next stage of preparation. Within 60 min after killing the animals, the ovarian tissue was homogenized in 0·1 M phosphate buffer pH 7·4 using a teflon pestle homogenizer. The homogenate was centrifuged at 10,000 *g* for 25 min. The supernatant was decanted and used as the enzyme source.

Incubation procedure

The 10,000 *g* supernatant of the ovarian homogenate was incubated in the presence of 0·1 M phosphate buffer pH 7·4, magnesium sulphate 50 μmoles, NADPH 0·6 μmoles and [7α-^{3}H] androstenedione 0·2 μCi as substrate to a total volume of 4 ml. When [6,7-^{3}H]-oestradiol-17β (0·15 μCi) was the substrate the 10,000 *g* supernatant of the ovarian homogenate was incubated in the presence of 0·1 M phosphate buffer pH 7·4, magnesium sulphate 50 μmoles, $NADP^+$ 0·06 μmoles or NAD^+ 0·75 μmoles, to a final volume of 4 ml. Incubations were carried out by addition of the substrate for 30 min at 37°C using a Dubnoff incubator with constant shaking in air. The incubations were stopped by addition of 0·25 ml acetic acid and freezing.

Extraction and isolation of steroids

The incubation mixture was extracted three times with 10 ml ethyl acetate, followed by one extraction with 5 ml ethyl acetate. The combined extracts were evaporated to dryness under nitrogen or at reduced pressure.

The products of the reaction were then separated using thin-layer chromatography on silica gel-G. When androstenedione was the substrate the thin-layer plates were chromatographed in benzene-ethyl acetate (3:2 v/v) together with standard reference. This solvent system and techniques separate androstene-

dione (R_F 0·56) and testosterone (R_F 0·32). In the experiments in which oestradiol-17β served as the substrate, the thin-layer plates were developed in chloroform-ethyl acetate (4:1 v/v). This solvent system separates oestradiol-17β (R_F 0·50) from oestrone (R_F 0·75). Location of the separated radioactive steroids was made possible by means of reference standard sprayed with scintillated liquid diluted (1:5 v/v) with methanol and then viewed under an ultraviolet lamp.

Measurement of the radioactivity

The area on thin-layer chromatographic plates containing the separated steroids was marked out and scraped into liquid scintillation vials. The scintillation fluid was prepared by dissolving 5 g of 2,5-diphenyloxazole and 50 mg of 1,4-bis-(5-phenyloxazolyl-2)-benzene in 1 l of dry toluene containing 5% methanol. 10 ml of it was added to each vial and the radioactivity was determined using a Packard Tricarb Model 2002 liquid scintillation spectrometer. With this instrument tritium was counted with an efficiency of 38%.

The products of the reactions were further identified by the following methods:

(a) Gas–liquid chromatography
(b) Mass spectrometry
(c) Preparation of derivatives
(d) Recrystallization to constant specific activity.

Gas–liquid chromatography

Aliquots eluted from the thin-layer plates were examined by gas chromatography using a Packard Model 871. This instrument was equipped with a flame ionization detector and had a column 1·96 m long and a 4 mm i.d. The stationary phase was 3% S.D. 30 on 100–120 mesh Gas Chrom Q. The column temperature was kept at 235°C with the detector at 245°C. Samples were introduced into the column with a 10 μl Hamilton syringe.

Mass spectrometry

An Hitachi Perkin–Elmer R.M.U. 6E. mass spectrometer was employed for mass spectrometric analysis. The sample eluted from the thin-layer plate was dissolved in acetone and introduced into the sample tube of the direct inlet system by means of a hypodermic syringe. The sample temperature was 130–170°C and the ion source temperature was kept at 250°C. Spectra were observed at 70 eV.

Methods for preparation of derivatives

Acetylation and chloroacetylation of steroids was carried out according to the methods of Zaffaroni and Burton[3] and Connell and Eik-Nes[4] respectively. The procedure of Brooks *et al.*[5] was employed for the benzoylation of steroids and the chromic acid technique described by Lieberman *et al.*[6] was used for the oxidation of testosterone to androstenedione.

Enzymic assay

The enzymic assay consisted of conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone and of [6,7-^{3}H]-oestradiol-17β to [6,7-^{3}H]-oestrone. Enzymic activity was expressed as a mμmoles product (testosterone or oestrone) formed per mg protein per minute. This was calculated from the data obtained from

conversion of added radioactive substrates (androstenedione or oestradiol-17β) to radioactive products (testosterone or oestrone) and the protein content of the enzyme solution. This assay was based on the assumption that the 10,000 g supernatant of mouse ovarian homogenate contains very little endogenous androstenedione or oestradiol-17β. The amounts of these endogenous steroids were disregarded, being negligible. To our knowledge, these steroids have never been detected in immature mouse ovaries.

Protein concentration was estimated by the method of Lowry *et al.*[7] using bovine serum albumin as standard. Hemoglobine was determined as pyridine hemochromogen by a method described by Rimington[8] and modified by Dodge *et al.*[9].

Results

(A) *Identification of* [7α-^{3}H]-*testosterone* The 10,000 g supernatant of ovarian homogenate was incubated with androstenedione. The steroids were extracted and chromatographed on silica gel-G plates as described under "Methods". The area corresponding to testosterone was eluted from thin-layer plates with methanol (3 × 4 ml) and taken to dryness. An aliquot of this residue was acetylated with acetic anhydride and pyridine (1 : 1 v/v). The free and acetylated testosterone were then subjected to gas chromatography. The retention times (t_R) of the substances injected in relation to pregnenolone acetate (internal standard t_R 25 min) were identical with the relative retention times of standard testosterone (0·64) and testosterone acetate (0·80).

The mass spectrum of testosterone acetate was found to be similar to the mass spectrum of authentic testosterone acetate. Characteristic peaks at m/e 330, 288, 270, 245, 228 and 124 appeared in both sample and standard testosterone acetate. Because of very small amounts of unknown compound available, the system was not saturated by the vapour of this compound throughout the scan and comparison of the relative intensities of the peaks with the standard had little meaning. However, agreement was fair to good in the high mass region and all prominent and isolated peaks in the standard had their counterpart in the unknown.

Aliquots of testosterone, eluted from thin-layer plates were further acetylated, chloroacetylated, benzoylated and oxidized to androstenedione. The testosterone derivatives and androstenedione were subjected to thin-layer chromatography and their R_F was found to be identical with the R_F of standard testosterone acetate, chloroacetate, benzoate and androstenedione.

(B) *Recrystallization to constant specific activity.* Proof that the substances reported in the present work as [7α-^{3}H]-testosterone and [6,7-^{3}H]-oestrone were radiochemically pure is presented in Table 1. After additions of appropriate steroids, the compounds were recrystallized from different solvents. After each recrystallization samples were taken for measurement of specific activities. It can be seen (Table 1) that the specific activities of all samples (crystals and mother liquors) remained constant through four crystallization and that no significant fall in specific activity occurred as the result of the first crystallization.

(C) *Cofactor requirement.* Table 2 shows the conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone in the presence of different pyridine nucleotides. The results indicate that NADPH is necessary for enzymic activity whereas NADH cannot be used as an electron donor. It can be seen that NADPH generated via $NADP^+$, Glc-6-P and Glc-6-P-dehydrogenase has the same effect

Table 1. Recrystallization of [7α-^{3}H]-testosterone and [6.7-^{3}H]-oestrone to constant specific activity*

	Recrystallization	Solvent	Specific activity (d.p.m./mg) Crystals	Mother Liquor
[7α-^{3}H]-testosterone:	After addition of carrier		203·789	
	1st	Ether/Petroleum-ether 40–60°	210·136	201·334
	2nd	Aqueous methanol	216·421	205·592
	3rd	Benzene/Hexane	218·947	210·060
	4th	Ether/Petroleum-ether 40–60°	210·647	210·197
[6.7-^{3}H]-oestrone:	After addition of carrier		13·684	–
	1st	Acetone/Petroleum-ether 40–60°	13·668	13·815
	2nd	Acetone/Hexane	13·236	13·684
	3rd	Aqueous methanol	12·894	12·657
	4th	Acetone/Petroleum-ether 40–60°	12·568	12·639

*Specific activity, d.p.m./mg after isolation from thin-layer chromatography and addition of carrier testosterone (22 mg) and oestrone (32 mg).

on [7α-^{3}H]-testosterone accumulation as when reduced triphosphopyridine nucleotide is added directly to the incubation mixture.

The pH dependence for the enzymic conversion of androstenedione to testosterone is illustrated in Fig. 1. The optimum pH range was between 6·5 and 7·5.

The conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone as a function of NADPH concentration is shown in Fig. 2. Under the experimental conditions employed it was found that the maximal conversion of androstenedione to testosterone was achieved when NADPH was used at a concentration of 0·15 mM. This concentration was employed in subsequent experiments.

The formation of [7α-^{3}H]-testosterone from [7α-^{3}H]-androstenedione by a 10,000 *g* supernatant of immature mouse ovarian tissue as a function of enzyme concentration is illustrated in Fig. 3. There is a linear relationship between the amount of testosterone formed and the concentration of enzyme. The formation of [7α-^{3}H]-testosterone from [7α-^{3}H]-androstenedione by a 10,000 *g* supernatant as a function of time is shown in Fig. 4.

Table 2. Cofactor requirement for the conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone in 10,000 *g* supernatant of immature mouse ovarian tissue

Cofactor	Testosterone (mμmoles × 10^{-4}/mg protein/min)
None	0·05
$NADP^+$ (0·6 μmoles)	0·05
NADPH (0·6 μmoles)	2·43
NADH (0·6 μmoles)	0·04
NADPH (0·6 μmoles) + NADH (0·6 μmoles)	2·27
NADPH generating system: ($NADP^+$ 1·2 μmoles) (Glc-6-P 5·5 μmoles) (Glc-6-P dehydrogenase 0·5 IU)	2·37

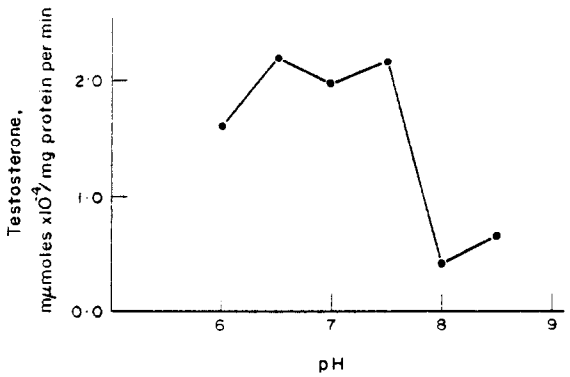

Fig. 1. The effect of pH on the conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone in a 10,000 *g* supernatant of immature mouse ovarian tissue. Incubation mixture: 3 mg protein in 0·1 M phosphate buffer 50 μmoles magnesium sulphate, 0·6 μmoles NADPH and 0·2 μCi[7α-^{3}H]-androstenedione (0·062 mμmoles). The total volume was 4 ml and the incubations were carried out at 37°C for 20 min.

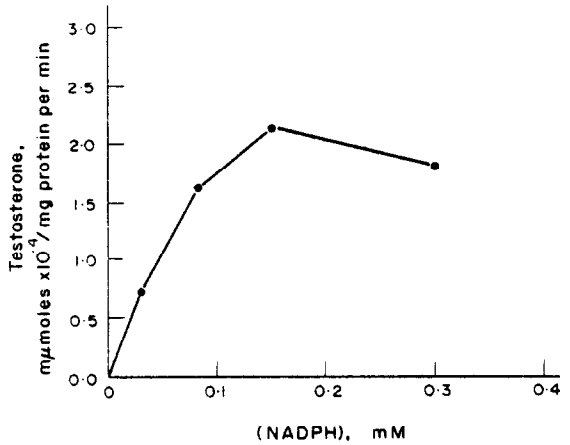

Fig. 2. Effect of NADPH on the conversion of [7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone in a 10.000 *g* supernatant of immature mouse ovarian tissue. Incubation mixture: 3 mg enzyme protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, increasing amounts of NADPH and 0·2 μCi [7α-^{3}H]-androstenedione (0·062 mμmoles). The total volume was 4 ml and the incubations were carried out at 37°C for 20 min.

(D) *Identification of* [6,7-^{3}H]-*oestrone*. The 10,000 *g* supernatant of ovarian homogenates was incubated with [6,7-^{3}H]-oestradiol-17β and $NADP^+$. The steroids were extracted and chromatographed on silica gel-G plates as previously described. The product of the enzymic reaction, considered to be [6,7-^{3}H]-oestrone was identified by the following methods:

(1) Thin-layer chromatography

(2) Formation of derivatives: acetylation, chloroacetylation and benzoylation

(3) Recrystallization to constant specific activity.

The derivatives were chromatographed on thin-layer plates and were found to have the same chromatographic mobility as standards oestrone acetate,

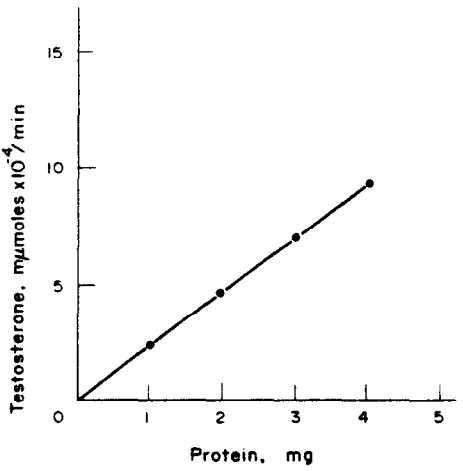

Fig. 3. The formation of [7α-³H]-testosterone from [7α-³H]-androstenedione by a 10,000 *g* supernatant of immature mouse ovarian tissue as a function of enzyme concentrations. The incubation mixture contained increasing amounts of protein enzyme in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 0·6 μmoles NADPH and 0·2 μCi [7α-³H]-androstenedione (0·062 mμmoles) to a total volume of 4 ml. Incubations were carried out in air at 37°C for 20 min.

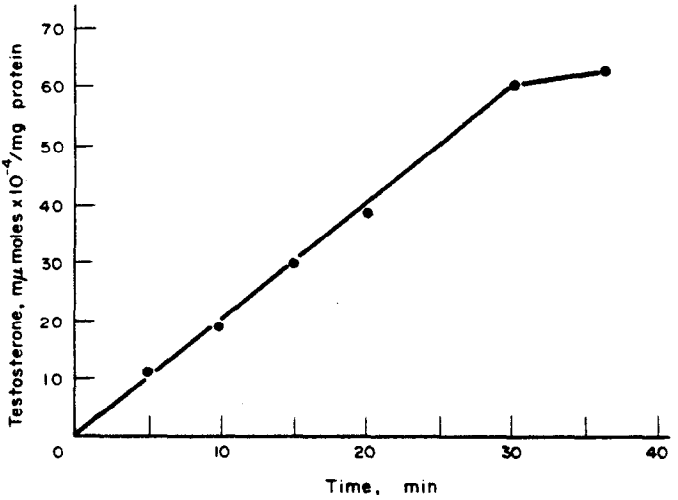

Fig. 4. Effect of time on the formation of [7α-³H]-testosterone from [7α-³H]-androstenedione by a 10,000 *g* supernatant of immature mouse ovarian tissue. Incubation mixture: 3 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 0·6 μmoles NADPH and 0·2 μCi [7α-³H]-androstenedione (0·062 mμmoles). The total volume was 4 ml and the incubations were carried out at 37°C for different times.

chloroacetate and benzoate. The recrystallization to constant specific activity of [6,7-³H]-oestrone is illustrated in Table 1.

(E) *Cofactor requirement.* The formation of [6,7-³H]-oestrone from [6,7-³H]-oestradiol-17β using varying amounts of $NADP^+$ and NAD^+ is shown in Figs. 5 and 6 respectively. Both oxidized pyridine nucleotides may be used as electron acceptors but $NADP^+$ was found to be the preferable cofactor for this reaction.

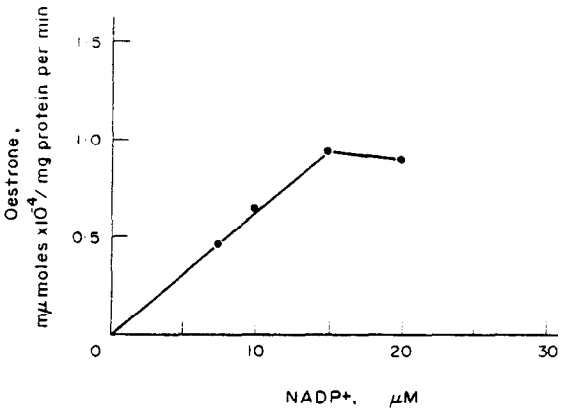

Fig. 5. Effect of NADP+ on the conversion of [6,7-^{3}H]-oestradiol-17β to [6,7-^{3}H]-oestrone by a 10,000 g supernatant of immature mouse ovarian tissue. Incubation mixture: 1 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, increasing amounts of NADP+ and 0·15 μCi [6,7-^{3}H]-oestradiol-17β (0·00375 mμmoles). The total volume was 4 ml and incubations were carried out in air for 20 min at 37°C.

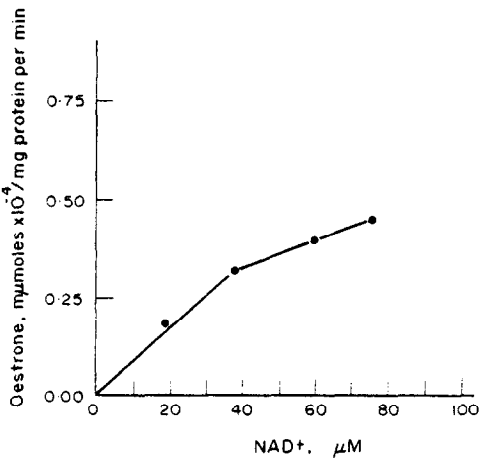

Fig. 6. Effect of NAD+ on the conversion of [6,7-^{3}H]-oestradiol-17β to [6,7-^{3}H]-oestrone by a 10.000 g supernatant of immature mouse ovarian tissue. Incubation mixture: 1 mg protein in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, increasing amounts of NAD+ and 0·15 μCi [6,7-^{3}H]-oestradiol-17β (0·00375 mμmoles). The total volume was 4 ml and incubations were carried out in air for 20 min at 37°C.

Figure 7 illustrates the formation of oestrone from oestradiol-17β in the presence of NADP+ at different time intervals. The reaction followed a linear time course for at least 15–20 min.

(F) *Incubation of mouse blood with* [6,7-^{3}H]-*oestradiol*-17β. Since an active 17β-hydroxysteroid dehydrogenase was described in rodent red blood cells [10, 11] the following experiments were carried out in order to evaluate to what extent the presence of erythrocytes in mouse ovaries may contribute to the enzymic activity.

Fresh heparinized mouse blood (300–600 μl) was homogenized in 0·1 M phosphate buffer pH 7·4 and centrifuged at 10.000 g for 25 min. The 10.000 g

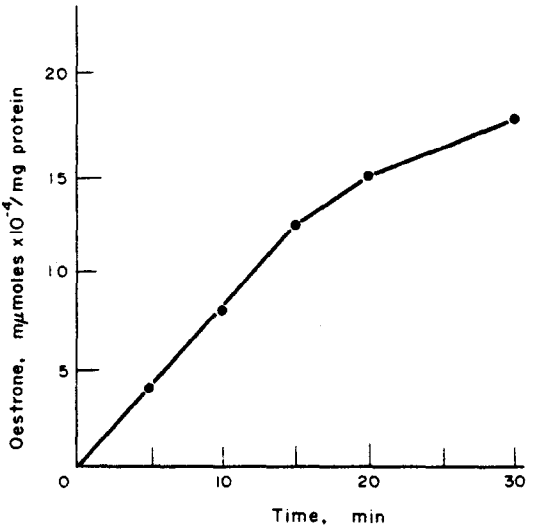

Fig. 7. Effect of time on the formation of [6,7-^{3}H]-oestrone from [6,7-^{3}H]-oestradiol-17β by a 10,000 *g* supernatant of immature mouse ovarian tissue. Incubation mixture: 1 mg protein enzyme in 0·1 M phosphate buffer pH 7·4, 50 μmoles magnesium sulphate, 0·06 μmoles $NADP^+$ and 0·15 μCi [6,7-^{3}H]-oestradriol-17β (0·00375 mμmoles). The total volume was 4 ml and incubations were carried out at 37°C for different times.

supernatant was incubated with [6,7-^{3}H]-oestradiol-17β and $NADP^+$ under identical conditions with those described for the ovarian tissue. No conversion of oestradiol-17β to oestrone was observed. Furthermore, hemoglobine was not detected in the 10,000 *g* supernatant of ovarian homogenate.

DISCUSSION

It has previously been demonstrated that immature rat ovarian tissue converts progesterone to oestradiol-17β[12] and pregnenolone to testosterone[13], thus indicating the presence of 17β-hydroxysteroid dehydrogenase in this tissue. Employing ovarian tissue of 28 day or older rats, Weisz and Lloyd[12] demonstrated the conversion of progesterone to oestradiol-17β and oestrone, whereas the ovarian tissue of 21 day old animals metabolized progesterone only to oestradiol-17β. This study seems to indicate that the ovarian tissue of 21 day old rats is incapable of transforming oestradiol-17β to oestrone.

The present investigation shows the ability of immature mouse ovarian tissue to convert[7α-^{3}H]-androstenedione to [7α-^{3}H]-testosterone and [6,7-^{3}H]-oestradiol-17β to [6,7-^{3}H]-oestrone. The products of the enzymic reactions were identified using thin-layer chromatography and derivative formation. Gas–liquid chromatography and mass spectrometry were employed for further identification of testosterone. The radiochemical purity of [7α-^{3}H]-testosterone and of [6,7-^{3}H]-oestrone was checked by recrystallization to constant specific activity. By the above methods of identification of the product of the enzymic reaction, it was unequivocally demonstrated that the immature mouse ovary contains a 17β-hydroxysteroid dehydrogenase which converts [7α-^{3}H]-androstenedione into [7α-^{3}H]-testosterone and [6,7-^{3}H]-oestradiol-17β into [6,7-^{3}H]-oestrone. An active 17β-hydroxysteroid dehydrogenase has been found in rodents[10, 11] and human red blood cells[14, 15].

The blood contamination of immature mouse ovarian tissue was found to be 1·92–3·52 μl/mg wet ovaries[16]. Since the most careful dissection of the ovarian tissue can not entirely eliminate blood contamination, it was necessary to exclude the possibility that the presence of erythrocytes in the ovaries may contribute to the 17β-hydroxysteroid dehydrogenase activity. It was observed that up to 600 μl of fresh heparinized mouse blood (see results) incubated with oestradiol-17β under identical conditions as those described for ovarian tissue was not capable to convert oestradiol-17β to oestrone. Furthermore, hemoglobine was not found in the 10,000 *g* supernatant of ovarian homogenate. These findings indicate that the procedure employed for the preparation of the enzyme from ovarian tissue neither hemolized nor extracted the 17β-hydroxysteroid dehydrogenase from the red blood cells.

This excludes the possibility that the source of 17β-hydroxysteroid dehydrogenase of a 10,000 *g* supernatant of ovarian homogenate was due to blood contamination and confirms the presence of this enzyme in immature mouse ovarian tissue.

In the present work it was not established whether the 10,000 *g* supernatant of immature mouse ovarian tissue contains a single 17β-hydroxysteroid dehydrogenase acting on both C_{18} and C_{19}-steroid substrates or two different enzymes. Since most mammalian 17β-hydroxysteroid dehydrogenase have not been adequately purified, the existence of different C_{18} and C_{19}-17β-hydroxysteroid dehydrogenase in steroidogenic tissue remains to be established.

In the present experiments a 10,000 *g* supernatant of immature mouse ovarian homogenate was selected as the source of enzyme since it was more efficient in converting [7α-^{3}H]-androstenedione into [7α-^{3}H]-testosterone than the total homogenate or other supernatants.

This 10,000 *g* supernatant of ovarian homogenate contains light mitochondria as well as microsomes. Since only small amounts of tissue were available for each experiment, a more precise location of this enzyme was not possible. Using rabbit ovarian homogenate Davenport and Mallette[17] demonstrated the presence of this enzyme in the soluble fraction.

As no attempts were made to separate the different elements of immature mice ovarian tissue, it was not possible to elucidate which compartments of the immature mouse ovary are mainly responsible for the formation of testosterone from androstenedione or of oestrone from oestradiol-17β. Work with ovarian tissue collected from sexually immature sows showed that 17β-hydroxysteroid dehydrogenase was more active in granulosa cells than in thecal cells[18].

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REFERENCES

1. Sulimovici S.: Ph.D. Thesis Edinburgh University, Scotland, U.K. (1968).
2. Sulimovici S. and Lunenfeld B.: *J. steroid Biochem.* **2** (1971) 203.
3. Zaffaroni A. and Burton R. B.: *J. biol. Chem.* **193** (1951) 749.
4. Connell G. M. and Eik-Nes K. B.: *Steroids* **12** (1968) 507.
5. Brooks R. V., Klyne W. and Miller E.: *Biochem. J.* **54** (1953) 212.
6. Lieberman S., Katzenellenbogen E. R., Schneider R., Studer P. E. and Dobriner K.: *J. biol. Chem.* **205** (1953) 87.

7. Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J.: *J. biol. Chem.* **193** (1951) 265.
8. Rimington C.: *Brit. Med. J.* **1** (1942) 177.
9. Dodge J. T., Mitchell C. and Hanahan D. J.: *Arch. biochem. Biophys.* **100** (1963) 119.
10. Portius H. J. and Repke K.: *Arch. Exp. Pathol. Pharmakol.* **239** (1960) 184.
11. Nicol M., Savoure N. and Rico S.: *C.R. Acad. Sci. (Paris)* Ser. D **268** (1969) 1552.
12. Weisz J. and Lloyd C. W.: *Endocrinology* **77** (1965) 735.
13. Beneviste R.: M.Sc. Thesis. The Hebrew University, Jerusalem, Israel (1969).
14. Jacobsohn G. M. and Hochberg R. B.: *J. biol. Chem.* **243** (1968) 2985.
15. Mulder E., Lamers-Stahlhofen G. J. M. and Van der Molen H. J.: *Biochem. J.* **127** (1972) 649.
16. Lunenfeld B. and Eshkol A.: *Vitams Horm.* **25** (1967) 137.
17. Davenport G. R. and Mallette L. E.: *Endocrinology* **78** (1966) 672.
18. Bjersing L. and Carstensen H.: *J. Reprod. Fert.* **14** (1967) 101.