Journal of Steroid Biochemistry, 1971, Vol. 2, pp. 203–213. Pergamon Press. Printed in Great Britain

STUDY OF Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE AND Δ^5-3-KETOSTEROID ISOMERASE IN IMMATURE MOUSE OVARIAN TISSUE

S. SULIMOVICI and B. LUNENFELD
Institute of Endocrinology, Tel Hashomer Government Hospital
and the Department of Life Sciences, Bar-Ilan University, Israel

(*Received* 30 *November* 1970)

SUMMARY

The transformation of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids, a two-stage reaction which requires the participation of a Δ^5-3β-hydroxysteroid dehydrogenase (EC 1.1.1.51) and Δ^5-3-ketosteroid isomerase (EC 5.3.3.1), has been studied in ovaries of immature mice. The substrates employed in this investigation were: 3β-hydroxy-5-pregnen-20-one (pregnenolone) and 3β-hydroxy-5-androsten-17-one (dehydroepiandrosterone).

The products of the enzymic reaction, progesterone and 4-androstenedione respectively, were identified by thin layer chromatography, gas–liquid chromatography and mass spectrometry. The radiochemical purity was established by recrystallization to constant specific activity.

The evidence accumulated is sufficient to establish that the ovaries of immature mice 'devoid of corpora lutea' are capable of converting exogenous pregnenolone to progesterone, and dehydroepiandrosterone to 4-androstenedione.

INTRODUCTION

THE oxidation of the Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids is catalysed in steroidogenic tissues by a NAD^+ dependent Δ^5-3β-hydroxysteroid dehydrogenase, and a Δ^5-3-ketosteroid isomerase[1]. The presence of these enzymes in steroid producing glands has been demonstrated both histochemically[2–5] and biochemically[6–9].

It is assumed that changes in concentration, relative activity, and inter and intracellular localization of this enzyme system may control the rate of steroid hormone biosynthesis. Therefore, various morphological changes in a steroid-producing gland may influence one of the above mentioned factors, and this, in turn, might affect steroidogenesis.

The ovary is an interesting organ for a study of the relationship between the morphological state of the tissue and its capacity to produce steroid hormones. Rubin *et al.*[10] investigated changes in Δ^5-3β-hydroxysteroid dehydrogenase activity in rat ovarian tissue. These authors found an increase in enzymic activity upon the appearance of the first corpus luteum. Luteinization of the prepuberal ovary by administration of pregnant mare's serum gonadotropin (PMSG) and human chorionic gonadotropin (HCG) was also accompanied by an increase in Δ^5-3β-hydroxysteroid dehydrogenase activity[11]. Studies by Ryan and Petro[12] showed that human ovarian follicles are capable of converting pregnenolone to progesterone. This conversion was found to be more active with granulosa cells than with theca cells. Granulosa cells of follicles undergoing atresia were found to be very active in Δ^5-3β-hydroxysteroid dehydrogenase[13]. Thus a relation-

ship clearly exists between the morphological state of the ovary and its capacity to produce Δ^4-3-ketosteroids.

Direct biochemical evidence for the presence of Δ^5-3β-hydroxysteroid dehydrogenase in immature rat ovarian tissue has been reported[10, 14, 15]. In these studies the dehydrogenase activity was measured by the increase in optical density at 240 nm caused by the formation of Δ^4-3-ketosteroids. For the final confirmation of the presence of the enzymes in immature ovaries, it is felt that the identification and characterization of the products of the enzymic reaction is necessary.

The present article is concerned with the capacity of the immature mouse ovarian tissue (devoid of corpora lutea) to convert pregnenolone to progesterone and dehydroepiandrosterone to 4-androstenedione. Criteria for the identification of the products of the enzymic reaction are also presented.

EXPERIMENTAL

Animals

Immature female Swiss albino mice were used for all experiments. The animals were housed in an air-conditioned room (20°C) and fed on Purina lab. chow and given water *ad libitum* up to the time of sacrifice. The animals varied in age from 21–25 days and in weight trom 10–13 g at the commencement of the experiment. Histological examination of ovaries of animals of this age showed no signs of ovulation. The components of such ovaries were follicles and interstitial cells only.

Radioactive substrates

[7α-^{3}H]Pregnenolone (14·70 Ci/mmol) and [7α-^{3}H]dehydroepiandrosterone (15·90 Ci/mmol) in benzene solution were purchased from the Radiochemical Centre (Amersham, England). The radiochemical purities were verified by thin-layer chromatography on silica gel-G, using benzene–ethyl acetate (4 : 1, v/v) together with standard reference samples. Each batch of [7α-^{3}H]pregnenolone and [7α-^{3}H]dehydroepiandrosterone respectively was tested in this way prior to use.

Chemicals

Nicotinamide adenine dinucleotide (NAD^+) was obtained from Sigma (England). Silica gel-G was purchased from Merck (Germany). Standard steroids: pregnenolone, progesterone, dehydroepiandrosterone and 4-androstenedione were acquired from Ikapharm (Israel). Scintillators for liquid scintillation 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 the killing of the animals, the

ovarian tissue was homogenized in 0·1 M phosphate buffer pH 7·4 with a loosely fitting all-glass homogenizer. The homogenization was carried out very carefully and no more than four or five passes were made. The homogenate was then freeze-dried by sublimation of the ice *in vacuo*. The lyophilized material was suspended in 0·1 M phosphate buffer pH 7·4 and centrifuged at 40,000 × *g* for 30 min. In all the experiments described, the 40,000 × *g* supernatant was used as the enzyme source.

Incubation procedure

The 40,000 × *g* supernatant of the lyophilized homogenate was incubated in the presence of 0·1 M phosphate buffer, pH 7·4, magnesium sulphate 50 μmol, NAD^+ 2μM, together with 0·1 μCi of [7α-^{3}H]pregnenolone or 0·1 μCi of [7α-^{3}H] dehydroepiandrosterone. The total volume of incubation was 2 ml. Incubations were performed at 37°C for 2 min, using a Dubnoff incubator, with constant shaking in air. The incubation period was terminated by the addition of 0·2 ml acetic acid and freezing.

Extraction and isolation of steroids

Following incubation with radioactive steroid substrates, the mixture was extracted three times with 10 ml ethyl acetate. The combined extract was washed with 5 ml of distilled water, dried with anhydrous sodium sulphate, filtered and evaporated to dryness under either nitrogen or air.

The products of the reactions were then separated using thin-layer chromatography on silica gel-G. When pregnenolone was the substrate, the thin-layer plates were developed in chloroform–ethyl acetate (8:2, v/v) together with standard reference. This solvent system and technique separates pregnenolone (R_F 0·41) from progesterone (R_F 0·56). In the experiments in which dehydroepiandrosterone served as the substrate, the thin-layer plates were chromatographed in benzene–methanol (9·5:0·5, v/v). This solvent system separates dehydroepiandrosterone (R_F 0·34) from 4-androstenedione (R_F 0·55). In these experimental conditions only radioactive progesterone and 4-androstenedione were formed, after the incubation of a 40,000 × *g* supernatant of mouse ovarian homogenate with radioactive pregnenolone and dehydroepiandrosterone respectively. The separated radioactive steroids and the reference standards, run on the thin-layer plates, were made visible by spraying with scintillation 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 as having an efficiency of 38%.

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

a. Ultraviolet absorption spectroscopy;
b. Gas–liquid chromatography;

c. Mass spectrometry;
d. Reduction of 4-androstenedione to testosterone;
e. Recrystallization to constant specific activity.

Ultraviolet absorption spectroscopy

The 40,000 × *g* supernatant of mouse ovarian homogenate was separately incubated with cold pregnenolone or dehydroepiandrosterone (10, 25, 50 and 100 μg) and NAD^+ (0·1 mM). The steroids were extracted and chromatographed on silica gel plates as described above. The area corresponding to progesterone after incubation with pregnenolone and the area of 4-androstenedione following incubation with dehydroepiandrosterone was eluted from the thin-layer plates with acetone and evaporated to dryness. 2 ml of methanol was added to the dry residue and the ultraviolet absorbing material was measured in a P.M.Q. II Zeiss spectrophotometer.

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 S.E. 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

A 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.

Reduction of 4-androstenedione to testosterone

4-androstenedione was reduced to testosterone by incubation at −15°C for 60 min in 1 ml methanol containing 100 mg anhydrous $NaBH_4$. Distilled water (5 ml) was added to the incubation mixture and the product was extracted with ether and applied to thin-layer chromatography. The chromatography was carried out in benzene–ethyl acetate (6:4, v/v). The testosterone was then eluted from the plates and acetylated with pyridine and acetic anhydride.

Enzymic assay

The enzymic assay was the same as previously described[16] and consisted of the conversion of [7α-^{3}H]pregnenolone to [7α-^{3}H]progesterone and the conversion of [7α-^{3}H]dehydroepiandrosterone to [7α-^{3}H]4-androstenedione. The enzymic activity, expressed as a percentage of the progesterone or 4-androstenedione formed per mg protein/min, was calculated from the data obtained from the conversion of the added radioactive Δ^5-3β-hydroxysteroid to radioactive Δ^4-3-ketosteroid and the protein content of the enzyme solution. This assay was based on the assumption that the 40,000 × *g* supernatant of immature mouse ovaries contained very little endogenous pregnenolone or dehydroepiandrosterone. The

amounts of endogenous pregnenolone and dehydroepiandrosterone were disregarded, being negligible. Protein concentration was estimated by the method of Lowry *et al.*[17] with bovine serum albumin used as standard.

RESULTS

A. *Identification of progesterone*

Samples of the product isolated following incubation of the 40,000 × *g* supernatant of ovarian homogenate with pregnenolone were eluted from the thin layer plates as described above. The ultraviolet spectrum was taken from 220–280 nm in methanol. A peak at 240 nm was found, indicating the formation of Δ^4-3-ketosteroid, presumably progesterone. When an aliquot of this material, after elution from the thin-layer plate, was subjected to gas chromatography, the retention time (t_R) of the substance injected, in relation to pregnenolone acetate (internal standard $t_R = 24$ min) was identical to the relative retention time of standard progesterone (0·67). The mass spectrum of this compound was found to be similar to the mass spectrum of authentic progesterone (Fig. 1).

B. *Identification of 4-androstenedione*

The 40.000 × *g* supernatant fluid of the homogenate of mouse ovarian tissue was incubated with cold dehydroepiandrosterone. The steroids were extracted and separated on thin-layer chromatography. The area corresponding to 4-androstenedione was eluted from the thin-layer plates and the ultraviolet spectrum taken in methanol. A peak at 240 nm due to Δ^4-3-ketosteroid, presumably 4-androstenedione, was observed. After elution from the thin-layer plate 4-androstenedione was further reduced to testosterone and the latter steroid acetylated as described above. The 4-androstenedione and the acetylated testosterone were injected into the gas–liquid chromatograph, with column and condition of operation the same as previously described. The retention time of the substances injected in relation to pregnenolone acetate (internal standard $t_R = 24$ min) were identical to the relative retention time of standard 4-androstenedione (0·58) and testosterone acetate (0·83). The mass spectrum of the 4-androstenedione isolated from the incubation was shown to be similar to that of the authentic compound (Fig. 1.).

C. *Recrystallization to constant specific activity*

Proof that the substances reported in the present study as [7α-^{3}H]progesterone and [7α-^{3}H]4-androstenedione were radiochemically pure is presented in Table 1. After additions of appropriate steroids, the compounds were recrystallized from four different solvents. It can be seen (Table 1) that the specific activities of all samples (crystals and mother liquors) remained constant through four crystallizations and that no significant fall in specific activity occurred as the result of the first crystallization.

D. *Time course of the enzymic reaction*

There is a linear relationship between the amount of progesterone formed and the concentration of enzyme (Fig. 2a). Analogous results were obtained when dehydroepiandrosterone was employed as substrate (Fig. 2b); however, dehydroepiandrosterone was metabolized faster than pregnenolone. The rate of formation

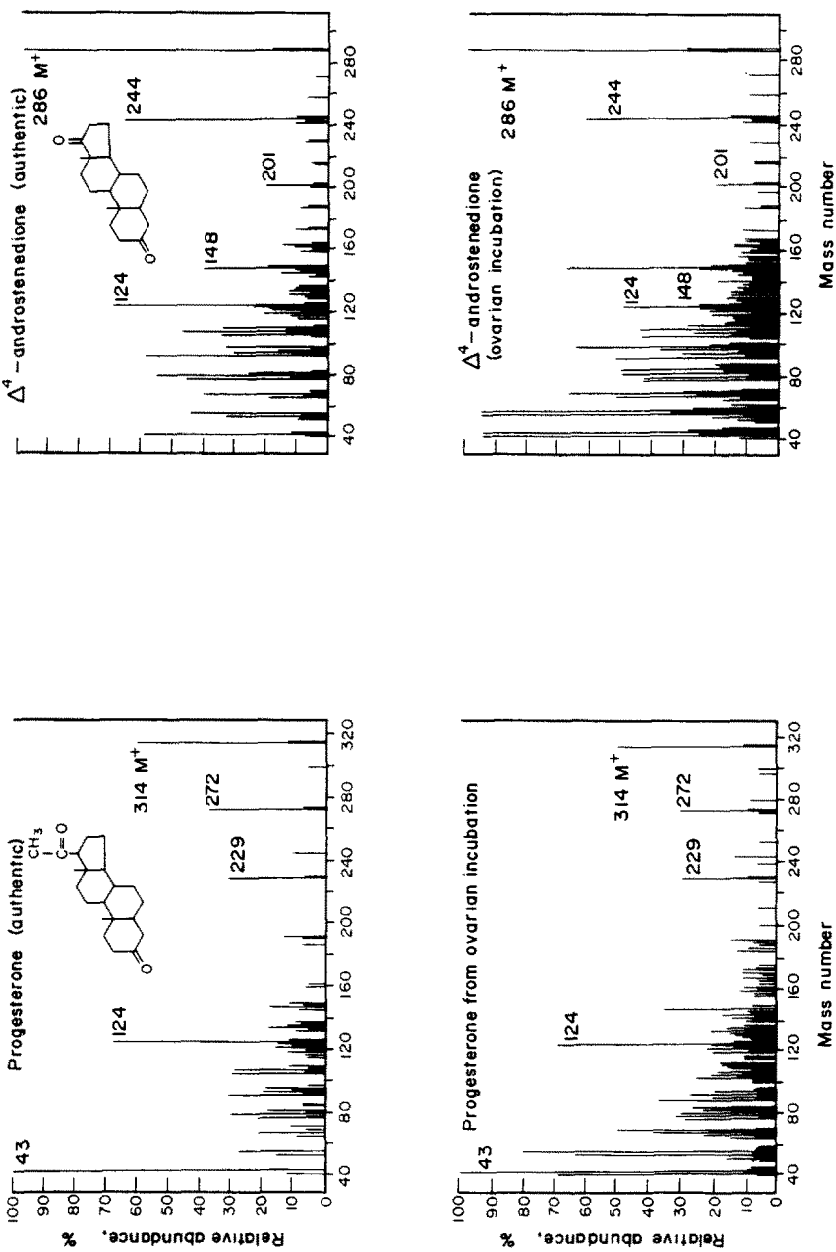

Fig. 1. Comparison of the mass spectra of authentic progesterone and 4-androstenedione and the compounds isolated from incubations of 40,000 × *g* supernatant of mouse ovarian tissue with pregnenolone and dehydroepiandrosterone respectively. The peaks m/e 43 (progesterone) and m/e 286 (4-androstenedione) were taken as 100%. All peaks below 5% have been omitted.

Table 1. Recrystallization of [7α-^{3}H] progesterone and [7α-^{3}H]4-androstenedione to constant specific activity*

[7α-^{3}H] progesterone				[7α-^{3}H] 4-androstenedione			
Recrystallization	Solvent	Specific activity (d.p.m./mg) Crystals	Mother liquor	Recrystallization	Solvent	Specific activity (d.p.m./mg) Crystals	Mother liquor
After addition of carrier	—	3700	—	After addition of carrier	—	2870	—
1st	Acetone/ hexane	3600	3040	1st	Acetone/ ligroin	2940	2500
2nd	Aqueous ethanol	3520	2920	2nd	Ethyl acetate	2820	2440
3rd	Ethyl acetate/ hexane	3590	2690	3rd	Aqueous ethanol	2880	2460
4th	Methanol	3600	3000	4th	Benzene/ hexane	2850	2450

*Specific activity, d.p.m./mg, after isolation from thin-layer chromatography and addition of carrier steroids (approx. 20 mg).

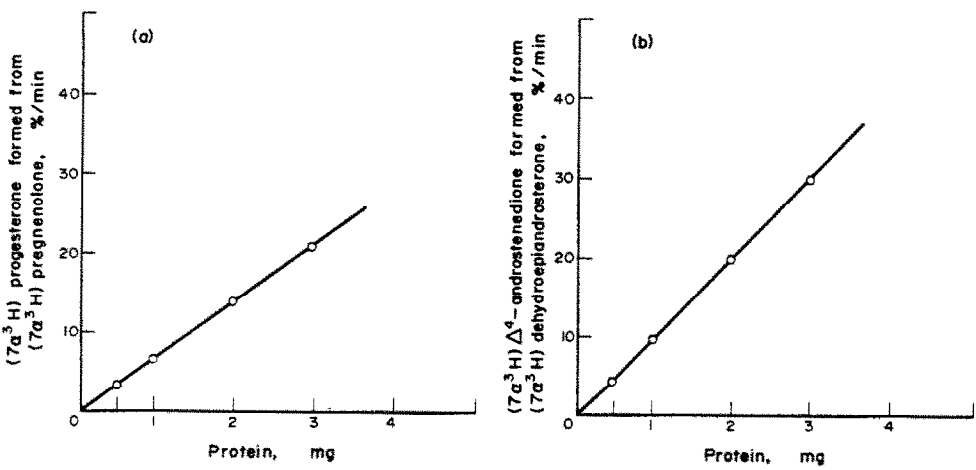

Fig. 2. The formation of progesterone from pregnenolone (a) and of 4-androstenedione from dehydroepiandrosterone (b) by a 40,000 $\times g$ supernatant of immature mouse ovarian tissue as a function of enzyme concentrations. The incubation mixture contained increasing amounts of enzyme protein in 0·1 M phosphate buffer pH 7·4, 50 μmol magnesium sulphate, 2 μM NAD^+, 0·1 μCi[7α-^{3}H] pregnenolone or 0·1 μCi[7α-^{3}H]-dehydroepiandrosterone, to a total volume of 2 ml. Incubations were carried out in air at 37°C for 2 min.

of 4-androstenedione was $0{\cdot}657 \times 10^{-6}$ mol/mg protein/min and that of progesterone was $0{\cdot}453 \times 10^{-6}$ mol/mg protein/min.

The conversion of [7α-^{3}H]pregnenolone to [7α-^{3}H]progesterone and of [7α-^{3}H]dehydroepiandrosterone to [7α-^{3}H]4-androstenedione at different con-

centrations of NAD^+ is shown in Figs. 3a and 3b respectively. The results demonstrate that the $40,000 \times g$ supernatant of mouse ovarian homogenate appears to contain endogenous NAD^+, since conversion to Δ^4-3-ketosteroids occurred without adding an exogenous co-factor.

Figure 4 (a and b) illustrates the formation of progesterone and 4-androstenedione at different time intervals using varying amounts of NAD^+. Zero kinetics were obeyed for 1–2 min at different concentrations of NAD^+.

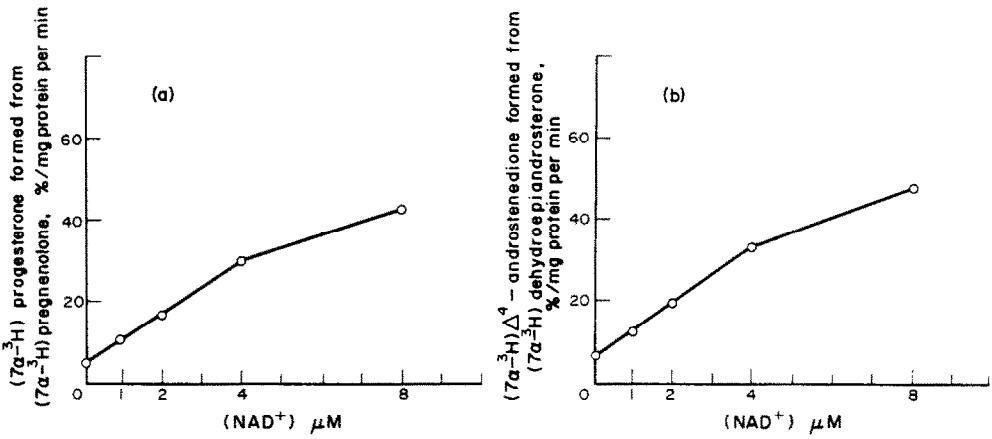

Fig. 3. Effect of NAD^+ on the conversion of pregnenolone to progesterone (a) and of dehydroepiandrosterone to 4-androstenedione (b) in a $40,000 \times g$ supernatant of immature mouse ovarian tissue. Incubation mixture: 1 mg enzyme protein in 0·1 M phosphate buffer pH 7·4, 50 μmol magnesium sulphate, increasing amounts of NAD^+ and 0·1 μCi[7α-^{3}H]pregnenolone or 0·1 μCi[7α-^{3}H]dehydroepiandrosterone. The total volume was 2 ml and the incubations were carried out at 37°C in air for 2 min.

DISCUSSION

This study demonstrated that the Δ^5-3β-hydroxysteroid dehydrogenase and Δ^5-3-ketosteroid isomerase were present in immature mouse ovarian tissue. The substrates employed, pregnenolone and dehydroepiandrosterone, were oxidized to progesterone and 4-androstenedione respectively in the presence of small amounts of NAD^+. In the present study the source of enzyme was a $40,000 \times g$ supernatant of the ovarian homogenate. This fraction appeared to contain endogenous NAD^+ since the conversion of the Δ^5-3β-hydroxysteroid to Δ^4-3-ketosteroids was achieved without adding any exogenous co-factor. The enzymic assay was carried out by means of radioactive precursors. However, the identification of the products of the reaction, progesterone and 4-androstenedione, was performed after incubation of the $40,000 \times g$ supernatant with cold steroid-substrate.

The dehydrogenase–isomerase system in endocrine tissue has been localized in the microsomal fraction[6]. However, a diffuse distribution of this enzyme in mitochondrial and microsomal fractions has been demonstrated for placenta[8] and ovarian tissue[16]. Since all the present experiments were performed in a $40,000 \times g$ supernatant of ovarian homogenate, intracellular localization of the enzymes was not possible. When intracellular localization of an enzyme is attempted, lyophilization prior to fractionation of the homogenate into subparticulate fractions must be avoided, since lyophilization and resuspension of

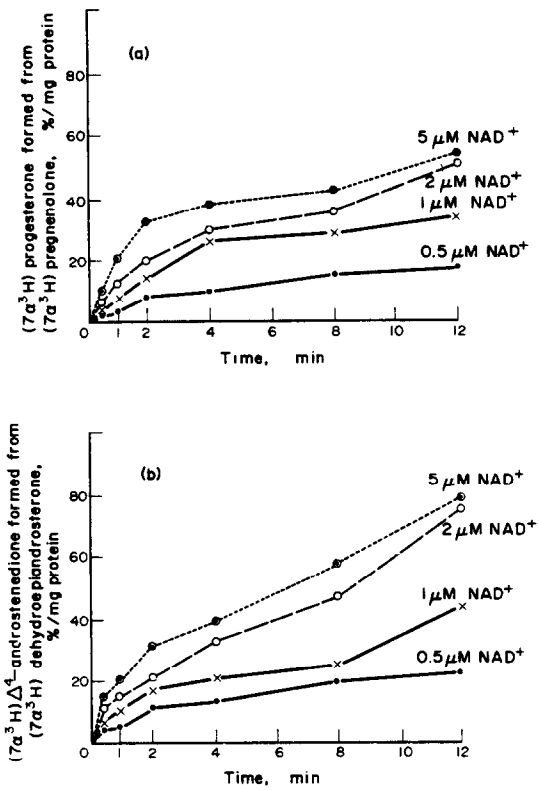

Fig. 4. The formation of progesterone from pregnenolone (a) and of 4-androstenedione from dehydroepiandrosterone (b) by a 40,000 × *g* supernatant of immature mouse ovarian tissue as a function of time and co-factor concentration. Incubation mixture: 1 mg enzyme protein in 0·1 M phosphate buffer pH 7·4, 50 μmol magnesium sulphate, increasing amounts of NAD^+ and 0·1 μCi[7α-^{3}H]pregnenolone or 0·1 μCi[7α-^{3}H]-dehydroepiandrosterone to a total volume of 2 ml. The incubations were carried out at 37°C in air for diverse periods.

the lyophilized material might lead to translocation of the enzyme. In these studies, the total homogenate was lyophilized to enable the collection of sufficient material for each experiment. This technique was used after it was established that under the experimental conditions described, lyophilization did not influence the conversion of Δ^5-3β-hydroxysteroids to Δ^4-3-ketosteroids.

Studies by Presl *et al.*[18], using histochemical methods, and by Rubin and coworkers[10, 14, 15], employing biochemical techniques, indicated the presence of Δ^5-3β-hydroxysteroid dehydrogenase in immature rat ovaries. The biochemical evidence of the above authors was based only on the increase in optical density at 240 nm caused by the formation of Δ^4-3-ketosteroids.

The data obtained in the present study by thin-layer chromatography, gas–liquid chromatography, ultraviolet absorption spectroscopy, and mass spectrometric analysis, as well as recrystallization to constant specific activity for the characterization of the products of the enzymic reaction (progesterone and 4-androstenedione), unequivocally confirm the presence of these enzymes in immature rodent ovaries devoid of corpora lutea.

Since no attempt was made to separate the granulosa, thecal and interstitial cells of the ovary, prior to homogenization, the intercellular localization of these enzymes was not studied. Although this tissue contained no corpora lutea, it could be claimed that the Δ^5-3β-hydroxysteroid dehydrogenase activity might be derived from atretic follicles which may be present in smaller or greater numbers in 21-day-old mice. Histochemical evidence in mouse ovarian tissue[5] supports the view that the main source of Δ^5-3β-hydroxysteroid dehydrogenase in the immature mouse is atretic follicles or follicles doomed to undergo atresia. Electron microscopic studies demonstrated that organelles of interstitial cells of immature rodents are indicative of steroid secretory function[19]. Histochemical studies in immature mice showed the appearance of formazan granules in interstitial cells after incubation with pregnenolone or dehydroepiandrosterone[20]. Therefore, the interstitial cells of immature rodents seem also to contain Δ^5-3β-hydroxysteroid dehydrogenase activity.

Although no direct evidence on secretion of biologically active steroids is available, indirect evidence seems to indicate such processes in infancy. At the age of 14 days, uterine weights of animals spayed at the age of 2 days were distinctly lower than normal[21]. Indication that ovarian steroids may play a role in gonadotropin feed-back mechanism in infancy can be derived from studies with animals placed in parabiosis, where it was shown that the infantile hypophysis following removal of the ovaries increased secretion of gonadotropins [22].

ACKNOWLEDGEMENTS

Our thanks are due to Dr. B. Sklarz of the Chemistry Department, Bar-Ilan University, for his help in performing the mass spectrometric analysis. The technical assistance of Mrs. V. Rotary is gratefully acknowledged. This research study was supported by Ford Foundation Grant No. 67–470.

REFERENCES

1. L. T. Samuels: In *Metabolic Pathways* (Edited by D. M. Greenberg), Academic Press, New York and London, Vol. 1 (1960) p. 431.
2. L. W. Wattenberg: *J. Histochem. Cytochem.* **6** (1958) 225.
3. H. Lewy, H. W. Deane and B. L. Rubin: *Endocrinology* **65** (1959) 932.
4. B. Goldberg, G. E. Jones and D. A. Turner: *Am. J. Obstet. Gynec.* **86** (1963) 349.
5. M. M. Ferguson: *J. endocr.* **32** (1965) 365.
6. K. F. Beyer and L. T. Samuels: *J. biol. Chem.* **219** (1956) 69.
7. J. Kowal, E. Forchielli and R. I. Dorfman: *Steroids* **3** (1964) 531.
8. S. S. Koide and M. T. Torres: *Biochim. biophys. Acta* **105** (1965) 115.
9. S. G. Cheatum and J. C. Warren: *Biochim. biophys. Acta* **122** (1966) 1.
10. B. L. Rubin, H. W. Deane, J. A. Hamilton and E. C. Driks: *Endocrinology* **72** (1963) 924.
11. B. L. Rubin and H. W. Deane: *Endocrinology* **76** (1965) 382.
12. K. J. Ryan and Z. Petro: *J. Clin. Endocr. Met.* **26** (1966) 46.
13. B. L. Lobel, R. M. Rosenbaum and H. W. Deane: *Endocrinology* **68** (1961) 232.
14. B. L. Rubin, J. A. Hamilton, T. J. Karlson and R. I. Tufaro: *Endocrinology* **77** (1965) 909.
15. B. L. Rubin: *Endocrinology* **83** (1968) 626.
16. S. Sulimovici and G. S. Boyd: *Eur. J. Biochem.* 7 (1969) 549.
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall: *J. biol. Chem.* **193** (1951) 265.
18. J. Presl, J. Jirasek, J. Horsky and M. Henzl: *J. Endocr.* **31** (1965) 293.
19. H. E. Stegner: *Workshop Meeting on Development of the Ovary in Infancy*, Birmingham 1969 (in press).
20. E. Rachamim: M.Sc.-Thesis, Bar-Ilan University, Israel (1970).

21. D. Price: *Anat. Rec.* **97** (1947) 519.
22. T. Martins and A. Rocha: *Endocrinology* **15** (1931) 421.
23. S. Sulimovici and B. Lunenfeld: *Hormone and Metabolic Research* (1971) (in press).