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# STUDIES ON ENZYME REACTIONS RELATED TO STEROID BIOSYNTHESIS—III. DISTRIBUTION OF THE TESTICULAR ENZYMES RELATED TO ANDROGEN PRODUCTION BETWEEN THE SEMINIFEROUS TUBULES AND INTERSTITIAL TISSUE

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SUMMARY

In order to examine intercellular distribution of several enzymes related to steroidogenesis in rat testicular tissue, the decapsulated tissue was manually separated into seminiferous tubule and interstitial cell fractions. When $^{14}C$-progesterone was incubated with the two fractions aerobically in the presence of NADPH, 17α-hydroxyprogesterone was obtained as the major product in case of interstitial cell fraction but no significant conversion of the substrate was detected by incubation with seminiferous tubule fraction. When 17α-hydroxyprogesterone was employed as substrate, testosterone was obtained as the main product by incubation with interstitial cell fraction, while in the case of seminiferous tubules, 17α,20α-dihydroxypregn-4-en-3-one was detected as the major metabolite but not $C_{19}$-steroids. From the above results, activities of 17α-hydroxylase and $C_{17}$–$C_{20}$ lyase were located in the interstitial tissue, while 20α-hydroxysteroid dehydrogenase was concentrated in the seminiferous tubules.

## INTRODUCTION

It had been accepted that testicular interstitial tissue was the site of androgen production in mammalians [1]. For biochemical experiments on the steroid metabolism in the testes, the whole organ had been generally employed with the assumption that enzyme activities demonstrated by the tissue originated in the interstitial tissue [2, 3]. Christensen and Mason [4] reported that the interstitial tissue was a principal source of androgen production, but showed that the seminiferous tubules are capable of producing significant amount of androgens from progesterone. On the other hand, the seminiferous tubules were demonstrated to be unable to convert cholesterol to androgen [5]. In this paper, rat seminiferous tubules and interstitial tissue were separated, and enzyme activities related to steroid metabolism were examined of these histologically different tissues.

The following trivial names were used in this text, progesterone, 4-pregnene-3,20-dione; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-dihydrotestosterone, 17β-hydroxy-5α-androstan-3-one; and NADPH, reduced form of nicotinamide adenine dinucleotide phosphate.

## EXPERIMENTAL

### *Separation of interstitial tissue and seminiferous tubules*

Rats of the Wistar strain, 3 months old, bred in this Institute, were sacrificed by decapitation. The testes were removed and placed in a Petri dish with 0·25 M sucrose solution at pH 7·4. The testes were separated into seminiferous tubule fraction and interstitial tissue fraction by the method of Hall *et al.* [5]. The seminiferous tubules were suspended in an ice-cold 0·25 M sucrose solution, and centrifuged at 800 *g* for 20 min. The precipitate was homogenized with a loose-fitting Teflon–glass homogenizer, and homogenates of the seminiferous tubules were employed as the enzyme source. On the other hand, suspension of the interstitial tissue was filtered through double layer of gauze, and unbroken interstitial tissue in the filtrate was homogenized, and used as homogenates of the interstitial tissue.

### *Radioactive steroids*

[4,$^{14}$C]-Progesterone (specific activity, 58·5 mCi/mmol) and [4,$^{14}$C]-17α-hydroxyprogesterone (specific activity, 37·7 mCi/mmol) were purchased from the Radiochemical Centre, Amersham, England. Purities of the radioactive steroids were confirmed by thin layer

chromatography immediately before use. The steroids were diluted with the corresponding non-radioactive preparations, so as to provide appropriate specific activities as substrates for enzyme assay.

*Incubation*

After each radioactive steroid was transferred to an incubation flask, 1 drop of propylene glycol was added to each flask. Shortly before incubation, the solvent was evaporated off under reduced pressure. Incubation mixture generally consisted the substrate steroid (30–32 nmol, 3·2–4·0 × $10^4$ c.p.m.), enzyme preparation in 0·25 M sucrose solution (2·0 ml), and NADPH (final concentration, 0·24 mM, Boehringer und Soehne, Mannheim, Germany), and its final vol. was adjusted as 5 ml. The mixture was then incubated at 37°C for 60 min under a mixture of oxygen and carbon dioxide (95:5, v/v).

*Extraction and isolation of the metabolites*

Immediately after the incubation, enzyme reactions were arrested by the addition of methylene dichloride (15 ml) to an incubation flask and the mixture was vigorously shaken to extract the steroids. The mixture was centrifuged at 400 **g** for 10 min, and the methylene dichloride layer was collected. The above procedure was repeated two more times in order to complete the extraction of steroids. The pooled extract was dried with anhydrous $Na_2SO_4$, and evaporated to dryness under reduced pressure. At this stage, 90–98% of the radioactivity initially added was recovered in the extract. An aliquot of the radioactive steroid extract was chromatographed on a thin layer coated with a mixture of silica gels G and GF (4:1, by weight) (Merck A. G., Darmstadt, Germany), using a benzene–acetone (4:1, v/v) solvent system at 15–20°C. After development of the thin layer plate, spots of the carrier steroids on the chromatogram were visualized under U.V. light (wavelength, 254 nm). Radioactive spots were detected by an autoradiographic method by exposing a sheet of X-ray film to the chromatogram for one week, and also by a windowless gas-flow counter with a scanning device (Vanguard Autoscanner 880 and 885, T.M.C., North Haven, Conn.). The detected spots of steroids were scraped off from the thin layer plate, packed as a small column, and steroids were extracted from the absorbent with a mixture of chloroform–ethanol (1:1, v/v).

*Identification of the metabolites*

Among the metabolites employed for the measurement of enzyme activities related to steroid transformation, 17α-hydroxyprogesterone, androstenedione and testosterone were identified according to our previous method [6], and 17α,20α-dihydroxy-4-pregnen-3-one was identified essentially by the same procedure as previously reported [7, 8].

*Quantitation of the metabolites*

Suitable aliquots of the extracts were evaporated to dryness in 20 ml glass vials (Wheaton Glass Co., Millville, N.J.) and dissolved in 11 ml of the toluene solution which contained 2,5-diphenyloxazole (PPO, 0·4%) and 1,4-bis-2-(5-phenyloxazolyl)benzene (POPOP, 0·01%). The radioactivity was measured with a liquid scintillation spectrometer (System 725, Nuclear-Chicago, Des Plaines, Ill.) for a time sufficient to reduce the counting error below 5%. The efficiency of counting $^{14}C$ was about 70%. The amounts of products were estimated in nmol by dividing the recovered radioactivities in products by the specific activities of the substrate administered.

*Measurement of protein*

Protein concentrations of the enzyme preparations, suitably diluted, were determined by the copper–Folin method [9], using a 1 cm light path at 750 nm.

*Histological examination*

Separated interstitial tissue and seminiferous tubules were centrifuged, then fixed with methanol and stained by the Giemsa's solution (Merck, A. G., Darmstadt, Germany).

## RESULTS

*Purities of the interstitial tissue and seminiferous tubules*

When isolated seminiferous tubules were examined under a light microscope, the surface of the tubules seemed smooth. But the fraction contained also the germinal cells which were squeezed out of broken tubules (Fig. 1). The interstitial tissue fraction used in this experiment was slightly contaminated with germinal cells, which was derived from the seminiferous tubules (Fig. 2).

*Distribution of 17α-hydroxylase*

[4,$^{14}$C]-Progesterone (32 nmol, 4 × $10^4$ c.p.m.) was incubated with the homogenates of seminiferous tubules (54 mg of protein) and interstitial tissue (38 mg of protein) in the presence of NADPH for 60 min, respectively. The conversion from progesterone to 17α-hydroxyprogesterone, androstenedione, and testosterone by both the fractions is shown in Table 1. About 80% of the progesterone added as the substrate was consumed by the interstitial tissue, and 17α-hydroxyprogesterone was obtained as the main product. When progesterone was incubated with the homogenate of

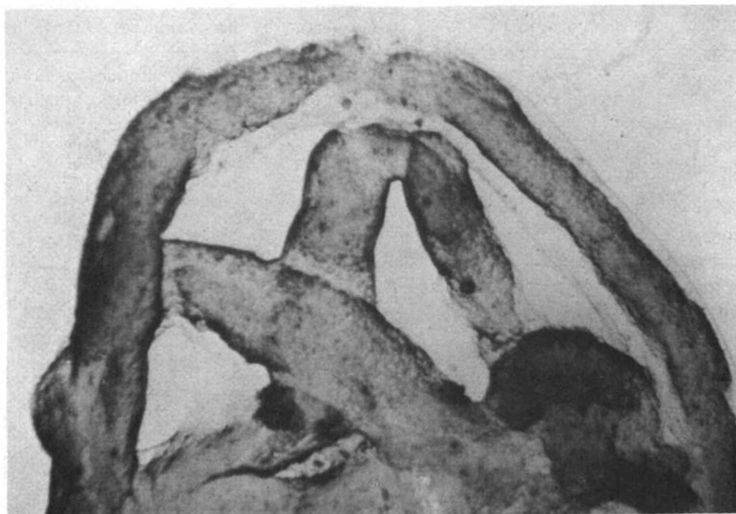

Fig. 1. Micrograph of a piece of seminiferous tubules that was isolated manually from the interstitial tissue ($\times$ 84).

the isolated seminiferous tubules, the major part of the substrate initially added was recovered unchanged. When the 17α-hydroxylase activity was defined as the sum of 17α-hydroxyprogesterone, androstenedione, and testosterone formed from the progesterone used as the substrate, specific activity of the 17α-hydroxylase in the interstitial tissue fraction was about 9 times as high as the one in the seminiferous tubule fraction.

*Distribution of $C_{17}$–$C_{20}$ lyase and 20α-hydroxysteroid dehydrogenase*

[4,$^{14}$C]-17α-Hydroxyprogesterone (30 nmol, $3{\cdot}2 \times 10^4$ c.p.m.) was incubated with the homogenates of the two testicular tissue fractions. By the interstitial tissue fraction, 17α-hydroxyprogesterone was mainly transferred to testosterone, but no 17α,20α-dihydroxypregn-4-en-3-one was produced. On the other hand, 17α-hydroxyprogesterone was directly converted by seminiferous tubules to 17α,20α-dihydroxypregn-4-en-3-one, but metabolized to androstenedione and testosterone in no significant amount. The $C_{17}$–$C_{20}$ lyase activity was defined as the sum of androstenedione and testosterone converted from the substrate, or 17α-hydroxyprogesterone, and the 20α-hydroxysteroid dehydrogenase activity was expressed as the yield of 17α,20α-dihydroxy-4-pregnen-3-one from the 17α-hydroxypro-

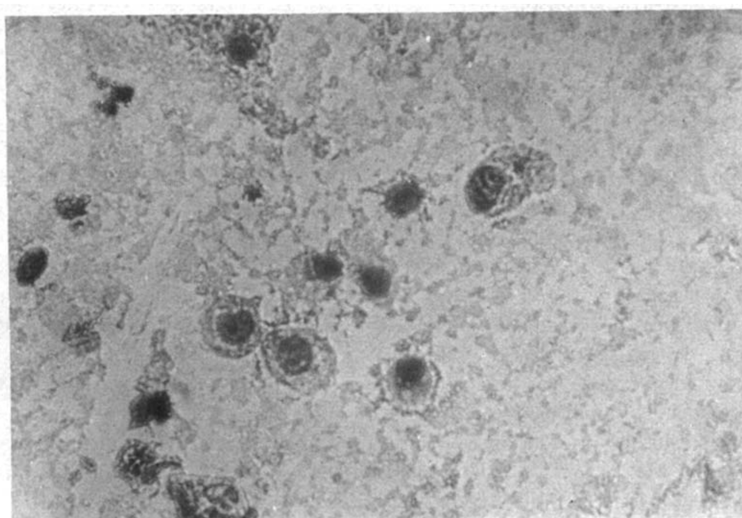

Fig. 2. Micrograph of interstitial tissue after separation of it from the seminiferous tubules ($\times$ 840).

Table 1. Metabolism of progesterone and 17α-hydroxyprogesterone by the seminiferous tubules and interstitial tissue

| | Interstitial tissue (38 mg of protein) | Seminiferous tubules (54 mg of protein) | Ratio* |
|---|---|---|---|
| Progesterone (substrate recovered) | 7·2† | 28·7 | |
| 17α-Hydroxyprogesterone | 11·3 | 1·2 | |
| Androstenedione | 1·5 | 1·2 | |
| Testosterone | 4·5 | 0·5 | |
| 17α-Hydroxylase activity‡ | 0·483 | 0·054 | 9·0 |
| 17α-Hydroxyprogesterone (substrate recovered) | 6·7 | 17·0 | |
| Androstenedione | 1·8 | 0·4 | |
| Testosterone | 12·3 | 1·7 | |
| 17α,20α-Dihydroxy-4-pregnen-3-one | 0·8 | 4·7 | |
| $C_{17}$–$C_{20}$ Lyase activity‡ | 0·368 | 0·039 | 9·4 |
| 20α-Hydroxysteroid dehydrogenase activity‡ | 0·022 | 0·089 | 0·3 |

* Rate of enzyme activity in interstitial tissue to the one in seminiferous tubules.
† nmol.
‡ nmol of products/mg of protein.

gesterone. The $C_{17}$–$C_{20}$ lyase activity was concentrated in the interstitial tissue 9·4 times more than the one in the semimiferous tubules (Table 1). On the other hand, the 20α-hydroxysteroid dehydrogenase was relatively more concentrated in the seminiferous tubules than in the interstitial tissue fraction.

## DISCUSSION

It is widely accepted that interstitial tissue of mammalian testis is the principal locale of androgen production by the following biochemical and histological evidences. (i) By local X-irradiation of 1000 R to the testes of rats, spermatogenesis was completely damaged in one month post-irradiation, but interstitial tissue and Sertoli cells remained histologically unchanged. The weights of seminal vesicles and ventral prostates of X-irradiated rats were found practically unchanged in comparison with those non-irradiated animals [1], suggesting that over all production and secretion of androgens were not significantly changed *in vivo* due to the X-irradiation. (ii) When human chorionic gonadotrophin was administered to a rat whose testicular glands were locally X-irradiated at 1000 R, the interstitial tissue showed specific hypertrophy, but no remarkable histological change was observed in the Sertoli cells. By biochemical analysis, the enzyme activities related to testosterone production from pregnenolone in the testes were found enhanced by the treatment of the gonadotrophin [1]. (iii) By the experimental bilateral cryptorchidism, the seminiferous tubules showed marked atrophy due to degeneration of cells in seminiferous epithelium, but Sertoli cells and interstitial tissue seemed histologically more resistant than spermatogenic cells to the elevated temperature. Specific activities of the enzymes related to production of testosterone from progesterone in testes increased by cryptorchidism 2–3 fold as much as the normal level, suggesting that these enzymes were located in the heat-resistant cells of testes [10]. (iv) After manual separation of the interstitial tissue from the seminiferous tubules of rat testicular tissue, progesterone was more efficiently converted to androgens by interstitial tissue than by seminiferous tubule fraction. From the above experimental results, the interstitial tissue was found to be primarily the site of androgen production, while the seminiferous tubules showed some activity of androgen production from progesterone *in vivo* [4] and the tissue culture [11]. In this connection, an interesting method has been reported for preparation of interstitial tissue fraction by the filtration of testicular tissue suspension of guinea pig testes through nylon monofilament mesh [12]. The interstitial tissue fraction thus prepared contained enriched activity of 17α-hydroxylase and histologically a number of interstitial tissue and cellular aggregates, with some erythrocytes and bits of cellular debris, but no tubular fragments. Distributions of the activities of the 17α-hydroxylase and $C_{17}$–$C_{20}$ lyase between the seminiferous tubules and interstitial tissue in the present experiment were agreeable with the previous finding as referred above.

Bell *et al.* [13] reported that progesterone was readily converted to androgen and reduced C-21 compounds by the seminiferous tubules of rat testes which

were separated from the adjacent interstitial tissue by dissection. They have proposed that the capacity of the Sertoli cells to produce androgens may be at least equal to that of the interstitial tissue, and 20α-hydroxysteroid dehydrogenase was localized in the Sertoli cells of rat testes. In the present experiment, the 20α-hydroxysteroid dehydrogenase was markedly concentrated in the seminiferous tubules. On the other hand, by histochemical examination, the 20α-hydroxysteroid dehydrogenase was selectively located in the interstitial tissue of the human testes [14]. From the previous results, the 20α-hydroxysteroid dehydrogenase of rat testes was located in the radio-[7] and heat-[10] resistant cells which were insensitive to human chorionic gonadotrophin [1, 15]. Also, the 20α-hydroxysteroid dehydrogenase would play a role of regulating androgen production in the following manner: 17α,20α-dihydroxy-4-pregnen-3-one which was produced from 17α-hydroxyprogesterone by the 20α-hydroxysteroid dehydrogenase was found as the competitive inhibitor in the course of conversion of 17α-hydroxyprogesterone to androstenedione by the microsomal $C_{17}$–$C_{20}$ lyase in the interstitial tissue [16]. It has been suggested that spermatogenesis can be maintained by the direct action of the androgen [17, 18] and 5α-dihydrotestosterone is the active form of androgen in the target organs [19, 20]. The $\Delta^4$-5α-hydrogenase was demonstrated in the seminiferous tubules of immature and mature rat testes [21, 22], but the conversion to the 5α-hydrogenated products from progesterone and 17α-hydroxyprogesterone was not detected in these experiments.

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