Reprinted from ENDOCRINOLOGY
Vol. 79, No. 2, August, 1966
pp. 406-410

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PRINTED IN U.S.A.

Cytochemical Study of Δ^5-3β-Hydroxysteroid Dehydrogenase in Testicular Cells Grown *in Vitro*[1]

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ABSTRACT. Cytochemical reaction for Δ^5-3β-hydroxysteroid dehydrogenase was studied in monolayers of testicular cells grown *in vitro*. Cells in cultures established from testicular tissue of immature rats were essentially negative for Δ^5-3β-hydroxysteroid dehydrogenase. Cells in cultures established from testicular tissue of similar animals pretreated with human chorionic gonadotropin (HCG) were strongly positive for Δ^5-3β-hydroxysteroid dehydrogenase. Addition of HCG to the culture medium failed to induce a positive reaction for this enzyme. These studies show that testicular cells which may be active in steroid biosynthesis can be grown *in vitro*. (*Endocrinology* **79:** 406, 1966)

IN THE COURSE of investigation designed to study the *in vitro* behavior of dissociated rat testicular cells, it was observed that some cells attach to the glass surface of the culture vessels, multiply and form a monolayer while others remain free-floating in the medium (1). The unattached cells were found to be predominantly germinal elements, spermatids, spermatocytes and spermatogonia. Microscopic study of the growing monolayer, using phase microscopy and bright light examination of stained monolayers or histologic sections of pellets prepared from the monolayers, failed to provide adequate information for positive identification of these cells. Wattenberg (2) and Baillie *et al.* (3) demonstrated that, under specific conditions, positive cytochemical reaction for Δ^5-3β-hydroxysteroid dehydrogenase is limited in the testes to the Leydig cells. This cytochemical reaction was utilized in the present study to characterize the testicular cells growing in culture in the form of monolayers.

Received February 24, 1966.
This work was supported in part by USPHS Grants HD 00399 and HD 01039.

[1] A preliminary account of this work was presented at the 47th Meeting of The Endocrine Society, 1965.

Materials and Methods

In the normal adult testes, only about 6% of the total weight of testicular tissue is contributed by Leydig cells (4). To enrich the testicular tissue with Leydig cells, 10-day-old male rats were injected subcutaneously with 50 IU human chorionic gonadotropin (HCG)[2] twice daily for a period of 10 days. Following this treatment, the animals were sacrificed and their testes were used to initiate cultures. A second group of cultures was derived from testicular tissue of intact 20-day-old rats. Half the cultures in both groups were grown in medium containing 5 IU/ml of HCG.

Tissue culture technique. Testicular tissue stripped of the tunica albuginea was minced into fragments less than 1 mm^3 in size and incubated in 0.25% trypsin in phosphate buffer pH 7.2 for 10–15 min at 31 C. Undigested tissue particles were allowed to settle out by gravity and the supernatant fluid containing predominantly single cells was spun at 900 rpm for 5 min. The cells were resuspended in culture medium and counted in a hematocytometer. Petri dishes, containing glass coverslips, were seeded with 12 ml of cell suspension containing 6 to 8×10^5 cells/ml and incubated for 10 days at 31 C in an atmosphere of 5% CO_2 and 95% air. The culture medium was maintained at pH 6.9–7.1 and was changed on the seventh day of incuba-

[2] Follutein, generously supplied by Dr. Aleck Borman of the Squibb Institute for Medical Research, New Jersey.

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tion. The composition of the culture medium has been described in detail elsewhere (5).

Cytochemical detection of Δ^5-3β-hydroxysteroid dehydrogenase. Coverslips, bearing the cells, were rinsed in Hanks balanced salt solution, frozen on dry ice for a few minutes and dried in a stream of cold air. The cytochemical reaction was carried out at 37 C in a solution containing 2 mg substrate, 5 ml propylene glycol, 10 mg tetranitro-blue tetrazolium, 20 mg nicotinamide, 25 mg diphosphopyridine nucleotide, 40 ml of 1/15M phosphate buffer (pH 7.2) and 25 ml distilled water. Incubation time of 100 min was selected as optimal on the basis of preliminary tests. Dehydroepiandrosterone, pregnenolone and 17α-hydroxypregnenolone were used as substrates. Following incubation the coverslips were rinsed in 1/15M phosphate buffer pH 7.2 and mounted with glycerol-gelatin. To check specificity of the reaction, several coverslips were incubated simultaneously in medium lacking the substrate. Several coverslips were rinsed in acetone prior to incubation, to remove

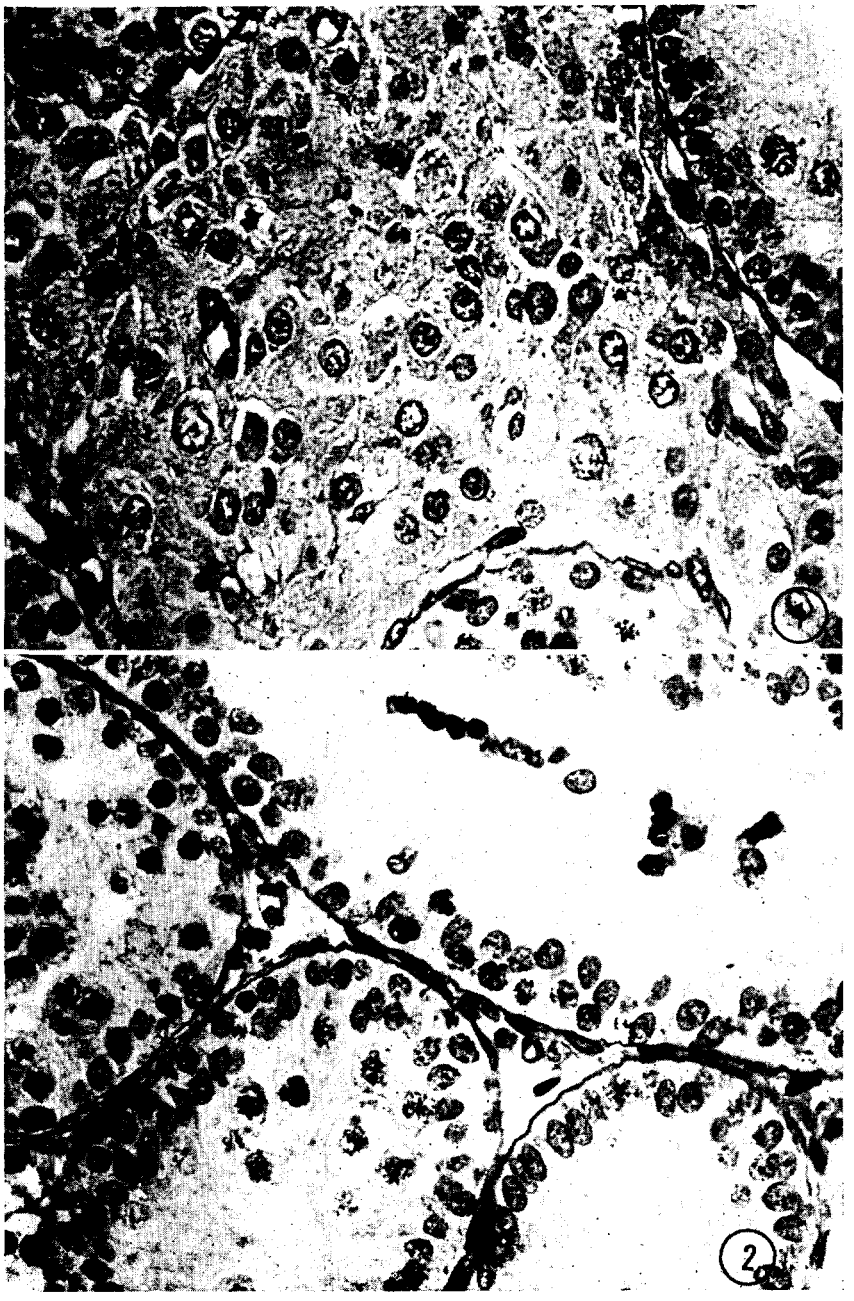

FIG. 1 and 2(×560). FIG. 1. Testis of a 20-day-old rat treated with HCG. Note hyperplasia of interstitial cells. FIG. 2. Testis of an untreated 20-day-old rat.

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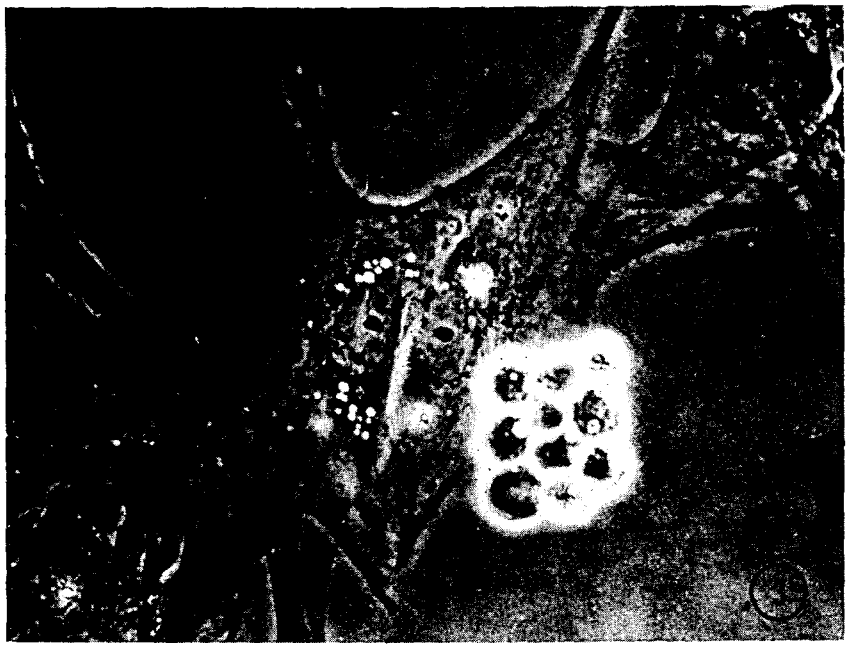

FIG. 3(×560). Four-day culture of testicular cells. Note clump of round, free-floating cells (germinal elements) and growth of cells on the glass surface.

free lipids. Frozen sections of adult rat testes served as positive controls.

Results

The histologic appearance of testicular tissue used to initiate the cultures is illustrated in Fig. 1 and 2. Testes obtained from animals treated with HCG show a marked preponderance of Leydig cells (Fig. 1) when compared to those from untreated animals (Fig. 2). However, the morphologic appearance of cultured cells originating from treated animals was similar to that of cultures from untreated animals. The cells were either spindle-shaped or had numerous long cytoplasmic processes. The nuclei contained from one to four nuclei of various sizes. Prior to change of medium, individual germ cells or their clusters were seen settled on the glass, but they never attached to the glass (Fig. 3). The dividing cells formed a loose network which, by the tenth day of growth, covered most of the glass surface. Presence of HCG in the medium caused the cultured cells to round up, producing numerous "holes" in the monolayer.

Δ^5-3β-Hydroxysteroid dehydrogenase in cultures of testicular cells of testes from rats treated with HCG. A total of 99 cultures from four separate experiments was examined. Most cells, whether grown with or without HCG in the culture medium, showed strongly positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase (Fig. 4). No significant difference was observed in intensity of the reaction or in the proportion of cells exhibiting the reaction among the three different steroids used as substrates.

Δ^5-3β-Hydroxysteroid dehydrogenase in cultures of testicular cells from untreated rats. In a total of 94 cultures examined, only an occasional cell revealed a weakly positive reaction (Fig. 5). Presence of HCG in the culture medium did not affect the reaction. Again, no qualitative or quantitative difference was observed among the three different substrates.

Discussion

While it is relatively easy to identify the Leydig cells in histologic preparations of rat testes, primarily due to their interstitial location, their identification in dissociated tissue is very difficult. In monolayer cultures of rat testicular cells, the Leydig cells

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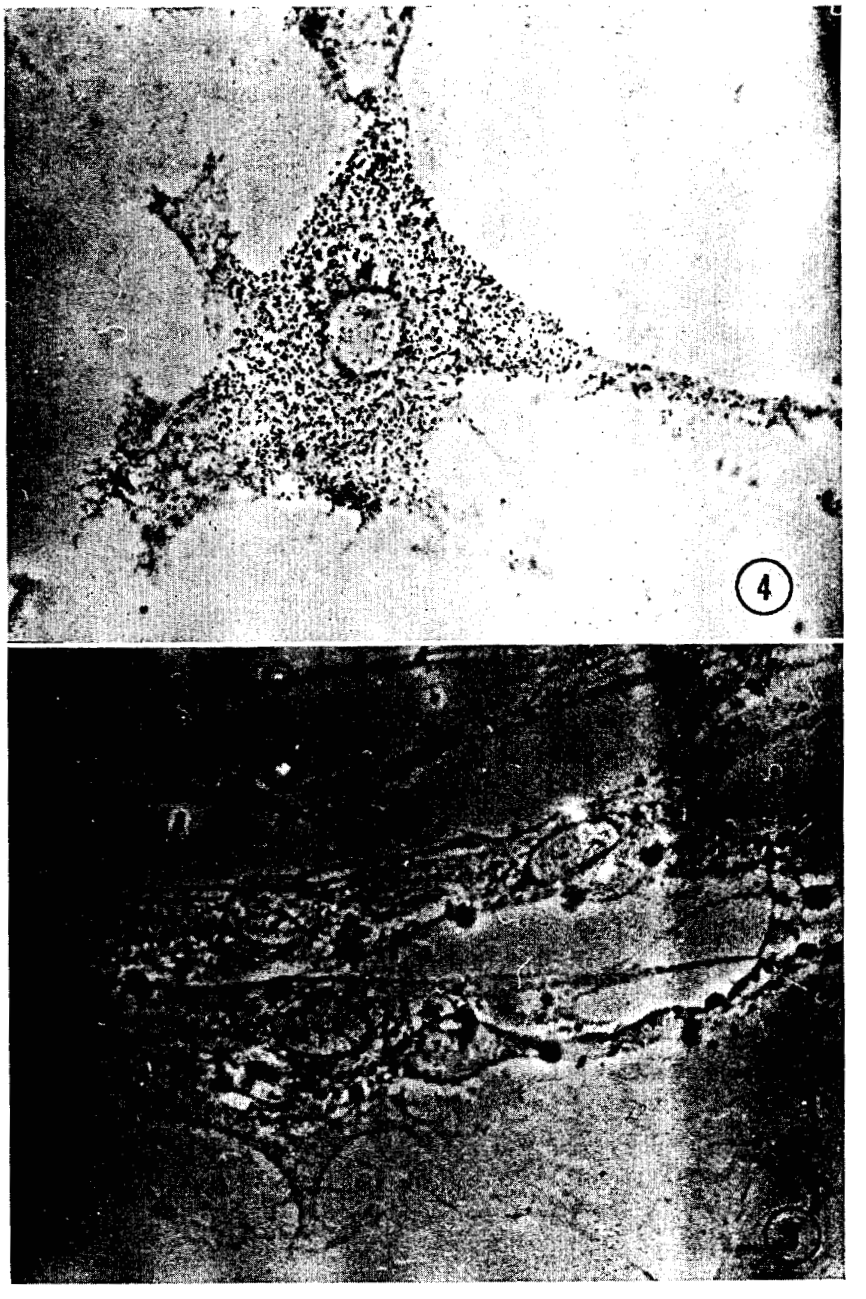

FIG. 4 and 5(×560). FIG. 4. A cytochemical reaction for Δ^5-3β-hydroxysteroid dehydrogenase in a cell from a monolayer derived from testes of rats treated with HCG. Note heavy deposits of formazan in the cytoplasm. FIG. 5. A cytochemical reaction for Δ^5-3β-hydroxysteroid dehydrogenase in a cellular monolayer derived from testes of untreated animals. Note occasional granules of formazan deposit.

could not be identified on a morphologic basis when examined with phase or bright light microscope. The question arose whether Leydig cells failed to survive under the culture conditions employed, or whether their morphologic appearance changed so that they could no longer be identified. Since it has been demonstrated that, in testes of rodents, the cytochemical reaction to Δ^5-3β-hydroxysteroid dehydrogenase appears to be specifically localized in the Leydig cells (2, 6–8), this reaction was employed to study the testicular cells growing in culture in form of a monolayer. The histochemical method utilizing tetranitro-blue tetrazolium as hydrogen acceptor distinctly demonstrates the sites of Δ^5-3β-hydroxysteroid dehydrogenase activity by the presence of abundant blue formazan granules in the cytoplasm of the cells. Removal of lipids by

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rinsing the slides in cold acetone did not change appreciably the characteristic reaction. The reaction was negative in cells incubated in a medium deprived of substrate, except for a few localized areas which showed a very slight reduction of tetrazolium. This is possibly due to the presence of alcohol dehydrogenases (9).

Niemi and Ikonen (8) have shown that, in testes from 21-day-old rats, only occasional interstitial cells show positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase, while in 24-day-old and older animals, in which puberty has set in, the reaction becomes stronger and appears in a majority of the interstitial cells. In monolayer cultures from testes of 20-day-old rats we also observed only an occasional cell showing weakly positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase. However, monolayers which originated from testes of rats pretreated with HCG showed a preponderance of cells strongly positive for Δ^5-3β-hydroxysteroid dehydrogenase. This suggests that the majority of cells in monolayers originating from testes of rats pretreated with HCG may be Leydig cells. Whether the monolayers derived from testicular tissue of untreated rats are predominantly composed of Leydig cell precursors which have not differentiated sufficiently to give a positive reaction, or whether they are composed of fibroblasts, cannot be determined at this time. The fact that, by *in vitro* treatment with HCG, we were unable to induce these cells to give a positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase does not rule out the possibility that they are Leydig cell precursors. Samuels and Helmreich (6) were also unable to induce an increase of Δ^5-3β-hydroxysteroid dehydrogenase activity in testicular slices from hypophysectomized animals incubated with chorionic gonadotropin, *in vitro*. The reasons for the failure of HCG to induce Δ^5-3β-hydroxysteroid dehydrogenase activity *in vitro* may be manyfold. It is doubtful that it is a function of time since the cultures were grown in the presence of HCG for ten days, similar to the time of *in vivo* treatment which was sufficient to cause a marked hypertrophy and hyperplasia of Leydig cells in immature testes. The possibility of toxic effect of HCG was considered, since the cells cultured in its presence did not appear to spread on glass as well as those grown without the hormone. However, presence of HCG in the culture medium did not seem to interfere with the positive reaction given by the cells originating from treated animals. The present study provides no clue to explain the failure of HCG to stimulate cells *in vitro* to give a positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase.

The results reported in this paper indicate that cultures of Leydig cells can be established *in vitro* and that, after ten days of growth and numerous cell divisions, these cells retain the ability to elicit a positive reaction for Δ^5-3β-hydroxysteroid dehydrogenase activity. If one assumes that this enzyme is necessary for steroidogenesis, and is found only in steroid-synthesizing cells, the ability to grow such cells in pure culture may offer a unique system for an *in vitro* study of steroid biosynthesis.

Acknowledgment

Acknowledgment is given to Mrs. J. E. Weisenfeld for her capable assistance in preparation of the cultures.

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