J. Steroid Biochem. Molec. Biol. Vol. 38, No. 4, pp. 523–531, 1991
Printed in Great Britain. 

0960-0760/91 $3.00 + 0.00


# REGULATION OF TESTICULAR DIFFERENTIATION AND TESTOSTERONE PRODUCTION IN THE FETAL MOUSE GONAD *IN VITRO*

TERUKO TAKETO,* JAMILAH SAEED, SYLVIE ROBERGE,† NOBORU MATSUO[1]‡ and SAMUEL S. KOIDE[1]
McGill University, Royal Victoria Hospital, Montreal, Canada H3A1A1 and
[1]The Population Council, New York, NY 10021, U.S.A.

*(Received 18 May 1990)*

**Summary**—The objective of the present study was to develop a chemically-defined medium in which early stages of testicular differentiation can be investigated in an organ culture system. Mouse gonadal primordia were explanted before and after initiation of morphological sex differentiation, i.e. 11 and 12 day of gestation (d.g.), respectively. We found that a combination of human albumin fraction, insulin (or IGF-I), and sodium pyruvate promoted testicular organization of gonadal explants of 11 d.g., but not those of 12 d.g. Insulin also increased the production of testosterone from testicular explants of 11 d.g., but not those of 12 d.g. For the younger explants, progesterone was more efficient than pregnenolone as a steroid precursor during the first day of culture, but the maximum effect of pregnenolone was much higher than that of progesterone in later stages. The responsiveness to human chorionic gonadotropin increased gradually along with testicular organization. The addition of either serum or pregnenolone prominently increased the activity of Δ5–3β hydroxysteroid dehydrogenase in testicular explants of 11 d.g., but not the number of positive cells as demonstrated by histochemical staining. These results suggest that insulin (or IGF-I) is required during the initial phase of testicular organization, which is reflected by an increase in testosterone production and sensitivity to gonadotropins.

## INTRODUCTION

Testosterone is an important steroid hormone produced by testes throughout gonadal development. The regulation of testosterone production has been well studied in postnatal and adult rats and, to a less degree, in fetal rats [1–3]. Although it has been suggested that testosterone is synthesized as early as the initial stage of testicular differentiation [4], its regulation at these early stages is not fully understood. We have been studying gonadal sex differentiation in the mouse, and found that fetal mouse testes secrete significant amounts of testosterone in culture despite their small size [5, 6]. Hence, the culture of fetal mouse gonads should provide an excellent system to study the role of hormones in testicular organization.

We have previously demonstrated that the mouse gonadal primordium explanted on 11 day of gestation (d.g.) does not initiate testicular differentiation *in vitro* unless the culture medium is supplemented with serum [7]. Once the development of the testicular primordium has advanced beyond a critical stage, equivalent to 12 d.g., it proceeds in minimum essential medium [5]. These results suggest that certain serum components are essential for the initiation of testicular differentiation *in vitro*. In our preliminary experiments to identify these essential serum components, we found that the components required for survival of germ cells in the testis cord are different from those for organization of the testis cord in the mouse [7]. We have shown that the low density lipoprotein fraction of human plasma possesses the activity to preserve germ cells [7]. The serum components required for testis cord organization remain to be identified.

It has been reported, on the other hand, that fetal calf serum impairs testicular organization of rat fetal testes in culture [8–10]. Their results differ from ours about the possible role of certain serum components in promoting testicular organization. It is conceivable that serum contains both promoting and inhibiting components for testicular organization, and that gonadal

*Author whom all correspondence should be addressed.
†Present address: Animal Science Department. Washington State University, Pullman, WA 99164-6332, U.S.A.
‡Present address: Kao Corp., Tochigi Research Laboratory, Tochigi, Japan.

primordia from different species or at different developmental stages may have varying sensitivities to both serum components. We found, in fact, that whole horse serum is inhibitory to the development of the fetal mouse testis explanted on 12 d.g. although to a much less extent than that reported for rat testicular explants (Taketo *et al.* unpublished data) [8, 10]. It is, therefore, important to develop a chemically defined medium not only for simplifying culture conditions but also promoting testicular development *in vitro*.

The purpose of the present study was to determine the combination of media components that promote testicular organization of the gonadal primordium explanted on 11 d.g., and to examine the effect of these hormones on the production of testosterone from gonadal explants. The results suggest that insulin (or IGF-I) is required during the initial phase of testicular organization, which is reflected by an increase in testosterone production and sensitivity to gonadotropins.

## EXPERIMENTAL METHODOLOGY

### *Chemicals*

Eagle's minimum essential medium (MEM), insulin, sodium pyruvate, penicillin G sodium, and streptomycin sulfate were purchased from GIBCO (Grand Island, N.Y., U.S.A.). Human serum albumin fraction was a gift from Dr Hao (New York Blood Center, N.Y., U.S.A.). Human growth hormone (hGH), fibroblast growth factor (FGF), and ceruloplasmin were donated by Dr J. Mather (Genetech, Calif., U.S.A.). Transferrin, epidermal growth factor (EGF) and selenous acid were purchased from Collaborative Research (Lexington, Mass., U.S.A.). Testosterone (T), progesteron (P4), pregnenolone (P5), follicle stimulating hormone (FSH), human chorionic gonadotropin (hCG), retinoic acid, retinol (vitamin A), ascorbic acid (vitamin C), $\alpha$-tocopherol (vitamin E), prostaglandin E1 (PGE1), prostaglandin F2$\alpha$ (PGF2$\alpha$), and somatostatin were purchased from Sigma (St Louis, Mo., U.S.A.). Hydrocortisone sodium succinate was purchased from Upjohn Co. (Kalamazoo, Mich., U.S.A.). Insulin-like growth factor-I (IGF-I) was purchased from Amersham Chem. (Oakville, Ont., Canada). Reagents for testosterone radioimmunoassay were purchased from RSL (Carson, Calif., U.S.A.).

### *Materials*

The mouse strain, Rockefeller University NCS (Swiss) was used throughout this study. For mating, three females were placed with a male in each cage overnight, and the presence or absence of copulation plugs was examined the next morning. The day when copulation plugs were found was designated as 0 d.g. Pregnant mice were sacrificed at either 11 or 12 d.g., and bilateral gonadal primordia with adjacent mesonephroi were immediately dissected from the fetuses. We have previously reported that all gonadal explants of 11 d.g. differentiate into either testes or ovaries within 5 days of culture in serum-containing media as ascertained by their chromosomal sex [7]. Hence, the sex of gonadal explants of 11 d.g. was identified after 7 days of culture. The sex of gonadal explants of 12 d.g. was indentified before culture by the presence or absence of testis cords under a dissecting microscope with transillumination [7].

### *Organ culture*

Each gonadal explant was cultured on a Nucleopore filter floating on 0.5 ml culture medium as described previously [11]. The basic culture medium was MEM containing Earle's balanced salts, 50 IU/ml penicillin G sodium, and 50 $\mu$g/ml streptomycin sulfate.

To evaluate the effect of an exogenous hormone on testicular organization, six pairs of gonadal explants of 11 d.g. were cultured for 7 days in one experiment. For each pair, one gonad was used as control (10% horse serum or a basic defined medium), and the other for testing the effect of hormones. Steroid hormones were dissolved at concentrations of 1–10 mg/ml in ethanol, and diluted to the final concentrations in the culture medium, which did not contain more than 1% ethanol. At the end of the culture period, serial sections from each pair of testicular explants were histologically examined to determine the progression of testicular organization. The experiments were repeated until, at least, 5 pairs of male gonadal explants in one condition were obtained. When more than half of the explants developed prominently better testicular structures than the control, the result was considered to indicate a promoting effect by the hormone.

To evaluate the effect of exogeneous hormones on testosterone production, gonadal explants were cultured in the basic medium supple-

mented with hormones for 3 days with a daily change of culture medium. The explants of 11 d.g. were further cultured in MEM supplemented with 10% horse serum for 4 more days. After culture, the gonadal sex was morphologically identified, and the data from testicular explants were analyzed. The spent culture media were placed in capped glass tubes and stored at −20°C until radioimmunoassay for testosterone was performed.

*Histological analysis*

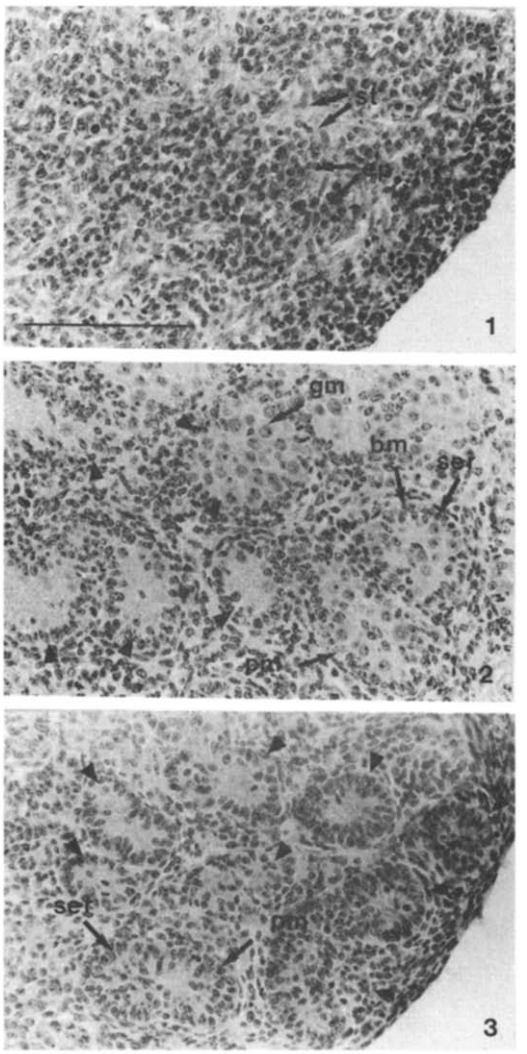
Gonadal explants were fixed in a freshly prepared mixture of acetic acid and absolute enthanol (1 : 3 v/v) for 4 h, embedded in paraffin, sectioned serially at 5 $\mu$m thickness, stained with hematoxylin and eosin, and observed with a light microscope (Zeiss, Axiophot).

*Histochemical staining for Δ5-3β-hydroxysteroid dehydrogenase (3β-HSDH)*

Gonadal explants were embedded in O.C.T. medium (Tissue-Tek) in liquid nitrogen, and serial sections of 10 $\mu$m thickness were cut with a cryostat. After air-drying on microscope slides overnight, sections were incubated in Levy's solution using dehydroepiandrosterone as a substrate [12]. Every 10th section was further stained with hematoxylin and eosin.

*Radioimmunoassay for testosterone*

Contents of testosterone (T) in the culture medium were determined by radioimmunoassay using [$^{125}$I]testosterone, rabbit antiserum against testosterone-19-carboxymethylether-BSA, and goat anti-rabbit gamma globulins. Cross-reactivity of the antiserum with 5α-dihydrotestosterone was 3.4%. The limit of sensitivity of testosterone assay was 0.1 ng/ml. Statistical analysis was performed using Student's *t*-test. The content of T was expressed as ng/ml culture medium. Since the size of fetal testis is consistent at each developmental stage, we did not determine the weight or protein concentration of each gonad.




Figs 1–3. Testicular explants of 11 d.g. after culture for 7 days in MEM supplemented with various hormones. The bar indicates the scale of 0.1 mm.

Fig. 1. MEM alone. Testis cord organization is absent although clusters of epithelial cells (ep) are seen among stromal cells (st).

Fig. 2. With 10% horse serum. Testis cords (indicated by arrowheads) are well organized, composed of fetal Sertoli cells (ser) along the basement membrane (bm) and germ cells (gm) in the center. Peritubular myoid-like cells (pm) differentiated outside the basement membrane.

Fig. 3. With 5 mg/ml albumin, 10 $\mu$g/ml insulin, and 110 $\mu$g/ml sodium pyruvate. Testis cords (indicated by arrowheads) are well organized, composed of fetal Sertoli cells (ser) and peritubular myoid-like cells (pm). Germ cells are absent.

## RESULTS

*Effects of hormones on testicular organization*

Figures 1–3 show representative structures of testicular explants of 11 d.g. after 7 days of culture. In MEM alone or MEM supplemented with one of the hormones listed in "Experimental Methodology", none of the gonadal explants developed characteristic differentiation structures (Fig. 1). In contrast, in the presence of 10% horse serum, testis cords were well organized in all testicular explants (Fig. 2). In MEM supplemented with both human albumin fraction at 5 mg/ml and insulin at 2–10 $\mu$g/ml, testis cords developed over a large area of gonadal explants.

The testis cord was usually thinner in diameter due to the absence of germ cells (Fig. 3). IGF-I at 1–10 ng/ml showed an effect on testicular organization similar to insulin (data not shown).

We examined whether other components would promote testicular organization from the gonadal explant of 11 d.g. when added to the culture medium supplemented with human albumin fraction and insulin. Addition of sodium pyruvate at a concentration of 110 μg/ml promoted testis cord organization (Fig. 3), whereas transferrin (0.1–0.5 μg/ml), ceruloplasmin (7 IU/ml), hGH (20 μg/ml), FGF (100 ng/ml), selenous acid (1–100 ng/ml), ascorbic acid (100 μg/ml), α-tocopherol (10 μg/ml), PGE1 (0.5 ng/ml), PGF2α (0.25 ng/ml), hydrocortisone (1–100 ng/ml), FSH (0.4–40 μg/ml), and somatostatin (0.01–1 μg/ml) showed a very weak effect, if any (data not shown). T (1–20 ng/ml), P4 (0.01–1 μg/ml), and P5 (0.01–0.1 μg/ml) promoted testicular organization in some series of experiments, but not in others. These steroid hormones also often increased the number of germ cells in the testis cord. Some hormones were inhibitory for testis cord organization at high concentrations, i.e. insulin at 200 μg/ml, EGF at 100 ng/ml, retinoic acid at 5 μg/ml, and retinol at 10 μg/ml.

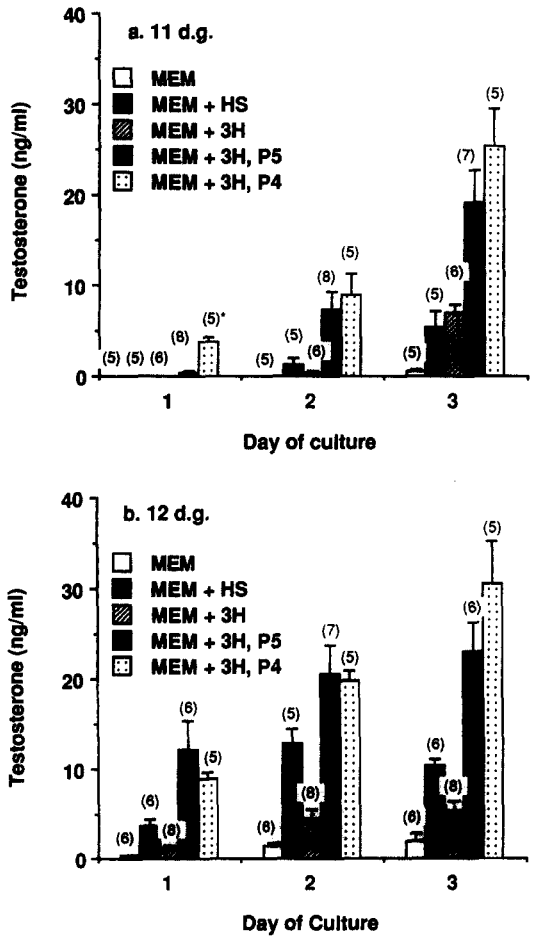



Fig. 4. T production from testicular explants of (a) 11 d.g. and (b) 12 d.g. The culture medium was supplemented with 10% horse serum (HS), 3H (human albumin, insulin and sodium pyruvate), 3H with pregnenolone (P5) or progesterone (P4) at 10 μg/ml. Each column indicates the mean value ± SE. The number in parentheses indicates the number of explants examined at each column. *Significantly different ($P < 0.001$) from the value with P5.

*Effects of serum and hormones on T production*

Testicular explants of 11 d.g. did not produce T when cultured in MEM alone for 3 days (Fig. 4a). The addition of 10% horse serum to the culture medium significantly increased T production during the third day of culture. The addition of a mixture of human albumin (5 mg/ml), insulin (10 μg/ml), and sodium pyruvate (110 μg/ml), designated as 3H, also increased T production comparable to the serum-containing medium.

Testicular explants of 12 d.g. cultured in MEM alone produced low but detectable amounts of T during the second and third day of culture (Fig. 4b). Addition of 10% horse serum increased T production throughout the culture period. The addition of 3H increased T production, but to a less degree than 10% horse serum.

*Effects of P4 and P5 on T production*

Since T production from the testicular explant of 12 d.g. was higher in the presence of serum than 3H, we studied whether the addition of T-precursor steroids would promote T production. Both P4 and P5 at 10 μg/ml significantly increased T production by the testicular explant of 11 d.g. except that P5 showed no effect during the first day of culture (Fig. 4a). In contrast, both steroids at 10 μg/ml increased T production from testicular explants of 12 d.g. throughout the culture period (Fig. 4b).

The dose–response of P4 and P5 to T production during the second day of culture was similar between testicular explants of 11 d.g. and those of 12 d.g. (Figs 5a and b). At a concentraion of 1 μg/ml, P4 was slightly more effective than P5 in increasing T production. On the other hand, the maximum effect of P5, at a concentration of 30 μg/ml, was significantly higher than that of P4.

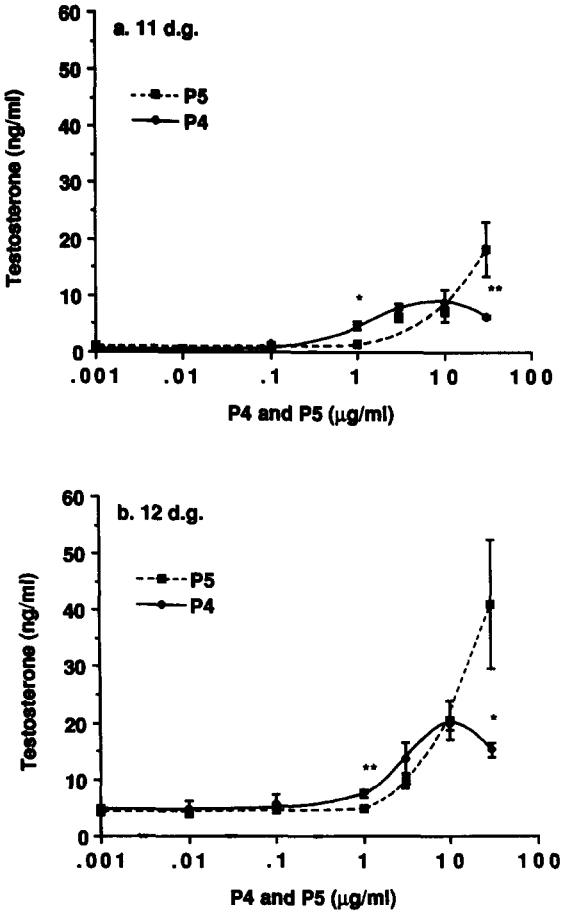



Fig. 5. Dose–response relationship of the effect of P4 and P5 on T production during the second day of culture from testicular explants of (a) 11 d.g. and (b) 12 d.g. The basic medium was MEM supplemented with 3H. P4 or P5 was added to the culture medium from the first day of culture. Each point indicates the mean value ± SE (5–9 explants were examined at each point). * and ** indicate significant differences from the value with P5 ($P < 0.01$ and 0.05, respectively).

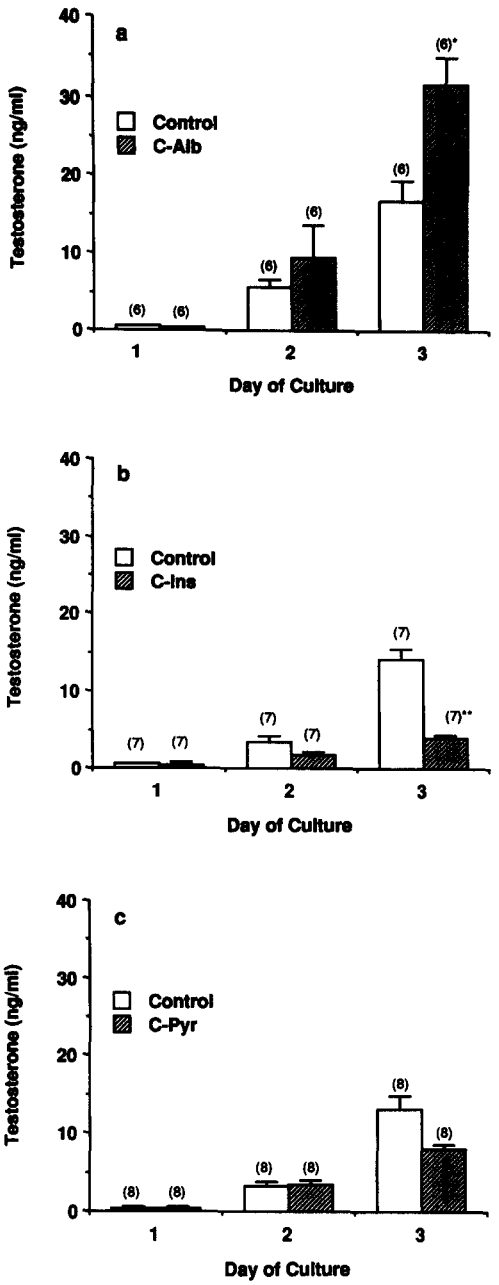



Fig. 6. Effects of omission of (a) human albumin (Alb), (b) insulin (Ins), and (c) sodium pyruvate (Pyr) on T production from testicular explants of 11 d.g. (hatched columns). Control medium (C) was MEM supplemented with 3H and P5 at 10 μg/ml (open columns). Each column indicates the mean value ± SE. The number in parentheses indicates the number of explants examined at each column. * and ** indicate significant differences from the control value ($P < 0.01$ and 0.001, respectively).

### *Effects of human albumin, insulin, and sodium pyruvate on T production*

Control testicular explants of 11 d.g. were cultured in the presence of 3H and P5 at 10 μg/ml. The contralateral explants were cultured in the absence of one of the hormones. The omission of human albumin fraction dramatically increased T production during the third day of culture (Fig. 6a). On the other hand, the omission of insulin reduced T production during the third day of culture (Fig. 6b). The omission of sodium pyruvate also decreased T production similar to insulin although the decrease was not statistically significant (Fig. 6c).

In contrast, T production from testicular explants of 12 d.g. in the presence of P5 at 1, 10, or 30 μg/ml was not influenced by the omission of either insulin or sodium pyruvate (data not shown). Omission of albumin, however, increased T production during the third day of culture more dramatically than that with testicular explants of 11 d.g. (data not shown).

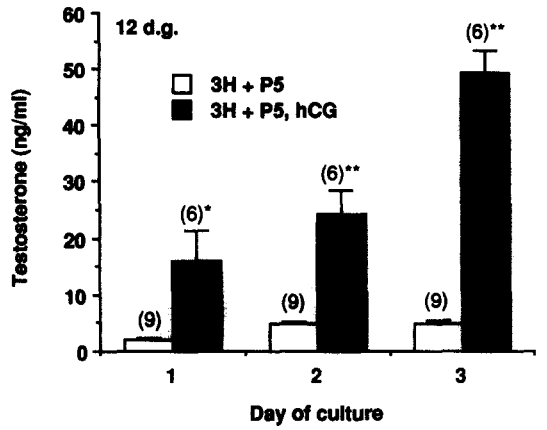



Fig. 7. Effects of hCG on T production from testicular explants of 12 d.g. Basic medium was MEM supplemented with 3H and P5 at 1 $\mu$g/ml (open column). hCG was added at concentration of 0.1 IU/ml (solid column). Each column indicates the mean value $\pm$ SE. The number in parentheses indicates the number of explants examined at each column. * and **, significantly different from the value with 3H + P5 alone ($P < 0.01$ and 0.001, respectively).

*Effects of hCG on T production*

Addition of hCG to the culture medium at a concentration of 0.5 IU/ml did not influence T production from testicular explants of 11 d.g. until the fourth day of culture in the presence of P5 at 10 $\mu$g/ml (data, not shown). On the other hand, addition of hCG at 0.1 IU/ml significantly increased T production from the testicular explant of 12 d.g. in the presence of P5 at 1 $\mu$g/ml (Fig. 7). The dose–response study shows that hCG was effective at the concentration of 0.01 IU/ml, and reached the maximum effect at 0.5 IU/ml (Fig. 8).

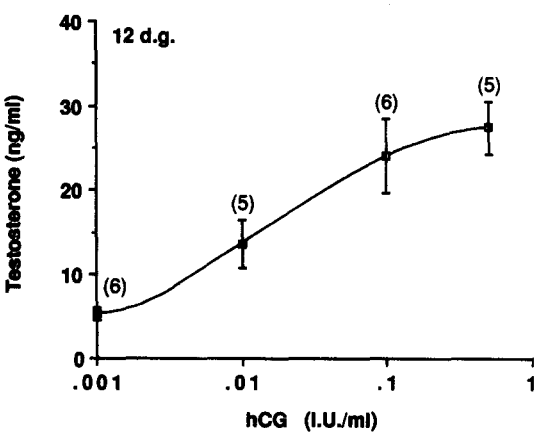



Fig. 8. Dose–response of the effect of hCG on T production from the testicular explant of 12 d.g. during the second day of culture. The basic medium was MEM supplemented with 3H and P5 at 1 $\mu$g/ml. hCG was added from the first day of culture. Each point indicates the mean value $\pm$ SE. The number in parentheses indicates the number of explants examined at each point.

*Effects of hormones on 3$\beta$-HSDH activity*

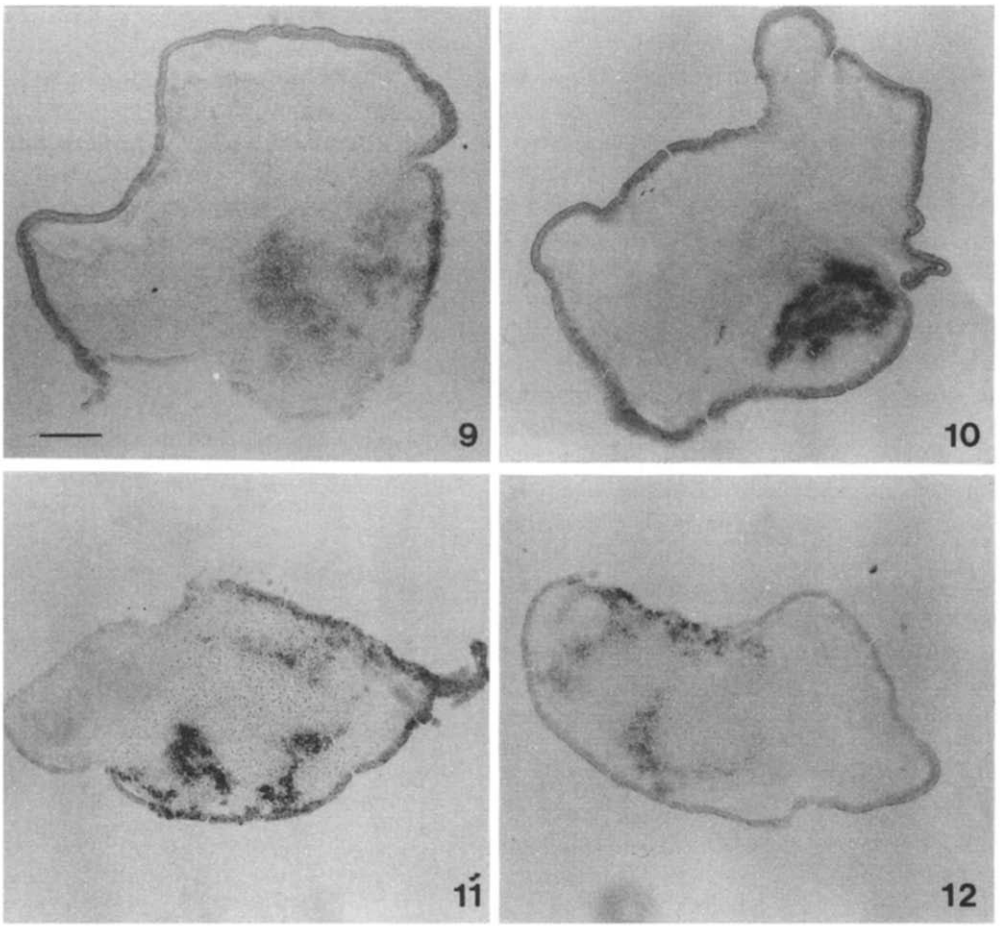
Weak 3$\beta$-HSDH activity was detected in testicular explants of 11 d.g. after culture for 7 days in MEM alone (Fig. 9). The enzymatic activity increased after culture in the presence of either horse serum or 3H with P5 at 10 $\mu$g/ml to a similar level (Figs 10 and 11). The addition of 3H or 3H with hCG at 0.5 IU/ml resulted in a medium intensity of staining (Fig. 12). In all explants examined except for those cultured in MEM alone, the enzymatic activity was distributed in the interstitium surrounded by testis cords and occasionally in the surface epithelium in the vicinity of the testis cord. The area of staining did not differ significantly in the presence of various hormones.

## DISCUSSION

The present study suggests that human albumin and IGF-I (or insulin) are the basic serum components required for organization of testis cords *in vitro*. Since effective concentrations of IGF-I were within the physiological range while those of insulin were high, IGF-I is presumably the active component of serum. In the present study, insulin was used as a component of basic medium since a large amount was required.

It has been known that insulin and transferrin are required as replacement for serum in the culture of most cell lines [13]. Thus, our present finding that insulin is essential for testicular organization *in vitro* is not surprising. Various effects of insulin and IGFs on neonatal and adult testes including those on T production have been reported [14–17]. However, in our system, the omission of insulin from the culture medium reduced T production from testicular explants of 11 d.g. whereas it did not influence T production from those of 12 d.g. It is noteworthy that the latter explants can undergo further morphological differentiation independent of these hormones [7]. Therefore, the effect of insulin appears to be critical for promoting the initial phase of testicular differentiation. This observation could be explained firstly, that insulin is required for testicular differentiation but not directly for T synthesis at an early stage of testicular development. Secondly, the fetal testis may produce IGF-I after differentiation and the effect of exogenous IGF-I or insulin may no longer be distinguished. It has been reported that the rat testis synthesizes IGF-I [18, 19].

Transferrin had no effect on testicular differentiation in our system. It is conceivable that




Figs 9–12. Testicular explants of 11 d.g. after culture for 7 days in MEM supplemented with various hormones. Histochemically stained for 3$\beta$-HSDH activity. The bar indicates the scale of 0.1 mm.

Fig. 9. MEM alone. Faint staining is seen over the whole gonadal area.

Fig. 10. With 10% horse serum. Intense staining is seen in the interstitium near the testis cords.

Fig. 11. With 3H and P5 at 10 $\mu$g/ml. Intense staining is seen in the interstitium of the testicular area.

Fig. 12. With 3H and hCG at 0.5 IU/ml. Medium intensity of staining is seen in the interstitium and surface area of the testicular region.

exogenous transferrin is not required since fetal Sertoli cells may produce considerable amounts of transferrin as reported for neonatal rat testes [20]. Nonetheless, it can be concluded that transferrin is not as essential as insulin for the initial phase of testicular differentiation.

The effect of human albumin fraction was contradictory: its addition to the culture medium promoted testicular organization while it suppressed T production. It is possible that the albumin fraction may have a dual action. Since the decrease in T production from the testicular explant of 11 d.g. was not as pronounced as that of 12 d.g., both actions may be nullified with the testicular explant of 11 d.g. Two contradictory activities of the albumin fraction may be attributed to different ingredients of this fraction since albumin is known to bind to small peptides, steroids, and fatty acids. We excluded the possibility that albumin may reduce the amount of free T in the culture medium because the addition of albumin fraction into the radioimmunoassay medium did not change the value of T contents (data not shown). Since the inhibitory effect of albumin fraction was observed only during the third day of culture, it may involve biological process of the active component in the gonadal explant. The albumin fraction used in the present study has been purified from Cohn fraction V, yet not free from minor contaminants. Further characterization of this fraction is necessary to understand its effect on testicular differentiation.

As steroid precursors, both P4 and P5 were effective in increasing T production except that

only P4 increased T production from the testicular explant of 11 d.g. during the first day of culture. It is conceivable that the activity of the enzyme converting P4 into T appears earlier than that converting P5 to P4 during initial stages of testicular differentiation. On the other hand, the maximum effect of P5 during the second day of culture was considerably higher than P4. These results may suggest that the T synthesis pathway via P4 develops early whereas another pathway from P5 to T without involving P4 synthesis may take over while testicular differentiation proceeds. Hence, P5 should be a better precursor of T production than P4, providing optimal conditions for the culture of fetal testes.

It has been known that hCG binds to LH/hCG receptors and stimulates T production from fetal mouse, rat and guinea pig testes [3, 21–23]. In the fetal rat, the increase in hCG/LH receptors correlates well with testosterone secretion *in vivo* and *in vitro* [3, 22]. On the other hand, in the fetal guinea pig, development of testosterone synthesis precedes hCG binding or development of LH responsiveness [21]. Our present results indicate that hCG can promote T production from the fetal mouse testis although the responsiveness appears slightly later than the initiation of T synthesis. This observation agrees with the previous report in the guinea pig. It remains to be determined whether the fetal mouse contains any gonadotropin-like material in early stages of testicular differentiation. It has been reported that gonadotropin-producing cells are not recognizable in the mouse pituitary until the 16th or 17th day of embryonic life [24]. Furthermore, it has been reported that mRNA for the $\beta$-chain of LH is present, whereas mRNA for the $\alpha$-chain common to all gonadotropins is absent in the rat placenta [25–27]. Hence, gonadotropins of maternal origin may play an important role in gonadal differentiation in mammals.

The histochemical staining for 3$\beta$-HSDH suggested that the steroidogenic activity was mainly localized to interstitial cells of the testicular explant. These cells are likely to be Leydig cells. It has been demonstrated that Leydig cells are detectable during the early stage of testicular organization in the mouse [28–30]. Since the number of steroidogenic cells did not apparently change in the presence of various hormones (serum, insulin, hCG, and P5) despite the different levels of T production, these hormones probably influence the enzymatic activity involved in T production but not the proliferation of Leydig cells. However, it is also possible that these hormones affect the differentiation of other cell types which influence Leydig cell functions.

In conclusion, the present results suggest that a combination of human albumin fraction, insulin (or IGF-I), and sodium pyruvate promotes testicular organization *in vitro* during the initial phase of sexual differentiation. In contrast, these hormones play a minor role in the morphological development or T production from testicular explants which have already initiated testicular organization.

*Acknowledgements*—This study was supported by Rockefeller Foundation Grant GA PS 8418 and NICHD Grant HD-12184 to S.S.K., and NICHD Grant HD-18669 and MRC (Canada) Grant MA-9740 to T. T.

## REFERENCES

1. Huhtaniemi I.: Endocrine function and regulation of the fetal and neonatal testis. *Int. J. Devl Biol.* **33** (1989) 117–123.
2. Warren D. W., Dufau M. L. and Catt K. J.: Hormonal regulation of gonadotropin receptors and steroidogenesis in cultured fetal rat testes. *Science* **218** (1982) 375–377.
3. Warren D. W., Huhtaniemi I. T., Tapanainen J., Dufau M. L. and Catt K. J.: Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. *Endocrinology* **114** (1984) 470–476.
4. George F. W., Catt K. J., Neaves W. B. and Wilson J. D.: Studies on the regulation of testosterone synthesis in the fetal rabbit testis. *Endocrinology* **102** (1978) 665–573.
5. Taketo T., Thau R. B., Adeyemo O. and Koide S. S.: Influence of adenosine 3′:5′ cyclic monophosphate analogues on testicular organization of fetal mouse gonads *in vitro*. *Biol. Reprod.* **30** (1984) 189–198.
6. Taketo-Hosotani T., Nishioka Y., Nagamine C. M., Villalpando I. and Merchant-Larios H.: Fertility of the B6.$Y^{DOM}$ sex-reversed female mice. *Development* **107** (1989) 95–105.
7. Taketo T., Seen C. D. and Koide S. S.: Requirement of serum components for the preservation of primordial germ cell in testis cords during early stages of testicular differentiation *in vitro* in the mouse. *Biol. Reprod.* **34** (1986) 919–924.
8. Agelopoulou R., Magre S., Parsavoudi E. and Jost A.: Initial phases of the rat testis differentiation *in vitro*. *J. Embryol. Exp. Morph.* **83** (1984) 15–31.
9. Jost A., Magre S. and Agelopoulou R.: Early stages of testicular differentiation in the rat. *Human Genet.* **58** (1981) 59–63.
10. Magre S. and Jost A.: Dissociation between testicular organogenesis and endocrine cytodifferentiation of Sertoli cells. *Proc. Natn Acad. Sci., U.S.A.* **81** (1984) 7831–7834.
11. Taketo T. and Koide S. S.: *In vitro* development of testis and ovary from indifferent fetal mouse gonads. *Devl Biol.* **84** (1981) 61–66.
12. Levy H. Deane H. W. and Rubin B. L.: Visualization of steroid-3$\beta$-ol dehydrogenase activity in tissues of intact and hypophysectomized rats. *Endocrinology* **65** (1959) 932–943.
13. Barnes D. and Sato G.: Methods for growth of cultured cells in serum-free medium. *Analyt. Biochem.* **102** (1980) 255–270.

14. Adashi E.: Insulin augmentation of testosterone production in a primary culture of rat testicular cells. *Biol. Reprod.* **26** (1982) 270-280.
15. Borland K., Mita M., Oppenheimer C. L., Blinderman L. A., Massague J., Hall P. F. and Czech M. P.: The action of insulin-like growth factors I and II on cultured Sertoli cells. *Endocrinology* **114** (1984) 240–246.
16. Lin T., Haskell J., Vinson N. and Terracio L.: Characterization of insulin and insulin-like growth factor I receptors of purified Leydig cells and their role in steroidogenesis in primary culture: a comparative study. *Endocrinology* **119** (1986) 1641–1647.
17. Mita M., Borland K., Price J. M. and Hall P. F.: The influence of insulin and insulin-like growth factor-I on hexose transport by Sertoli cells. *Endocrinology* **116** (1985) 987–992.
18. Casella S. J., Smith E. P., Van Wyk J. J., Joseph D. R., Hynes M. A., Hoyt E. C. and Lund P. K.: Isolation of rat testis cDNAs encoding an insulin-like growth factor I precursor. *DNA* **6** (1987) 325–330.
19. Ritzén E. M.: Chemical messengers between Sertoli cells and neighbouring cells. *J. Steroid Biochem.* **19** (1983) 499–504.
20. Skinner M. K. and Griswold M. D.: Sertoli cells synthesize and secrete transferrin-like protein. *J. Biol. Chem.* **255** (1980) 9523-9525.
21. Brinkmann A. O. and van Straalen R. J. C.: Development of the LH-response in fetal guinea pig testes. *Biol. Reprod.* **21** (1979) 991–997.
22. Gangnerau M.-N., Funkenstein B. and Picon R.: LH/hCG receptors and stimulation of testosterone biosynthesis in the rat testis: changes during foetal development *in vivo* and *in vitro*. *Molec. Cell. Endocr.* **28** (1982) 499–512.
23. Pointis G. and Mahoudeau J. A.: Responsiveness of foetal mouse testis to gonadotrophins at various times during sexual differentiation. *J. Endocr.* **74** (1977) 149–150.
24. Sano M. and Sasaki E.: Embryonic development of the mouse anterior pituitary studied by light and electron microscopy. *Z. Anat. Entwickl.-Gesch.* **129** (1969) 195–222.
25. Carr F. E. and Chin W. W.: Absence of detectable chorionic gonadotropin subunit messenger ribonucleic acids in the rat placenta throughout gestation. *Endocrinology* **116** (1985) 1151–1157.
26. Tepper M. A. and Roberts J. L.: Evidence for only one $\beta$-luteinizing hormone and no $\beta$-chorionic gonadotropin gene in the rat. *Endocrinology* **115** (1984) 385–391.
27. Wurzel J. M., Curatola L. M., Gurr J. A., Goldschmidt A. M. and Kourides I. A.: The luteotropic activity of rat placenta is not due to a chorionic gonadotropin. *Endocrinology* **113** (1983) 1854–1857.
28. Pehlemann F. W. and Lombard M. N.: Differentiation of ovarian and testicular interstitial cells during embryonic and post-embryonic development in mice. Ultrastructural observations. *Cell. Tissue Res.* **188** (1978) 465–480.
29. Russo J. and de Rosas J. C.: Differentiation of the Leydig cell of the mouse testis during the fetal period —an ultrastructural study. *Am. J. Anat.* **130** (1971) 461–480.
30. Scheib D. and Lombard M. N.: Histoenzymological investigations on the $\Delta$5-3$\beta$-hydroxysteroid dehydrogenase activity in the embryonic and post-natal mouse testis. Normal testis *in vivo* and *in vitro* and explanted with hypothesis. *Arch. d'Anat. Micro. Morph. Exp.* **60** (1971) 205–218.