

# Temporal changes in rat Leydig cell function after the induction of bilateral cryptorchidism

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**Summary.** Adult rats were made bilaterally cryptorchid and studied at intervals of 3, 7, 14 or 21 days to study temporal changes in Leydig cell function. Serum FSH and LH levels were measured and the cross-sectional area of the Leydig cells assessed by morphometry. The function of the Leydig cells was judged by the binding of <sup>125</sup>I-labelled hCG to testicular tissue *in vitro* and the testosterone response of the testis to hCG stimulation *in vitro*. By 3 days after cryptorchidism, the binding of labelled hCG to testicular tissue was significantly decreased compared to that of controls, but the testes were able to respond to hCG stimulation *in vitro*. At 7, 14 and 21 days after cryptorchidism, an enhanced testosterone response was observed and the size of the Leydig cells was significantly greater than that of the controls, which indicated increased secretory activity by the cryptorchid testis. Although serum FSH levels were significantly elevated after 3 days of cryptorchidism, serum LH levels did not rise until 7 days, thereby suggesting that the loss of receptors is unlikely to result from down-regulation by LH. The reduced testosterone response of the cryptorchid testis *in vivo* to low doses of hCG and the enhanced response at high doses are probably related to the reduced blood flow to the cryptorchid testis and the decreased sensitivity of the Leydig cells induced by LH/hCG receptor loss.

## Introduction

Surgically induced cryptorchidism results in a progressive degeneration of the germ cells of the testis (Clegg, 1963). Bilateral cryptorchidism also results in some degree of Leydig cell failure (Kerr, Rich & de Kretser, 1979b); 1 month after the induction of cryptorchidism, serum LH levels were elevated, serum testosterone levels were significantly lower or unchanged, and a diminished testosterone response to hCG stimulation was observed *in vivo* compared to control animals. However, the Leydig cells were hypertrophied and their cytological features were suggestive of increased steroid secretion consistent with the enhanced secretory activity observed in response to maximal hCG stimulation *in vitro* (de Kretser, Sharpe & Swanston, 1979). A marked reduction in the ability of the cryptorchid testis to bind labelled hCG *in vitro* was associated with the changes in Leydig cell function (de Kretser *et al.*, 1979). It was consequently postulated that the increased LH secretion may have secondarily affected Leydig cell function, causing hypertrophy and down-regulating the number of LH/hCG receptors which, in turn, may have altered the sensitivity of the testis to hCG stimulation *in vivo*.

In this study we have examined Leydig cell function after the induction of bilateral cryptorchidism to establish the temporal relationship between the elevation of serum gonadotrophin levels and the changes in the steroidogenic secretory capacity and hCG binding *in vitro*. Morphometric analyses were also performed to assess the increase in Leydig cell size.

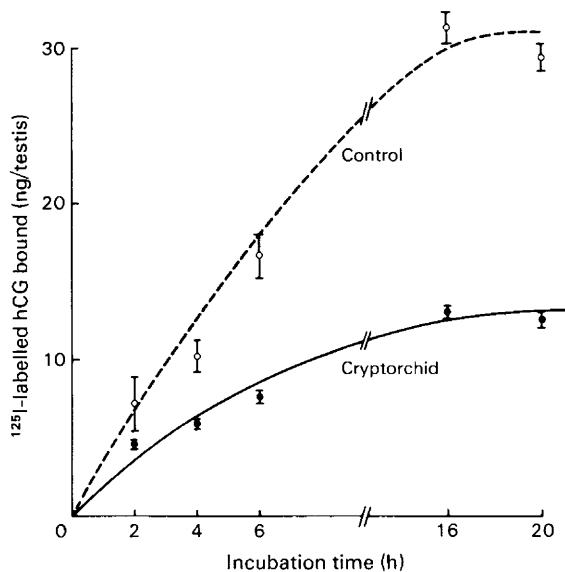
## Materials and Methods

### Animals

Mature Sprague-Dawley rats aged 2–3 months were made surgically cryptorchid by relocation of the testis into an abdominal position and closure of the inguinal canal. Animals were killed by decapitation 3, 7, 14 or 21 days after the induction of bilateral cryptorchidism and trunk blood was collected and stored at  $-20^{\circ}\text{C}$  until assayed for LH, FSH and testosterone. Control animals were sham-operated by opening the scrotum and gently displacing the testes into the abdomen without closure of the inguinal canal.

### Measurement of *in-vitro* binding of $^{125}\text{I}$ -labelled hCG

The testes were excised, weighed, decapsulated and placed in ice-cold phosphate-buffered saline (PBS), pH 7.2. The testes were homogenized by hand using a glass-teflon homogenizer and were centrifuged at 20 000 g for 15 min. The pellet was resuspended, centrifuged again and stored in 1 ml PBS/0.1% BSA at  $-20^{\circ}\text{C}$  before use. After thawing, the pellets were dispersed at a concentration of 200 mg/ml in PBS. Highly purified hCG (CR121: activity 9286 i.u./mg, kindly provided by the National Pituitary Agency, NIH, Bethesda, Maryland, U.S.A.) was iodinated with chloramine T to a specific activity of 30–50  $\mu\text{Ci}/\mu\text{g}$  by the method of Greenwood, Hunter & Glover (1963). Triplicate 100- $\mu\text{l}$  aliquots of the suspension were incubated with 100  $\mu\text{l}$   $^{125}\text{I}$ -labelled hCG (100 000 c.p.m.) and 50  $\mu\text{l}$  PBS buffer for 16 h at  $22^{\circ}\text{C}$ . The non-specific binding of  $^{125}\text{I}$ -labelled hCG was determined by the addition of an excess of hCG (100 i.u. in 50  $\mu\text{l}$  PBS; Pregnyl: Organon) instead of buffer alone. The incubation was terminated by the addition of 3 ml 0.9% (w/v) NaCl, the tubes were centrifuged at 1500 g for 30 min and the resulting radioactivity in the pellet was measured in a Packard gamma spectrometer. The lower speed of centrifugation resulted in only a 0.5–1.0% decrease in  $^{125}\text{I}$ -labelled hCG bound to tissue when compared to centrifugation at 20 000 g; consequently the lower speed was used routinely because of increased capacity of the centrifuge. The LH/hCG binding was expressed as ng hormone bound per testis.



**Text-fig. 1.** The effect of time on the binding of  $^{125}\text{I}$ -labelled hCG to testicular homogenates from normal and cryptorchid rat testes. Each point represents the mean  $\pm$  s.e.m. of 5 replicates.

The possibility that the presence of degenerating germ cells in the homogenates of testes from cryptorchid rats may alter the kinetics of  $^{125}\text{I}$ -labelled hCG, in comparison to preparations from control rats, was evaluated by performing a time-course of binding at 22°C for 16 h for both preparations. Although the total binding to homogenates from cryptorchid testes were lower, the kinetics did not differ from those for control testicular homogenates (Text-fig. 1).

#### *Stimulation of testosterone production by hCG in vitro*

The testes were removed, weighed, decapsulated and placed in 2 ml Krebs-Ringer-bicarbonate buffer (Dawson, Elliot & Jones, 1969) containing 1 mg glucose/ml (KRBG). Testosterone production in response to hCG was determined by methods previously published (de Kretser *et al.*, 1979). At 2 weeks after the induction of cryptorchidism, the in-vitro testosterone response to different doses of hCG (2–1500 mi.u./ml: (Pregnyl: Organon) was determined. Unless indicated the concentration of hCG used in the studies was 700 mi.u./ml.

#### *Studies on Leydig cells isolated from cryptorchid rats*

Preliminary attempts to isolate Leydig cells from rats 21 or 28 days after inducing cryptorchidism by the method of Mendelson, Dufau & Catt (1975) met with considerable difficulty. This presumably resulted from increased collagen accumulation in the peritubular tissue of cryptorchid rats (Kerr *et al.*, 1979a) requiring more extensive incubation with collagenase. These extensive incubations (30–60 min) resulted in poor Leydig cell viability, especially from normal rats. However, since the present study demonstrates changes in Leydig cell function within 7 days of inducing cryptorchidism, i.e. before collagen accumulation is increased, Leydig cells were obtained by incubation with collagenase (Sigma Type 1), 0.25 mg/ml, for 15 min from control and cryptorchid testes. The binding of  $^{125}\text{I}$ -labelled hCG and the in-vitro response to 700 mi.u. hCG were then assessed.

#### *In-vivo dose-response to hCG*

In view of previous results (Kerr *et al.*, 1979b), which demonstrated poor response of the cryptorchid testis to hCG stimulation *in vivo* but hyper-responsivity to hCG *in vitro*, a dose-response test to hCG stimulation *in vivo* was performed. Normal rats and 21-day-cryptorchid rats were injected s.c. with 5, 50 or 200 i.u. hCG and killed by decapitation 60 min later. This time was chosen because earlier studies in normal rats and those with spermatogenic damage from other causes showed this to be the time to achieve the maximal initial response (Rich, Kerr & de Kretser, 1979). Similar data are available for cryptorchid rats (unpublished). The sera were separated and stored at –20°C until assayed for testosterone.

#### *Radioimmunoassay of serum LH, FSH and testosterone*

FSH and LH were measured by double-antibody radioimmunoassays using methods previously described (Lee, de Kretser, Hudson & Wang, 1975). The FSH assay utilized an antiserum raised to purified human FSH and showed less than 0.5% cross-reactivity with rat LH and TSH. Purified rat FSH (NIAMDD-FSH-I3) was iodinated with lactoperoxidase and used as tracer and NIAMDD-FSH-RP1 as standard. The antiserum (NIAMDD-LH-S4) was used to measure rat LH and purified rat LH (NIAMDD-LH-I4) was used as both tracer and standard. The intra-assay variation ranged from 7 to 9% for both assays. All samples from the study were measured in the same assay.

Testosterone was measured by a specific radioimmunoassay similar to that described by Corker & Davidson (1978). Incubation media were assayed directly for testosterone content

because ether-extracted and unextracted samples gave similar results. The anti-serum to testosterone was raised in sheep against testosterone-3-carboxymethyloxime coupled to bovine serum albumin and showed a 98% cross-reactivity with 5 $\alpha$ -dihydrotestosterone. The intra-assay coefficient of variation was 6.8% and the inter-assay coefficient of variation was 16%.

### Histology

The testes of rats that had been cryptorchid for 3, 7, 14 or 21 days were fixed by perfusion of a mixture of glutaraldehyde (5% by vol.), formaldehyde (4% by vol.) and 2,4,6-trinitro-cresol (0.05% by vol.) buffered to pH 7.4 with 0.2 M-sodium cacodylate as previously described (Kerr & de Kretser, 1975). Following post-fixation in osmium tetroxide, the small pieces of testicular tissue were dehydrated and embedded in a mixture of Epon : Araldite (1 : 1, v/v). Sections 0.5–1  $\mu$ m in thickness were stained with toluidine blue and examined with a Leitz Orthoplan microscope. The cross-sectional area of Leydig cells, selected at random, was measured using a Leitz Image Analyser. Equal numbers of Leydig cells ( $n = 300$ ) were measured from 3 animals in each of the 4 groups.

### Statistical analysis

The results were subjected to analysis by Student's *t* test except for the data in Table 1 concerning serum FSH and the incremental rise of testosterone to hCG stimulation. Due to the variance in the latter increasing with the magnitude of the mean, the data were log-transformed and subjected to analysis of variance.

## Results

There was a marked reduction in testis weight after the induction of cryptorchidism (Table 1), such that at 21 days the cryptorchid testis was approximately one quarter of the weight of the control testis. There was no further decline in testis weight at 21 days after cryptorchidism.

**Table 1.** The effect of cryptorchidism of rats on testicular weight, serum FSH and LH concentrations, binding of <sup>125</sup>I-labelled hCG and incremental rise of testosterone production

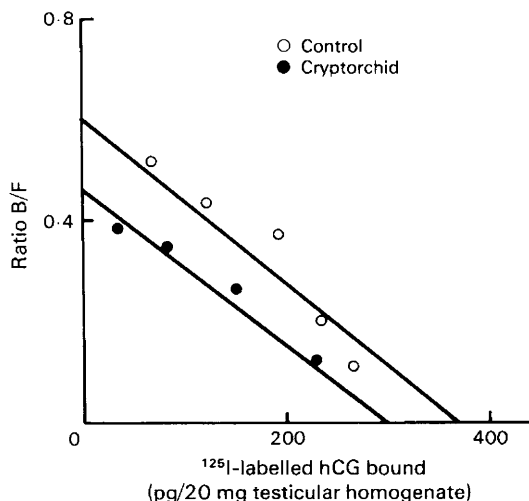
	Days after induction of cryptorchidism			
	3	7	14	21
Testis wt (g)				
Control	1.53 $\pm$ 0.06	1.15 $\pm$ 0.06	1.44 $\pm$ 0.06	1.63 $\pm$ 0.04
Cryptorchid	1.54 $\pm$ 0.06	1.06 $\pm$ 0.11*	0.47 $\pm$ 0.04*	0.42 $\pm$ 0.03*
Serum LH (ng/ml)				
Control	1.02 $\pm$ 0.11	0.69 $\pm$ 0.11	0.47 $\pm$ 0.08	0.76 $\pm$ 0.09
Cryptorchid	0.82 $\pm$ 0.11	1.47 $\pm$ 0.22*	1.72 $\pm$ 0.14*	1.50 $\pm$ 0.09*
Serum FSH (ng/ml)				
Control	492 $\pm$ 27	421 $\pm$ 18	402 $\pm$ 21	544 $\pm$ 22
Cryptorchid	767 $\pm$ 32*	638 $\pm$ 53**	855 $\pm$ 50**	1093 $\pm$ 126**
Binding of <sup>125</sup> I-labelled hCG (ng/testis)				
Control	16.9 $\pm$ 1.3	13.3 $\pm$ 0.5	12.7 $\pm$ 0.5	10.3 $\pm$ 2.8
Cryptorchid	11.9 $\pm$ 1.1**	4.3 $\pm$ 0.5**	3.4 $\pm$ 0.4**	2.0 $\pm$ 0.2**
Incremental rise in testosterone to hCG (ng/testis)				
Control	350 $\pm$ 70	530 $\pm$ 70	520 $\pm$ 80	190 $\pm$ 60
Cryptorchid	260 $\pm$ 60**	1100 $\pm$ 140**	1680 $\pm$ 260**	1560 $\pm$ 320**

Values are mean  $\pm$  s.e.m. for 8 rats/group.

\*  $P < 0.05$ , \*\*  $P < 0.005$  compared to controls.

Serum FSH levels were significantly increased within 3 days and continued to rise above control values with increasing time after the induction of cryptorchidism (Table 1). Serum LH levels show considerable variability in control animals, presumably related to episodic secretion, but a significant ( $P < 0.05$ ) rise in serum LH above basal levels was not apparent until 7 days after cryptorchidism; values then remained elevated throughout the study (Table 1).

The binding of labelled hCG to testicular tissue from 3-day-cryptorchid rats was significantly ( $P < 0.01$ ) lower than that for controls. Thereafter the binding capacity of the cryptorchid testis showed a further decline, reaching 19% of control values at 21 days of cryptorchidism (Table 1). Scatchard analyses of the results after cryptorchidism for 7 days show that the loss of binding was not associated with a change in the affinity of the hormone for the receptor (Text-fig. 2). No significant difference of the slopes was found either by calculating the errors and using Student's  $t$  test or by the use of a bioassay programme to test parallelism of the regression lines (Finney, 1964).



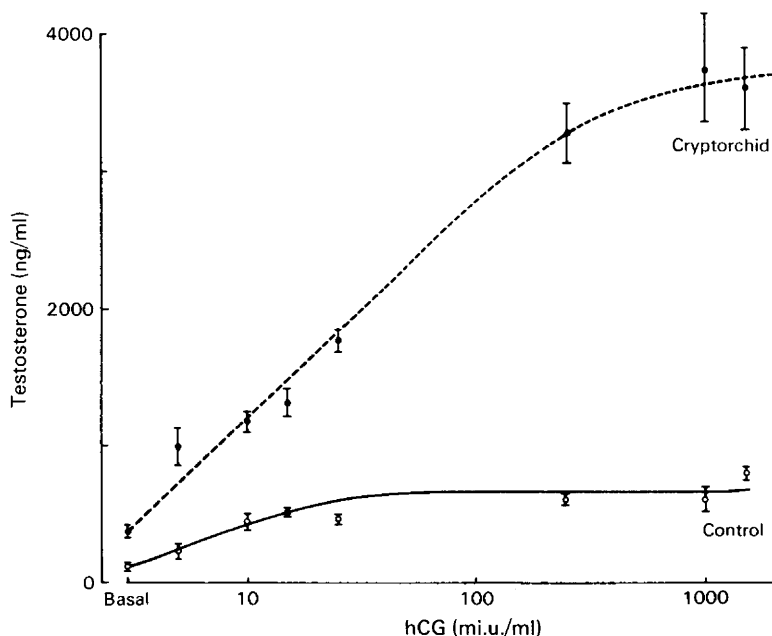
**Text-fig. 2.** Scatchard analysis of the binding data obtained using testicular tissue from control and 7-day-cryptorchid animals. Each point represents the mean of triplicate determinations from pooled samples of testicular tissue from the 5 animals in each group.

By 3 days after the induction of cryptorchidism, the testes were able to respond to hCG *in vitro* by a rise in testosterone production. There was no difference in testosterone production by control and cryptorchid testes at 3 days, but testes from 7-, 14- and 21-day-cryptorchid animals showed a significantly ( $P < 0.01$ ) enhanced response to hCG stimulation *in vitro* compared to control testis (Table 1). The results in Text-fig. 3 demonstrated that at all doses of hCG the capacity of the testis to produce testosterone *in vitro* was greater for the cryptorchid testes. Calculation of the dose required to stimulate a half-maximal increase was 23.5 i.u./ml for the cryptorchid testis and 4.5 i.u./ml for control groups.

The size of the Leydig cells was significantly ( $P < 0.05$ ) enlarged within 3 days of the induction of cryptorchidism and the increase was maintained (Table 2).

#### Response of isolated Leydig cells

Leydig cells isolated from testes 7 days after the induction of cryptorchidism demonstrated a significantly greater response of testosterone to hCG stimulation than did cells from control rats (Table 3). The binding of <sup>125</sup>I-labelled hCG to Leydig cells from cryptorchid rats was reduced to 45% of that for Leydig cells from control rats (Table 3).



**Text-fig. 3.** The effect of increasing concentrations of hCG on testosterone production *in vitro*. Values are mean  $\pm$  s.e.m. (per ml of incubation buffer) for 5 testes.

**Table 2.** The effect of cryptorchidism in rats on the cross-sectional area of the Leydig cells

Days after cryptorchidism	Total cross-sectional area ( $\mu\text{m}^2$ )	
	Control	Cryptorchid
3	108 $\pm$ 6	159 $\pm$ 9*
7	109 $\pm$ 6	156 $\pm$ 6*
14	111 $\pm$ 5	154 $\pm$ 9*
21	103 $\pm$ 4	166 $\pm$ 4*

Values are mean  $\pm$  s.e.m. for 300 cells in each of 3 rats/group.

\* Significantly different from control value:  $P < 0.01$ .

**Table 3.** The *in-vitro* responses of Leydig cells isolated from the testes of rats cryptorchid for 7 days

	No. of test-tubes	Control	Cryptorchid
Testosterone production (ng/ $10^6$ cells)			
Basal	5	3.5 $\pm$ 0.1	3.3 $\pm$ 0.3
hCG-stimulated	5	4.5 $\pm$ 0.1	6.2 $\pm$ 0.2*
$^{125}\text{I}$ -labelled hCG binding (pg/ $10^6$ cells)			
	3	48.1 $\pm$ 1	21.8 $\pm$ 0.9*

Values are mean  $\pm$  s.e.m.; each test-tube contained  $10^6$  cells from a different animal.

\* Significantly different from control value,  $P < 0.001$ .

*In-vivo dose-response of testosterone to hCG stimulation*

Basal serum testosterone levels in controls and 21-day-cryptorchid rats did not differ (Table 4). At a dose of 5 i.u. hCG serum testosterone levels were significantly greater in control animals than in cryptorchid rats, but the reverse was true with 200 i.u. hCG. There was no difference with 50 i.u. hCG.

**Table 4.** The dose-response relationship between the levels of serum testosterone and the dose of hCG administered to control and 21-day-cryptorchid rats

Dose of hCG (i.u.)	Serum testosterone (ng/ml)	
	Control	Cryptorchid
0	2.0 ± 0.2	1.9 ± 0.2
5	16.4 ± 0.9	8.0 ± 1.2**
50	18.3 ± 2.2	21.9 ± 2.5
200	17.6 ± 1.5	27.5 ± 3.8*

Values are mean ± s.e.m. for 5 rats/group.

Significantly different from control values, \*  $P < 0.05$ , \*\*  $P < 0.001$ .

### Discussion

This study demonstrates that the reduction in the number of available testicular LH/hCG receptors in the cryptorchid rat is not directly related to an immediate change in peripheral LH concentrations. Serum LH values were unchanged 3 days after the induction of bilateral cryptorchidism and were not significantly elevated until 7 days of cryptorchidism. However, an initial significant reduction in the binding of labelled hCG to cryptorchid testis *in vitro* was first observed at 3 days and continued to decline to lower levels after 21 days of cryptorchidism compared to controls. The decline in hCG binding was also found in Leydig cells isolated from cryptorchid testes and incubated with the tracer in the absence of seminiferous tubules and degenerating germ cells. These results, and the demonstration that the kinetics of the binding reaction were similar in both control and cryptorchid testicular homogenates (Text-fig. 1), exclude the possibility that the loss of binding was due to damage of the iodinated hCG by lysosomal products released from degenerating germ cells.

This temporal pattern is not consistent with the explanation that the decreased binding is the result of the increased serum LH levels after cryptorchidism. Scatchard analysis demonstrates that the change in hCG binding is due to a loss of receptors rather than any change in the affinity of the hormone-receptor interaction. Further studies are necessary to determine the mechanism by which hCG binding is decreased following cryptorchidism.

Despite the loss of LH/hCG receptors, the cryptorchid testis is able to respond in an exaggerated manner to hCG stimulation *in vitro* and does not demonstrate the desensitizing effect found after the loss of hCG receptors induced by high doses of hCG (Tsuruhara, Dufau, Cigorruga & Catt, 1977). This provides further evidence that the loss of receptors following hCG is not the cause of the desensitization, in keeping with the data of Haour & Saez (1978) who showed that cycloheximide could prevent the loss of LH receptors caused by large doses of hCG but did not prevent the desensitization process. The hypertrophy of the Leydig cells precedes the enhanced production of testosterone by the cryptorchid testis and correlates with previous ultrastructural observations of an increase in the organelles involved in steroid biosynthesis (Kerr *et al.*, 1979b). The finding that preparations of Leydig cells isolated from

cryptorchid testes show an exaggerated hCG-induced testosterone response is evidence that this phenomenon is not the result of any change in the steroid metabolism of damaged seminiferous tubules. The dose of hCG required to cause half-maximal stimulation of the cryptorchid testis is greater than that required by the control testis and may be a measure of decreased sensitivity consistent with the loss of LH/hCG receptors. Examination of the dose-response relationship of testosterone to hCG stimulation *in vitro* provided results different from those previously obtained (de Kretser *et al.*, 1979) in that the present data show hyper-responsiveness of the cryptorchid testis at low doses of hCG. It is possible that the use of hemi-testes in the previous study may be the result of these differences as Dufau, Catt & Tsuruhara (1971) have shown that mechanical disruption of the testis can result in decreased testosterone production. The results described herein are reproducible and are identical to those obtained using testes from rats irradiated as fetuses to cause testicular damage (Rich & de Kretser, 1979).

Our previous studies (Kerr *et al.*, 1979b; de Kretser *et al.*, 1979) have demonstrated a paradox between the *in-vivo* hypo-responsiveness of the Leydig cell to hCG stimulation and the hyper-responsiveness *in vitro*, a difference attributed to the reduced blood flow to the cryptorchid testis (Damber, Bergh & Janson, 1978) and supported by the fact that at low doses the control rats demonstrated a greater response to hCG *in vivo* than did the cryptorchid rats. However, at high doses of hCG the cryptorchid rats showed a greater testosterone production than controls and it is possible that the large doses of hCG have compensated for the reduced blood flow to the cryptorchid testis and overcome the decreased sensitivity resulting from the loss of LH/hCG receptors, both of which contribute to the hyporesponsiveness of the cryptorchid testis at lower doses of hCG.

The enhanced *in-vitro* production of testosterone in response to hCG by cryptorchid testes is difficult to reconcile with a decreased activity of the cholesterol side-chain cleavage enzyme and 3 $\beta$ -hydroxysteroid dehydrogenase, and diminished androgen production as demonstrated by the conversion of precursor steroids (Inano & Tamaoki, 1968; Le Vier & Spaziani, 1968; Wisner & Gomes, 1978). However, we have demonstrated a significant stimulation of pregnenolone, androstenedione and oestradiol production by cryptorchid testes during *in-vitro* stimulation (de Kretser *et al.*, 1979). It is possible that the *in-vitro* studies using precursor conversion techniques do not accurately reflect the capacity for steroid production from endogenous unlabelled precursors. Furthermore, the hypertrophy of the Leydig cells and the increase in the organelles involved in steroid biosynthesis (Kerr *et al.*, 1979b) support the observations in this study of increased steroidogenic capacity of these cells.

Other models of spermatogenic damage (i.e. rats treated by fetal irradiation, prolonged administration of hydroxyurea or chronic feeding of a vitamin A deficient diet) also result in elevated LH levels, hyporesponsiveness to hCG *in vivo*, Leydig cell hypertrophy and hyperresponsiveness to hCG *in vitro* and loss of LH receptors (Rich, Kerr & de Kretser, 1979; Rich & de Kretser, 1979). It is therefore unlikely that the changes in Leydig cell function are due solely to increased intra-abdominal temperature, and a control mechanism operating between the tubule and intertubular compartments of the testis could be involved.

Aoki & Fawcett (1978) proposed a local control mechanism to explain the hypertrophic appearance of Leydig cells which surrounded localized areas of tubule damage induced by anti-fertility agents. They suggested that the damaged portions of the seminiferous tubules might elaborate a product which results in the stimulation of Leydig cells, or that the Leydig cells might be released from the inhibitory effects of an agent secreted during normal testicular function. It is well established that cryptorchidism causes a severe disruption of spermatogenesis (Nelson, 1951) and also alters the structure and function of the Sertoli cells as demonstrated by decreased androgen-binding protein production (Kerr *et al.*, 1979a). Either component could be involved in the postulated control mechanism. Our present results show that the hypertrophy and hyperresponsiveness of the Leydig cells precede the rise in serum LH, and it is therefore likely that other factors such as local mechanisms may be involved.



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