J. steroid Biochem. Vol. 22, No. 3, pp. 293–298, 1985
Printed in Great Britain.

0022-4731/85 $3.00 + 0.00

CHANGES OF OESTROGEN RECEPTOR LEVELS IN LEYDIG CELLS FROM MICE AND RATS DURING CULTURE

ELS M. J. J. BERNS, ALBERT O. BRINKMANN, FOCKO F. G. ROMMERTS, EPPO MULDER and HENK J. VAN DER MOLEN
Department of Biochemistry (Division of Chemical Endocrinology), Medical Faculty, Erasmus University Rotterdam, The Netherlands

(*Received* 4 *January* 1984)

Summary—Two functional properties of Leydig cells in culture, i.e. LH-stimulated steroidogenesis and nuclear oestrogen receptor levels have been investigated. Leydig cells isolated from testes of immature rats and mature mice maintained their responsiveness to LH during 48–72 h of cell culture, although the mouse Leydig cells appeared to be less responsive to LH after 72 h of culture. In contrast, nuclear oestrogen receptor levels in both types of Leydig cells declined to 10–20% of the initial value after 24 h in culture. In the 48–72 h culture period nuclear oestrogen receptor levels recovered to 75% of the initial value only in Leydig cells from immature rats, whereas the nuclear oestrogen receptor levels in Leydig cells from mature mice remained low. These data demonstrate that during *in vitro* culture of Leydig cells, preservation of LH responsiveness does not necessarily warrant that other Leydig cell parameters e.g. nuclear oestrogen receptors also remain unaltered.

INTRODUCTION

The suppressive action of oestrogens on testicular steroidogenesis *in vivo* has led to many speculations about a physiological role of oestradiol in testicular function [1, 2, 3]. Studies with hypophysectomized animals have clearly shown that the inhibitory action of oestrogens on the testis can be direct [4, 5] and is at least expressed at the level of the microsomal enymes: 17α-hydroxylase and C_{17-20}-lyase [6, 7]. Specific oestrogen receptors and oestrogen-induced translocation of these receptors to the nucleus have also been observed in Leydig cells [8, 9]. These observations suggest that Leydig cells may be considered as target cells for oestrogens.

Several studies have shown that the inhibition of the testicular conversion of progesterone to testosterone after administration of hCG is accompanied by a depletion of cytosolic oestrogen receptors [10, 11, 12] and it has been suggested that testicular oestradiol is an intracellular mediator in one of the steps of the "desensitization" process [13, 14]. Brinkmann *et al.*, however, reported that oestrogen receptor translocation does not always result in an inhibition of microsomal enzymes involved in testicular steroid production [15]. As a result of these contradictory observations, the function of the oestrogen receptor remains questionable.

Various investigators have attempted to study the *in vitro* effects of physiological concentrations of oestradiol on isolated cells but no consonant results have been obtained [16, 17]. Although the LH dependent steroid production in Leydig cells from immature rats and mature mice is not significantly changed during culture [18, 19], certain functional properties of Leydig cells, however, can change, e.g. loss of LH receptor [18] and increased sensitivity to adenosine and related compounds [20]. It could also be possible that the oestrogen receptor binding activity may change during culture. Oestrogen receptor levels have therefore been measured in isolated Leydig cells from mouse and immature rats during several days in culture. For characterization of the functional properties of these cells the LH-dependent steroid production has been measured.

EXPERIMENTAL

Materials

[2,4,6,7-^{3}H]Oestradiol-17β (sp. act. 91.5 Ci/mmol) was obtained from New England Nuclear Group Corp. (Boston, MA, U.S.A.). Unlabelled steroids were purchased from Steraloids Inc., Pawling, NY, U.S.A. The radiochemical purity of the labelled oestradiol-17β was verified by thin-layer chromatography.

Ovine luteinizing hormone (NIH-LH-S20) was a gift from the NIAMDD, Bethesda, MD, U.S.A. Cyanoketone (2α-cyano-4,4′-17α-trimethyl-17β-hydroxy-5-androsten-3-one), an inhibitor of 3β-hydroxysteroid dehydrogenase activity, is a former product of Stirling-Winthrop, New York, U.S.A. SU-10603 (7-chloro-3,4-dihydro-2-(3-pyridyl)-1-(2H)-naphthalenone), an inhibitor of 17α-hydroxylase activity, was a gift from Ciba-Geigy, Basle, Switzerland.

Cells and cell culture

Isolation of Leydig cells

Rat. Immature Wistar rats, 23–27-days old, were killed with CO_2. Eight to twelve decapsulated testes

were incubated at 37°C in 50 ml plastic conical tubes (Falcon) containing 10 ml minimal essential medium (with fungizone, 600 ng/ml, streptomycin 100 μg/ml, and penicillin, 100 U/ml = MEM), with 1% foetal calf serum (FCS, Gibco) and 10 mg collagenase (Worthington, 135 U/mg). The tubes were placed in a water bath at 37°C with their long axis parallel to the shaking direction and were shaken for 20 min (90 cycles/min). The suspension was diluted with 25 ml saline. After allowing the tissue debris to sedimentate for 5 min, the supernatant was collected and centrifuged at 108 *g* for 10 min at room temperature. The cells were washed twice with MEM. Cells were suspended in MEM with 1% FCS, and divided over Costar flasks (75 cm^2). Each flask contained 10^7 interstitial cells. 20–30% of the attached cells were Leydig cells, as established by histochemical detection of 3β-hydroxysteroid-dehydrogenase activity. The flasks were placed in an atmosphere of 5% CO_2 in air at 37°C and the cells were cultured without renewal of culture medium for 0, 5, 24, 48 or 72 h.

Mouse. Mice, Swiss outbred, 6–12 weeks, were killed with CO_2. After decapsulation, the testes of the mice were placed in minimal essential medium (2 ml/testis). The testes were carefully drawn into a 20 ml syringe through a silicone tubing (4 mm i.d.) for five times. This procedure was repeated with a syringe provided with tubing 2 mm i.d. The suspension obtained was filtered through a 30 μm nylon gauze and the filtrate was divided in equal portions over culture flasks (Costar 75 cm^2). Each flask contained 10^7 interstitial cells. Twenty per cent of the cells were Leydig cells, as established by histochemical detection of α-naphthyl esterase activity. The flasks were incubated in an atmosphere of 5% CO_2 in air at 32°C for 0, 5, 24, 48 and 72 h without renewal of the medium.

LH-stimulated testosterone production by mouse Leydig cells isolated by collagenase dispersion, as described in the previous section for the immature rat Leydig cells, was not different from the production by mechanically isolated cells. Since the isolation by mechanical means is reproducible, quicker and less expensive, this method has been adopted for all the culture experiments with mouse Leydig cells.

Biochemical procedures

Nuclear receptor studies

For each experiment 4 flasks (about 10^7 cells/flasks) were used. The attached cells were washed twice with phosphate buffered saline, and incubated for 1 h at 37°C (rat) or 32°C (mouse) in the presence of 10 nM [^{3}H]oestradiol. In parallel incubations, the same amount of cells was incubated with 10 nM [^{3}H]oestradiol and 1 μM diethylstilboestrol (DES). After incubation, the medium was discarded and the cells were processed for isolation of nuclear receptors essentially as described by Mulder *et al.* [21]. Briefly, the cells were removed from the flasks by scraping and were subsequently homogenized at 1°C in buffer A (containing 50 mM Tris–HCl, 1.5 mM EDTA, 1.5 mM dithiothreitol; pH 7.4). From this point all procedures were performed at 1°C. The homogenate was centrifuged at 800 *g* for 10 min, the 800 *g* pellet was resuspended in buffer and washed 3 times with buffer A. (The buffer of the second wash contained 0.2, Triton X-100.) The nuclear (800 *g*) pellet was extracted with buffer B (50 mM Tris–HCl, 0.4 M KCl, 1.5 mM EDTA and 1.5 mM dithiothreitol; pH 8.5 at 1°C) for 60 min. The nuclear extract was centrifuged at 105,000 *g* for 30 min.

Since mouse Leydig cells attach only after 24 h, even in the presence of fetal calf serum, the cells were not washed during 0–24 h of cell culture, in order to prevent cell losses. Oestrogen receptor levels were assayed in interstitial cells in suspension at 0, 5 and 24 h. Fetal calf serum was omitted in these experiments because it will reduce the uptake of oestradiol. At the indicated time intervals cells suspended in medium without FCS were incubated for 1 h at 32°C with [^{3}H]oestradiol. After incubation, the medium and cells were collected and centrifuged for 10 min at 100 *g*. The cells were washed twice with MEM, homogenized in a glass–glass potter with a motor-driven pestle and centrifuged at 800 *g* for 5 min. The 800 *g* pellets were resuspended in buffer A and processed for isolation of nuclear receptors.

Protamine sulphate precipitation assay

Receptors were estimated essentially as described by Chamness *et al.* [22] with addition of pyridoxal phosphate (final concentration 10 mM) [23]. The KCl concentration during precipitation was below 0.04 M. Nuclear receptors were expressed as fmol/10^7 Leydig cells.

Sucrose gradient centrifugation

Nuclear extracts (200 μl) were layered on 4.1 ml of a 5–20% sucrose gradient, prepared in buffer B, containing 0.4 M KCl. After centrifugation in a Beckman L5-65B centrifuge at 2°C for 18 h at 260,000 *g* in a SW-60 rotor, the bottom of the tube was pierced and fractions of 7 drops were collected. Radioactivity was measured in each fraction. γ-Globulin (7.2 S), [^{14}C]bovine serum albumin (4.6 S) and ovalbumin (3.6 S) were used as sedimentation markers.

Steroid production

Before each experiment the medium of the cells was renewed with fresh medium without FCS and the cells were processed for steroid production as described below.

Rat. LH-stimulated pregnenolone production by rat Leydig cells was studied during 1 h in the presence of 5 μM cyanoketone and 19 μM SU-10603 and a maximal stimulating dose of LH (100 ng/ml). Media were collected and stored immediately at −20°C until analysis. The amount of pregnenolone secreted into the medium was measured by radioimmunoassay [24].

Steroid productions were expressed as $\mu g/h \times 10^7$ Leydig cells.

Mouse. Testosterone production by mouse Leydig cells was measured during 1 h in the presence of LH (100 ng/ml). Media were collected and stored immediately at −20°C. The amount of testosterone secreted into the medium was measured by radioimmunoassay [25]. Steroid productions were expressed as $\mu g/h \times 10^7$ Leydig cells.

Other procedures

DNA content of the 800 *g* pellet was determined according to Giles and Myers[26], using calf thymus DNA as standard.

RESULTS

Oestrogen receptor content during culture

Nuclear oestrogen receptors were measured at different time intervals during cell culture. The nuclear extract obtained from freshly isolated Leydig cells, after incubation for 1 h with [^{3}H]oestradiol, contained 25 ± 7.5 fmol oestrogen receptor/10^7 rat Leydig cells and 50 ± 6 fmol oestrogen receptor/10^7 mouse Leydig cells respectively. The radioactivity in both nuclear extracts sedimented in sucrose gradients as a 5 S peak (Fig. 1 only shown for rat Leydig cells). When the cells were kept in culture for 24 h specific [^{3}H]oestradiol binding in the nuclear extract was decreased (see Fig. 1). The nuclear oestrogen receptor levels from cultured immature rat and mature mouse Leydig cells decreased within 5 h to approx. 60 and 30% respectively of the initial value at 0 h (Fig. 2). A further decrease to 10–20% was observed after 24 h of cell culture. When the Leydig cells were kept in culture for 48–72 h, the nuclear receptor levels of rat cells were restored to 75% of the initial value, whereas in mouse Leydig cells the levels remained low (Fig. 2).

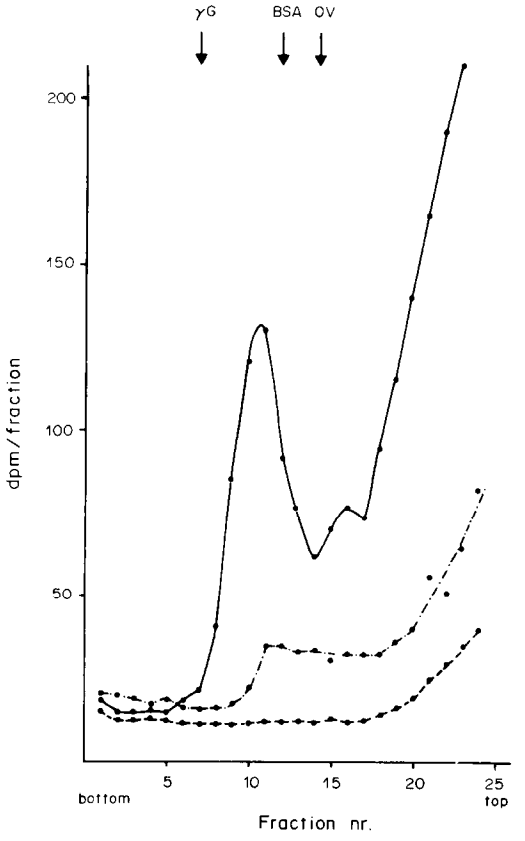

Fig. 1. Sucrose gradient sedimentation profiles of oestrogen receptors extracted with 0.4 M KCl from nuclei of rat interstitial cells after 1 h of cell culture (●——●) and after 24 h of cell culture (●—·—●). The cells were incubated for 1 h at 37°C with 10 nM [^{3}H]oestradiol in the absence (●—·—●) or presence of a 100-fold molar excess of diethylstilboestrol (●— —●). The sucrose gradients contained 0.4 M KCl. Gamma globulin (γ-G, 7.2 S), bovine serum albumine (BSA, 4.6 S) and ovalbumin (OV, 3.6 S) were used as sedimentation markers.

Effects of LH in culture

Leydig cells isolated from the testes of immature rats and mature mice were cultured for 72 h. At different time intervals after the start of the culture, the steroidogenic activity of the cells was measured. The LH stimulated steroid production by freshly isolated Leydig cells was 0.9 ± 0.2 μg pregnenolone/10^7 rat Leydig cells and 9.3 ± 2.2 μg testosterone/10^7 mouse Leydig cells. Leydig cells of both species maintained their responsiveness to LH with respect to pregnenolone or testosterone production respectively during the whole culture period (Fig. 3). A 15-fold stimulated steroid production was achieved by LH treatment. This stimulation factor was not altered during the culture period, except for mouse Leydig cells at 72 h (Fig. 3, panel B).

The DNA content during culture

The DNA content of the rat and mouse cells did not change significantly during the 72 h culture period (Fig. 4).

Exchange studies

Underestimation of nuclear oestrogen receptors can occur if exchange of radioactive ligand with endogenously bound oestradiol is incomplete. Exchange studies, where interstitial cells from immature rats were preincubated for 1 h with 1 nM oestradiol and subsequently incubated with 10 nM [^{3}H]oestradiol also for 1 h, showed that 80% of the unlabelled ligand can be exchanged with the tritiated ligand (Table 1).

Table 1. Effect of preincubation with 1 nM oestradiol on nuclear oestrogen receptor levels in cultured Leydig cells from immature rats

	fmoles receptor/10^7 cells
Without preincubation	21.7
Preincubation with 1 nM oestradiol	17.6

Receptor levels were estimated after incubation of the cells with 10 nM [^{3}H]oestradiol with or without a 100-fold molar excess of DES. Results are represented as means of two estimations.

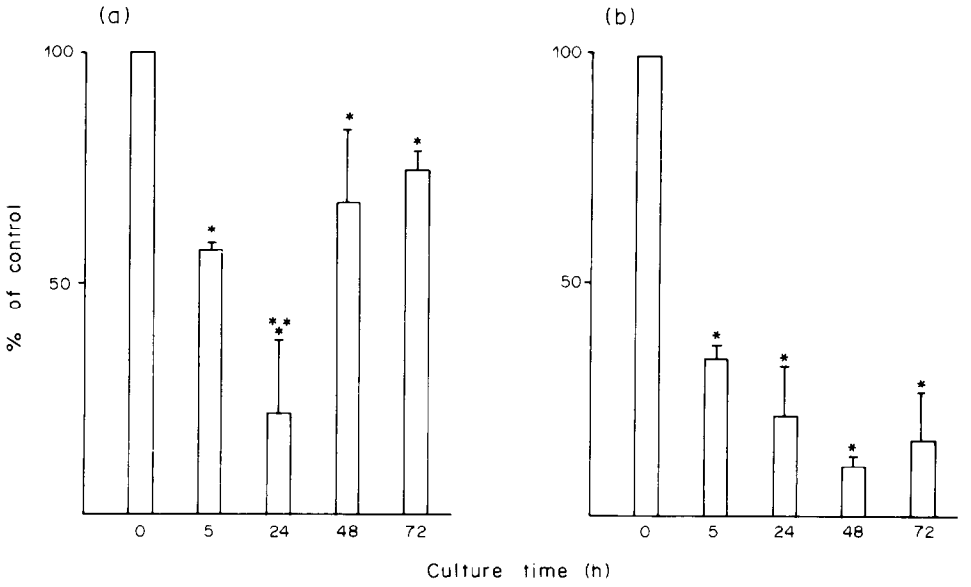

Fig. 2. Effect of culture time on nuclear oestrogen receptor levels of rat (panel A) and mouse (panel B) interstitial cells. Nuclear receptors were estimated by protamine sulphate precipitation assay at the indicated time periods. Nuclear receptor levels at time zero 25 ± 7.5 fmol/10^7 rat Leydig cells and 50 ± 6 fmol/10^7 mouse Leydig cells was taken as 100%. Each point represents the mean $\pm$ SD of 3–8 estimations. (*$P < 0.005$ when compared with 0 h, **$P < 0.025$ when compared with 5 and 48 h 2-tailed Student's t-test).

DISCUSSION

A transient decrease in nuclear oestradiol receptor binding activity was observed when Leydig cells from immature rats were cultured during a period of 72 h, while LH responsiveness with respect to steroidogenesis did not change significantly during the same period, only mouse Leydig cells appeared to be less responsive to LH after 72 h of culture. In cultured mouse Leydig cells nuclear oestrogen receptors were also decreased after 24 h in culture. But in contrast to the rat Leydig cells, receptor levels stayed at a decreased level during the next 24 h in culture, while LH responsiveness of the cells was not affected. The observed decreases in oestrogen receptor levels were not due to decreases in the number of interstitial cells,

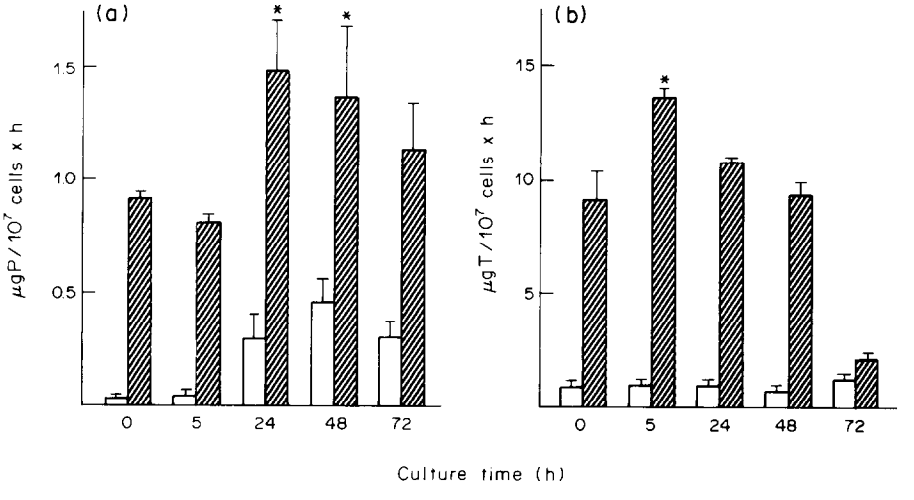

Fig. 3. Effect of culture time on LH-stimulated pregnenolone (P) production by rat (panel A) and LH-stimulated testosterone (T) production by mouse (panel B) interstitial cells. Steroid production was estimated for 1 h in fresh medium as described in the Experimental section at the indicated time periods. Steroid productions are expressed as μg steroid per hour per 10^7 Leydig cells. Open bars represent basal production and hatched bars represent LH-stimulated production. Each point represents the mean $\pm$ SD of 3–6 estimations. (*$P < 0.005$ when compared with 0 h).

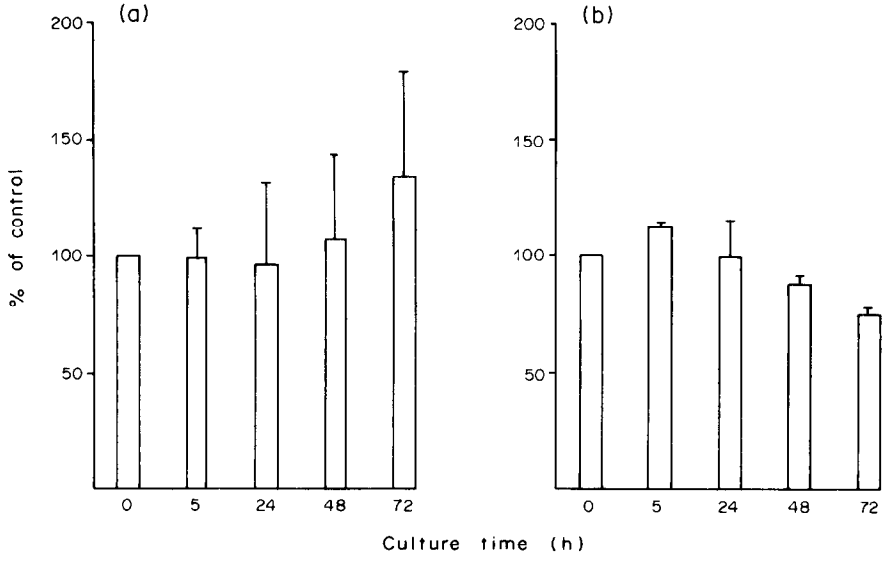

Fig. 4. Effect of culture time on DNA content in the culture flasks of rat (panel A) and mouse (panel B) interstitial cells. DNA content was estimated as described in the Experimental section at the indicated time periods. Each point represents the mean ± SD of 3–8 estimations.

because the DNA content of the cell cultures did not change significantly throughout the whole culture period (72 h).

We have considered the possibility that methodological artifacts might have influenced the actual nuclear oestrogen receptor levels. For example if damage to the cells would have occurred during the isolation procedure or during culture, receptor leakage out of the cytoplasm could not be excluded. However, under these conditions also leakage of other cellular components could be expected. Since LH-stimulated steroid production, which requires intact cells [27], is not changed, the presence of damaged cells and the ensuing receptor leakage is very unlikely.

The observation that basal steroid production by immature rat and mature mouse interstitial cells as well as the DNA-content of the cultures did not change during the whole culture period (72 h) strongly argues against a possible change in the proportion of steroidogenic cells. This is further supported by the observed unchanged LH-stimulated steroid production. The observed decline in the oestrogen receptor content, therefore, is not due to changes in the proportion of steroidogenic cells.

Underestimation of nuclear oestrogen receptors can also occur if exchange of radioactive ligand with bound oestradiol (produced either endogenously or by contaminating Sertoli cells) is incomplete. The exchange studies, however, showed that 80% of the unlabelled ligand can be exchanged with the tritiated ligand.

The present results show that during culture of the cells the binding properties of the oestrogen receptor change, resulting in a temporal (rat Leydig cell) or permanent (mouse Leydig cell) inactivation of the binding site. Alterations of binding activities of oestrogen receptors by phosphorylation have been described by Migliaccio[28], and the observed changes in binding activity might be explained by changes in intracellular phosphorylation. The observed decrease in receptor binding activity during the first 24 h in culture could also be explained by receptor breakdown. A 50% decrease in nuclear receptor was found after 5 h of cell culture of both cell types. Whether this binding reflects the half life of the oestradiol receptor in Leydig cells, remains to be proven but it is in agreement with the half life of about 4 h reported for the oestrogen receptor in MCF-7 cells [29]. Specific changes in protein synthesis or protein breakdown in Leydig cells during *in vitro* culture could therefore also be an explanation for the (temporal) changes in oestrogen binding activity. Moger *et al.* observed an altered binding of the cytoplasmic oestrogen receptor in testicular tissue incubated during several hours *in vitro* [16]. Similarly, Peck *et al.* found a rapid decrease in oestrogen receptor binding activity in excised uteri during organ culture [30]. Oestrogen receptors in the mammary tumour cell line MCF-7, however, appeared to be stable under *in vitro* conditions [31]. These observations indicate that the oestrogen receptor is unstable under primary culture conditions, whereas in permanent cell lines the oestrogen receptor is stable.

The increase in nuclear receptor levels observed at 48 h in immature rat Leydig cells might reflect new synthesis of oestrogen receptor molecules or a reactivation of receptor molecules from a non-steroid binding form to a steroid binding form. More experiments are needed to elucidate the exact mechanism of the increase in receptor levels.

The rapid decrease in oestrogen receptor levels could be a plausible explanation for the observed oestrogen insensitivity of Leydig cells *in vitro* [16, 17]. According to the present results effects of oestrogens on isolated Leydig cells can only be studied *in vitro* after it has been verified that oestrogen receptor properties are normal.

Acknowledgements—We wish to thank the National Institute of Health, Endocrinology Study Section, Bethesda (U.S.A.), for gifts of ovine luteinizing hormone. We thank Dr M. A. Blankenstein (Department of Biochemistry, Rotterdam Radiotherapeutic Institute, Dr Daniel den Hoed Clinic) for his continuous interest in our work and constructive discussions.

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