LUTROPIN REGULATION OF STEROIDOGENESIS AND SPECIFIC PROTEIN SYNTHESIS IN RAT LEYDIG CELLS

Proefschrift
ter verkrijging van de graad van doctor in de geneeskunde
aan de Erasmus Universiteit te Rotterdam
op gezag van de rector magnificus Prof.Dr.B.Leijnse
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaats vinden
op vrijdag 11 november 1977
des namiddags te 3.00 uur

door
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geboren te Delft.

Drukkerij de Vries-Rotterdam

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Dit proefschrift werd bewerkt in het Instituut Biochemie II (Chemische Endocrinologie) van de Faculteit der Geneeskunde, Erasmus Universiteit te Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door steun van de stichting voor Medisch Wetenschappelijk Onderzoek FUNGO.

"We must note, however, that when a small principle changes, usually many of the things which depend upon it undergo an accompanying change. This is clear with castrated animals, where, although the generative part alone is destroyed, almost the whole form of the animal thereupon changes so much that it appears to be female or very nearly so, which suggests that it is not merely in respect of some casual part or some casual faculty that an animal is male or female. It is clear, then, that "the male" and "the female" are a principle".

Aristotle:
Generation of animals I

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INTRODUCTION AND SCOPE OF THE THESIS

1.1 Lutropin regulatory mechanism of steroidogenesis

In the testis steroidogenesis takes place in the Leydig cells (Hooker, 1970) and is under the control of lutropin, a glycoprotein hormone with a mol. wt. of about 30,000 consisting of 2 nonidentical subunits, which is secreted by the anterior pituitary (Hall, 1970). The main steroid secreted by the testis of the adult rat is testosterone (Eik-Nes, 1970). Cholesterol is the precursor for the synthesis of steroid hormones and its conversion into pregnenolone is stimulated by lutropin (Hall, 1970). The further conversion of pregnenolone into testosterone is not under the control of lutropin.

The present evidence indicates that the lutropin or choriogonadotropin controlled steroidogenic regulatory mechanisms (human choriogonadotropin is a glycoprotein hormone with a mol. wt. of about 40,000, consisting of 2 nonidentical subunits, which is produced by the placenta) consist of the following cascade of events. The first is the binding of lutropin to specific receptors, located in the plasma membrane of the cell (Catt et al., 1974). Full stimulation of steroidogenesis is already obtained when less than 1% of these receptors are occupied with lutropin (Catt and Dufau, 1973a). Next activation of adenylate cyclase occurs, resulting in elevation of the cellular level of cyclic AMP (Murad et al., 1969; Kuehl, 1970; Hollinger, 1970; Rommerts et al., 1972; Cooke et al., 1972b; Catt et al., 1972b). Activation of protein kinase is the primary function by which cyclic AMP controls metabolic functions in eukaryotic cells and it has been demonstrated that in Leydig cells activation of protein kinase occurs with the same doses of lutropin, needed for stimulation of steroidogenesis (Cooke et al., 1976b).

Inhibition of protein or RNA synthesis results in an inhibition of the lutropin stimulation of steroidogenesis, suggesting that both these processes are necessary (Hall and

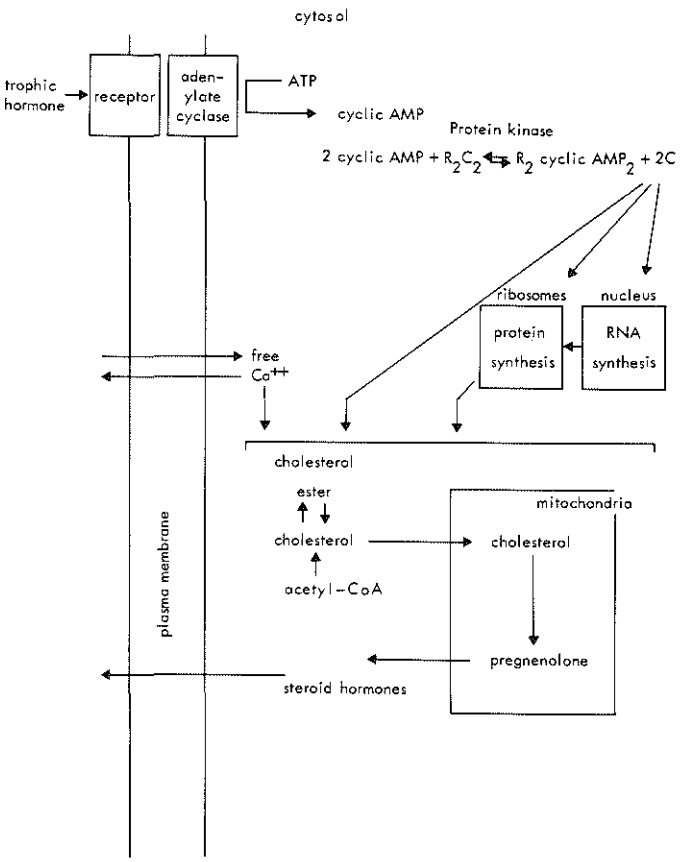

Figure 1.1 Hypothetical model for the regulation of steroidogenesis by trophic hormone

RC : protein kinase holoenzyme

C : catalytic subunit

R : regulating subunit

Eik-Nes, 1962; Shin, 1967; Sakamoto et al., 1973; Cooke et al., 1975a,b; Mendelson et al., 1975). After maximal stimulation of steroidogenesis by lutropin addition of protein synthesis inhibitors such as cycloheximide decrease steroidogenesis to basal values, the reaction following first order kinetics (half life 13 min) (Cooke et al., 1975a). These results indicate that the continuous synthesis of a protein(s) with a short half life equal to or less than 13 min is needed for the stimulation of steroidogenesis by lutropin. A scheme summarizing these events is given in figure 1.1.

1.2 Scope of this thesis

In this thesis the role of protein synthesis in the lutropin regulatory mechanism of steroidogenesis in rat testis Leydig cells has been investigated. It was decided to study this by investigating specific proteins newly synthesized in the presence of lutropin. Before specific protein synthesis in Leydig cells could be investigated, however, it was necessary to modify the Leydig cell preparation which was then available. Previous work had been carried out on interstitial tissue obtained by wet dissection of rat testis (Rommerts et al., 1973b). Although this preparation was enriched in Leydig cells with respect to total testis, it was inhomogeneous and it also gave a variable response to trophic hormone stimulation. This problem was solved by the preparation of single cell suspensions from total testis tissue. However these suspensions only contained 6% Leydig cells. In order to increase the chance of finding lutropin controlled specific proteins synthesized in Leydig cells it was decided to purify the Leydig cells. This part of the work is described in chapter 3, section 3,4 and appendix paper I. To investigate the incubation conditions, best suited for maximal lutropin stimulation of steroidogenesis the effect of some additions to the incubation medium were more closely investigated. This is described in chapter 3, section 6 and with respect to Ca^{++} in appendix paper II.

The effect of lutropin and cycloheximide on specific protein synthesis is described in chapter 4, section 2 and appendix papers III and IV. It was found that lutropin stimulates the synthesis of a protein with an apparent mol. wt. of 21,000 (LH-IP) in Leydig cells from mature rats. In chapter 4, section 3 and appendix paper V investigations into the regulation of the synthesis of this protein by lutropin are described in more detail. To investigate the biological role of LH-IP it is necessary first to isolate and purify the protein. Because the amount of Leydig cells obtained from intact rat testis is too low for such an isolation and purification procedure, the presence of LH-IP was investigated in a rat Leydig cell tumour which can be obtained in much greater quantities. However, with this preparation it was found that lutropin had no detectable effect on the synthesis of LH-IP, but stimulated the synthesis of 2 other proteins with apparent mol. wt.'s of 27,000 and 29,000.

Finally the effect of lutropin on specific protein synthesis in Leydig cells from immature rats and in tumour Leydig cells were compared. This part of the work is described in chapter 4.4 and appendix paper VI.

SUMMARY OF THE LITERATURE ON THE LUTROPIN REGULATION OF STEROIDOGENESIS

2.1 Introduction

The relationship between the testes and sexual function was recognized by Aristotle ('Generation of animals', ± 300 B.C.). In 1849 Berthold directly demonstrated the androgenic potency of testicular transplants in preventing the atrophy of the comb in castrated cockerals. 78 Years later the first androgenically potent lipid extract from bull testicules was described by McGee (1927).

In the adult male androgens can maintain spermatogenesis and the structural integrity of the secondary sex organs. In order that a compound may qualify as an androgen it must stimulate the growth of 3 test organs: a) the capon comb, b) the prostate and c) the seminal vesicle and it must be effective in restoring sexual activity in castrated male animals (Eik-Nes, 1970). The main androgen secreted by the mature rat testis is testosterone (Eik-Nes, 1970).

The testis is an ovoid organ surrounded by the tunica albuginea (a thick connective tissue capsule), and is composed of seminiferous tubules and interstitial tissue (figure 2.1). The seminiferous tubules contain myoid, endothelial, Sertoli and germinal cells and their function is to produce spermatozoa which are transported to the epididymis for further maturation. The interstitial tissue consists of a framework of loose fibrous connective tissue that supports blood and lymph vessels and nerves. It contains fibroblasts, macrophages, plasma cells, lymphocytes and the interstitial cells of Leydig (referred to as Leydig cells named after their discoverer Von Leydig, 1857). Bouin and Ancel (1903) were one of the first investigators to recognize that the Leydig cells are the site of androgen production and since then an overwhelming amount of supporting evidence has been obtained, e.g. destruction of tubular cell types does not result in markedly diminished androgen levels, animals bearing Leydig cell tumours have elevated levels of androgens, the locali-

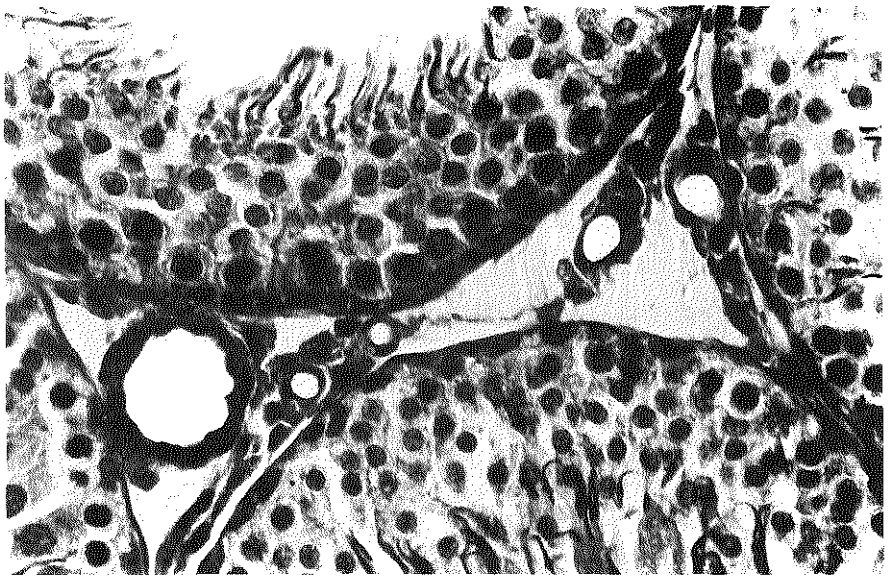

Figure 2.1 Photomicrograph of rat testis tissue, stained with Periodic Acid Schiff and hematoxylin. This section (750x magnification) shows part of circular shaped seminiferous tubules which contain Sertoli cells and germ cells and in between the seminiferous tubules blood and lymph vessels and Leydig cells

zation in the Leydig cells of certain enzymes involved in steroidogenesis has been demonstrated histochemically and biochemically and the production of testosterone by isolated Leydig cells has been demonstrated in vitro (see for review: Hooker, 1970; and further Christensen and Mason, 1965; Cooke et al., 1972a; Cooke et al., 1974; Catt et al., 1973b; Moyle and Ramachandran, 1973; van Damme et al., 1974; van der Molen et al., 1975).

It cannot be excluded that some androgen production takes place in the seminiferous tubules (Christensen and Mason, 1965; Hooker, 1970; Bell et al., 1975). However the evidence for this is not convincing especially with regard to de novo steroid synthesis (Hall et al., 1969). The low androgenic capacity of seminiferous tubules demonstrated by some workers can be explained by contamination with Leydig cells which are difficult to remove from the tubules even after

extensive washing (Hovatta et al., 1974).

Smith (1927) and later Greep (1936-37) established the role of pituitary secretions in the regulation of both the endocrine and exocrine function (production of spermatozoa) of the testis. It is now well accepted that the two trophic hormones follitropin and lutropin secreted by the pituitary have separate sites of action in the testis; follitropin acts on the seminiferous tubules while lutropin controls Leydig cell function (Hall, 1970; Dorrington et al., 1972; Cooke et al., 1974).

In the following sections of this chapter the current state of knowledge about the lutropin regulation of steroidogenesis in testis Leydig cells will be reviewed.

2.2 Biosynthesis of the steroid hormones and the regulation of their synthesis

2.2.1 Cholesterol: the precursor for testosterone biosynthesis

Testosterone is synthesized from cholesterol (Eik-Nes, 1970). Most of the cholesterol is synthesized within the testis (Eik-Nes, 1975) which is in contrast to the situation observed in the adrenal gland, where exogenous cholesterol accounts for 80% of that used for the more active steroidogenesis. The cellular uptake of cholesterol by the adrenal gland is stimulated by corticotropin (Dexter et al., 1970; Gwynne et al., 1976). Regulation of the intracellular cholesterol level may be of importance for testosterone production. Control of testicular cholesterol synthesis is still poorly understood although there is some evidence that testosterone regulates cholesterol synthesis through feedback inhibition (Eik-Nes, 1970; Neaves, 1975). Within the Leydig cell, cholesterol is present in the free and esterified form. In the mouse Leydig cell the bulk of free cholesterol is present in the microsomal fraction while esterified cholesterol is mainly stored in lipid droplets (Aoki and Massa, 1975). Hydrolysis of fatty acid esterified cholesterol is required before it can be converted into pregnenolone (Moyle et al., 1973). Injection of lutropin into the mouse results in a decrease

in the esterified cholesterol pool in the testis, but no marked change in the concentration of free cholesterol occurs (Bartke, 1971; Pokel et al., 1972; Aoki and Massa, 1975). Moyle et al. (1973a) demonstrated that in mouse tumour Leydig cells lutropin stimulates the hydrolysis of esterified cholesterol into free cholesterol. Activation of adrenal cholesterol esterase has been shown to occur by phosphorylation, which is one of the possible mechanisms of action of trophic hormone stimulation (Naghshineh et al., 1974; Wallat et al., 1976; Beckett and Boyd, 1977). In rat testis interstitial cells most of the cholesterol is present in the free form (van der Molen et al., 1972; Hafiez and Bartke, 1972) and in contrast to the mouse testis, administration of choriogonadotropin does not have a detectable effect on the free or esterified cholesterol pools in rat testis (van der Molen et al., 1972). One explanation for these results may be that the amount of cholesterol converted into androgens is small compared to the total cholesterol pool (van der Vusse, 1975). Until now precise knowledge is lacking about the localization and size of the cholesterol pool used as substrate for conversion into androgens in the rat testis Leydig cell.

2.2.2 The conversion of cholesterol into pregnenolone

It is generally accepted that the conversion of cholesterol into pregnenolone, which takes place in the mitochondrion, is the rate-limiting step in the overall production of androgens and that this conversion is under the control of lutropin (Hall, 1966; Hall and Young, 1968; Hall, 1970). Several authors have suggested that corticotropin or lutropin stimulate steroidogenesis in their respective target organs by regulating the mitochondrial precursor pool of cholesterol available for the side chain cleavage enzyme (Kahnt et al., 1974; Boyd et al., 1975).

However the results of Farese and Prudente (1977a) indicate a more direct effect of trophic hormone on the cholesterol side chain cleavage enzyme complex. They found that

the degree of stimulation of pregnenolone production by corticotropin in solubilized cholesterol side chain cleavage enzyme preparations and in intact mitochondrial preparations were comparable.

Caron et al. (1975) demonstrated that a partially purified cyclic AMP dependent protein kinase caused a 20-74% stimulation of the activity of the cholesterol side chain cleavage enzyme activity reconstituted from corpus luteum cytochrom P_{450} and purified adrenodoxin reductase.

Administration of cycloheximide in vivo prevents the trophic hormone stimulation of pregnenolone production in mitochondrial preparations from the adrenal, corpus luteum and testis (Koritz and Kumar, 1970; Jefcoate et al., 1974; van der Vusse et al., 1975a). Farese and Prudente (1977a) showed that this effect of cycloheximide on stimulated pregnenolone production is abolished by solubilization of the cholesterol side chain cleavage enzyme and they therefore suggested that a cycloheximide sensitive protein is required to overcome a barrier which exists in the intact mitochondria and restrains cholesterol side chain cleavage. This is in agreement with earlier suggestions for a role of a cycloheximide sensitive protein in the translocation or the transportation of cholesterol within the mitochondrion (Simpson et al., 1972; Boyd et al., 1975). This conclusion, however, does not agree with the findings reported by Mahaffee et al. (1974) namely that cycloheximide does not prevent the mitochondrial accumulation of cholesterol brought about by corticotropin in the rat adrenal. Further these authors concluded that cycloheximide blocks the steroidogenic process at a site that is not the principle site of regulation. A similar conclusion is reported by Bell et al. (1973) who suggested that cycloheximide acts primarily upon the cholesterol hydroxylases rather than upon events mediating the transport of cholesterol.

2.2.3 Conversion of pregnenolone into testosterone

The conversion of pregnenolone into testosterone takes place outside the mitochondria in the endoplasmic reticulum and is not under the control of lutropin (Hall, 1968; van der Molen and Eik-Nes, 1971). No effect of cycloheximide on the conversion of pregnenolone into corticosterone has been observed in the adrenal gland (Davis and Garren, 1968).

2.3 Mechanism of action of trophic hormones

2.3.1 Role of membrane receptors

It is generally accepted that the coupling of a trophic hormone to its receptor is the first event in the stimulation of steroidogenesis. Using autoradiographic and immunohistological methods a receptor for lutropin and choriogonadotropin has been demonstrated to be located in the Leydig cell (De Kretser et al., 1971a; De Kretser et al., 1971b; Castro et al., 1972; Dal Lago et al., 1975; Dal Lago et al., 1976). Sucrose density gradient centrifugation has revealed the localization of this receptor in the plasma membrane (Catt et al., 1974). Other arguments that the lutropin receptor is localized in the plasma membrane are: the stimulation of steroidogenesis in vitro by incubation with lutropin coupled to Sepharose, which excludes intracellular effects of the trophic hormone (Dufau et al., 1971) and the fact that incubation of Leydig cells with trypsin resulted in a loss of lutropin binding sites (Steinberger, 1973). Binding studies with Leydig cell homogenates and solubilized receptors have shown that one type of receptor with high affinity and limited capacity is present (Catt et al., 1972a; Dufau and Catt, 1975). However, these studies do not exclude the presence of another type of receptor with a very low capacity or receptors having similar physical characteristics but different biological functions as indicated by the results of Moyle and Ramachandran (1973). These authors demonstrated that dilution of Leydig cells after preincubation

with lutropin was sufficient to terminate the stimulatory action of lutropin on cyclic AMP production, whereas the same procedure failed to affect the steroidogenic response to lutropin. Removal of lutropin from tumour Leydig cells with lutropin antiserum resulted in an almost immediate cessation of steroidogenesis (Moyle et al., 1971) indicating that the continuous occupation of receptors by lutropin is necessary for stimulation of steroidogenesis. They postulated therefore the presence of 2 functionally different types of receptors. Moyle (1975) found this postulation strengthened by the results, which he obtained with choriogonadotropin derivatives lacking various sugar residues. Whereas sequential removal of the sialic acid, galactose, N-acetyl glucosamine and mannose residues led to a progressive increase in the effective dose of the hormone required to stimulate steroidogenesis, it resulted in a loss in the ability of the hormone to stimulate cyclic AMP accumulation.

The presence of different types of receptors can explain the fact that a maximum response in steroidogenesis is obtained at concentrations of lutropin resulting in less than 1% occupancy of the receptors (Catt and Dufau, 1973a). However, assuming the presence of only one single type of receptor has led to the concept of spare receptors (Catt and Dufau, 1973a). It has been proposed that these receptors function as storage for lutropin (Catt and Dufau, 1973a) or means by which the probability will be increased that circulating hormone will be concentrated by the target cell to reach an adequate level of receptor occupancy to initiate steroidogenesis (Catt and Dufau, 1973a). The concept of spare receptors has been questioned by Ryan and Lee (1976) because the optimal conditions for a biological response are different from the optimal conditions for the binding assays and this may have led to misinterpretation of the obtained data. With mouse tumour Leydig cells different results were obtained, lutropin binding was found to be proportional to the lutropin provoked steroidogenic response (Moudgal et al., 1971). Differences in the assay for lutropin binding may explain the

observed differences between dose of lutropin - lutropin binding relationship and dose of lutropin - steroidogenic response relationship in rat testis Leydig cells (Catt and Dufau, 1973a) and in mouse tumour Leydig cells (Moudgal et al., 1971). The first authors determined specific binding by incubation of tissue with ^{125}I-hCG in presence or absence of excess unlabelled choriogonadotropin, whereas Moudgal et al. (1971) determined specific binding by incubation of the tissue with lutropin followed by washing the tissue 5 times at 0^{o}C. The lutropin retained in the pellet was determined with a radioimmunoassay and was referred to as specifically bound lutropin.

Gonadotropin receptors of rat testis have been solubilized and purified 15000 fold to 50% theoretical purity (Charreau et al., 1974; Dufau and Catt, 1975a; Dufau et al., 1975b). The isolated receptor exhibited the properties of a molecule with a mol. wt. of approximately 200,000 (Dufau et al., 1973a; Dufau et al., 1974a). The number of lutropin receptors in mature rat testis is under the control of the pituitary, because 3 days after hypophysectomy a 50% decrease in receptor concentration occurred (Thanki and Steinberger, 1976). Replacement therapy with lutropin, follitropin or testosterone propionate failed to maintain lutropin binding at the pre-hypophysectomy level (Thanki and Steinberger, 1976). According to the results of Hsueh et al. (1976a) this loss of receptors does not occur in every Leydig cell; these authors found that a 50% reduction in cells containing immunofluorescence labelling of gonadotropin receptors occurred 3 days after hypophysectomy.

Regulation of gonadotropin receptors in mature and immature rat testis by circulating homologous hormone has been reported (Hsueh et al., 1976b; Chen and Payne, 1977a; Haour et al., 1977; Sharpe, 1977; Hsueh et al., 1977). A single injection of choriogonadotropin or lutropin results in a decrease in available and absolute number of receptor sites.

A rapid (after 3 hours) and prolonged (approximately 4-5 days) loss of receptors was observed after high doses of gonadotropins and a delayed but equally prolonged loss of receptors to 30% of the control value occurred after low doses. In the latter case only 8% of the available receptors were occupied by the administered hormone (Hsueh et al., 1976b; Chen and Payne, 1977a; Haour and Saez, 1977; Sharpe, 1977; Hsueh et al., 1977). Ascoli and Puett (1977) reported an intracellular uptake of ^{3}H-labelled lutropin by testis cells 60 min after injection of the hormone and they suggest that the whole hormone receptor complex is internalized presumably via endocytosis and that the endocytic vesicles fuse with primary lysosomes where catabolism then occurs. By this mechanism the hormone action on the target cell is terminated and this may explain the loss of receptors described above some hours after injection of the trophic hormone. The results described by Ascoli and Puett (1977) may explain the older histological observations of a cytoplasmic localization of lutropin (Castro et al., 1970; De Kretser et al., 1971a; De Kretser et al., 1971b). In these studies Leydig cells were incubated in the presence of labelled lutropin for 30 min to several hours during which time uptake of the hormone could have taken place.

2.3.2 Role of cyclic AMP, cyclic GMP and Ca^{++}

Sandler and Hall (1966) were the first to demonstrate the stimulation of testosterone production in rat testis by the addition of cyclic AMP. Since then this observation has been confirmed by other investigators (Connell and Eik-Nes, 1968; van der Molen and Eik-Nes, 1971; Catt et al., 1972b). Catt et al. (1972b) demonstrated that db-cyclic AMP, an analogue of cyclic AMP, stimulates testosterone production in rat testis about 50 times more effectively than cyclic AMP. Sandler and Hall (1966) reported that the effect of cyclic AMP on steroidogenesis was located beyond the synthesis of

cholesterol. Van der Molen and Eik-Nes (1971) demonstrated that it acted at steps prior to the formation of pregnenolone, suggesting an effect on the conversion of cholesterol into pregnenolone. As stated in section 2.2 this conversion is the rate-limiting step in the overall production of androgens and is under the control of lutropin (Hall, 1970). In 1968 Connell and Eik-Nes reported that the addition of theophylline, a competitive phosphodiesterase inhibitor, to rabbit testis slices increased the production of testosterone, suggesting that cyclic AMP is involved in this stimulation. Addition of lutropin to rat testis stimulates the adenylate cyclase activity (Murad et al., 1969; Kuehl et al., 1970; Hollinger, 1970). Hollinger (1970) and Eik-Nes (1971) reported the localization of adenylate cyclase in the nuclear and mitochondrial fraction of the testis, however no marker enzymes for the different subcellular fractions were included in these studies. Pulsinelli (1972) using marker enzymes for plasma membranes and mitochondria, found the adenylate cyclase primarily associated with the plasma membranes. Testis mitochondria, which were free of plasma membrane contamination have been reported to contain a lutropin sensitive adenylate cyclase (Sulimovici et al., 1973, 1974, 1975). However,gonadotropins did not augment the production of pregnenolone in testicular mitochondria, containing adenylate cyclase activity (Pulsinelli and Eik-Nes, 1970). Addition of lutropin to testis preparations results in increased levels of cyclic AMP in the tissue and in the medium (Rommerts et al., 1972; Cooke et al., 1972b; Catt et al., 1972b; Dufau et al., 1973b; Moyle and Ramachandran, 1973; Dorrington and Fritz, 1974). This increase in cyclic AMP level occurs within 1-3 min after the addition of lutropin and precedes the increase in testosterone production (Rommerts et al., 1972; Dufau et al., 1973b; Moyle and Ramachandran, 1973). Addition of phosphodiesterase inhibitors like theophylline or 3-isobutyl-1-methylxanthine, potentiates the effect of lutropin or choriogonadotropin on cyclic AMP levels and on testosterone production (Dufau

et al., 1973b; Catt and Dufau, 1973a; Dufau et al., 1974b; Cooke et al., 1976b). All these results suggest a role for cyclic AMP as second messenger in the lutropin regulatory mechanism of steroidogenesis.

Another way to test whether cyclic AMP is involved in the stimulation of steroidogenesis has been by the use of choleratoxin, which has been shown to mimic the action of hormones at their target cells through stimulation of cyclic AMP production (Sahours and Cuatrecasas, 1975; Haksar and Peron, 1975; Palfreyman and Schulster, 1975). Cooke et al. (1977a) demonstrated that in rat testis Leydig cells incubated with increasing doses of choleratoxin, testosterone and cyclic AMP production were increased in a parallel fashion, indicating that cyclic AMP is involved here in the stimulation of steroidogenesis. However the lowest dose of lutropin needed for stimulation of cyclic AMP production is one order of magnitude higher than needed for stimulation of testosterone production (Catt and Dufau, 1973a; Rommerts et al., 1973a; Moyle and Ramachandran, 1973). Because of this discrepancy in the amount of lutropin needed for an increase in cyclic AMP levels and stimulation of testosterone production, the obligatory requirement of cyclic AMP in the action of lutropin in steroidogenesis in the testis has been questioned. It has been suggested that at low concentrations of lutropin other substances may act as second messengers e.g. cyclic GMP or Ca^{++}.

No increase in cyclic GMP levels have been detected after addition of lutropin to Leydig cells (Williams et al., 1976a; M. Pueta Cuva, unpublished results) and addition of cyclic GMP to Leydig cells did not stimulate steroidogenesis (Williams et al., 1976a). These results indicate that cyclic GMP is not a mediator of lutropin stimulated steroidogenesis in rat testis Leydig cells. Rasmussen (1970) has drawn attention to the possible role of Ca^{++} as second messenger in hormone controlled processes. Van der Vusse et al. (1976) have shown that Ca^{++} can mimic the effect of lutropin on pregnenolone production in isolated rat testis mitochondria. In the adrenal gland it has been demonstrated that the pre-

sence of Ca^{++} in the incubation medium is required for full stimulation of steroidogenesis (Birmingham et al., 1953; Sayers et al., 1972; Rubin et al., 1972). All these results may imply a direct role of Ca^{++} in the trophic hormone regulatory mechanism of steroidogenesis, although a permissive role for Ca^{++} cannot be excluded. Concerning the corticotropin stimulation of steroidogenesis in adrenal cells Ca^{++} has been shown to be involved in the transmission of the signal arising from corticotropin-receptor interaction to the adenylate cyclase (Lefkowitz et al., 1970; Rubin et al., 1972; Sayers et al., 1972; Haksar and Peron, 1973; Kowal et al., 1974), in protein synthesis (Farese, 1971a; Farese and Prudente, 1977b), in conversion of cholesterol into pregnenolone (Simpson et al., 1972; Arthur et al., 1976) and in the release of steroids (Rubin et al., 1972).

Before more can be said about the role of Ca^{++} in the trophic hormone regulatory mechanism of steroidogenesis it is necessary to know more about the amount of free and bound Ca^{++} in the different cellular compartments under different stimulatory conditions. At this moment this knowledge is completely lacking.

Another explanation for the observed discrepancy between lutropin dose-cyclic AMP response relationship and lutropin dose-testosterone response relationship may be that only a small amount of cyclic AMP is involved in the stimulation of steroidogenesis and/or that at low concentrations of lutropin a translocation of cyclic AMP from one compartment to another compartment is involved. That this may be true is indicated by the recent results of Dufau et al. (1977) who demonstrated that there was an increase in the number of occupied cyclic AMP binding sites in Leydig cells at all lutropin concentrations that gave a steroidogenic response.

2.3.3 Role of protein kinase

Since activation of protein kinase is the primary function by which cyclic AMP controls metabolic functions in eukaryotic cells, several authors have measured activation of cyclic AMP dependent protein kinase in testis cells as a function of lutropin concentrations and have compared this relationship with the lutropin dose-testosterone relationship. Podesta et al. (1976) reported that activation of protein kinase activity in rat testis Leydig cells by lutropin parallelled the increase in cyclic AMP levels in these incubations and at low doses of lutropin, which evoked a response in testosterone production, no activation of protein kinase could be observed. In contrast Cooke et al. (1976b) observed an activation of cyclic AMP dependent protein kinase with all doses of lutropin which also gave a response in testosterone production, whereas a rise in cyclic AMP levels was detected only at higher doses of lutropin. In these determinations histone has been used as substrate for protein kinase and the possibility exists that this histone is a relatively poor substrate for the specific protein kinase involved in the regulation of steroidogenesis. Therefore it seems more appropriate to determine phosphorylation of endogenous substrates in intact cells in the presence of different doses of lutropin. This type of experiments has been performed by Cooke et al. (1977b). These authors reported that in rat testis Leydig cells lutropin stimulates the phosphorylation of 3 proteins with apparent mol. wt.'s of 14,000, 58,000 and 76,000. Phosphorylation of these proteins with different doses of lutropin parallelled the stimulation of testosterone production and reached a maximum approximately 20 min after the addition of the hormone to

the cells. These observations favour a role for cyclic AMP as a second messenger in the mechanism of action of lutropin, although it is also possible that activation of protein kinase has been caused by factors other than cyclic AMP.

The relation of the phosphorylated endogenous proteins to the stimulation of steroidogenesis is still obscure. The lutropin stimulated phosphorylation of more than one protein can point to a pleiotropic regulation of steroidogenesis. In other steroidogenic tissues more data have been obtained about the possible role of protein phosphorylation. In the adrenal phosphorylation of ribosomal proteins has been reported after addition of corticotropin (Murakami and Ichii, 1973; Roos, 1973). In addition it has been shown that phosphorylation activates adrenal cholesterol esterase (Naghshinah et al., 1974; Wallat and Kunan, 1976; Becket and Boyd, 1976) and activates a reconstituted cholesterol side chain cleavage enzyme complex in the bovine corpus luteum (Caron et al., 1975).

2.3.4 Role of protein and RNA synthesis

Hall and Eik-Nes (1962) were the first to show the inhibitory effects of protein synthesis inhibitors such as puromycin on the lutropin stimulated steroidogenesis in rabbit testis preparations. Since then their observations have been confirmed by other authors (Shin, 1967; Sakamoto et al., 1973; Cooke et al., 1975a; Mendelson et al., 1975). The effect of protein synthesis inhibitors on lutropin stimulation of steroidogenesis is due to their effect on protein synthesis and not due to other toxic effects as supported by the parallelism of inhibition of protein synthesis and testosterone production with different doses of the inhibitors puromycin and cycloheximide (Cooke et al., 1975a). These last authors could not find an effect of chloramphenicol, an inhibitor of mitochondrial protein synthesis, on steroidogenesis, which may indicate that only cytoplasmic protein

synthesis is involved. This is in contrast with the finding of Hall and Eik-Nes (1962) namely that chloramphenicol has an inhibitory effect on steroidogenesis in rabbit testis. However,these last authors measured the amount of ^{14}C-acetate incorporated into testosterone while Cooke et al. (1975a) measured mass of testosterone produced with a radioimmunoassay method and it is possible that chloramphenicol influences the conversion of ^{14}C-acetate into cholesterol, a precursor of testosterone. Addition of cycloheximide to Leydig cell preparations, after full stimulation with lutropin, decreased the stimulated testosterone production to basal levels (Cooke et al., 1975a; Mendelson et al., 1975). This decrease followed first order kinetics with a half life of 13 min (Cooke et al., 1975a) indicating that for the stimulation of steroidogenesis the continuous synthesis of a protein with a short half life is necessary. The involvement of such a protein in the stimulation of steroidogenesis by trophic hormone has also been reported for the adrenal gland (Garren et al., 1965; Schulster et al., 1974, 1970; Lowry and McMartin, 1974), corpus luteum (Hermier et al., 1971), and the Graafian follicle (Lieberman et al., 1975). The action of this protein may be located in the mitochondrion as has already been discussed in section 2 of this chapter. Garren et al. (1965) have proposed a model in which the regulation of steroidogenesis by trophic hormones is mediated by the synthesis of a regulatory protein with a short half life (figure 2.2A). It has been suggested that the time period between the addition of corticotropin to the adrenal cells and the increase in steroid secretion is too short (<24s) for new protein synthesis and therefore that the synthesis of the protein with short half life is not under the control of trophic hormone but that a protein is continuously synthesized and is then activated in the presence of the hormone (figure 2.2B) (Schulster et al., 1974; Lowry and McMartin, 1974). The available evidence however,does not exclude a third possibility namely that the protein(s) with a short half life plays a permissive

- = inhibition, + = stimulation.

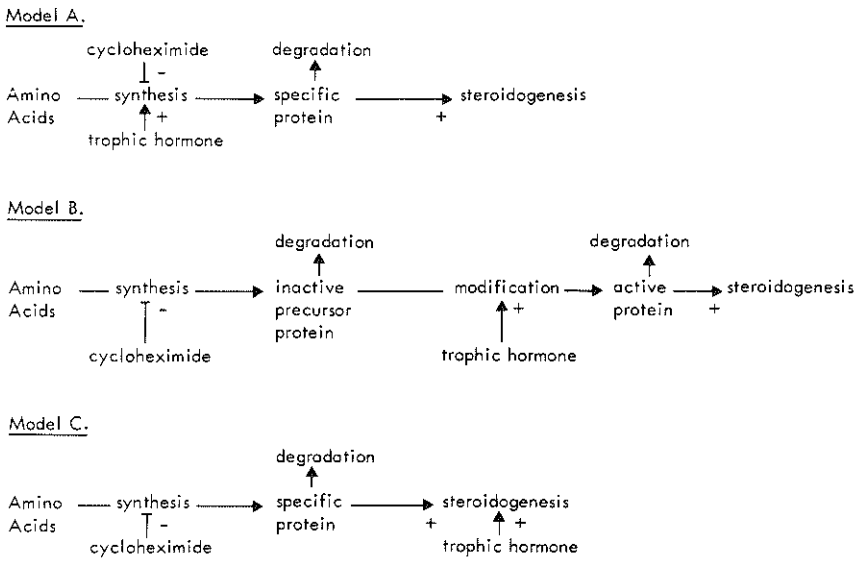

Figure 2.2 Hypothetical models for the role of protein synthesis in the regulation of steroidogenesis

role in the trophic hormone stimulation of steroidogenesis (figure 2.2C).

In adrenal cells steroidogenesis is not inhibited by RNA synthesis inhibitors, indicating that new mRNA synthesis is not inhibited necessary for stimulation of steroidogenesis. This is in contrast to the testis because several authors have described inhibition of lutropin stimulated steroidogenesis by RNA synthesis inhibitors such as actinomycin D and cordycepin (Shin and Sato, 1971; Mendelson et al., 1975; Cooke et al., 1975b). In the study of Mendelson the inhibition of the stimulation of steroidogenesis by actinomycin D was highest when added at the start of the incubation, and became progressively less when added at later times during the incubation period (Mendelson et al., 1975), indicating that new mRNA synthesis is involved in the lutropin stimulation of testosterone production. The RNA synthesis involved in the lutropin stimulation of steroidogenesis in rat testis Leydig cells, may be necessary for translation of the protein(s) with a short half life or for translation of other proteins, which are not involved in the corticotropin stimulation of steroidogenesis in adrenal cells. At this moment however,not enough data are available to make definite conclusions.

ISOLATION AND PURIFICATION OF LEYDIG CELLS

3.1 Introduction

In order to study the possible role of specific protein synthesis in lutropin regulatory mechanism(s) of steroidogenesis in the rat testis several restrictions apply to the type of testis preparations that could be used, e.g.:

1. It must contain a lutropin sensitive steroidogenic system.
2. It must be homogeneous, so that specific protein synthesis under different stimulatory conditions can be compared with each other.
3. The protein synthesis system must be as specific as possible for the Leydig cells. This means that the number of other cells containing active protein synthesizing systems must be kept as low as possible.
4. The number of Leydig cells must be sufficiently high for the detection of specific protein synthesis.
5. The procedure for cell preparation must be carried out in a reasonable time period and must be reproducible, so that it is suitable to use as a routine procedure.

Investigations into the lutropin regulation of steroidogenesis have been carried out in many different ways, e.g.:

1. In incubation systems of short duration (Hall and Eik-Nes, 1962; Christensen and Mason, 1965; Dufau et al., 1971; Cooke et al., 1972; Moudgal et al., 1972; Van Damme et al., 1974).
2. In tissue or cell cultures (Steinberger et al., 1967; Shin, 1967; De Kretser et al., 1971; Inano et al., 1972).
3. Superfusion of whole testis (Satyaaswaroop and Gurpide, 1974) or of isolated parts of it (Cooke et al., 1975).

4. In situ perfusion of intact testis (Eik-Nes, 1970).
5. In vivo (Chen and Payne, 1977; Hsueh et al., 1977; Sharpe, 1977).

In the studies described in this thesis an incubation system of short duration was chosen and not long term tissue or cell cultures because our main interest was the short term regulation of steroidogenesis by lutropin.

The purpose of the following sections is to review the different testis cell preparations which are available for studying effects of lutropin on specific protein synthesis.

First of all methods which are available for identifying Leydig cells are discussed (section 3.2). Then the various Leydig cell preparations are evaluated with respect to the criteria discussed above (section 3.3-3.5). In section 3.4 methods are described to obtain enriched Leydig cell suspensions, which were found to fulfil all 5 criteria. In section 3.5 the pros and cons of a Leydig cell tumour as an experimental model are summarized. Next some requirements of the incubation medium are discussed. Finally the steroidogenic capacity and sensitivity towards lutropin of the different Leydig cell preparations are compared with each other.

3.2 Identification of Leydig cells

The Leydig cell in mammals is a relatively large, polyhedral cell. The nucleus is spherical or ovoid and contains one or more nucleoli. The chromatin, present in granules, is predominantly distributed towards the periphery of the nucleus. The Leydig cell can be identified on the basis of its morphology or on the basis of specific proteins present in the Leydig cell and not in other testicular cell types. These marker proteins can be detected under the light microscope by histochemical, immunofluorescent or autoradiographic tech-

niques.

Identification of Leydig cells on basis of their morphology can be done by phase contrast, light or electron microscopy after fixation and suitable staining. According to Meistrich et al. (1973) identification of Leydig cells in suspension by phase contrast microscopy on a quantitative basis is not possible. Determination of the number of Leydig cells in suspension by light microscopy has been reported but this is not easily carried out (Meistrich et al., 1973; Davies and Schuetz, 1975). The most frequently used method for the identification of Leydig cells is 3β-hydroxysteroid dehydrogenase histochemistry (Steinberger et al., 1966; 1967; Hovatta et al., 1974; Dufau and Catt, 1975b; Wiebe, 1976). Steinberger et al. (1967) pointed out that once the Leydig cells are removed from the interstitial areas of the testis that this is the only way of detecting them. Histochemical studies have shown that the 3β-hydroxysteroid dehydrogenase is almost exclusively located in the Leydig cells (Wattenberg, 1958; Levy et al., 1959; Niemi et al., 1962, 1963). However some activity is also present in the peritubular myoid cells (Wiebe, 1976; De Kretser et al., 1971). Hovatta et al. (1974) detected some cells showing a positive histochemical reaction for 3β-hydroxysteroid dehydrogenase in the peritubular cells of teased tubules. They found that these cells were different from myoid cells, which showed a weaker activity. The predominantly interstitial localization of 3β-hydroxysteroid dehydrogenase, as demonstrated histochemically has been confirmed biochemically, using interstitial tissue and seminiferous tubules separated from each other by wet dissection of rat testis (see section 3.3) (van der Vusse et al., 1974).

Another way of identifying Leydig cells is by means of their specific lutropin receptors (Hsueh et al., 1976). These receptors are specifically localized in the Leydig cells (see section 2.3.1) although lutropin receptors have

also been detected on peritubular cells (Castro et al., 1972). Hsueh et al. (1976) reported a good agreement in the number of Leydig cells in cell suspensions, when estimated by immunofluorescence labelling of lutropin receptors and 3β-hydroxysteroid dehydrogenase histochemistry.

There are various other histochemical or histological methods that have been used for the identification of Leydig cells e.g. Nieminen et al. (1975) reported the specific histochemical localization of aminopeptidase in guinea pig Leydig cells using N-L-arginyl-2-naphtylamine and N-L-valyl-2-naphtanylamine as substrates and Darzynkievicz and Gledhill (1973) described the specific uptake of ^{3}H-ATP by fixed Leydig cells from rat testis. A marker used to identify different cell types in the testis is the nonspecific esterase (Niemi and Ikonen, 1963; Niemi et al., 1966). Two functionally different nonspecific esterases have been demonstrated in the testis; one type, detected using naphtyl acetate as substrate, is located in the Leydig cells whereas the other type, with indoxyl acetate as substrate, is located in the Sertoli cells and in some Leydig cells (Niemi et al., 1966). Esterases in the Leydig cells can be measured biochemically with p-nitrophenylpropionate as substrate (Niemi et al., 1966). These results were confirmed by Rommerts et al. (1973b) on testis tissues isolated by wet dissection, who found 50 times more p-nitrophenyl esterase activity in the interstitium than in the seminiferous tubules using p-nitrophenyl acetate as a substrate.

Because it is difficult to guarantee absolute specificity for Leydig cells with any of these markers, it was decided to use 4 different methods for identification, i.e. 3β-hydroxysteroid dehydrogenase histochemistry, Periodic Acid Schiff-staining (Roosen-Runge, 1959; Baillie, 1961), p-nitrophenyl esterase activity and testosterone production (section 2.1). Of these 4 markers Periodic Acid Schiff-staining appears to be the least specific for Leydig cells because other testicular cells are also stained by this reagent (Hooker, 1970). This lack of specificity was

also indicated in our experiments because in the different Leydig cell preparations tested, there were more Periodic Acid Schiff positive cells than cells containing 3β-hydroxysteroid dehydrogenase. The Periodic Acid Schiff-staining was therefore omitted as a marker in later experiments.

3.3 Testis preparations containing Leydig cells

3.3.1 Whole testis

Various preparations of whole testes have been used to investigate lutropin regulation of steroidogenesis e.g. decapsulated intact testis (Catt and Dufau, 1973a; Dufau et al., 1973b), small pieces of whole testes (Rommerts et al., 1972), testis slices (Hall and Eik-Nes, 1962; Connell and Eik-Nes, 1968) and teased testes (Dufau et al., 1971; Rommerts et al., 1973a). The last 3 experimental preparations guarantee a better contact between the medium and testis cells than the first one. Dufau et al. (1971) reported that teasing of the rat testis resulted in a substantial loss of the steroidogenic response to trophic hormones as compared with the intact testis. However,Rommerts et al. (1973a) did not find much difference between lutropin stimulated testosterone production in teased and unteased testes. These contradictory findings could be explained by two opposite effects of teasing: i.e. damage to the Leydig cells, resulting in loss of steroidogenic response to lutropin and better stimulation due to improved contact between the medium containing the lutropin and the Leydig cells. Differences in the degree of teasing may have resulted in a preponderance of one of these effects.

These whole testis preparations did not appear to be a good experimental model, because they contain only a small number of Leydig cells and therefore do not fulfil to criterum number 3 in section 3.1, namely that the preparation must contain as few as possible other cell types with an active protein synthesizing system.

3.3.2 Interstitial tissue

A method of preparing cells enriched in Leydig cells was originally reported by Christensen and Mason (1965). These authors described a mechanical method for the separation of the tubular and interstitial compartments, by microdissection of rat testis. Microscopic examination of the interstitial tissue revealed only slight contamination with tubular material. By microdissection of X-irradiated rats Yokoe et al. (1971) obtained interstitial tissue containing more than 95% Leydig cells as was judged by histological examination. The number of Leydig cells in the interstitial tissue obtained by wet dissection of rat testis was found by Rommerts et al. (1973b) to be 4-8 times higher than in total testis tissue, using p-nitrophenyl esterase as a marker. However, when compared with total testis tissue, the amount of testosterone produced by this interstitial tissue was lower than could be expected on the basis of this increased number of Leydig cells (Rommerts et al., 1973a; Cooke et al., 1974). It was concluded that the lower production of testosterone may have been due to damage of Leydig cells. A second disadvantage of this preparation became apparent during preliminary experiments on specific protein synthesis, namely that different parts of the dissected interstitial tissue contained different amounts of contaminating tubular cells. This type of Leydig cell preparation did not therefore fulfil criteria 2 as stated in section 3.1 namely that it must be homogeneous.

3.3.3 Testis cell suspensions

The use of cell suspensions guarantee a homogeneous distribution of Leydig cells in different incubations. Two different procedures have been described to obtain such a suspension:

1. a mechanical method, described by Van Damme et al. (1974) in which mouse testes are cut into small pieces and incubated in Eagles medium containing 2% calf serum, for 10 min at room temperature with continuous stirring with a magnetic

stirrer. The medium is then filtered and contains lutropin sensitive Leydig cells. However,it was found that if rat testes were used instead of mouse testes, Leydig cells were obtained which were insensitive to lutropin.

2. The second method is based on enzymic treatment of the testes. For this purpose the use of 2 different enzymes have been reported: trypsin and collagenase. Incubation of rat testis with collagenase has been found to result in Leydig cell suspensions responsive to lutropin and choriogonadotropin (Catt et al., 1973b; Moyle and Ramachandran, 1973; Dufau et al., 1974b).The use of trypsin however resulted in cells which did not show specific lutropin binding, indicating that destruction of the receptor sites had taken place. Only after incubation of the cells for some time the binding capacity improved (Steinberger et al., 1973). We therefore adopted the method using collagenase for the preparation of Leydig cell suspensions essentially as described by Moyle and Ramachandran (1973) (appendix paper I). However this method resulted in Leydig cell suspensions containing only 6% Leydig cells as determined by the use of 3β-hydroxysteroid dehydrogenase as marker for the Leydig cells. This was much lower than the percentage of Leydig cells in cell preparations obtained by collagenase treatment of rat testis as reported by the group of Dufau and Catt, i.e. about 40%. This group also determined the number of Leydig cells by 3β-hydroxysteroid dehydrogenase histochemistry and by immunofluorescence labelling of the choriogonadotropic receptors (Dufau and Catt, 1975b; Hsueh et al., 1976a). The amount of testosterone produced by their Leydig cell preparations in the presence of maximum amounts of trophic hormone is about 4-21 ng testosterone/10^6 cells/3 hours (Williams et al., 1976a; Williams et al., 1976b), which is comparable with the amount of testosterone produced by our cell preparation containing only 6% Leydig cells, namely 10.6 ng testosterone/10^6 cells/3 hours (see table 3.2). Because of the low amounts of Leydig cells in our cell preparations, they did not fulfil the third criterium in 3.1, namely that the Leydig cell pre-

paration must contain as few as possible other cell types, with an active protein synthesis. It was therefore decided to investigate methods to purify the Leydig cells.

3.4 Methods for enrichment of Leydig cell suspensions

The interstitial tissue obtained by wet dissection as mentioned in section 3.3 is enriched in Leydig cells and could be used as a source for the preparation of Leydig cell suspensions. However the dissected interstitial tissue has the disadvantage that the steroidogenic response of the Leydig cells to lutropin is partially impaired, probably due to damage to the cells and it was therefore decided to abandon this method as a method for the purification of Leydig cells. In this section several other methods are described which could potentially be used to obtain enriched Leydig cell preparations.

3.4.1 Selective destruction of testicular cells

The main contaminating cell types in the Leydig cell suspensions obtained by incubation of rat testis with collagenase are various germinal cells and therefore it can be expected that the use of testis in which the germinal cells have been destroyed will result in cell preparations containing a higher proportion of Leydig cells. Selective destruction of germinal cells has been obtained with the use of chemical agentia (Panatelli, 1975) by experimental cryptorchidy (VandeMark and Free, 1970), by X-irradiation (Ellis, 1970; Oakberg, 1975) or by feeding the rats with a diet deficient in essential fatty acids (Ahluwalia et al., 1968; van der Molen et al. 1971). Testis cell suspensions from rats with defective spermatogenesis resulted in higher steroidogenic activities compared with testis cell suspensions from intact rat testes (appendix paper I). However, a drawback of these procedures may be that some damage to the Leydig cells may have occurred by this treatment.

Because good methods for enrichment of Leydig cells in testis cell preparation were developed during our study, which were less laborious and lacked this disadvantage, selective destruction of testicular cells was not chosen for further experimentation.

3.4.2 Purification of Leydig cells from testis cell suspensions

a. Theoretical background

The separation of different classes of particles or cells from each other is based on differences in their physical and/or chemical characteristics e.g. mass, density, charge and properties of the plasma membrane. An important group of separation methods is based on differences in distance covered in a certain period by particles or cells separated by a gravitational, centrifugal or electrical force. When a centrifugal force is exerted on an ideal particle (spherical, rigid, smooth, uncharged, unhydrated and constant in size and density) its velocity will be expressed by the following equation:

$$v = \frac{a^2 (Dp-Dm)\omega^2 r}{18\eta}$$

where a = diameter of the particle (cm)
Dp = density of the particle ($g.cm^{-3}$)
Dm = density of the surrounding fluid ($g.cm^{-3}$)
η = viscosity of the fluid (poises)
ω = angular velocity ($radians.sec^{-1}$)
r = radial distance from axis of rotation (cm)

Boone et al. (1968) demonstrated that mammalian cells tend to obey this sedimentation equation when centrifuged in a Ficoll solution. This means that mammalian cells tend to

behave as ideal particles under such conditions. Consideration of the equation described above reveals the two basic principles by which cells can be separated by centrifugation.

b. Separation of testis cells on the basis of their density

When cells are centrifuged through a solution with increasing density their velocities will decrease until they are zero when the density of the surrounding fluid is equal to the density of the cells. By centrifugation of testis cells through a discontinuous density gradient of Ficoll-Metrizoate it was demonstrated that lutropin responsive Leydig cells had a higher buoyant density than most of the contaminating testis cells (appendix paper I). This is in agreement with the results of Meistrich and Trostle (1975) using mouse testis cells. They found that only spermatogonia, late spermatids and spermatozoa had higher buoyant densities than Leydig cells. However,because of the hypertonicity of the Renografin gradient used by these authors, true cell buoyant densities were not determined.When we centrifuged the testis cells (containing 6% Leydig cells) through a Ficoll-Metrizoate discontinuous gradient a 13 times purification of Leydig cells was obtained (approximately 78% of the cells were Leydig cells as was determined by 3β-hydroxysteroid dehydrogenase histochemistry). Although by this procedure cell suspensions were obtained containing a high proportion of Leydig cells a variant of this procedure for purification of Leydig cells, based on the same principle (see section below) was preferred for routine experiments. This gave less purified Leydig cell suspensions (60%) but it was less time-consumi ınd gave a higher total number of Leydig cells.

When testis cells were centrifuged through a Ficoll solution with a density inbetween the density of the Leydig cells and the large amount of contaminating testicular cells the Leydig cells were sedimented to the bottom of the tube whereas most of the other cell types floated to the top of the Ficoll solution. By this procedure the proportion of Leydig cells in the cell suspensions increased from 6% to 35% as determined by 3β-hydroxysteroid dehydrogenase histo-

chemistry. No adverse effects of this procedure was observed on the steroidogenic capacity of the Ficoll purified Leydig cells. These cells were further purified to 60% for routine experiments (see section c).

Erythrocytes are the most abundant contaminating cells after centrifugation through Ficoll. Several methods have been described in literature for removal of erythrocytes e.g. agglutination of erythrocytes followed by centrifugation or sedimentation, specific lysis of the erythrocytes and density gradient centrifugation. We have tried several of these methods. When testis cells were centrifuged through a 13% Ficoll solution (with a density of 1.053 g/ml) under which had been introduced a 20% Ficoll solution, the Leydig cells were found at the interface of the 13% and 20% Ficoll solutions whereas most of the erythrocytes sedimented to the bottom of the tube. However total removal of erythrocytes was not possible by this method. Removal of erythrocytes by specific lysis was performed as described by Roos and Loos (1970), which consisted of incubating the cell suspensions in a solution of NH_4Cl (155 mM), $KHCO_3$ (10 mM) and EDTA 0.1 mM pH 7.4 for 10 min at $0^{\circ}C$. This method resulted in total removal of erythrocytes from the cell suspensions, but the testosterone production of the Leydig cells was decreased by 25% indicating that the Leydig cells were also damaged. Erythrocytes can also be removed by perfusion of the testis as described by Frederik et al. (1973). Using this procedure no erythrocytes were found in the Leydig cell suspensions and no adverse effects on the Leydig cells were observed, however,this method is time-consuming and therefore unsuitable for routine experimentation. Because it was demonstrated that protein synthesis was very low in rat blood cells (appendix paper III) it was decided not to remove the erythrocyte contamination from the Leydig cell suspension.

c. Separation of testis cells on the basis of their diameter

After centrifugation through Ficoll solutions (density (1.053 g/ml) all sedimented cells have a density higher than 1.053 g/ml. When these sedimented cells are centrifuged

through a medium with a density much lower than 1.053 g/ml, the sedimentation rate will become more dependent on differences in cell diameter.

It was found that Leydig cells were one of the fastest sedimenting testis cells in media with lower densities. This was shown by separation of testis cells by velocity sedimentation at 1 g (Meistrich et al., 1973; Davis and Schuetz, 1975). We have made use of this characteristic of the Leydig cells for their further purification. The testis cells were first sedimented by centrifugation through the Ficoll solution, followed by centrifugation for a very short time (2 min) through a 6% Dextran solution (density of 1.023 g/ml). It was found that the Leydig cells were among the first cells to sediment (appendix paper I). By this procedure it was possible to purify the Ficoll purified Leydig cells further from 35% to about 60%, as determined by 3β-hydroxysteroid dehydrogenase histochemistry. The purified Leydig cells obtained in this way were responsive to lutropin and could be obtained in sufficient quantities which made analytical studies of specific protein synthesis possible. In addition the whole procedure of purification only took 75-90 min. This Leydig cell preparation fulfilled all the criteria stated in section 3.1 and it was therefore decided to use it for further experimentation.

d. Other methods for the purification of Leydig cells

Another possible way of purifying Leydig cells is to make use of the specific lutropin receptors which are present in the plasma membrane. This could be achieved by affinity chromatography in which the Leydig cells are specifically retained on a column containing lutropin coupled to sepharose. This type of separation method has been reviewed by Robbins and Scheerson (1974). Precautions must be taken to prevent stimulation of the cells by this method and separation of the coupled hormone and the Leydig cells must be carried out under conditions, which do not damage the cells, this would exclude the use of low pH (Dufau et al., 1972) or high salt

concentrations (Chen and Payne, 1977), conditions which have been used previously to dissociate lutropin-Leydig cell receptor complexes. Immunofluorescence labelling of the lutropin receptors (Hsueh et al., 1976a) followed by separation of the labelled and unlabelled cells with a cell sorter could also be used to purify Leydig cells. Another possibility would be to make use of the fact that Leydig cells are the only testis cells which form rosettes with lymphocytes (Rivenson et al., 1974). These cell rosettes could then be easily separated from the other cells by velocity sedimentation or centrifugation.

3.5 Leydig cell tumours

Several investigators have used Leydig cell tumours as a source of Leydig cells (Moyle et al., 1971; Shin and Sato, 1971; Inano et al., 1972; Yang et al., 1974; Jull et al., 1974; Wolff and Cooke, 1977). Recently we obtained a transplantable rat Leydig cell tumour which has lutropin receptors and produces androgens. Histological and histochemical examination revealed the presence of only one cell type in addition to blood and connective tissue cells. Several differences between these tumour Leydig cells and Leydig cells from mature rat testis became apparent during our study.

The amount of testosterone was only a small fraction of all the steroids produced and in addition lutropin stimulated the synthesis of proteins in the tumour Leydig cells which were different from those in Leydig cells from adult rats (appendix paper VI). Other investigators have also reported abnormalities in tumour Leydig cells: e.g. loss of lutropin receptors (Shin et al., 1968; Wolff and Cooke, 1977) and changes in the steroids secreted (Shin et al., 1968; Jull et al., 1973; Wolff and Cook, 1977). The Leydig cell tumour we obtained met all the requirements stated in section 3.1: namely it possessed a lutropin sensitive steroidogenesis, a high purity of Leydig cells and the cells could be obtained

in large quantities.

3.6 Incubation conditions

Krebs Ringer bicarbonate buffer (Umbreit et al., 1964) is the most widely used medium for short term incubations with Leydig cell preparations. In the present study when Eagles medium (Eagle, 1959) was used instead of Krebs Ringer bicarbonate buffer it was found that similar results were obtained with respect to lutropin stimulated testosterone production. Van Damme et al. (1974) reported that addition of calfs' serum to Eagles medium resulted in a 3-fold increase in lutropin sensitivity of steroidogenesis in mouse testis cells compared with 1.5 times increase in the case of addition of calfs' serum to Krebs Ringer bicarbonate buffer. In contrast to their findings we could not observe a change in lutropin sensitivity of steroidogenesis in rat testis Leydig cells by addition of calfs' serum to any of these media and even a decrease in total testosterone production was found.

As described in section 2.3.2 the presence of Ca^{++} in the incubation medium is a prerequisite for maximum corticotropin stimulation of steroidogenesis on adrenal cells. For Leydig cells it has been reported that omission of Ca^{++} from the medium resulted in only a slight decrease in trophic hormone stimulation of testosterone production (Mendelson et al., 1975). However we observed a 60% decrease in lutropin stimulated steroidogenesis in rat testis Leydig cells in the absence of Ca^{++}. Under these same conditions basal testosterone production was unchanged. Addition of Ca^{++} to the medium restored the testosterone response to lutropin within 30 min, indicating that the effect of Ca^{++} was not due to irreversible damage of the Leydig cells. Activation of cyclic AMP dependent protein kinase by lutropin was not decreased by omission of Ca^{++} from the incubation medium, suggesting that

Ca^{++} may be involved in steroidogenesis at a stage beyond the lutropin receptor-adenylate cyclase-protein kinase system (appendix paper II). Also in adrenal cells it has been postulated that Ca^{++} is involved in processes beyond the formation of cyclic AMP, although in these cell types a role of Ca^{++} has also been suggested for the transmission of the signal arising from corticotropin receptor interaction to the adenyl cyclase (see section 2.3.2). In all further experiments reported in this thesis a Ca^{++} concentration of 2.5 mM was used in the incubation medium.

3.7 Testosterone production in Leydig cell preparations

Leydig cell preparations can be characterized by their testosterone production and its sensitivity to lutropin. Table 3.1 summarizes the testosterone production in different

Table 3.1 Comparison of testosterone production in different Leydig cell preparations (ng testosterone/mg protein/hour)$^{+}$ in the presence or absence of added lutropin.

	testosterone production (ng/mg protein/hour) control	added lutropin^{+}	reference
total testis tissue	0.5	3.0	1
interstitial tissue (obtained by wet dissection)	2.4	6.7	2
Dextran purified Leydig cells	10.2	295.9^{++}	3

ref.: 1. Rommerts et al., 1973a
2. Cooke et al., 1975
3. Janszen et al., 1976a

$^{+}$ Leydig cell preparations were incubated during 2-4 hours. For comparison purposes linear production rate during this period is assumed and the total production is divided by the incubation time.

$^{++}$ 10^6 cells in these preparations correspond with 0.196 $\pm$ 0.028 mg protein (mean $\pm$ s.d., n=6).

Table 3.2 Comparison of testosterone production and amount of Leydig cells in Leydig cell suspensions used by different groups of investigators. Number between parentheses refers to the reference.

group	testosterone production (ng T/10^6 cells/hour)+		% of Leydig cells
	control	+trophic hormone	
Catt and Dufau	0.2-1.1	1.3-7 (1)	± 40(2)
Moyle and Ramachandran	0.1-3	5-24(3)	
Cooke and Janszen:			
crude preparation	0.5	3.5 (1)	± 6 (6)
purified preparation	2.0	58 (5)	± 59(6)

+Testis cells were incubated during a 2-3 hours period. For comparison purposes linear production rate during this period has been assumed and the total production is divided by the incubation time.

1. Williams and Catt, 1976a; Williams et al., 1976b.
2. Determined by 3β-hydroxysteroid dehydrogenase histochemistry (Dufau and Catt, 1975b) and by immunofluorescence labelling of lutropin receptors (Hsueh et al., 1976a).
3. Moyle and Ramachandran, 1973; Ramachandran and Sairam, 1975.
4. Janszen et al., 1976a.
5. Janszen et al., 1976b.
6. Determined by 3β-hydroxysteroid dehydrogenase histochemistry (Janszen et al., 1976a).

Leydig cell preparations. Dextran purified Leydig cell suspensions gave the highest production rates. This was most probably due to the presence of higher amounts of Leydig cells per mg protein, a good contact between the Leydig cells and the medium containing the lutropin and a better integrity of the Leydig cells. In table 3.2 the results from various groups of investigators are compared. The highest and lowest values quoted in the literature of the respective groups are given. The values reported by the group of Catt and Dufau are comparable with the values reported by us, whereas the estimated number of Leydig cells in their preparations is 6-7 times higher than in our crude preparations. The reason for this discrepancy is not known. It is possible that their preparations contain more lutropin unresponsive Leydig cells. Purification of our crude Leydig cell suspension by the 2 centrifugation steps described in section 3.4 resulted in a higher number of Leydig cells in the cell suspensions and concomitantly in a higher steroidogenic activity. In table 3.3 the testosterone production in Dextran purified Leydig cell suspensions is compared with the testosterone production in vivo and in vitro of total testis homogenates

Table 3.3 Comparison of testosterone production in Leydig cell suspensions, total testis homogenate and by testis in vivo. All production rates are standardized per total testis.

	testosterone production (nmol/testis equivalent/hour)		reference
	control	lutropin treated	
in vivo	5.4		1
in vitro:			
total testis homogenate	4.8	19.8	2
Dextran purified Leydig cells	1.0	11.1	3

+These cell preparations contain 59% Leydig cells as determined with 3β-hydroxysteroid dehydrogenase histochemistry and it is assumed that a rat testis contains $32.4 \cdot 10^6$ Leydig cells (Christensen, Testis Workshop, Toronto, 1977).

1. De Jong et al., 1973.
2. Van der Vusse, 1975.
3. Janszen et al., 1976b.

under control conditions and when stimulated before isolation. The control production of the cell suspension is lower than the control production in vivo and from the total testis homogenates. This may be due to the fact that in vivo some lutropin is present while it is probably better to compare the production of testosterone in hypophysectomized animals with the control production of the cell suspension. Stimulation with lutropin resulted in comparable testosterone production rates in testis homogenates and in the Leydig cell suspensions, which is an indication that the isolation and purification procedure does not markedly impair the lutropin regulatory mechanism in the Leydig cells.

The minimal dose of lutropin needed for stimulation of steroidogenesis in vitro is approximately 1 ng/ml and maximal stimulation is obtained with 10-100 ng/ml (Janszen et al., 1976a). These concentrations of lutropin are in the same range as the physiological plasma concentrations determined in the male Wistar rat i.e. 10-50 ng/ml (van Beurden, 1977).

One of the discrepancies between the lutropin stimulation of steroidogenesis in vivo and in vitro is the longer time needed in vitro to observe stimulation of steroidogenesis after addition of trophic hormone. Eik-Nes observed an increase in secreted steroids 3-6 min after the addition of choriogonadotropin to perfused dog testis, whereas lutropin stimulation of steroidogenesis in different Leydig cell preparations in vitro takes at least 20 min (table 3.4). However,preincubation of Leydig cell suspensions for 1 hour results in a decrease of this lag phase to less than 5 min (Cooke et al., 1977a). The reason for this observation may be due to repair of some damage to Leydig cells which occurred during the isolation procedure or alternatively to loss of some essential factor which is resynthesized during the preincubation period.

It may be concluded that Dextran purified Leydig cells after stimulation with physiological amounts of lutropin in vitro produce amounts of testosterone comparable with those

Table 3.4 Time needed for trophic hormone stimulation of steroidogenesis in different testis preparations.

	time needed for first detectable increase in steroidogenesis	reference
perfusion of dog testis in vitro:	<3-6 min	1
total testis tissue	60 min	2
interstitial tissue	30-60 min	3
Leydig cell suspension		
without preincubation	20-30 min	4,5
with 1 h preincubation	<5 min	5

1. Eik-Nes, 1975.
2. Rommerts et al., 1972.
3. Rommerts et al., 1973a.
4. Janszen et al., 1976a.
5. Cooke et al., 1977a.

produced in vivo and therefore provide a good model for studying lutropin regulatory mechanisms.

PROTEIN SYNTHESIS AND LUTROPIN REGULATION OF STEROIDOGENESIS

4.1 Introduction

On the basis of experiments with protein synthesis inhibitors it has been proposed that the continuous synthesis of a protein with a short half life is necessary for the lutropin stimulation of testosterone production in rat testis Leydig cells (Cooke et al., 1975a; Mendelson et al., 1975). In section 2.3.4 three possible models (figure 2.2) which have been proposed to explain the possible role of this protein in the regulation of steroidogenesis in the testis and other organs have been discussed. These possibilities concerned:
A) regulation of steroidogenesis by trophic hormones mediated by de novo synthesis of a protein with a short half life.
B) in the presence of the trophic hormone an inactive protein with a short half life is converted into an active form with short half life.
C) a protein with a short half life plays a permissive role in the stimulation of steroidogenesis by trophic hormones.

One way to discriminate between models A versus B and C is to identify the specific protein(s) involved. This can be carried out for example by investigation of the effect of different protein fractions from the steroidogenic tissue on various enzymatic reactions involved in steroidogenesis (Farese, 1971b; Kan and Ungar, 1973) followed by investigation of the half life of the active protein(s) and its regulation by trophic hormone. A second way is to investigate the synthesis of specific proteins with a short half life in steroidogenic cells using labelled amino acid precursors (Grower and Bransome, 1970). After the existence of such a protein has been demonstrated, it has to be isolated and its role in the regulation of steroidogenesis has to be determined.
Using the first method Farese (1971) demonstrated increased steroidogenic activity in control adrenal mitochondrial preparations after addition of the 60,000 g supernatant from

corticotropin treated adrenal glands. The formation of the corticotropin induced factor in the supernatant was blocked with puromycin and possessed the properties of a protein(s). However in this study a control experiment to determine the steroidogenic activity of the 60,000 g supernatant was not carried out. In a later publication the same authors demonstrated that homogenization of adrenal glands can result in solubilization of the cholesterol side chain cleavage enzyme complex (Farese and Prudente, 1977a), which may explain the earlier found activity of this protein fraction. Kan and Ungar (1973) have also described an adrenal protein factor, which stimulates cholesterol side chain cleavage, but which is not under the control of corticotropin.

The second approach was followed by Grower and Bransome (1970) who incubated corticotropin stimulated and control mouse adrenocortical cells with ^{3}H-leucine and separated the ^{3}H-labelled proteins of the cytosol by polyacrylamide gel electrophoresis followed by counting the amount of ^{3}H in the different fractions of the polyacrylamide gel. These authors observed a transient increase in radioactivity in one protein fraction and at the same time a transient decrease in another protein fraction. No further information has been published about the nature of these proteins and their eventual relationship to steroidogenesis. Using a similar approach Rubin et al. (1974) and Laychock and Rubin (1974) have described the corticotropin induction of 4 specific proteins in the perfusate of cat adrenal with apparent mol. wt.'s of respectively 12,500, 48,000, 58,000 and 70,000. No further information about the role of these proteins has been published.

In our study it was decided to follow the second approach. This was carried out by incubation of dextran purified Leydig cells with lutropin for various periods of time followed by addition of radioactive amino acids to the medium to label newly synthesized proteins. After incubation of the cells, total protein was extracted and separated by SDS-polyacrylamide gel electrophoresis. Radioactivity in the separated proteins was determined by counting slices of the

polyacrylamide gel in a liquid scintillation counter or by autoradiography (appendix papers III and IV).

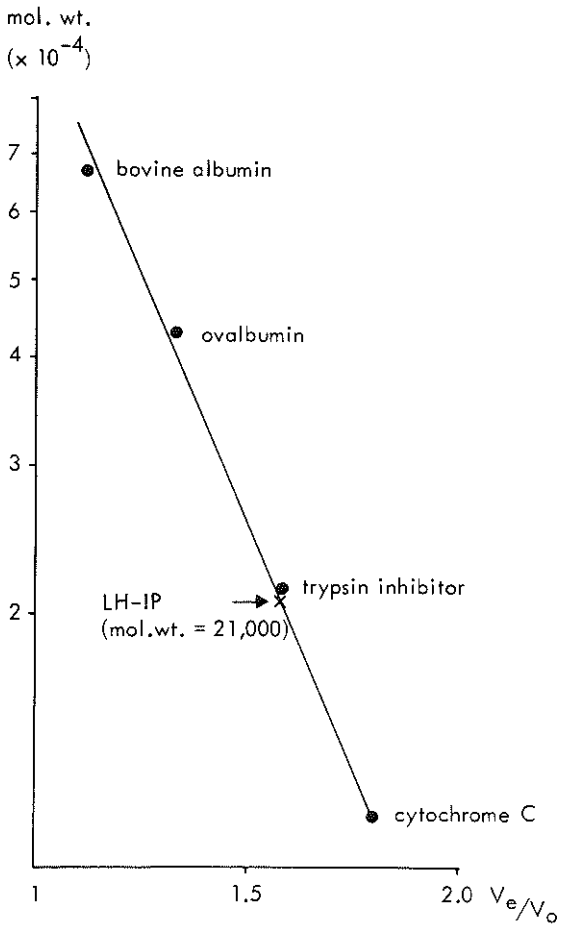

Figure 4.1 Calibration curve for the estimation of the mol. wt. of lutropin induced protein (LH-IP). Data were obtained from gel filtration experiments on Sephadex G-100 and transposed to a single calibration curve. As eluant was used 0.15 M $(NH_4)HCO_3$ pH 7.4. The presence of LH-IP was demonstrated by SDS-polyacrylamide gel electrophoresis of the various fractions followed by autoradiography as described in the materials and methods section in appendix papers III and IV.

4.2 Effect of lutropin and cycloheximide on specific protein synthesis

4.2.1 Lutropin induced protein in rat Leydig cells

The results of experiments carried out to investigate the effect of lutropin and cycloheximide on specific protein synthesis in rat testis Leydig cells are presented in appendix papers III and IV. Using ^{35}S-methionine increased synthesis of a protein with an apparent mol. wt. of 21,000 was observed 2 hours after the addition of lutropin to the Leydig cells and the synthesis was at a maximal rate 2-3 hours later. The synthesis of this protein (referred to as LH-IP) was only stimulated by lutropin and was not due to increased levels of testosterone or to contaminating amounts of follitropin in the lutropin preparation. This protein was only detected in the enriched Leydig cell preparation (60% Leydig cells) and not in seminiferous tubules or in rat blood cells and it could hardly be detected in a crude Leydig cell preparation (6% Leydig cells). It was therefore concluded that in the rat testis LH-IP is synthesized only in the Leydig cells. Subcellular fractionation of the Leydig cells indicated that the protein is present in the cytosol fraction. Determination of the mol. wt. of LH-IP by SDS-PAGE and Sephadex-G100 chromatography (figure 4.1) gave identical values, indicating that under the conditions used for Sephadex chromatography LH-IP is present as a monomer. Incubation of the cells for 30 min in the presence of cycloheximide after labelling of the proteins with ^{35}S-methionine, did not result in a decrease in radioactivity in LH-IP indicating that the half life of LH-IP is longer than 30 min. It can be concluded therefore that LH-IP is not identical to the postulated protein with a short half life ($t_{\frac{1}{2}} = \pm$ 13 min) that is involved in the lutropin stimulation of steroidogenesis.

At this moment one can only speculate about the role of LH-IP in rat testis Leydig cells. LH-IP may be involved in

the regulation of steroidogenesis, in the trophic effects of lutropin or possibly in other lutropin regulated processes in Leydig cells. If LH-IP is involved in lutropin stimulation of steroidogenesis it is difficult to explain why stimulation of the synthesis of LH-IP can be detected only 2 hours after addition of lutropin to the cells whereas steroidogenesis is already stimulated within 5-30 min (figure 4.2). It is possible that at the start of the incubation enough LH-IP is present for the lutropin stimulation of steroidogenesis and that new synthesis is only required sometime later when the amount of LH-IP becomes rate-limiting. This hypothesis implies that other factors regulated by lutropin are also necessary for the stimulation of steroidogenesis.

It is also possible that LH-IP is a negative regulator of lutropin stimulation of steroidogenesis. Decreases in testosterone production after prolonged stimulation with lutropin have been found, e.g. injection of choriogonadotropin or lutropin into mature and immature rats results in a initial rise in plasma testosterone level followed by a decrease some hours later (Hsueh et al., 1976b, Sharpe, 1976; Haour and Saez, 1977). During in vitro incubation of Leydig cells with lutropin the testosterone production also increases and then after 2-3 hours decreases. In the adrenal gland inhibitory factors of steroidogenesis in cell-free fractions have been described (Farese, 1971a; Ungar et al., 1973). Recently Honn and Chavin (1977) found that actinomycin D increased corticotropin stimulated steroidogenesis in the human adrenal gland and these authors suggested that actinomycin D prevents the accumulation of a mRNA species which may direct the synthesis of a steroidogenic inhibitory protein. The found decrease in testosterone production may also be due to a lack of steroid precursor. However,administration of choriogonadotropin to rats did not have a detectable effect on total amount of cholesterol in the testis (van der Molen et al., 1972), although precise knowledge is lacking about the cholesterol pool used for steroidogenesis; this pool may be small and

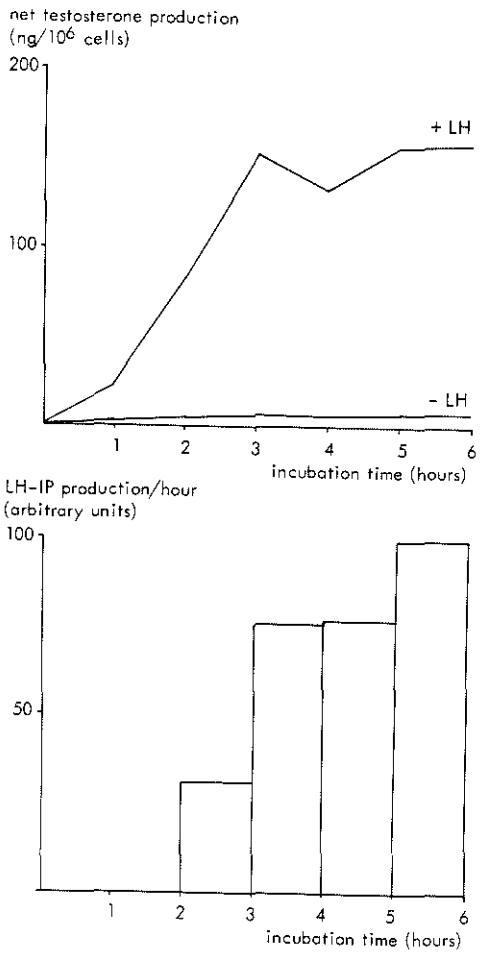

Figure 4.2 Time course of lutropin stimulation of testosterone and LH-IP production in rat Leydig cells. For testosterone production the Dextran purified Leydig cells were incubated as described in the materials and methods section of appendix paper III, in the presence or absence of added lutropin (100 ng/ml) and at the given times testosterone level was determined and the zero time level was subtracted. In the case of LH-IP synthesis Dextran purified Leydig cells were incubated for different time periods with added lutropin, then ^{35}S-methionine was added to the cells and the incubation was continued for 1 hour. The amount of newly synthesized LH-IP was estimated as described in appendix paper V.

could be exhausted soon after stimulation. A third possibility to explain the decrease in production rate of testosterone is a decrease in the signal arising from the hormone receptor interaction. Several investigators have reported a decrease in the number of lutropin receptor sites in the testis several hours after administration of the hormone in vivo (section 2.3; Sharpe, 1976,1977; Hsueh et al., 1976b, 1977; Chen and Paine, 1977; Haour and Saez, 1977). Sharpe (1977) demonstrated that administration of cycloheximide blocked these processes, suggesting that protein synthesis may play a role in this decrease. Again it is possible that LH-IP is involved in such a process.

LH-IP may also be related to the trophic effects of lutropin on Leydig cells. Long term treatment (5 days) of rats with human choriogonadotropin has been demonstrated to result in an increase in steroid production in testis homogenates and mitochondrial preparations (van der Vusse et al., 1975a). Purvis et al (1973) reported an increase in activity of the microsomal cytochrome P_{450} dependent enzymes 17α-hydroxylase and steroid C17,20-lyase after long term treatment with human choriogonadotropin and van der Vusse et al. (1975a) demonstrated an increase in 3β-hydroxysteroid dehydrogenase activity after such a treatment. The lack of effect of lutropin on specific protein synthesis within 2 hours after its addition to the cells as observed in this study, may suggest that model A in figure 2.2 (regulation of steroidogenesis by trophic hormones is mediated by de novo synthesis of a protein with a short half life) does not apply to the lutropin regulation of steroidogenesis in rat Leydig cells. However, it is possible that we have missed such a protein and therefore more research is needed using a protein separation system with a higher resolving power than the SDS-polyacrylamide gel electrophoresis system used in this study e.g. 2-dimensional polyacrylamide gel electrophoresis system (O'Farrell,1975) and/or using other radioactive amino acids.

4.2.2 A protein with a short half life present in rat Leydig cells

The presence of a protein with a short half life in Leydig cells was investigated by incubation of the Leydig cells in the presence of cycloheximide, after labelling of the proteins with ^{35}S-methionine, for different time periods (appendix paper III). It was found that a rapid decrease occurred in the amount of radioactivity in a protein with apparent mol. wt. of 33,000. The half life of this protein (referred to as P33) was calculated to be approximately 11 min. This corresponds to the value of 13 min calculated from the decrease in lutropin stimulated testosterone production after addition of cycloheximide to Leydig cells (Cooke et al., 1975a). This protein was not detected in seminiferous tubules and rat blood cells and was hardly detectable in crude Leydig cell preparations (containing 6% Leydig cells). It was concluded therefore that it was specifically present in the Leydig cells. Subcellular fractionation revealed that this protein was present in the particulate fraction of the cell. A more precise localization was not possible because of the lability of the protein, which made prolonged fractionation studies difficult. No effect of lutropin on the synthesis, half life or mol. wt. of P33 was observed. After hypophysectomy of the rats for 16 days, P33 could still be detected.

According to model 2.2B the inactive regulator protein with a short half life is converted into an active form in the presence of trophic hormone. This modification may be achieved by splitting off part of the molecule, by phosphorylation etc. However, as already shown above, no effect of lutropin on the mol. wt. of P33 was observed and after incubation of the cells with lutropin no incorporation of ^{32}P into this protein could be detected (Cooke et al., 1977b). Therefore, if the disappearance of P33 is responsible for the effect of protein synthesis inhibitors on lutropin stimulated testosterone production, model 2.2C would seem to be the most appropriate, namely that a protein with a short half

life plays only a permissive role in the trophic hormone stimulated steroidogenesis.

A protein with a short half life (12 min) is ornithine decarboxylase, which catalyses the rate-limiting step in the synthesis of polyamines (Russell and Snyder, 1969). However, it is not identical with P33 because the mol. wt. of ornithine decarboxylase is approximately 75,000 (Jänne and Williams-Ashman, 1971). Recently it has been shown that choriogonadotropin increases ornithine decarboxylase activity in mouse testis cells after a lag period of 2-3 hours (Mobius et al., 1977). It is also interesting to note that in other tissues proteins with a short half life may be involved in the stimulation of prostaglandin production in transformed mouse fibroblasts (Pong et al., 1977) and in protein degradation (Epstein et al., 1975).

4.3 Lutropin regulation of LH-IP synthesis

The mechanisms involved in the lutropin regulation of LH-IP synthesis were investigated in more detail and the results obtained are reported in appendix paper V. A close correlation was found between the dose of lutropin needed for the stimulation of steroidogenesis and for the stimulation of LH-IP synthesis. Incubation of Leydig cells with db-cyclic AMP and choleratoxin with amounts which stimulated testosterone production also stimulated LH-IP synthesis, and addition of MIX (a phosphodiesterase inhibitor) to Leydig cells potentiated the effect of submaximal doses of lutropin on steroidogenesis and on LH-IP synthesis. From these results it was concluded that steroidogenesis and LH-IP synthesis are stimulated by lutropin at least partly by a common pathway, both involving cyclic AMP (see section 2.3.2). In eukaryotic cells regulation of the metabolic processes by cyclic AMP involves protein kinase activation. Lutropin stimulates the phosphorylation of 3 different proteins with apparent mol. wt.'s of 76,000, 58,000 and 14,000 (Cooke et al., 1977b). The phosphorylation of one or more of these proteins may be involved in the lutropin regulatory mechanism of LH-IP synthesis. At this moment,however,the subcellular localization

and biological function of these phosphoproteins are unknown. Stimulated phosphorylation of ribosomal protein by trophic hormone and cyclic AMP has been reported for other steroidogenic tissue such as the adrenal gland (Roos, 1973; Murakami and Ichii, 1973; Ichii et al., 1974) and the corpus luteum (Azhar et al., 1975b). This phosphorylation may result in altered ribosomal function and may be an indication of trophic hormone control of the synthesis of specific proteins.

In order to investigate whether LH-IP synthesis is regulated by lutropin at the level of translation or at the level of transcription Leydig cells were incubated with a dose of actinomycin D which inhibited 89-93% of RNA synthesis. When actinomycin D was added directly to the cells or 1 hour after the addition of lutropin, the stimulation of LH-IP was prevented. Addition of actinomycin D to the cells 4-5 hours after the start of the incubation no longer had an effect on the lutropin stimulation of LH-IP synthesis. These results could be interpreted as evidence that mRNA synthesis is necessary for the lutropin stimulation of LH-IP synthesis. However several aspecific effects of actinomycin D on cellular metabolism such as direct inhibition of protein synthesis have been described (Scott and Tomkins, 1975). In the present study a correction was made for the aspecific direct inhibitory effect of actinomycin D on protein synthesis because the synthesis of LH-IP was measured relative to the synthesis of another protein not affected by lutropin and it is assumed that actinomycin D affects at the same degree the synthesis of LH-IP and this protein. This is illustrated by the lack of effect of actinomycin D when it was added to the cells after full stimulation of LH-IP synthesis. However, in the experiments where actinomycin D was added at the start of the incubation other aspecific effects of actinomycin D may have caused the prevention of lutropin stimulation of LH-IP synthesis. For that reason these experiments must be repeated with different RNA synthesis inhibitors having different sites of action or even better the mRNA formed for LH-IP should be measured directly e.g. by using a mRNA translational system.

Incubation of the Leydig cells for 3 hours in the presence of actinomycin D after stimulation of LH-IP synthesis, did not result in a decrease in LH-IP synthesis, indicating that under these conditions the mRNA coding for LH-IP is stable and has a half life longer than 4 hours. Therefore synthesis of LH-IP may occur long after stimulation of the Leydig cells by lutropin. This may be the cause of the active synthesis of LH-IP which occurred in the controls of some experiments. Evidence that this reflected synthesis of LH-IP and not of some other protein with the same mol. wt. was obtained in experiments in which choriogonadotropin was administered to hypophysectomized rats. Leydig cells from these rats showed maximal LH-IP synthesis which could not be stimulated further by incubation of the cells with lutropin. Synthesis of a protein with the same apparent mol. wt. as LH-IP was always observed, even in rats hypophysectomized for 16 days. However,other methods will have to be used, e.g. other separation techniques or immunological methods, to confirm that there is only one single protein with a mol. wt. of 21,000 which is affected by these various conditions.

Hormonal induction of specific proteins has been studied in great detail for model systems which are under steroid hormone control (see review Tata, 1976; O'Malley, 1976). Less work has been done on cyclic AMP induced specific protein synthesis in eukaryotic cells (Wicks, 1974). The involvement of cyclic AMP may be by stimulation of the mRNA synthesis via phosphorylation of nuclear proteins. In the ovary cyclic AMP dependent translocation of cytoplasmic protein kinase to nuclear acceptor sites has been reported (Jungman et al., 1974, 1977; Spielvogel et al., 1977). However,in testis interstitial tissue Cooke and van der Kemp (1976) could not detect lutropin induced translocation of soluble protein kinase to another subcellular compartment. It is possible that only a small part of total kinase activity is translocated or that the cyclic AMP dependent protein kinase is already present in the nucleus.

Chiu et al. (1976) have observed an increase in testicular RNA-polymerase activity and chromatin template activity

after administration of choriogonadotropin to hypophysectomized rats. Reddy and Villee (1975) detected an increase in uridine incorporation into poly A containing mRNA in interstitial tissue 2 hours after the administration of choriogonadotropin to immature rats. However,these authors did not investigate these effects in interstitial cells from mature rat testis.

In conclusion the data described in this section indicate that the lutropin stimulation of LH-IP synthesis is mediated by cyclic AMP and may involve new mRNA synthesis. In figure 4.3 a hypothetical model is given for this lutropin regulatory mechanism.

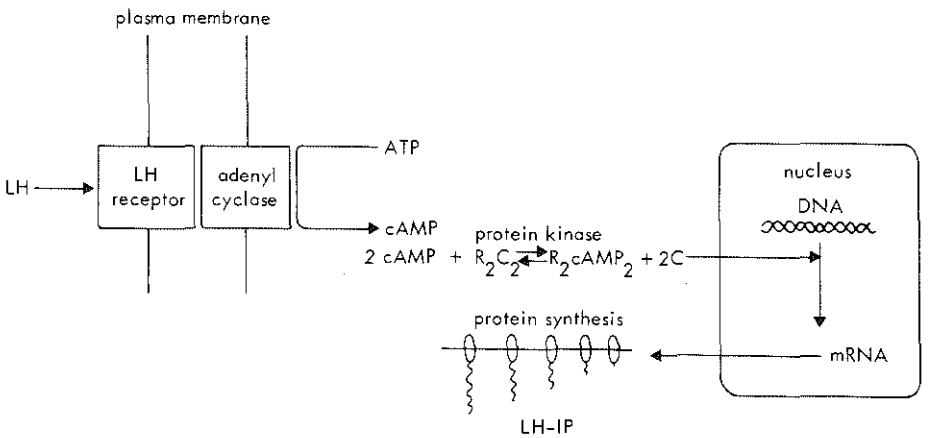

Figure 4.3 Hypothetical model for regulatory mechanism of lutropin on LH-IP synthesis

RC : protein kinase holoenzyme

C : catalytic subunit

R : regulating subunit

4.4 Comparison of lutropin stimulation of specific protein synthesis in Leydig cells from immature rats, mature rats and in tumour Leydig cells

The effect of lutropin on specific protein synthesis was investigated in Leydig cells from immature rats and in tumour Leydig cells and this was compared with the lutropin effect on specific protein synthesis in Leydig cells from mature rats. The results of this work are presented in appendix paper VI. Two hours after addition of lutropin to Leydig cells from immature rats the synthesis of proteins with apparent mol. wt.'s of 29,000, 27,000, 21,000 and 11,000 was stimulated and with high amounts of lutropin the synthesis of a protein with apparent mol. wt. of 13,000 decreased. In tumour Leydig cells lutropin stimulated the synthesis of two proteins with apparent mol. wt.'s of 29,000 and 27,000 (paper VI, table 4).All these proteins have half lives longer than 30 minutes and are therefore not identical with the postulated protein with a short half life. It is possible that the proteins present in the 3 different types of Leydig cells with the same apparent mol. wt.'s are similar. However, conclusive evidence can only be obtained when other properties of these proteins have been measured. In addition absence of an effect of lutropin on the synthesis of certain proteins in certain Leydig cell preparations does not necessarily reflect that these proteins are not synthesized in these cells. If all the detected lutropin sensitive proteins are involved in the lutropin regulation of steroidogenesis in these cells then the lutropin regulatory mechanism of steroidogenesis could be different in these different Leydig cell types. Differences in testosterone metabolism have been reported (van Beurden et al., 1976; appendix paper VI) and it has been suggested that the lutropin regulatory mechanism in Leydig cells of immature rats is different from that in Leydig cells of mature rats (van Beurden, 1977). None of the lutropin sensitive proteins detected in the Leydig cells from mature rats, immature rats and in tumour Leydig cells are identical to

trophic hormone sensitive proteins detected in other steroidogenic systems. The proteins secreted by the cat adrenal after stimulation with corticotropin for example have quite different mol. wt.'s (Rubin et al., 1974; Laychock and Rubin, 1974). Nothing is known about the nature of the corticotropin sensitive proteins in the adrenal, reported by Grower and Branssome (1970) and therefore it is not possible to compare them with the lutropin sensitive proteins in the rat Leydig cells.

In conclusion in Leydig cells from immature rat testis several lutropin sensitive proteins have been found with apparent mol. wt.'s of 11,000, 13,000, 21,000, 27,000 and 29,000. In Leydig cells from mature rat testis one protein with an apparent mol. wt. of 21,000 (LH-IP) is controlled by lutropin whereas in tumour Leydig cells lutropin regulates the synthesis of 2 proteins with apparent mol. wt.'s of 27,000 and 29,000.

SUMMARY

Testosterone production in the male rat testis takes place in the Leydig cells and is under the control of lutropin, which is secreted by the anterior pituitary. The stimulation of testosterone production by lutropin is inhibited by protein synthesis inhibitors such as cycloheximide and puromycin. According to literature after maximal stimulation by lutropin of testosterone synthesis in rat testis Leydig cells addition of cycloheximide decreases testosterone production to control values, the reaction following first order kinetics (half life 13 min).

These data indicate that the continuous synthesis of a protein(s) with a short half life equal or less than 13 min is involved in the stimulation of Leydig cell steroid synthesis by lutropin. The purpose of this study was to investigate the role and properties of the protein(s) involved in the lutropin regulation of testosterone production.

In order to carry this out it was necessary to develop a method for obtaining a lutropin sensitive, enriched Leydig cell preparation. This is described in chapter 3. Dissociation of the testis cells with collagenase resulted in a testis cell suspension containing 6% Leydig cells as was determined with 3β-hydroxysteroid dehydrogenase histochemistry. It was found that most of the contaminating cell types in this suspension had buoyant densities much below that of Leydig cells and these cell types could therefore be removed by centrifugation through a Ficoll solution with a density in between the density of these contaminating cells and that of the Leydig cells. The sedimented cells contained 35% Leydig cells. Centrifugation of these cells for a very short time (2 min) at 100xg through a 6% Dextran solution (density of 1.023 g/ml) resulted in a preferential sedimentation of the Leydig cells. The testis cells obtained by this procedure consisted of approximately 60% Leydig cells. The testosterone production rate by these cells after stimulation with physiological amounts of lutropin was comparable with the in vivo

production rate from the testis. It was also established that Ca^{++} is necessary to obtain full stimulation of steroidogenesis by lutropin.

It was decided first to investigate whether lutropin sensitive specific protein synthesis could be detected in the Leydig cells (chapter 4, section 2). Leydig cells were incubated with lutropin for various periods of time, then ^{35}S-methionine was added and the incubation was continued for some time in order to label the newly synthesized proteins. After incubation the proteins were extracted and separated by SDS polyacrylamide gel electrophoresis followed by autoradiography of the dried gel to detect the radioactivity incorporated into the proteins. Incubation of the Leydig cells from mature rat testis for 2 hours in the presence of lutropin resulted in increased synthesis of a protein with an apparent mol. wt. of 21,000 (referred to as LH-IP).

Five hours after the addition of lutropin to the cells the synthesis of LH-IP was maximally stimulated. This protein has a half life longer than 30 min and is present in the cytosol of the Leydig cells, probably as a monomer. A close correlation was observed between the doses of lutropin needed for stimulation of testosterone production and for the stimulation of LH-IP synthesis. The synthesis of LH-IP was also stimulated by the addition of db-cyclic AMP and choleratoxin to the Leydig cells in concentrations that stimulated testosterone production. Addition of MIX, a phosphodiesterase inhibitor, to the Leydig cells potentiated the effect of submaximal doses of lutropin on steroidogenesis and on LH-IP synthesis. These results indicate that the stimulatory effect of lutropin on LH-IP synthesis is mediated by cyclic AMP. Addition of actinomycin D, an RNA synthesis inhibitor, to the Leydig cells at the start of the incubation prevented the stimulation of LH-IP synthesis by lutropin, whereas addition of actinomycin D at later times after the start of the incubation resulted in a progressively smaller inhibitory effect. No effect could be observed when actinomycin D was added

after 5-6 hours. These results may be an indication that mRNA synthesis is involved in the lutropin stimulation of LH-IP synthesis. Administration of choriogonadotropin to hypophysectomized rats also resulted in increased synthesis of LH-IP.

Incubation of Leydig cells from immature rat testis for 1-2 hours with lutropin resulted in increased synthesis of 4 different proteins with apparent mol. wt.'s of 11,000, 21,000, 27,000 and 29,000 and at higher concentrations of lutropin (100 ng/ml) a decrease in the synthesis of a protein with an apparent mol. wt. of 13,000 was found. Incubation of tumour Leydig cells for 2 hours in the presence of lutropin resulted in increased synthesis of 2 different proteins with apparent mol. wt.'s of 27,000 and 29,000. Stimulation of specific protein synthesis occurred with the same concentrations of lutropin that stimulated steroidogenesis. Addition of actinomycin D to the cells at the start of the incubation prevented the lutropin stimulation of specific protein synthesis in both Leydig cell types, which may suggest that mRNA synthesis is necessary for their stimulation. All these proteins have half lives longer than 30 min and are therefore not identical to the postulated protein with a short half life, which is involved in the lutropin stimulation of steroidogenesis in rat testis Leydig cells. However a protein whose synthesis is not influenced by lutropin but has a short half life (11 min) was detected in Leydig cells from adult rats. This protein has an apparent mol. wt. of 33,000 and is located in the particulate fraction of the Leydig cells. No effect of lutropin on its synthesis, half life or mol. wt. could be detected.

Our results suggest, that the regulation of steroidogenesis by lutropin is not mediated by the induction or activation of a protein with a short half life (model 2.2 A and B), but that such a protein may only play a permissive role (model 2.2 C). However, a protein with a short half life which is under the control of lutropin may have been missed with the separation and detection methods used in this study. More research is needed using better separation methods to confirm this conclusion.

At this moment the biological functions of the lutropin and cycloheximide sensitive proteins present in the Leydig cells are unknown and further research is required to determine whether these proteins are involved in the lutropin regulatory mechanism of steroidogenesis.

SAMENVATTING

Testosteronproduktie in de testis van de mannelijke rat vindt plaats in de Leydig cellen. Deze produktie wordt gereguleerd door lutropin, dat uitgescheiden wordt door de hypofyse voorkwab. De stimulering van de testosteronproduktie door lutropin wordt geremd door eiwitsyntheseremmers zoals cycloheximide en puromycine. Wanneer na maximale stimulatie van de testosteronproduktie door lutropin cycloheximide aan de cellen toegediend wordt dan vermindert de testosteronproduktie tot het basale niveau. Deze afname volgt een 1e orde kinetiek (halfwaarde tijd: 13 min.). Deze gegevens tonen aan dat de voortdurende synthese van een of meer eiwitten met een korte halfwaarde tijd nodig is voor de stimulering van de steroidsynthese in Leydig cellen door lutropin. Het doel van deze studie was de rol en eigenschappen van de eiwitten, welke betrokken zijn bij de regulatie van de steroidsynthese door lutropin te onderzoeken.

Om dit onderzoek uit te voeren was het noodzakelijk om een methode te ontwikkelen voor het verkrijgen van een lutropin gevoelig preparaat verrijkt aan Leydig cellen. Dit gedeelte van het werk is beschreven in hoofdstuk 3. Het van elkaar losmaken van de testiscellen met behulp van collagenase resulteerde in een suspensie van testiscellen welke 6% Leydig cellen bevatte. Dit werd bepaald met behulp van 3β-hydroxysteroid dehydrogenase histochemie. Het bleek dat de meeste cellen in deze suspensie dichtheden bezaten, welke veel lager waren dan die van de Leydig cellen. Daarom was het mogelijk deze cellen te verwijderen door de testiscellen te centrifugeren in een Ficoll oplossing met een dichtheid tussen die van de Leydig cellen en de rest in. Van de neergedraaide cellen waren 35% Leydig cellen. Bij zeer kort centrifugeren (2 min.) van deze cellen bij 100 g. in een 6% Dextran oplossing (dichtheid: 1,023 g./ml.) werden voornamelijk de Leydig cellen neergedraaid. Door deze werkwijze werd een celpreparaat verkregen dat voor 60% uit Leydig cellen bestond. De testosteronproduktie van deze cellen na stimulatie met fysiologische

hoeveelheden lutropin was vergelijkbaar met de testosteronproduktie van de testis in vivo. Bovendien werd vastgesteld dat voor een volledige stimulatie van de testosteronproduktie door lutropin de aanwezigheid van Ca^{++} in het inkubatiemedium noodzakelijk was.

Het onderzoek naar de aanwezigheid van lutropin gereguleerde specifieke eiwitsynthese in Leydig cellen van volwassen ratten is beschreven in hoofdstuk 4. Hiertoe werd lutropin aan een Leydig celsuspensie toegediend en na verschillende tijden werden de nieuw gesynthetiseerde eiwitten gemerkt met ^{35}S-gemerkt methionine. Na de inkubatie werden de eiwitten geïsoleerd en gescheiden door middel van SDS-polyacrylamide gel elektroforese, gevolgd door autoradiografie van de gedroogde gel om de radioaktiviteit in de eiwitten te meten. Wanneer deze Leydig cellen gedurende 2 uur geïnkubeerd werden in aanwezigheid van lutropin dan werd de synthese van een eiwit met een schijnbaar molekuulgewicht van 21000 (verder genoemd LH-IP) verhoogd. De synthese was 5 uur na toediening van lutropin aan de cellen maximaal gestimuleerd. Dit eiwit heeft een langere halfwaarde tijd dan 30 min. en is in het cytosol van de cel aanwezig, waarschijnlijk als monomeer. De LH-IP-produktie werd gestimuleerd met dezelfde hoeveelheden lutropin, welke nodig zijn voor de stimulatie van de testosteronproduktie. Hetzelfde gold ook voor de stimulatie met db-cAMP en choleratoxin. Toevoegen van MIX, een remmer van de fosfodiesterase, aan de Leydig cellen versterkte het effekt van submaximale hoeveelheden lutropin op zowel de testosteronsynthese als op de LH-IP-synthese. Deze resultaten wijzen erop dat het stimulerend effekt van lutropin op de LH-IP-synthese tot stand komt via het cyclisch AMP. Toediening van actinomycin D, een RNA-syntheseremmer, aan de Leydig cellen aan het begin van de inkubatie voorkwam de stimulering van de LH-IP-synthese door lutropin. Echter naarmate het actinomycin D later toegevoegd werd, werd het remmend effekt kleiner. Geen effekt van actinomycin D werd gezien wanneer het 5-6 uur na het begin van de inkubatie toegevoegd werd. Deze resultaten kunnen

erop duiden dat voor de stimulatie van de LH-IP-synthese door lutropin synthese van mRNA nodig is. Toediening van choriogonadotropin aan gehypofysektomeerde ratten had een verhoogde LH-IP-synthese tot gevolg.

Inkubatie van Leydig cellen van jonge ratten gedurende 1-2 uur in aanwezigheid van lutropin had een verhoogde synthese van 4 verschillende eiwitten tot gevolg met schijnbare molekuulgewichten van 11000, 21000, 27000 en 29000 en bij hogere lutropinkoncentraties werd een verlaging van de synthese van een eiwit met een schijnbaar molekuulgewicht van 13000 gevonden. Wanneer tumor Leydig cellen geïnkubeerd werden gedurende 2 uur in aanwezigheid van lutropin dan werd een verhoogde synthese van 2 eiwitten met schijnbare molekuulgewichten van 27000 en 29000 gevonden. Stimulering van de specifieke eiwitsynthese vond plaats met dezelfde hoeveelheden lutropin, welke ook de steroidsynthese stimuleerden. Wanneer bij het begin van de inkubatie actinomycin D aan de cellen toegediend werd, dan werd de lutropin gestimuleerde specifieke eiwitsynthese in beide Leydig celtypen voorkomen. Dit zou erop kunnen duiden dat voor deze stimulatie mRNA-synthese noodzakelijk is. Al deze eiwitten hebben een halfwaarde tijd welke langer is dan 30 min. en daarom zijn geen van deze eiwitten identiek aan het gepostuleerde eiwit met een korte halfwaarde tijd ($\lesssim$ 13 min.), welke betrokken is bij de lutropin gestimuleerde steroidsynthese in ratte Leydig cellen. Er werd echter in de Leydig cellen van volwassen ratten een eiwit ontdekt met een halfwaarde tijd van ongeveer 11 min. De synthese van dit eiwit stond echter niet onder invloed van lutropin. Dit eiwit heeft een schijnbaar molekuulgewicht van 33000 en is aanwezig in de deeltjesfraktie van de Leydig cel. Er kon geen effekt van lutropin gevonden worden op de halfwaarde tijd of molekuulgewicht van dit eiwit.

Onze resultaten suggereren, dat de regulatie door lutropin van de steroidsynthese niet verloopt via de induktie of aktivering van een eiwit met een korte halfwaarde tijd (model 2.2 A en B), maar dat een dergelijk eiwit wel aanwezig moet zijn wil stimulatie door lutropin plaats kunnen vinden (model 2.2C) Met de scheidings- en bepalingstechnieken, gebruikt in dit onderzoek kan echter een eiwit met een korte halfwaarde tijd, dat onder de kontrole van lutropin staat, gemakkelijk gemist zijn en daarom is verder onderzoek nodig, waarbij betere scheidingsmethoden gebruikt worden, om deze konklusie te bevestigen.

Op dit moment zijn de funkties van de lutropin en cycloheximide gevoelige eiwitten in de Leydig cellen onbekend, en meer onderzoek is vereist om te bepalen of deze eiwitten betrokken zijn in het lutropin regulatiemechanisme van de steroidproduktie.

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NON STANDARD ABBREVIATIONS

ACTH	- adrenocorticotropin
cyclic AMP	- adenosine 3':5'-monophosphate
cyclic GMP	- guanosine 3':5'-monophosphate
db-cyclic AMP	- N^6-2'-0-dibutyryl-adenosine-3':5'-monophosphate
FSH	- follitropin
g	- relative centrifugal force
HCG	- human choriogonadotropin
LH	- luteinizing hormone/lutropin
LH-IP	- lutropin-induced protein
Metrizoate	- 3-acetamido-2,4,6-triiodo-5-(N-methylacetamido)-benzoate
min	- minute
MIX	- 3-isobutyl-1-methylxanthine
mol. wt.	- molecular weight
mRNA	- messenger ribonucleic acid
n	- number of determinations
P33	- protein with mol. wt. 33,000
s	- second
s.d.	- standard deviation
SDS	- sodium dodecyl sulphate

TRIVIAL AND SYSTEMATIC NAMES USED IN THIS WORK

cholesterol	- 5-cholesten-3β-ol
corticosterone	- 4-pregnen-11β,21-diol-3,20-dione
pregnenolone	- 5-pregnen-3β-ol-20-one
testosterone	- 4-androsten-17β-ol-3-one
adenylate cyclase	- ATP pyrophosphase-lyase (cyclizing) (EC 4.6.1.1)
aminopeptidase	- amino-acyl dipeptide hydrolase (EC 3.4.1.3)
cholesterol esterase	- sterol-ester hydrolase (EC 3.1.1.13)
cholesterol side chain cleavage enzyme complex	- cytochrome P_{450} containing enzyme complex catalyzing the conversion of cholesterol into pregnenolone and isocaproic acid (NADPH dependent)
collagenase	- clostridiopeptidase A (EC 3.4.4.19)
17α-hydroxylase	- steroid, reduced-NADP: oxygen oxidoreductase (17α hydroxylating) (EC 1.14.1.7)
3β-hydroxysteroid dehydrogenase	- 3β-hydroxysteroid:NAD(P^+) oxido-reductase (EC 1.1.1.51)
C17,20-lyase	- cytochrome P_{450} containing enzyme complex catalyzing the conversion of 17α-hydroxyprogesterone into androstenedione and acetate (NADPH dependent)
nonspecific esterase	- carboxylic-ester hydrolase (EC 3.1.1.1)
ornithine decarboxylase	- L-Ornithine carboxy-lyase (EC 4.1.1.17)
protein kinase	- ATP: protein phosphotransferase (EC 2.7.1.37)
trypsin	- (EC 3.4.4.4)

NAWOORD

In dit proefschrift is het werk beschreven dat uitgevoerd is van augustus '73 tot medio '77 op de afdeling Biochemie II van de Erasmus Universiteit te Rotterdam.
Dit proefschrift zou nooit in de huidige vorm tot stand zijn gekomen zonder de medewerking van vele anderen. Enkelen wil ik hier met name noemen en hiervoor bedanken.
Mijn promotor, Brian Cooke, voor de prettige samenwerking, de kritische begeleiding en voor zijn nooit aflatende ijver om de manuskripten in begrijpelijk Engels om te zetten.
Henk van der Molen voor de kritische diskussies over het werk en de vele versies van de verschillende manuskripten.
Ria van Driel, die ondanks de vele routine in de praktische uitvoering van de proeven haar goede humeur wist te bewaren.
Willie Bakhuizen en Marja Decae voor het typen van de manuskripten.
Pim Clotscher voor het lopende houden van de in dit werk gebruikte apparatuur.
De andere medewerkers van Biochemie II voor de suggesties welke gegeven zijn tijdens de vele informele en formele besprekingen.
Verder wil ik nog de beide coreferenten, Dr. J.W. Hekkelman en Dr. H.R. Scholte bedanken voor het kritisch beoordelen van het proefschrift.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 24 november 1944 geboren in Delft. Na het behalen van het getuigschrift gymnasium β in 1963 aan het St. Stanislascollege te Delft werd begonnen met de studie biologie aan de R.U. te Leiden. In 1966 werd het kandidaatsexamen k' behaald en in 1969 het doctoraalexamen met als hoofdvak biochemie (Prof.Dr. L. Bosch) en als bijvakken dierfysiologie (Prof.Dr. H. Wolvekamp) en microbiologie (Dr. J. Wessels). Hij was in 1967 verbonden als studentassistent aan de afdeling Dierfysiologie en in '68/'69 aan de afdeling Microbiologie. In de periode '70/'72 was hij werkzaam op de afdeling Biochemie II van het Unilever Research Laboratorium te Vlaardingen en vanaf augustus 1973 is hij werkzaam als wetenschappelijk medewerker op de afdeling Biochemie II van de Faculteit der Geneeskunde van de Erasmus Universiteit te Rotterdam, alwaar het in dit proefschrift beschreven werk werd verricht.

APPENDIX PAPERS

J. Endocr. (1976), **70**, 345–359 *Printed in Great Britain*

PURIFICATION AND CHARACTERIZATION OF LEYDIG CELLS FROM RAT TESTES

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(*Received* 18 *September* 1975)

SUMMARY

An LH-responsive Leydig cell preparation (containing 6 ± 2 % Leydig cells) was obtained by collagenase treatment of rat testis. Centrifugation of this cell preparation through a 13 % Ficoll solution for 10 min at 1500 ***g*** resulted in a four times purification of the Leydig cells, with a concomitant increase in steroidogenic activity. Addition of 0·2 % albumin to the 13 % Ficoll solution, adjusted to 280 mosmol/l, resulted in a further twofold purification of the Leydig cells paralleled by a twofold increase in steroidogenic activity. Centrifugation of these Ficoll-albumin-purified Leydig cells through a 6 % dextran solution for 2 min at 100 ***g*** resulted in a further 1·7 times purification of the Leydig cells. A combination of the two centrifugation steps resulted in a 12·5 times purification of Leydig cells compared with the original crude cell suspension, while an increase in steroidogenic activity of 22·5 times was obtained. This final cell preparation contained 59 ± 17 % Leydig cells (mean ± S.D., $n = 6$). The recovery of Leydig cells was 29 %.

Collagenase treatment of testes deficient in spermatogenesis resulted in a cell preparation with the same steroidogenic activity as Ficoll-purified cells from normal testes. Centrifugation of these cells through a 13 % Ficoll solution gave only a limited increase in the steroidogenic activity. Isopycnic centrifugation of the crude cell preparation on a discontinuous Ficoll metrizoate gradient resulted in two discrete peaks of Leydig cells, one peak at a density of 1·039–1·055 g/ml and one at a density of 1·068–1·088 g/ml. Both types of cells produced testosterone. In the presence of LH, cyclic AMP production in both types of Leydig cells increased, but testosterone production was only increased by LH in the 'denser' Leydig cells and not in the 'light' Leydig cells.

No difference in sensitivity to LH could be observed between the Leydig cell preparations of different purity. Using a 60 min pre-incubation period the highest testosterone response was obtained with 100–1000 ng LH/ml. The same maximum testosterone response was obtained with 10–100 ng LH/ml when the pre-incubation period was omitted.

INTRODUCTION

The available evidence indicates that follicle-stimulating hormone (FSH) and luteinizing hormone (LH) have separate sites of action in the testis, and these hormones can activate adenyl cyclase and protein kinases in the tubular and interstitial compartment respectively (Castro, Alonso & Mancini, 1972; Cooke, van Beurden, Rommerts & van der Molen, 1972; Dorrington & Fritz, 1974; Means, 1974; Cooke & van der Kemp, 1976). Furthermore, it has been shown that cytoplasmic protein synthesis is necessary for LH stimulation of testosterone production (Hall & Eik-Nes, 1962; Shin & Sato, 1971; Sakamoto, Matsukura,

Tada, Watanabe & Imura, 1973; Cooke, Janszen, Clotscher & van der Molen, 1975; van der Vusse, Kalkman, van Winsen & van der Molen, 1975). Several studies on the localization of these testicular processes have used isolated interstitial tissue and seminiferous tubules which were separated from each other by wet dissection (Christensen & Mason, 1965; Rommerts, van Doorn, Galjaard, Cooke & van der Molen, 1973*b*). Although with this procedure seminiferous tubules can be obtained relatively free of interstitial tissue, the dissected interstitial tissue contains small but variable amounts of contaminating cells from the seminiferous tubules. This was demonstrated by electrophoresis of the proteins from dissected interstitial tissue after incubation with labelled amino acids; extra peaks of radioactivity were found due to protein synthesis in contaminating seminiferous tubular cells, furthermore the degree of contamination varied from one sample of dissected interstitial tissue to another (F. H. A. Janszen, B. A. Cooke & H. J. van der Molen, unpublished observations). Hence isolated interstitial tissue obtained in this way is unsuitable for studying certain aspects of LH action if replicate uniform aliquots of interstitial tissue are required (e.g. for incorporation of labelled amino acids into specific proteins using the double-labelling technique (Barnea & Gorski, 1970)).

A suspension of dissociated rat testicular Leydig cells should provide a more uniform preparation than the interstitium obtained after dissection of the testis.

Recently it has been reported that after collagenase treatment of decapsulated testes, Leydig cell preparations can be obtained, which respond to LH (Moyle & Ramachandran, 1973; Dufau, Mendelson & Catt, 1974; Van Damme, Robertson & Diczfalusy, 1974). However, using essentially the method of Moyle & Ramachandran (1973), we have obtained Leydig cell preparations which were highly contaminated with spermatogenic cells. Although this preparation is uniform, it is most probably unsuitable for studying specific LH-induced processes in Leydig cells. The purpose of the present study was to investigate methods for the further purification of these cell suspensions, obtained after collagenase treatment of the testes.

MATERIALS AND METHODS

Materials

Ovine LH (ovine, NIH-S18) was a gift from the NIAMDD, Bethesda, Maryland, U.S.A. The collagenase type 1 was purchased from Sigma Chemical Company, St Louis, Missouri, U.S.A., the Ficoll 400 from Pharmacia Fine Chemicals A.B., Uppsala, Sweden, the lima bean trypsin inhibitor from Boehringer Mannheim GmbH, the sodium metrizoate (i.e. the sodium salt of 3-acetamido-2,4,6-triiodo-5-(*N*-methylacetamido)-benzoic acid) from Neygaard & Co AS, Oslo, Norway, and the bovine albumin fraction V from Fluka A.G., Buchs, Switzerland.

Animals

The intact adult male rats were from the Wistar strain, sub-strain R-Amsterdam, and were between 3 and 5 months old. Testes with defective spermatogenesis were obtained from rats deficient in essential fatty acids (EFA) (van der Molen & Bijleveld, 1971; Rommerts *et al.* 1973*b*), from prenatally irradiated rats (Ellis, 1970) and from cryptorchid rats (VanDemark & Free, 1970).

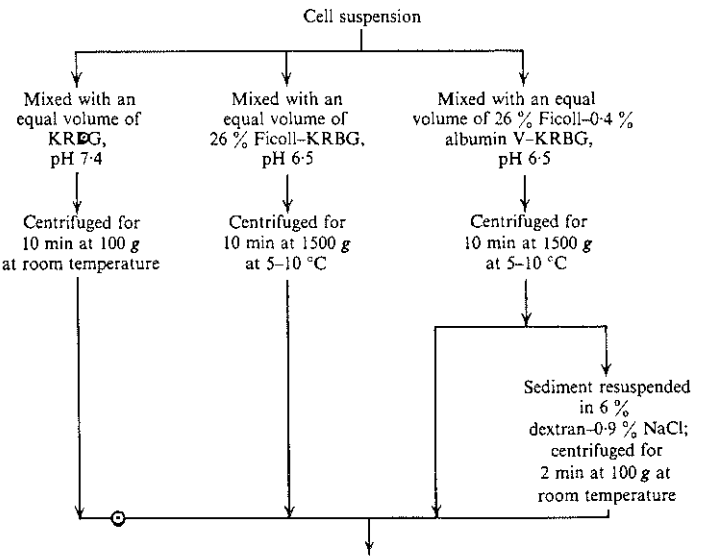
The EFA-deficient rats (obtained from Unilever Ltd, Vlaardigen, The Netherlands) were fed during weaning on a diet lacking in essential fatty acids and were 12 months old. Pregnant rats (18–20 days after conception) were irradiated with 150 rad. The rats were about 80 days old when used. Rats (3-5 months old) were made bilaterally cryptorchid by surgical fixation of the testes in the abdomen 3-5 weeks before use. After treatment, the weight of the testes was reduced by 30–40 % in the case of EFA-deficient rats and more than 50 % in

the case of the irradiated and cryptorchid rats. Histological investigations showed that most tubules were devoid of spermatogenic cells.

Isolation of cells

Rats were killed by decapitation and the testes were immediately removed and decapsulated. Two testes were then incubated in 7 ml Krebs–Ringer bicarbonate buffer containing 0·2 % glucose (KRBG) at pH 7·4 and 1 mg collagenase/ml under an atmosphere of $O_2:CO_2$ (95:5, v/v) at 37 °C for 18 min. Plastic incubation tubes of 40 ml capacity with tight-fitting caps were used. They were placed longitudinally in the water bath and were shaken with a frequency of 75 cycles/min. After incubation, 15 ml 0·9 % NaCl were added to each tube. The tubes were inverted several times and then left for 10 min at room temperature. The supernatant was carefully syphoned off with the aid of Tygon tubing and was filtered through 60 μm nylon gauze. The cell suspension was then centrifuged according to Table 1. Cell densities were determined by counting the nucleated cells in a haemocytometer.

Table 1. *Procedure for the centrifugation of rat testicular Leydig cells*



The supernatants were discarded and the sediments were resuspended in KRBG (pH 7·4) containing 1 % bovine serum albumin V and 0·1 mg lima bean trypsin inhibitor/ml.

KRBG, Krebs–Ringer bicarbonate buffer, containing 0·2 % glucose.

Preparation of density gradients

Discontinuous density gradients were prepared from high- and low-density stock solutions. For the composition of these stock solutions see Table 2.

The different densities of the gradient were obtained by mixing different volumes of the two solutions. The discontinuous density gradient was prepared in 50 ml polycarbonate tubes by successive layering of 7 layers of 5 ml of the solutions with decreasing densities: e.g. 1·088, 1·075, 1·068, 1·055, 1·049, 1·039 and 1·020 g/ml on each other. The cells (8×10^7) obtained by centrifugation for 10 min at 100 *g* in KRBG pH 7·4 (see above) were suspended

in 8 ml KRBG (pH 7·4), 0·2 % albumin and Ficoll-sodium metrizoate solution of low density (1 : 1, v/v) and layered on top of the gradient. The gradients were centrifuged for 40 min at 1500 *g* at 0 °C in a Sorvall centrifuge. After centrifugation, a 1 ml sample was removed with an Eppendorf pipette from the different cell layers at the interface of the successive densities. These samples were mixed with 5 vols KRBG pH 7·4 and centrifuged for 10 min at 100 *g* at room temperature. By this method 25 % of the total number of cells applied to the gradient could be collected. The recovery of phenyl esterase activity in these cells was 76 ± 8 % (mean ± S.D., $n = 3$). The sediments were resuspended in KRBG (pH 7·4), containing 1 % bovine serum albumin and 0·1 mg lima bean trypsin inhibitor/ml.

Table 2. *Composition of high- and low-density Ficoll–metrizoate stock solutions (essentially according to Loos & Roos, 1974)*

	High density	Low density
Ficoll 400	16·93 g	2·43 g
Sodium metrizoate solution (32·8 %)	17·5 ml	12·0 ml
0·175 M-Tris-HCl (pH 7·4 at 0 °C)	12·3 ml	11·6 ml
Krebs–Ringer solution*	—	17·5 ml
Albumin V	200 mg	200 mg
D-Glucose	200 mg	200 mg
Distilled water was added to give a final volume of 100 ml		
Refractive index (at room temperature)	1·3673	1·3442

The osmolarity was measured with a Knauer osmometer and was adjusted to 270–300 mosmol/l by adding solid NaCl.

* Composition: 125 mM-NaCl, 5 mM-KCl, 1·2 mM-$MgSO_4$, 35 mM-Tris and 1 mM-NaH_2PO_4 (pH 7·4 at 0 °C).

Incubation of the cell suspension

The cells were either pre-incubated for 1 h at 32° C under an atmosphere of O_2:CO_2 (95:5, v/v) or were directly incubated. The incubations were carried out in plastic tubes in volumes of 0·2–0·3 ml with a cell density of about 3×10^6 cells/ml at 32 °C under O_2:CO_2 (95:5, v/v) either in the absence or presence of added LH, with continuous shaking at 100 cycles/min.

Leydig cell markers

The following markers for Leydig cells were used: testosterone production; phenyl esterase activity (Niemi, Härkönen & Ikonen, 1966; Rommerts *et al.* 1973*b*); 3β-hydroxysteroid dehydrogenase histochemistry (Levy, Deane & Rubin, 1959; Niemi & Ikonen, 1963) and the periodic acid–Schiff's (PAS) reaction (Roosen-Runge & Anderson, 1959; Baillie, 1961; Niemi & Ikonen, 1963). They were determined as follows.

Testosterone production

After incubation, testosterone was extracted from the cells plus medium and determined by radioimmunoassay as described by Verjans, Cooke, de Jong, de Jong & van der Molen (1973).

Phenyl esterase activity

Phenyl esterase activity was determined as decribed by van der Vusse, Kalkman & van der Molen (1975).

Periodic acid–Schiff's staining

A drop of the cell suspension was dried on a glass slide in the presence of Bouin's fluid. The dried cell suspensions were fixed in Bouin's fluid for several days. Staining with periodic acid–Schiff's reagent was done according to Hotchkiss (1948).

Histochemical demonstration of 3β-hydroxysteroid dehydrogenase activity

A drop of the cell suspension was frozen on a glass slide either directly or after mixing with a drop of 6 % dextran in 0·9 % NaCl solution to obtain better preservation of cell morphology. The cell suspension was stored at −20 °C for several days, but never for longer than 2 weeks. Just before use it was dried in air, and 3β-hydroxysteroid dehydrogenase activity was demonstrated according to the method described by Levy, Deane & Rubin (1959). Instead of dehydroepiandrosterone dissolved in propylene glycol, epiandrosterone (3β-hydroxy-5α-androstan-17-one) dissolved in dimethylformamide was used as substrate (Wiebe, 1974).

RESULTS

Purification of Leydig cells by centrifugation through Ficoll solutions

After centrifugation of the cell preparation obtained by collagenase treatment of rat testes through different concentrations of Ficoll, the steroidogenic activity with and without added LH was increased several times (Fig. 1). The highest steroidogenic activity was obtained with Ficoll concentrations of 12·6–13·7 %. The same pattern was obtained when in similar experiments phenyl esterase activity was used as a marker for Leydig cells. A Ficoll concentration of 13 % was therefore chosen for subsequent experiments. Using this Ficoll concentration, 11 % of the cells in the original suspension were recovered, whereas 50 % of the steroidogenic activity and phenyl esterase activity in the original cell suspension was still present. The recovery of the steroidogenic and phenyl esterase activity decreased in the cell

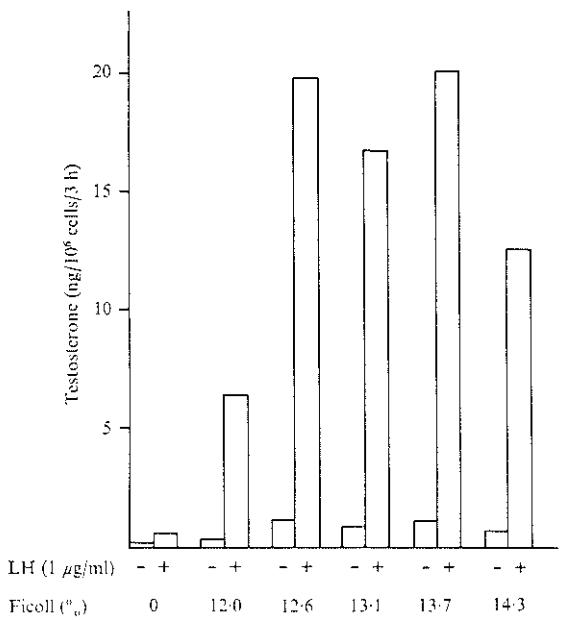


Fig. 1. Steroidogenic activity of sedimented testicular cells of rats, centrifuged for 10 min at 1500 *g* through various concentrations of Ficoll. The cells were first pre-incubated for 60 min.

preparations obtained after centrifugation through the higher Ficoll concentrations. Centrifugation for periods longer than 10 min (up to 40 min) did not increase the recovery of Leydig cells. In Tables 3 and 4 mean values of several experiments are given for the steroidogenic activity, phenyl esterase activity, proportion of cells stained with periodic acid–Schiff's reagent and proportion of cells showing 3β-hydroxysteroid dehydrogenase activity.

Table 3. *Basal and LH-stimulated testosterone production in isolated, unpurified and Ficoll-purified Leydig cells of rats* (*means* ± S.E.M.)

	Testosterone (ng/10^6 cells/3 h)	
	Control	+1 μg LH/ml
Unpurified cells	1·5 ± 0·1 (5)	10·6 ± 1·9 (5)
Ficoll-purified cells	4·6 ± 0·8 (5)	42·6 ± 4·1 (5)

Number of observations in parentheses.

Table 4. *Characteristics of unpurified and Ficoll-purified Leydig cells of rats* (*means* ± S.E.M.)

	Unpurified cells	Ficoll-purified cells
3β-Hydroxysteroid dehydrogenase-containing cells (%)	6·4 ± 1·5 (8)	27·6 ± 4·8 (8)
Periodic acid–Schiff positive cells (%)	7·6 ± 2·7 (8)	36·0 ± 5·1 (7)
Phenyl esterase activity (μmol nitrophenol/min/10^6 cells)	0·150 ± 0·33 (9)	0·549 ± 0·130 (9)

Number of observations in parentheses.

The results from all four markers indicate that the proportion of Leydig cells was increased approximately four times after centrifugation through a 13 % Ficoll solution as compared with centrifugation through KRBG alone.

Effect of osmolarity and albumin concentration

The osmolarity of the 13 % Ficoll solution in KRBG (pH 6·5) was found to be 320 mosmol/l, which was higher than the osmolarity of 283 ± 2 (S.E.M.) mosmol/l ($n \geq 6$) reported for rat serum (Williams, Kraft & Shortman, 1972). Lowering the osmolarity of the Ficoll solution to

Table 5. *Effect of adding 0·2 % albumin to 13 % Ficoll solution on the characteristics of the sedimented rat testicular cells* (*means* ± S.D.)

	Testosterone (ng/10^6 cells/3 h)		3β-Hydroxysteroid dehydrogenase-containing cells (%)
	Control	+1μg LH/ml	
13 % Ficoll (320 mosmol/l)	3·5 ± 2·6 (4)	18·3 ± 7·9* (4)	20 ± 10 (3)
13 % Ficoll, 0·2 % albumin V (280 mosmol/l)	5·4 ± 2·0 (4)	38·6 ± 13·3* (4)	36 ± 8** (3)

Number of observations in parentheses.
The increase (mean ± S.D.) of the steroidogenic activity in the four experiments was 206 ± 39 %.
* $P < 0.005$: compared with control value.
** $P < 0.005$: compared with the value from 13 % Ficoll only.

280 mosmol/l did not influence the steroidogenic activity of the sedimented cells. Addition of 0·2 % albumin to the Ficoll solution at this osmolarity resulted in a twofold increase of the steroidogenic activity of the sedimented cells. This increase was accompanied by a concomitant increase in the number of cells containing 3β-hydroxysteroid dehydrogenase activity (Table 5). Higher albumin concentrations (0·5 and 1·0 %) in the Ficoll solution abolished the purification effects, and the steroid production rates were the same for cell preparations purified by centrifugation through a Ficoll solution alone or through Ficoll containing 1·0 % albumin. The addition of 0·2 % albumin to the 13 % Ficoll solution with an osmolarity of 320 mosmol/l did not have any effect on the steroidogenic activity of the sedimented cells (data not shown).

Effect of Ficoll on steroidogenic activity

To investigate possible adverse effects of the Ficoll on the steroidogenic activity, the cell suspension obtained by centrifugation for 10 min at 100 *g* in KRBG was pre-incubated for 30 min at 32 °C in a 13 % Ficoll solution. In the control experiment the cell suspension was pre-incubated for 30 min in KRBG. The Ficoll was removed by centrifugation (10 min at 100 *g*) of the cells after addition of excess KRBG. The cells were then incubated with and without added LH (1 μg/ml). In the control experiment the LH-stimulated testosterone production was 9·6 ± 0·1 ng/10^6 cells/3 h, and in the cell suspension pre-incubated with Ficoll was 8·1 ± 0·3 ng/10^6 cells/3 h.

Incubations in the presence of 0, 1·8 and 3·6 % Ficoll gave LH-stimulated testosterone productions of 7·0 ± 1·4, 9·5 ± 0·4 and 7·5 ± 0·2 ng/10^6 cells/3 h respectively (the results are the mean ± range of duplicate incubations), thus indicating that Ficoll does not inhibit steroidogenesis.

Table 6. *Effect of centrifugation of a Ficoll–albumin purified rat testicular cell suspension through 6 % dextran–0·9 % NaCl solution for 2 min at 100 g (means* ± S.D.)

	Leydig cell content* (%)	Purity of Leydig cell preparation compared with control (%)	Recovery of Leydig cells* (%)	Testosterone production/10^6 cells/2 h (+LH) compared with control (%)
Control, sediment (10 min, 100 *g*)	36 ± 11 (6)	100 (6)	100 (6) (0·97 × 10^6 cells)†	100 (4)
Sediment (2 min, 100 *g*)	59 ± 17 (6)	168 ± 34 (6)	46 ± 10 (6) (0·44 × 10^6 cells)†	267 ± 77 (4)
Supernatant (2 min, 100 *g*)	31 ± 13 (6)	85 ± 14 (6)	50 ± 16 (6) (0·46 × 10^6 cells)†	33 ± 9 (4)
Recovery (supernatant + sediment after 2 min at 100 *g*)			94 ± 17 (6)	94 ± 17 (4)

Number of observations in parentheses.

* Number of Leydig cells was determined with the use of 3β-hydroxysteroid dehydrogenase histochemistry.

† Mean value of six observations.

Effect of centrifugation through dextran solutions

An additional purification of the Leydig cells centrifuged through Ficoll–albumin solutions was obtained by centrifugation through a 6 % dextran–0·9 % NaCl solution for 2 min at 100 *g*. In the control experiment cells were centrifuged for 10 min at 100 *g* in the 6 % dextran–0·9 % NaCl solution. After centrifugation, 87 ± 19 % (mean ± S.D., $n = 6$) of the cells were recovered. The percentage of 3β-hydroxysteroid dehydrogenase-containing cells in the sediment was increased by 64 % while the steroidogenic activity, as measured in the presence of added LH, increased by 167 % (Table 6). In two experiments the phenyl esterase activity was measured and it was found to parallel the proportion of cells containing 3β-hydroxysteroid dehydrogenase activity in the control, sediment and supernatant; 0·95 ± 0·25, 1·80 ± 0·10 and 0·75 ± 0·05 μmol/min/10^6 cells containing phenyl esterase activity and 41·5 ± 3·5 %, 74·5 ± 0·5 % and 40·0 ± 1·0 % 3β-hydroxysteroid dehydrogenase-containing cells respectively (figures are the mean ± range of two experiments). In a separate study the influence of centrifugation time was investigated. The percentage of 3β-hydroxysteroid dehydrogenase-containing cells and specific phenyl esterase activity was determined in the sediment and supernatant after centrifugation of the cells for 1, 2 or 4 min at 100 *g* through a

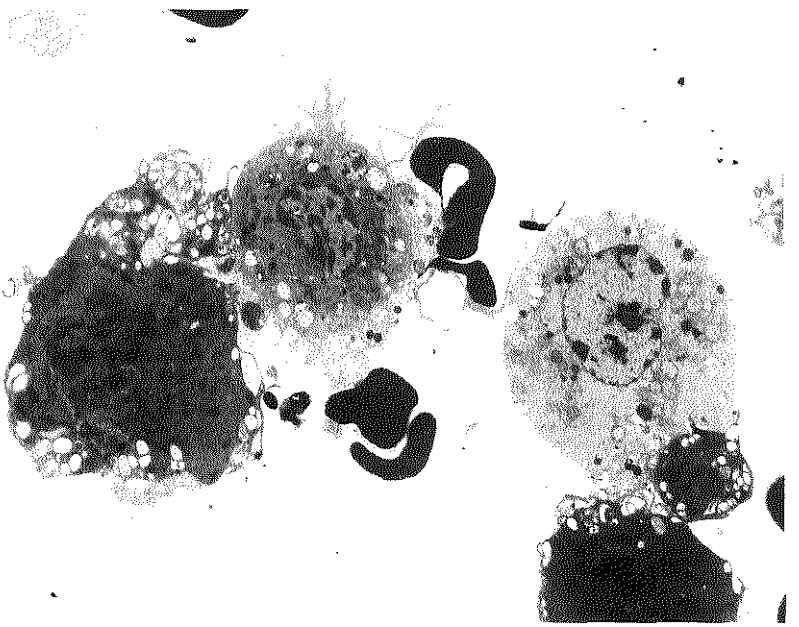


Fig. 2. Electron micrograph of dextran-purified Leydig cells. Purified cells were fixed in glutaraldehyde buffered with phosphate, post-fixed in buffered osmium tetroxide, dehydrated through graded ethanol solutions and embedded in Epon. Sections were stained with uranyl acetate and lead citrate. (Magnification × 2017.)

6 % dextran solution. Both Leydig cell markers gave almost identical results, therefore the mean purification factor and the recovery of the sedimented Leydig cells determined with these markers are given. After 1 min of centrifugation, the purification of the sediment was 1·8× and the recovery 22 %; after 2 min of centrifugation, 1·5× and 65 % respectively and after 4 min of centrifugation, 1·1× and 90 % respectively.

An electron micrograph of dextran-purified cells is shown in Fig. 2.

Leydig cell preparations from rat testes with defective spermatogenesis

Testosterone production by crude unpurified cell preparations from testes with defective spermatogenesis was in all cases higher than the production by crude cell preparations of intact rat testes, and was in the same range as the testosterone production of Ficoll-purified cell suspensions from intact rat testes. Centrifuging the cell suspensions from these pre-treated rats through a 13 % Ficoll-KRBG solution resulted in only a small increase in steroidogenic activity (Table 7).

Table 7. *Testosterone production in the absence and presence of added LH in unpurified and 13 % Ficoll-purified cells from rat testes, deficient in spermatogenesis (means ± range of duplicate incubations)*

	Testosterone (ng/10^6 cells/3 h)			
	Unpurified		Ficoll-purified	
	Control	+1μg LH/ml	Control	+1μg LH/ml
Essential-fatty-acid-deficient rats	1·8 ± 0·1	23·8 ± 0·7	3·7	64·5 ± 5·9
Irradiated rats				
Expt 1	5·6 ± 0·20	34·9 ± 2·1	—	—
Expt 2	2·4 ± 0·0	22·0 ± 1·6	4·9 ± 1·2	30·9 ± 0·7
Expt 3	10·5 ± 0·3	123·7	11·5 ± 0·4	179·5 ± 6·7
Cryptorchid rats				
Expt 1	5·5 ± 0·7	41·3 ± 0·5	—	—
Expt 2	3·7 ± 0·2	36·0 ± 2·5	5·6 ± 0·3	49·9 ± 6·5

Density distribution of rat testicular Leydig cells

As described above, crude Leydig cell preparations can be purified by centrifugation through a 13 % Ficoll solution (density 1·053 g/ml). Leydig cells are therefore probably among the cells with the highest buoyant density in the testicular cell preparation. In order to determine the exact buoyant density of the Leydig cells, centrifugation was carried out through a discontinuous density gradient of Ficoll in KRBG (pH 6·5) containing 0·2 % albumin, with an osmolarity of 260–290 mosmol/l. Two discrete peaks of cells containing 3β-hydroxysteroid dehydrogenase and phenyl esterase activity were found. One peak at a low density was found in the fraction where 80 % of the nucleated cells were present, and the second peak was observed at a considerably higher density. To exclude the possibility of adherence of Leydig cells to other cell types in peak I by the Ficoll, density gradients of Ficoll-metrizoate mixtures (Loos & Roos, 1974) without Ca^{2+} were used. The discontinuous density gradient was prepared as described in the Materials and Methods section. 3β-Hydroxysteroid dehydrogenase histochemistry, phenyl esterase activity and testosterone production were used as markers for the Leydig cells.

Fig. 3 shows the values of the measured parameters in the different fractions in one of the four identical experiments carried out. The fraction with density 1·039–1·049 g/ml contained 84 % of the nucleated cells. Two discrete peaks of cells containing 3β-hydroxysteroid

activity and phenyl esterase activity were again detected. Peak I in fractions 2–3 (density 1·049–1·055 g/ml) and peak II in fractions 5, 6 and 7 (density 1·068–1·088 g/ml). Basal testosterone production in fraction 2 (peak I) was 0·21 ± 0·20 ng testosterone/10^6 cells/2 h and in fraction 6 (peak II) 3·79 ± 1·47 ng testosterone/10^6 cells/2 h (mean ± S.D., $n = 4$). However, in the presence of LH a stimulation of testosterone production was found only in peak II. Fractions 2 and 6 contained most of the 3β-hydroxysteroid dehydrogenase-containing cells. In fraction 2 the percentage of 3β-hydroxysteroid dehydrogenase-containing cells was 5·2 ± 5·7 % and in fraction 6, 78·2 ± 10·2 % ($n = 4$). Peak I contained 56 ± 28 % of the total Leydig cells and peak II 44 ± 28 % ($n = 4$). In two experiments in which cyclic

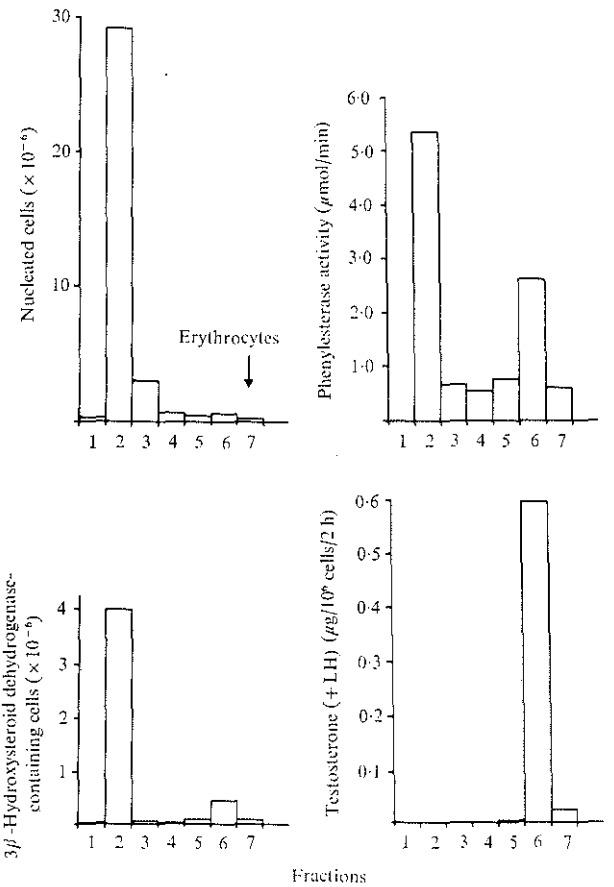


Fig. 3. Density distribution of rat testicular Leydig cells. The number of nucleated cells, phenyl esterase activity, proportion of cells containing 3β-hydroxysteroid dehydrogenase and LH-stimulated testosterone production (1 μg LH/ml) per 10^6 cells, not pre-incubated, are shown in the various fractions of a discontinuous Ficoll–metrizoate gradient, centrifuged for 40 min at 1500 g at 0 °C. Fractions 1 to 7 had densities of 1·020, 1·039, 1·049, 1·055, 1·068, 1·075 and 1·088 g/ml respectively.

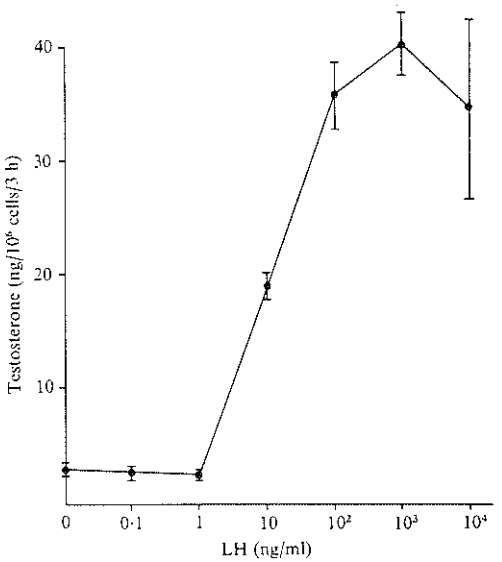


Fig. 4. LH dose-response curve for testosterone production by Leydig cell preparations, purified by centrifugation for 15 min at 1500 *g* through a 13 % Ficoll solution. The cells were pre-incubated for 60 min at 32 °C. Values are means ± S.D of three observations.

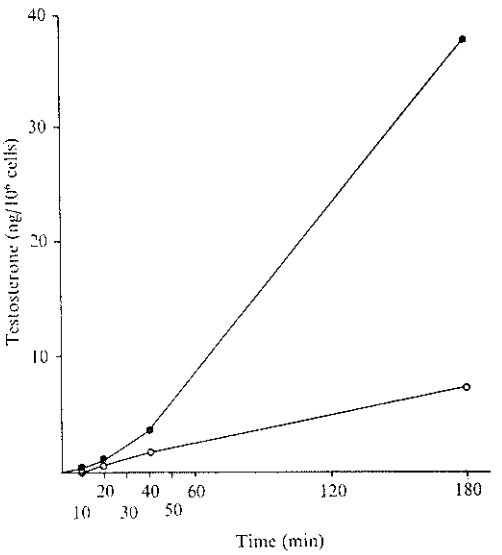


Fig. 5. Time course of testosterone production by Leydig cells purified by centrifugation for 10 min at 1500 *g* through a 13 % Ficoll solution. The cells were pre-incubated for 60 min at 32 °C. ●, 1 μg LH/ml; ○, control.

AMP was measured, cyclic AMP production increased in the presence of LH in peak I (fraction 2) from 0·18 to 3·5 ng/10^6 cells/2 h in one experiment and in the other experiment from 0·30 to 0·54 ng/10^6 cells/2 h, and in peak II from 0·10 to 10·94 ng/10^6 cells/2 h and from 0·74 to 35·17 ng/10^6 cells/2 h.

Dose-response and time curves after LH stimulation

Figure 4 shows an LH dose–response curve for testosterone production in cells purified by centrifugation through a 13 % Ficoll-KRBG solution. These cells were first pre-incubated for 1 h. The minimal dose of LH which was required to give a detectable increase in testosterone production was between 1 and 10 ng LH/ml. Maximal response was obtained with 100–1000 ng LH/ml. No difference in sensitivity towards LH could be detected in the crude unpurified Leydig cell preparation compared with the various purified preparations. Omission of the 1 h pre-incubation time did not change the maximal testosterone response in the presence of excess LH. However, it did change the sensitivity of the cells to LH. Without pre-incubation a maximum response was obtained with between 10 and 100 ng LH/ml. With a maximal dose of LH an increase in testosterone production was detectable 20 min after the addition of the trophic hormone. Maximal response was not reached even 40 min after the addition of LH (Fig. 5).

DISCUSSION

The concomitant presence of basal testosterone production, phenyl esterase activity and 3β-hydroxysteroid dehydrogenase-containing cells at two discrete density regions after isopycnic centrifugation of the crude Leydig cell preparation indicates the presence of two types of Leydig cells: 'light' Leydig cells with a density of 1·039–1·055 g/ml and 'dense' Leydig cells with a density of 1·068–1·088 g/ml. The absence of an LH response in the 'light' Leydig cells shows that these two types of cells differ not only in physical characteristics but also in function. As an explanation of these observations it may be considered that (1) the 'light' Leydig cells are damaged cells of which the LH regulation mechanism is impaired by the preparation and/or centrifugation procedure, or (2) the 'light' and 'dense' cells are two functionally different sub-populations of Leydig cells. Damage to cells can result in a higher or lower buoyant density (Shortman, 1968). However, it is difficult to explain how this can result in such a clear-cut change in density as seen by the two distinct peaks of activity. The existence of different sub-populations of Leydig cells in the mature rat testis has not previously been reported, although different generations of Leydig cells in immature and mature rat testes have been described (Roosen-Runge & Anderson, 1959; Niemi & Ikonen, 1963). The existence of Leydig cells with LH receptors and an adenyl cyclase system but unresponsive to the trophic hormone in immature rat testis has been reported (Frowein & Engel, 1973). In this respect the 'light' Leydig cells could perhaps resemble immature rat testicular Leydig cells, which have survived in the mature testis.

The results from the centrifugation procedures used in this study for the purification of Leydig cells may be explained on the basis of the buoyant density of the Leydig cells. Centrifugation of the crude cell preparation through a 13 % Ficoll solution, with a density of 1·053 g/ml, separated approximately 90 % of the cells with a lower density (mostly spermatocytes and spermatids) from the sedimented denser cells, which included the Leydig cells. However, the correlation between the four Leydig cell markers in the control and Ficoll-purified cell preparations indicated that 'light' and 'dense' Leydig cells were not separated from each other by this centrifugation step. This might have resulted from the formation of cell aggregates and adherence of the 'light' and 'dense' Leydig cells to each other. Lowering the pH of the Ficoll solution from 7·4 to 6·5 decreased cell aggregation, but it was not absent

as seen by phase contrast microscopy. The further purification obtained (approximately 2 times) by addition of 0·2 % albumin to the 13 % Ficoll solution was partly due to a decrease in total number of cells that sedimented (75 %) and partly due to sedimentation of more Leydig cells (30 %). Loos & Roos (1974) have also reported higher cell recoveries from continuous Ficoll-Isopague gradients by the addition of albumin and they suggested this was probably due to a lowering of the non-selective cell loss.

The next purification step, centrifugation for 2 min at 100 *g* through a 6 % dextran solution, was obtained probably more on a basis of the size of the cells than on their buoyant densities. The proportion of Leydig cells, as determined by phenyl esterase activity and 3β-hydroxysteroid dehydrogenase histochemistry, increased 1·7 times in the sediment, while the steroidogenic activity (measured in the presence of LH) increased 2·7 times in the sediment. It is possible that this separation between a larger proportion of LH-responsive cells in the sediment and less LH-responsive cells in the supernatant corresponds to a separation between the so-called 'dense' and 'light' Leydig cells respectively.

A testosterone response to LH was detectable only 20 min after the addition of LH, and it took more than 40 min to obtain a maximum response. Similar results have also been reported for the time course of the testosterone response of total testicular tissue *in vitro* (Rommerts, Cooke, van der Kemp & van der Molen, 1972), dissected interstitial tissue in static incubations (Rommerts, Cooke, van der Kemp & van der Molen, 1973*a*) and in a superfusion system (Cooke *et al.* 1975), and for Leydig cell preparations (Moyle & Ramachandran, 1973; Catt & Dufau, 1975). However, the testosterone response *in vivo* is much faster (Eik-Nes, 1970; van der Vusse, Kalkman, van Winsen & van der Molen, 1975). The cause of this time difference between stimulation *in vivo* and *in vitro* is still unclear and obviously requires further investigation with respect to the mechanism of LH action on steroidogenesis. For the adrenal, this situation is discussed by Schulster & Jenner (1975). A maximum response of testosterone was obtained with about 100 ng LH/ml. By omission of the 1 h pre-incubation period the same maximum amount of testosterone was produced with about 10 ng LH/ml, which is comparable with the results of Moyle & Ramachandran (1973). The decrease in sensitivity of the Leydig cells during pre-incubation might have been caused by loss of LH receptor sites due to the action of proteolytic enzymes still present.

In conclusion the present study has demonstrated that by a simple two-step centrifugation procedure it is possible to increase the number of Leydig cells 12·5 times and steroidogenic activity 22·5 times in the preparation obtained by collagenase treatment of rat testes. The resulting preparation, which contains approximately 60 % Leydig cells, is functionally responsive to physiological amounts of LH and can be used to investigate many aspects of the intracellular mechanisms of testicular steroidogenesis.

The authors would like to thank Dr P. M. Frederik for the preparation of electron micrographs of Leydig cells and are also grateful to the National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland, U.S.A., for gifts of sheep LH. This work was financially supported in part by the Dutch Foundation for Medical Research (FUNGO).

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Biochem. J. (1976) **160**, 433–437
Printed in Great Britain

The Effect of Calcium Ions on Testosterone Production in Leydig Cells from Rat Testis

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(*Received* 17 *May* 1976)

Leydig-cell suspensions, prepared from rat testes, were incubated with different amounts of Ca^{2+} with and without added luteinizing hormone. The basal testosterone production in the absence of luteinizing hormone was unaffected by the Ca^{2+} concentration in the incubation medium. The luteinizing hormone-stimulated testosterone production, however, was progressively decreased in the absence of Ca^{2+} to one-third of that with 2.50mM-Ca^{2+}. This decrease in luteinizing hormone-stimulated testosterone production was independent of the different concentrations of luteinizing hormone (0–10μg/ml) used and could be restored by the addition of Ca^{2+} to the incubation medium. The restoration of the stimulation was achieved within 30min after the addition of Ca^{2+} to the medium. Activation of cyclic AMP-dependent protein kinase by luteinizing hormone was not decreased by omission of Ca^{2+} from the incubation medium, suggesting that Ca^{2+} may be involved in steroidogenesis at a stage beyond the luteinizing hormone receptor–adenylate cyclase–protein kinase system.

The regulation of steroidogenesis by trophic hormones such as adrenocorticotropin (corticotropin, ACTH) and luteinizing hormone (lutropin, LH) most probably involves stimulation of cyclic AMP production and protein kinase activation in their respective target organs (Schulster, 1974; Cooke & van der Kemp, 1976). The continuous synthesis of a protein (or proteins) is also necessary for this regulation (Garren *et al.*, 1965; Cooke *et al.*, 1975). However, the involvement of other processes cannot be excluded. For example, it has been established that the presence of extracellular Ca^{2+} is a prerequisite for full stimulation of corticosteroid synthesis by adrenocorticotropin in the adrenal gland (Birmingham *et al.*, 1953; Sayers *et al.*, 1972; Rubin *et al.*, 1972). Much less work has been carried out on the effect of extracellular Ca^{2+} in other steroidogenic tissues. Mendelson *et al.* (1975) reported that omission of Ca^{2+} from the incubation medium resulted in only a small decrease in the stimulation by human choriogonadotropin of testosterone production in rat testis Leydig cells. However, Van der Vusse *et al.* (1976) have reported that the stimulation of pregnenolone production by rat testis mitochondria after administration of luteinizing hormone *in vivo* could be completely mimicked by the addition of Ca^{2+} to the incubation medium of mitochondria. This apparent discrepancy between the effect of Ca^{2+} on steroid production by testis Leydig cells and by isolated mitochondria made us decide to investigate further the role of Ca^{2+} in the stimulation of testosterone production by luteinizing hormone in the rat testis by using a purified Leydig-cell preparation.

Materials and Methods

Sheep luteinizing hormone (NIH S18) was a gift from the NIAM, Bethesda, MD, U.S.A.

Crude collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A.

EGTA [ethanedioxybis(ethylamine)tetra-acetic acid] and bovine serum albumin (fraction V) were obtained from Fluka A.G., Buchs, Switzerland, and the Ficoll 400 was from Pharmacia Fine Chemicals A.B., Uppsala, Sweden.

Adult male Wistar rats substrain R-Amsterdam (3–5months old) were used in this study.

Isolation and incubation of Leydig cells

The Leydig-cell suspensions were prepared and purified as described by Janszen *et al.* (1976). Briefly this method includes incubation of the testes with collagenase and centrifugation of the cell suspension through 13% Ficoll/0.2% bovine serum albumin in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964) containing 0.2% glucose, pH6.5, for 10min at 1500*g* followed by centrifugation of the sedimented cells through 6% Dextran T 250 in 0.9% NaCl solution for 2min at 100*g*.

After purification, the sedimented cells containing about 60% Leydig cells were resuspended in Krebs–Ringer bicarbonate buffer, pH 7.4, from which the Ca^{2+} was omitted, but which contained 0.1% bovine serum albumin and 2 mM-EGTA, and then pre-incubated for 30 min at 32°C under an atmosphere of O_2+CO_2 (95:5) to remove all the bound Ca^{2+}. After preincubation the cells were sedimented by centrifugation for 5 min at 100*g* and resuspended in Krebs–Ringer glucose buffer containing different concentrations of Ca^{2+} in 0.1 mM-EGTA and 0.1% bovine serum albumin. The cells were then incubated as described in the Results section at 32°C under an atmosphere of O_2+CO_2 (95:5). Cell densities were about 1×10^6 cells/ml and were determined by counting the nucleated cells in a haemocytometer.

Testosterone production

After incubation, the testosterone was extracted from the cells plus medium with ethyl acetate (2×2 ml) and determined by radioimmunoassay as described by Verjans *et al.* (1973).

Protein kinase assay

Protein kinase activity was determined as described by Cooke & van der Kemp (1976) in the presence and absence of 0.6 μM-cyclic AMP and the protein kinase activity ratio (ratio of protein kinase activity without added cyclic AMP/activity with added cyclic AMP) was calculated.

Results

Effect of extracellular Ca^{2+} on the testosterone production of isolated Leydig cells

Preincubated Leydig cells were incubated in Krebs–Ringer bicarbonate buffer, pH 7.4, containing either 0, 1.25 or 2.5 mM-Ca^{2+} with or without added luteinizing hormone, at a dose that gave maximum stimulation of testosterone production (100 ng/ml).

The basal testosterone production without added luteinizing hormone was not significantly changed in the absence of Ca^{2+} (Table 1). However, in the absence of extracellular Ca^{2+} the stimulation of the testosterone production by luteinizing hormone was only one-third of the control values.

Influence of dose of added luteinizing hormone on the testosterone production in the absence or presence (2.5 mM) of extracellular Ca^{2+}

Preincubated Leydig cells were incubated in the absence or presence of Ca^{2+} with different doses of luteinizing hormone. The absence of Ca^{2+} lowered the testosterone response in the presence of the different doses of luteinizing hormone tested (Fig. 1).

Table 1. *Influence of the concentration of Ca^{2+} in the medium on the testosterone production in isolated Leydig cells in the absence or presence of 100 ng of added luteinizing hormone/ml*

Values are means ± S.D. for the numbers of experiments in parentheses and are percentage syntheses compared with the control incubation containing 2.5 mM-Ca^{2+}.

Ca^{2+} in incubation medium (mM)	Testosterone synthesis (%)	
	−Luteinizing hormone	+Luteinizing hormone
0	125 ± 56% (10)	36 ± 14% (11)*
1.25	129 ± 41% (3)	79 ± 15% (4)*
2.50	100 (10)	100 (11)

* Significantly different from control.

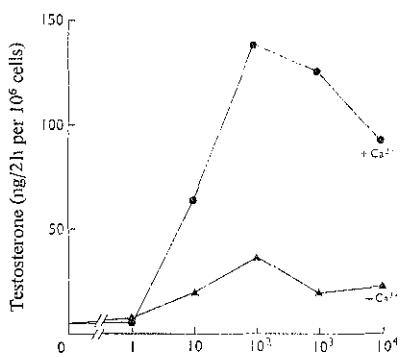

Fig. 1. *Luteinizing hormone dose–response curve of testosterone synthesis by isolated Leydig cells in the absence or presence of Ca^{2+}*

Leydig cells were incubated for 2 h in the absence (▲) or presence (●) of Ca^{2+} (2.5 mM) in the presence of different doses of luteinizing hormone (0–10 μg/ml). Means of duplicate incubations are given.

Time-course of testosterone synthesis in isolated Leydig cells in the absence or presence of extracellular Ca^{2+}

Preincubated Leydig cells were incubated in the absence or presence of Ca^{2+} and in the absence or presence of added luteinizing hormone (100 ng/ml) for different time-periods. No difference was detected in amounts of testosterone in all four types of incubation after 15 min (Fig. 2). After 30 min a stimulation

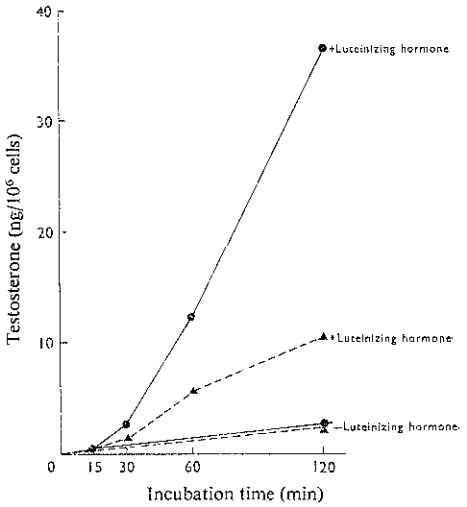

Fig. 2. *Time-course of testosterone production by Leydig cells incubated with or without Ca^{2+} with or without added luteinizing hormone*

Leydig cells were incubated for different time-periods up to 120min without Ca^{2+} (▲), with and without luteinizing hormone (100ng/ml) and with Ca^{2+} (2.5mM) (●) with and without luteinizing hormone. Means of duplicate incubations are given.

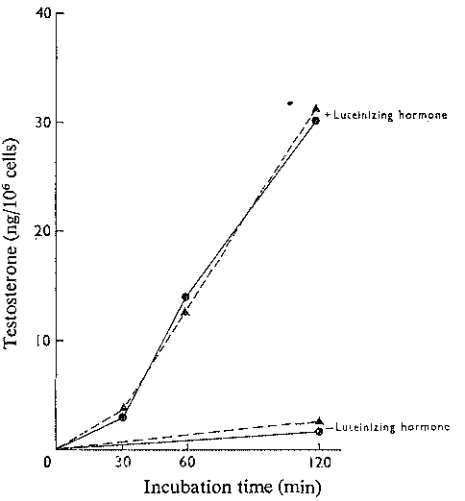

Fig. 3. *Time-course of testosterone production by Leydig cells in the presence of Ca^{2+} with or without luteinizing hormone, preincubated without or with Ca^{2+}*

Preincubated Leydig cells were incubated for 2h without (▲) or with Ca^{2+} (2.5mM) (●). After this incubation period Ca^{2+} was added (final concentration 2.5mM) to the cells incubated without Ca^{2+} and the Leydig cells were incubated for a further 2h without or with luteinizing hormone (100ng/ml). The amount of testosterone in cells plus medium was determined at different times after the addition of Ca^{2+} and luteinizing hormone. Means of duplicate incubations are given.

of the testosterone production was found in the presence of luteinizing hormone. The stimulation was less in the absence of Ca^{2+}. This decrease in the stimulation of the testosterone production remained during the rest of the incubation period.

Influence of re-addition of Ca^{2+} on the luteinizing hormone-stimulated testosterone synthesis in isolated Leydig cells after incubation without Ca^{2+}

Preincubated Leydig cells were incubated for 2h with or without Ca^{2+} in the absence of luteinizing hormone. After this incubation period, Ca^{2+} was added to the incubation medium without Ca^{2+} to give a final concentration of 2.5mM. The cells were then incubated with or without luteinizing hormone (100ng/ml) for 2h. Testosterone production was virtually the same after adjustment of the Ca^{2+} concentration to the control value (Fig. 3). This effect was already seen at 30min (the first time-interval studied) after the addition of the Ca^{2+} and luteinizing hormone. However, stimulated testosterone production did not reach the control value in all experiments after the addition of Ca^{2+} to the incubation medium. In six experiments the percentage of stimulated testosterone production during the first 2h incubation period in the absence of Ca^{2+} was 39 (S.E.M. ±7)% of the control value, and after the addition of Ca^{2+} this value became 71 (S.E.M. ±12) % of the control value during the second 2h incubation period. These values were significantly ($P<0.005$) different from each other.

Influence of extracellular Ca^{2+} on the activation of protein kinase activity in isolated Leydig cells

Leydig cells were prepared as described in the Materials and Methods section, except that the centrifugation through Dextran was omitted in order to obtain enough cells for the determination of testosterone production and protein kinase activity in the same cell preparation. The preincubated cells were incubated in Krebs–Ringer glucose buffer with or without 2.5mM-Ca^{2+} and with 0.1mM-EGTA. For the determination of protein kinase activity the cells were incubated with or

Table 2. *Influence of extracellular Ca^{2+} on the activation of protein kinase activity in isolated Leydig cells*

Values are means±S.D. with numbers of incubations in two separate experiments in parentheses. Luteinizing hormone when present was at a concentration of 100ng/ml.

Ca^{2+} in incubation medium (mM)	Testosterone production (ng/2h per 10^6 cells)		Protein kinase activity ratio	
	−Luteinizing hormone	+Luteinizing hormone	−Luteinizing hormone	+Luteinizing hormone
2.50	1.4±0.4*(5)	135±15.4 (6)	0.17±0.09 (4)	0.76±0.10 (6)
0	2.4±1.8 (5)	71.4± 8.9 (6)	0.21±0.05 (4)	0.72±0.10 (6)

without luteinizing hormone for 20min, and for the determination of testosterone production cells were incubated for 120min. In the absence of Ca^{2+} the luteinizing hormone-stimulated testosterone production decreased to about one-half of that in the presence of 2.5mM-Ca^{2+}. However, omission of Ca^{2+} did not affect luteinizing hormone-stimulated protein kinase activity; in both the presence and the absence of Ca^{2+}, luteinizing hormone increased the protein kinase activity ratio from 0.2 to 0.75 (Table 2). The total protein kinase activity determined in the presence of excess of cyclic AMP was the same in all incubations [39.6 (S.D. ±12.4, $n = 20$) pmol of ^{32}P incorporated/10^6 cells].

Discussion

The results obtained in the present investigation clearly show that maximum luteinizing hormone stimulation of testosterone production in rat testis Leydig cells can only be obtained in the presence of Ca^{2+}. This requirement for Ca^{2+} was demonstrated at all doses of luteinizing hormone used; in the absence of Ca^{2+} the luteinizing hormone-stimulated testosterone production was decreased to about one-third of that obtained in the presence of 2.5mM-Ca^{2+}. These results are in contrast with those of Mendelson *et al.* (1975), who have reported that omission of Ca^{2+} from the incubation medium decreased the testosterone response only slightly at high doses of human choriogonadotropin and not at lower doses. In the adrenal for maximum stimulation of steroidogenesis by adrenocorticotropin the presence of Ca^{2+} in the medium is also necessary (Birmingham *et al.*, 1953; Farese, 1971; Rubin *et al.*, 1972; Sayers *et al.*, 1972; Haksar & Peron, 1973; Bowyer & Kitabchi, 1974; Kowal *et al.*, 1974; Wishnow & Feist, 1974). However, the decrease in the adrenal steroid production by omission of Ca^{2+} in the medium could be overcome at least partly by using higher amounts of adrenocorticotropin (Sayers *et al.*, 1972; Haksar & Peron, 1973; Bowyer & Kitabchi, 1974; Kowal *et al.*, 1974). From these latter results it was concluded that Ca^{2+} may be involved in the transmission of the signal arising with the adrenocorticotropin–receptor–adenylate cyclase system. Bowyer & Kitabchi (1974) reported for adrenal cells that lowering the Ca^{2+} concentration in the incubation medium also decreased the corticosteroid response to dibutyrylcyclic AMP, indicating that in adrenal cells Ca^{2+} may also be important after the elaboration of the second messenger. In their experiments the concentration of dibutyryl cyclic AMP necessary to achieve half-maximum steroid response did not change with decreasing Ca^{2+} concentrations; the corticosterone production in the absence of Ca^{2+} decreased to the same extent with different concentrations of dibutyryl cyclic AMP. In this respect the effect of Ca^{2+} concentration on the luteinizing hormone dose–response curve in rat Leydig cells may be compared with the effect of Ca^{2+} on the dibutyryl cyclic AMP dose–response curve in rat adrenal cells. Further, the present study has shown that luteinizing-hormone activation of protein kinase in the Leydig cells is not affected by omission of Ca^{2+} from the medium, so the effect of Ca^{2+} is then most probably after the activation of protein kinase. One objection to the results for the protein kinase activity could be that the enzyme activity was measured only 20min after the addition of luteinizing hormone. However, as shown in Figs. 2 and 3, the effect of Ca^{2+} on the stimulation of steroidogenesis is obtained within 30min of incubation. It is not possible to conclude which of the processes involved in the regulation of testosterone synthesis, after the activation of protein kinase, have been affected by the omission of Ca^{2+} from the medium; it may be protein synthesis, as suggested by Farese (1971), or at the mitochondrial level, as suggested by Van der Vusse *et al.* (1976). Another possible explanation is that Ca^{2+} is necessary for the production of certain substrates for testosterone synthesis, which become limited when higher amounts of testosterone are produced. If this were true, one would expect that omission of Ca^{2+} had a greater effect on high testosterone production rates rather than on lower ones. This was shown not to be the case; even 30min after addition of luteinizing hormone, when testosterone production was still low, a smaller production in the absence

of Ca^{2+} compared with the control was already apparent. Also with submaximum doses of luteinizing hormone, testosterone production was markedly decreased compared with the control.

Another explanation that has to be considered is that the decrease in luteinizing-hormone stimulation of testosterone synthesis could have been caused by damage to the cells through the absence of Ca^{2+} in the incubation medium. This possibility was investigated by measuring testosterone synthesis with and without luteinizing hormone after the addition of Ca^{2+} to the Leydig-cell suspension, which had been preincubated for 2h in the absence of Ca^{2+}. In all experiments the addition of Ca^{2+} considerably increased the testosterone production and in some of the experiments testosterone production was restored to control values within 30min after the addition of Ca^{2+} to the cells. This indicates that damage to the cells did not take place or only to a limited extent.

In conclusion, it is apparent from the present study that Ca^{2+} is necessary for luteinizing-hormone stimulation of testosterone biosynthesis in testis Leydig cells. Its site of action is probably after the activation of protein kinase by luteinizing hormone. However, its precise mode of action remains to be elucidated.

We are grateful to the National Institutes of Health, Endocrinology Study Section, Bethesda, MD, U.S.A., for gifts of sheep lutropin. This work was financially supported in part by the Dutch Foundation for Medical Research (FUNGO). The technical assistance of Miss L. M. Lindh with the determination of the protein kinase activity is gratefully acknowledged.

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LH INDUCTION OF A SPECIFIC PROTEIN (LH-IP) IN RAT TESTIS LEYDIG CELLS

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Received 25 September 1976

1. Introduction

The available evidence suggests that LH stimulation of testosterone production in rat testis Leydig cells involves protein(s) with a short half life. This evidence is based on the effects of inhibitors of protein and RNA synthesis on LH stimulated testosterone production [1–3], particularly the rapid effect of cycloheximide, which causes a decrease in testosterone synthesis following first order kinetics with a half life of 13 min [4]. Recent work in our laboratory has shown that two proteins which are synthesized in rat testis Leydig cells and which can be detected using polyacrylamide gel electrophoresis, may be important in the regulation of testosterone production by LH; one of these proteins has a short half life (about 11 min) and is present in the particulate fraction of the Leydig cell, but is not under the influence of LH; the other protein (referred to as LH-IP, LH-induced protein) can be detected 2 h after the addition of LH to Leydig cells and has a half life longer than 30 min [5]. We now wish to report that the second protein (LH-IP) can be induced by LH or dibutyryl-cAMP but not by testosterone or follicle stimulating hormone (FSH). Dose response studies have also shown that the induction of LH-IP and the stimulation of testosterone production require approximately the same concentrations of LH. Incubation of the Leydig cells with actinomycin D prevented the induction of LH-IP by LH.

2. Materials and methods

Ovine FSH (NIH-FSH-S9) and ovine LH (NIH-LH-S18) were gifts from the Endocrinology Study Section, National Institute of Health, Bethesda, Maryland, USA. [^{35}S]Methionine (280 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Elipten-phosphate (an inhibitor of cholesterol side-chain cleavage) was a gift from CIBA, Basel, Switzerland.

Leydig cell suspensions from rat testis were prepared and purified by centrifugation through Ficoll and Dextran solutions as described before [6]. Leydig cells were incubated in Krebs-Ringer solution pH 7.4 containing 0.2% glucose, 0.1% bovine serum albumin fraction V and amino acid mixture lacking in methionine under an atmosphere of 95% O_2/5% CO_2 with LH as indicated in the text and then proteins were labelled by addition of [^{35}S]methionine for 30 minutes.

In order to control whether the cells were stimulated by LH, Leydig cells were incubated in parallel experiments with or without added LH (100 ng/ml) for 2 h. After this incubation period testosterone was extracted and determined as described before [7]. Testosterone production (mean ± S.E.M.) in the absence of added LH was 4.0 ± 0.4 ng/10^6 nucleated cells (n = 15), in the presence of LH 115.8 ± 14.4 ng/10^6 nucleated cells (n = 15) and in the presence of LH and elipten-phosphate 3.3 ± 1.5 ng/10^6 nucleated cells (n = 3).

After incubation of the cells, 10 vol. cold (0°C) Krebs-Ringer buffer without bovine serum albumin was added and the cells were sedimented by centrifugation for 10 min at 100 × g at 4°C. The supernatant was discarded and the sedimented cells were resuspended in a glycine–sodium dodecyl sulphate buffer (0.1 M glycine, 0.1 M NaCl, 0.01 M EDTA, 0.1% sodium dodecyl sulphate and 0.01 M β-mercapto

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ethanol) pH 8.5. The suspension was heated at 100°C for 10–15 min in glass tubes and after cooling, acetone (4 vol.) was added. The water–acetone mixture was stored overnight at –20°C. The precipitated proteins were sedimented by centrifugation for 10 min at 1500 × *g* at 4°C and dissolved in Tris–glycerol buffer (0.05 M Tris, 10% glycerol, 1% sodium dodecyl sulphate and 1% β-mercaptoethanol) pH 6.8 and heated at 100°C for 2 min. Electrophoresis was carried out in 10% and 15% discontinuous SDS polyacrylamide slab-gels according to Laemli [8]. Before drying the gels, they were impregnated with 2,5-diphenyl oxazole [9]. The gels were then exposed to Kodak X-ray film RP 14 for 1–2 weeks.

3. Results

3.1. *Influence of incubation time*

Two hours after addition of LH to Leydig cells increased incorporation of [^{35}S]methionine could be observed in a protein band with a mol. wt of approximately 21 000 (LH-IP) (fig.1). Only in one out of 6 experiments with intact and hypophysectomized animals was this LH-increased incorporation detectable one hour after addition of LH to the cells. About four hours after addition of LH the labelling of LH-IP seemed to be at a maximal level.

3.2. *Effect of different doses of LH*

When Leydig cells were incubated with 1 ng LH/ml for 3 h the incorporation of [^{35}S]methionine in LH-IP was only slightly increased compared with the control. With 10 ng LH/ml a clear increase in ^{35}S-incorporation in LH-IP was evident while maximal labelling was obtained with 100–1000 ng LH/ml (fig.2).

3.3. *Effect of testosterone and FSH*

To investigate whether the increase in LH-IP labelling after addition of LH was due to the higher level of testosterone in LH-stimulated Leydig cells, testosterone (100 ng/10^6 nucleated cells) was added to Leydig cells and incubated for 3 h. No change in ^{35}S-incorporation of LH-IP as compared with control cells was observed (fig.3). In other experiments

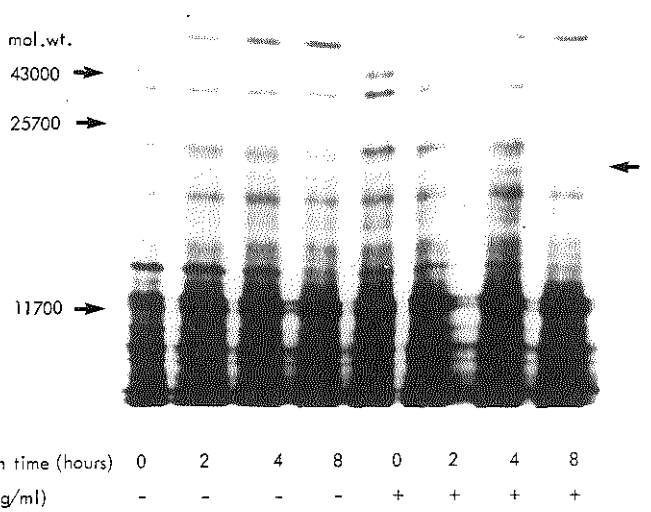

Fig.1. Time course of LH induction of LH-IP. Leydig cells were incubated with or without added LH (100 ng/ml) for 0, 2, 4, and 8 h followed by incubation with [^{35}S]methionine for 30 min. Proteins were separated by electrophoresis on 10–15% discontinuous SDS–polyacrylamide gel. The following mol. wt markers were used: ovalbumin, chymotrypsinogen and cytochrome *c*. In this figure only the 15% part of the gel is shown.

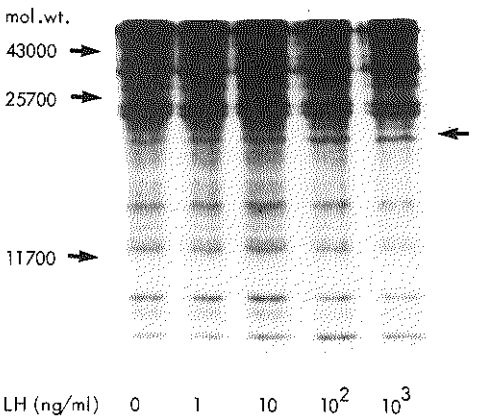

Fig.2. LH dose response relationship of LH-IP synthesis. Leydig cells were incubated with different doses of LH for 3 h followed by incubation with [^{35}S]methionine for 30 min.

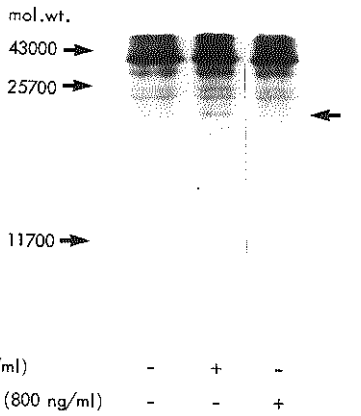

Fig.3. Effect of testosterone on LH-IP synthesis. Leydig cells were incubated without or with LH (100 ng/ml) or with testosterone (± 100 ng/10^6 nucleated cells) for 3 h followed by incubation with [^{35}S]methionine for 30 min.

elipten-phosphate (300 μg/ml) was added to LH-stimulated Leydig cells to prevent the synthesis of testosterone [10]. However, under these conditions the LH-stimulated labelling of LH-IP was not inhibited. Addition of FSH (100 ng/ml) to the Leydig cells instead of LH did not stimulated the labelling of LH-IP.

3.4. *Effect of dibutyryl-cAMP*

Addition of 0.1 mM dibutyryl-cAMP instead of LH

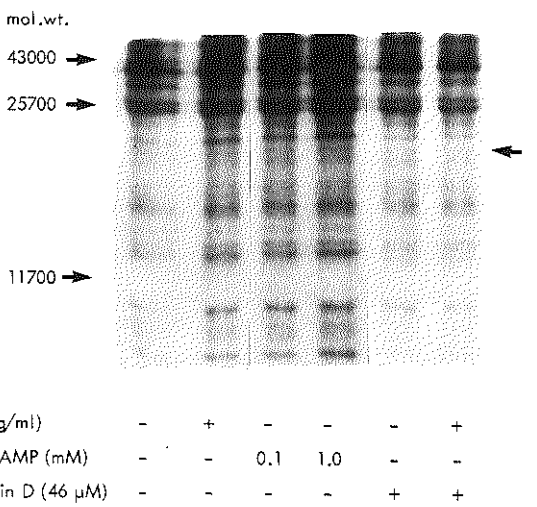

Fig.4. Effect of dibutyryl-cAMP or actinomycin D on synthesis of LH-IP. Leydig cells were incubated with or without LH alone or in combination with actinomycin D or with dibutyryl-cAMP for 3 h followed by incubation with [^{35}S]methionine for 30 min.

to Leydig cells gave a submaximal stimulation of testosterone production while addition of 1 mM dibutyryl-cAMP gave about the same stimulation of testosterone production as 100 ng LH/ml. As can be seen in fig.4 both concentrations of dibutyryl-cAMP stimulated the ^{35}S-incorporation in LH-IP.

3.5. *Effect of actinomycin D*

Addition of actinomycin D (46 μM) to Leydig cells inhibits 89–93% of RNA synthesis [2]. Addition of this amount of actinomycin D to LH-stimulated Leydig cells prevented the LH-stimulated labelling of LH-IP (fig.4).

4. Discussion

From the present results it may be concluded that an increased incorporation of [^{35}S]methionine in a protein with a mol. wt of 21 000 (LH-IP) can be detected 2 h after addition of LH to Leydig cells. This increase of ^{35}S-incorporation most probably reflects protein synthesis and was not due to the effects of increased synthesis of testosterone or contaminating amounts of FSH in the LH preparation. Dibutyryl-cAMP also increases the incorporation of the methionine into LH-IP, therefore it is probable that the effect of LH on the synthesis of LH-IP is mediated by increased cAMP production. Actinomycin D was found to inhibit the LH-stimulated synthesis of LH-IP, which suggests that this stimulation of protein synthesis is probably mediated by increased synthesis of new mRNA. In another study [5] it has been shown that LH-IP is located specifically in the Leydig cells and not in other cell types of the rat testis and that its half life is longer than 30 min.

These results raise the question about the possible role of LH-IP in the Leydig cell. Certain aspects would support a role of this protein in the LH-stimulation of testosterone production in Leydig cells, namely:

1. The close correlation between the LH dose response relationship of LH-IP synthesis and testosterone production; the lowest dose of LH required for stimulation of the synthesis of this protein as well as for stimulation of the testosterone production was about 1 ng/ml and maximum response was obtained with about 100 ng/ml LH [6].
2. RNA synthesis is apparently required for LH stimulation of testosterone production up to 150 min after addition of LH to Leydig cells [3].

However 2 points may be raised against an obligatory role of LH-IP in testosterone production:

1. The long half life (more than 30 min) of LH-IP, which is not in accordance with the rapid effect of cycloheximide [4].
2. LH-IP could be detected only 2 h after addition of LH Leydig cells, which is much later than the first stimulation of testosterone production, which can already be detected within 5–30 min [11] after addition of LH.

Therefore further work will be necessary to investigate the possible role of LH-IP in the effect of LH on testosterone production in rat testis Leydig cells.

Acknowledgements

We are grateful to the National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland, USA for gifts of sheep LH and FSH. This work was financially supported in part by the Dutch Foundation for Medical Research (FUNGO).

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Biochem. J. (1977) **162**, 341–346
Printed in Great Britain

Specific Protein Synthesis in Isolated Rat Testis Leydig Cells

INFLUENCE OF LUTEINIZING HORMONE AND CYCLOHEXIMIDE

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(*Received* 27 *August* 1976)

The effect of luteinizing hormone (luteotropin) and cycloheximide on specific protein synthesis in rat testis Leydig cells has been investigated. Proteins were labelled with either [^{14}C]leucine, [^{3}H]leucine or [^{35}S]methionine during incubation with Leydig-cell suspensions *in vitro*. Total protein was extracted from the cells and separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. No detectable increase in the synthesis of specific proteins could be observed after incubation of Leydig cells with luteinizing hormone for up to 1 h. However, after a 2 h incubation period, an increase in [^{35}S]methionine incorporation was observed in a protein with an apparent mol.wt. of 21000 (referred to as 'protein 21'). When, after labelling of this protein with [^{35}S]-methionine, Leydig cells were incubated for another 30 min with cycloheximide, no decrease in radioactivity of this protein band was observed, indicating that it does not have a short half-life. However, another protein band was detected, which after incubation with cycloheximide disappeared rapidly, the reaction following first-order kinetics, with a half-life of about 11 min. This protein, with an apparent mol.wt. of 33000 (referred to as 'protein 33'), was found to be located in the particulate fraction of the Leydig cell, and could not be demonstrated in other rat testis-cell types or blood cells. No effect of luteinizing hormone on molecular weight, subcellular localization or half-life of protein 33 was observed. A possible role for protein 33 and protein 21 in the mechanism of action of luteinizing hormone on testosterone production in Leydig cells is discussed.

Previous work has shown that luteinizing hormone (luteotropin) specifically stimulates synthesis of cyclic AMP (see the review by Rommerts *et al.*, 1974), activation of protein kinase (Cooke & Van der Kemp, 1976; Cooke *et al.*, 1976) and production of testosterone in rat testis Leydig cells *in vitro* (see the review by Dufau & Catt, 1975). It was also shown that protein synthesis may play a role in the luteinizing-hormone stimulation of steroidogenesis (Hall & Eik-Nes, 1962; Shin, 1967; Moyle *et al.*, 1971; Cooke *et al.*, 1975; Mendelson *et al.*, 1975). Addition of the protein-synthesis inhibitors cycloheximide and puromycin to Leydig-cell suspensions inhibits luteinizing-hormone stimulation of testosterone production to the same extent as protein synthesis. After maximal stimulation of testosterone synthesis in rat testis Leydig cells by luteinizing hormone, addition of cycloheximide decreases testosterone production to control values, the reaction following first-order kinetics (half-life 13 min) (Cooke *et al.*, 1975). These results indicate that the continuous synthesis of protein with a short half-life equal to or less than 13 min is involved in the stimulation of Leydig-cell steroid synthesis by luteinizing hormone. Similar results have been reported for other steroid-synthesizing tissues, e.g. the adrenal gland (Ferguson, 1963; Garren *et al.*, 1965; Schulster *et al.*, 1970; Rubin *et al.*, 1973; Lowry & McMartin, 1974), Graafian follicle (Tsafriri *et al.*, 1973; Younglai, 1975) and corpus luteum (Hermier *et al.*, 1971).

Garren *et al.* (1965) proposed a model in which the regulation of steroidogenesis by corticotropin (ACTH) in the adrenal gland was mediated by the synthesis of a protein with a short half-life. Since then, however, no further proof for such a synthesis has been demonstrated. On the basis of kinetic data, Schulster *et al.* (1974) and Lowry & McMartin (1974) rejected the hypothesis that corticotropin would stimulate the production of a specific protein, and these authors proposed an alternative model in which the regulation of steroidogenesis by corticotropin in adrenal cells was mediated by the activation of a protein with a short half-life. At present there is insufficient evidence to determine which of the proposed models is correct or even to exclude a third possibility, that a protein with short half-life is involved in the regulatory mechanism of steroidogenesis by trophic hormones, and that this protein is as such not affected by the trophic hormone.

The present study was undertaken to obtain information about the proposed regulatory protein in the testis. This has been achieved by incubation of

isolated Leydig cells with radioactively labelled amino acids in the presence or absence of luteinizing hormone, followed by separation of the labelled protein by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Protein patterns of control and luteinizing-hormone-stimulated cells were compared by using either the double-isotope-labelling technique with [^{14}C]- and [^{3}H]-leucine or radioautography of [^{35}S]methionine-labelled proteins. The presence of proteins with a short half-life was investigated by incubation of Leydig cells with cycloheximide after labelling of the proteins with [^{35}S]methionine. Part of the present work was described in a Short Communication given at the sixth British/Dutch Endocrine Meeting (on 7 September 1976) at the University of Bristol, Bristol, U.K.

Materials and Methods

Materials

Sheep luteinizing hormone (NIH S18) was a gift from the NIAM, Bethesda, MD, U.S.A. Crude collagenase was purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. [^{14}C]-Leucine (350mCi/mmol), [^{3}H]leucine (50Ci/mmol) and [^{35}S]methionine (250Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Bovine serum albumin (fraction V) was obtained from Fluka AG, Buchs, Switzerland, and Ficoll 400 from Pharmacia Fine Chemicals A.B., Uppsala, Sweden. Soluene 350 was obtained from Packard Instrument Co., Downers Grove, IL, U.S.A. Adult male Wistar rats sub-strain R-Amsterdam, 3–5 months old, were used.

Methods

Leydig-cell preparation. Leydig-cell suspensions were prepared and purified as previously described by Janszen *et al.* (1976). Briefly this method consists of incubating decapsulated testes with collagenase for 18–40min at 37°C (until the tubules are fully dispersed). The cell suspension is then centrifuged for 10min at 1500g through a 13% (w/v) Ficoll/0.2% albumin solution in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964), containing 0.2% glucose, pH6.5, followed by centrifugation of the sedimented cells for 2min at 100*g* through a 6% (w/v) dextran solution. Approx. 60% of the nucleated cells obtained were Leydig cells. The sedimented cells were resuspended in Krebs–Ringer bicarbonate buffer containing 0.2% glucose and 0.1% bovine serum albumin (fraction V) and incubated at 32°C with continuous shaking under an atmosphere of O_2+CO_2 (95:5). All procedures were carried out in plastic tubes.

Extraction and separation of proteins. After incubation of the cells, a tenfold excess of Krebs–Ringer buffer without bovine serum albumin at 0°C was added, and the cells were sedimented by centrifugation for 10min at 100*g* at 4°C. The supernatant was discarded and the sedimented cells were resuspended in a glycine/sodium dodecyl sulphate buffer (0.1M-glycine, 0.1M-NaCl, 0.01M-EDTA, 0.1% sodium dodecyl sulphate and 0.01M-β-mercaptoethanol), pH8.5. The suspension was heated at 100°C for 10–15min in glass tubes and, after heating, acetone (4vol.) was added. The water/acetone mixture was stored overnight at −20°C. The precipitated proteins were sedimented by centrifugation for 10min at 1500*g* at 4°C, and dissolved in Tris/glycerol buffer (0.05M-Tris, 10% (v/v) glycerol, 1% sodium dodecyl sulphate and 1% β-mercaptoethanol), pH6.8, and heated at 100°C for 2min.

No labelled proteins could be detected in the water/acetone mixture after sedimentation of the protein. Electrophoresis was carried out in 10 and 15% (w/v) (continuous and discontinuous) sodium dodecyl sulphate/polyacrylamide cylindrical gels, or in slab gels by the method of Laemli (1970).

After electrophoresis in cylindrical gels (6mm×90 mm), the gels were pushed out of the tubes, frozen on solid CO_2, and 1mm thick sections were obtained with a Mickle gel slicer. The proteins were extracted from the slices by incubation with 0.5ml of Soluene 350 for 3h at 60°C. Methoxyethanol/toluene scintillation liquid (80g of naphthalene, 4g of 2,5-diphenyloxazole, 40mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene, 0.5 litre of toluene, 0.5 litre of methoxyethanol) was added and the radioactivity was counted in an Isocap 300 liquid-scintillation counter using a double-labelling programme for ^{3}H and ^{14}C. The counting efficiency for both labels was in the order of 30%.

For radioautographic detection of [^{35}S]methionine-labelled proteins, electrophoresis was carried out on slab gels (140mm×160mm×1mm) with a current of 20mA/gel under continuous cooling with running tap water. After electrophoresis, the gels were fixed in a methanol/water/acetic acid (5:4:1, by vol.) mixture for at least 60min. The gels were stained with 1% Amido Black in 7% (v/v) acetic acid for 30min and de-stained by several washings with a methanol/water/acetic acid (30:63:7, by vol.) mixture. To improve the detection efficiency of the ^{35}S label in some experiments, gels were impregnated with 2,5-diphenyloxazole (as described by Bonner & Laskey, 1974). The gels were dried (at 70–80°C) on a Bio-Rad gel-slab dryer under continuous heating. Gels were exposed to Kodak X-ray film RP-14, usually for about 1 week. The radioautograms were scanned with a Vitatron TLD 100 densitometer. The correlation between the amount of radioactivity in the gel and the densitogram of the exposed X-ray film was tested with a radioautogram of known increasing amounts of ^{35}S. The ratio of the density of each band divided by the density of a band with a mol.wt. of approx. 43000 (which was shown to be unaffected

by the different incubation conditions used) was calculated. This ratio was used for a quantitative evaluation of the observed protein bands.

Results

Synthesis of proteins in Leydig cells incubated in the absence or presence of luteinizing hormone

Leydig cells prepared by centrifugation of the cell suspension through 13% Ficoll/0.2% bovine serum albumin in Krebs–Ringer buffer, were incubated for 60min with and without added luteinizing hormone (100ng/ml); this amount of luteinizing hormone gives maximum stimulation of testosterone production (Janszen *et al.*, 1976). [^{3}H]Leucine was then added to luteinizing-hormone-stimulated cells and [^{14}C]leucine to control cells, and the incubations were continued for 30min. Both incubations were combined and the proteins were extracted and submitted to electrophoresis in cylindrical gels. No difference in the separation pattern of incorporated leucine was observed between control and luteinizing-hormone-stimulated cells (Fig. 1). Also, with shorter labelling times (5 and 10min), no significant difference between the radioactive patterns of control and

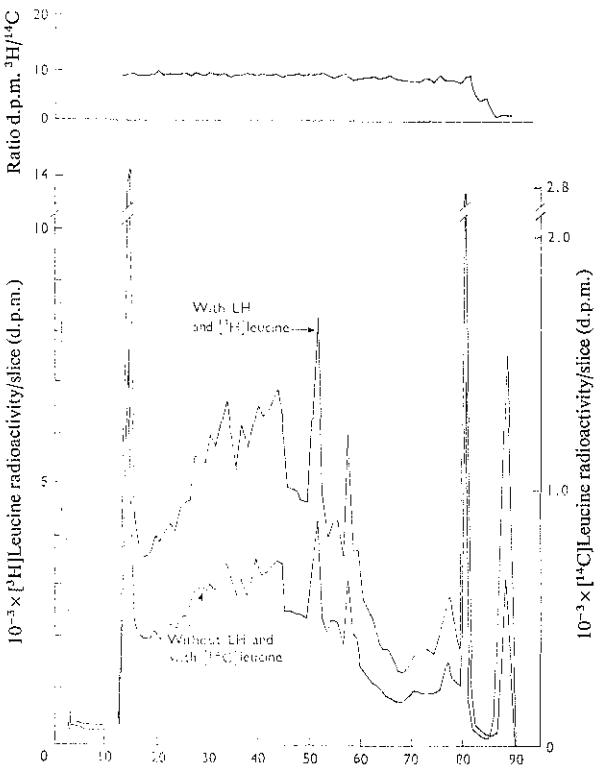


Fig. 1. *Synthesis of Leydig-cell proteins in the absence or presence of luteinizing hormone, and their separation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*

Leydig cells were incubated for 60min with or without added luteinizing hormone (100ng/ml) followed by incubation with [^{3}H]leucine (100μCi/ml) and [^{14}C]leucine (10μCi/ml) respectively. Electrophoresis was performed on a stacking gel (3%) from slice numbers 1–12, and 10% gel from slice numbers 13–90. To detect changes in the synthesis of a specific protein the ratio of ^{3}H and ^{14}C radioactivities (d.p.m.) in each slice was calculated. The mean (±S.D.) value of the ratio for slices numbered 13–82 was 9.51±0.41. This ratio did not exceed the mean ± twice the S.D. in any of the slices. In slices numbered 83–90 the amount of ^{14}C label increased relative to the ^{3}H label. This increase was independent of the presence of luteinizing hormone. Abbreviation: LH, luteinizing hormone.

luteinizing-hormone-stimulated cells was observed. In other experiments, Leydig cells were purified by centrifugation of the cell suspension through Ficoll and dextran solutions. These cells were incubated with or without luteinizing hormone (100ng/ml) for 0, 1, 2 and 3h, followed by incubation with [^{35}S]-methionine for 30min. After incubation, the proteins were extracted and separated by electrophoresis on sodium dodecyl sulphate / polyacrylamide slab gels. Radioactivity was detected by radioautography.

No new ^{35}S-labelled protein band was observed after incubation with luteinizing hormone for up to 1h [Plate 1(*a*, *c*, *e* and *g*)]. Similar results were also obtained after subcellular fractionation before electrophoresis, to improve the sensitivity of detection. Only after incubation of Leydig cells with luteinizing hormone for 2h or more was an increase observed in [^{35}S]methionine incorporation into a protein band with an apparent mol.wt. of approx. 21000 [Plate 1(*i*, *j*, *k*, *l*, *m*, *n*, *o* and *p*), band A]. For convenience this protein is referred to as 'protein 21'.

Effect of cycloheximide on [^{35}S]methionine-labelled proteins

Cells were incubated with cycloheximide (25μg/ml), which inhibits 95% of protein synthesis, for different time-periods up to 30min, to investigate the possibility that a protein with long half-life is converted into a protein with short half-life after incubation with luteinizing hormone. No difference between luteinizing-hormone-stimulated and control cells was observed. However, it was found that an intensely labelled band disappeared very quickly after addition of cycloheximide [Plate 2(*a*, *b*, *c*, *d*, *e*, *f* and *g*)]. The rate of decrease followed first-order kinetics and a half-life of 11min was calculated. Incubation of Leydig cells in the presence of luteinizing hormone did not change this half-life. The decrease in the amount of the protein in the cell did not correspond with the appearance of this protein in the incubation medium. The apparent mol.wt. of the protein was determined to be approx. 33000. For convenience it is referred to as 'protein 33'. Incubation of Leydig cells in the presence of luteinizing hormone did not result in a detectable change in molecular weight of protein 33.

When Leydig cells were incubated with cycloheximide (25μg/ml) for 30min after the appearance of protein 21 in the presence of luteinizing hormone, no decrease in amount of radioactivity of this protein band was observed [Plate 2(*k*, *l*, *o* and *p*)].

Cellular localization of proteins 33 *and* 21

Seminiferous tubules, obtained by wet dissection of rat testis (Rommerts *et al.*, 1973), were incubated with [^{35}S]methionine, and the proteins were separated by sodium dodecyl sulphate / polyacrylamide-gel electrophoresis. No protein with a mol.wt. of 33000 and a short half-life was observed (Plate 3). When seminiferous tubules were incubated for 3h with luteinizing hormone (100ng/ml) or testosterone (800ng/ml), followed by incubation for 30min with [^{35}S]methionine, it was not possible to detect the appearance of protein 21. Similar results were obtained with rat blood cells. In a cell preparation containing 6% Leydig cells instead of 60% (as used in above study), the presence of protein 33, or the appearance of protein 21 after incubation for 3h with luteinizing hormone, were hardly detectable.

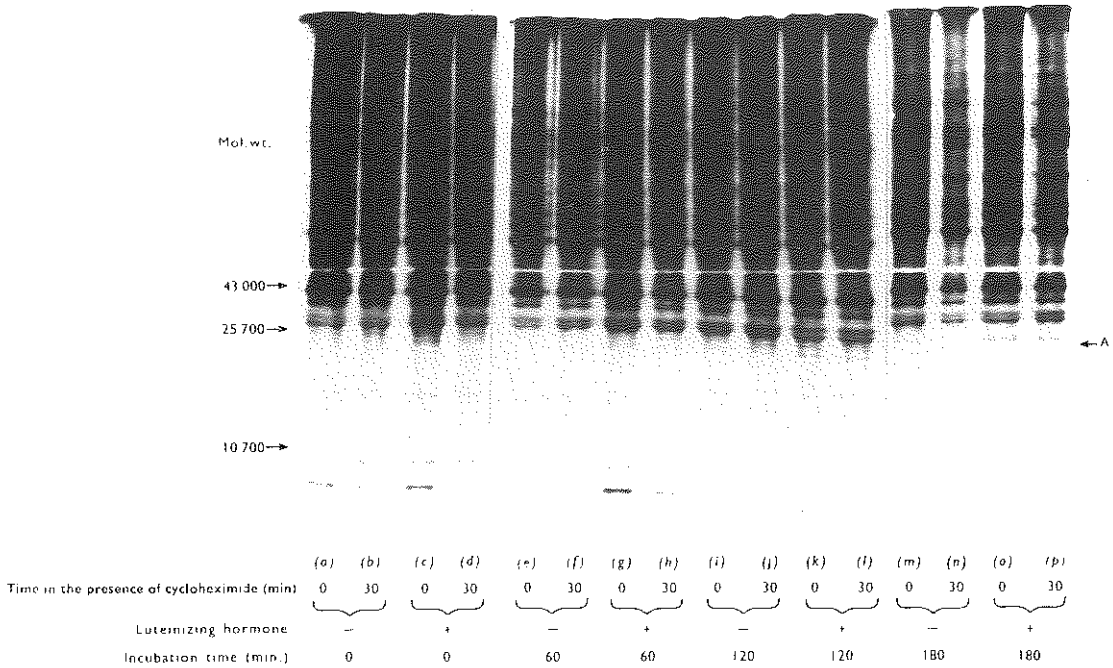
Subcellular localization of protein 33

The subcellular localization of protein 33 was determined by homogenization of the cells and subcellular fractionation of the homogenate. Extensive washing of the fractions was omitted, because it was decided to work quickly to minimize degradation of the labile protein by proteinases. The different subcellular fractions were characterized by using the following markers: DNA, monoamine oxidase, carboxyl esterase and lactate dehydrogenase (Table 1). Protein 33 was mainly present in the 10min/500*g*

Table 1. *Distribution of DNA, monoamine oxidase, carboxyl esterase and lactate dehydrogenase in subcellular fractions of a rat testis Leydig-cell homogenate*

The concentrations of the markers are been expressed as relative specific activity (ratio of percentage recovered activity to the percentage of recovered protein).

	Relative specific activity			
	Sediment			
Markers	10min, 100*g*	10min, 500*g*	10min, 15000*g*	Supernatant 10min, 15000*g*
DNA	3.07	5.30	0.60	0.25
Monoamine oxidase	2.03	2.63	2.70	–
Carboxyl esterase	0.24	0.72	1.16	1.17
Lactate dehydrogenase	0.46	0.46	0.38	1.41
Protein	17%	4%	21%	58%

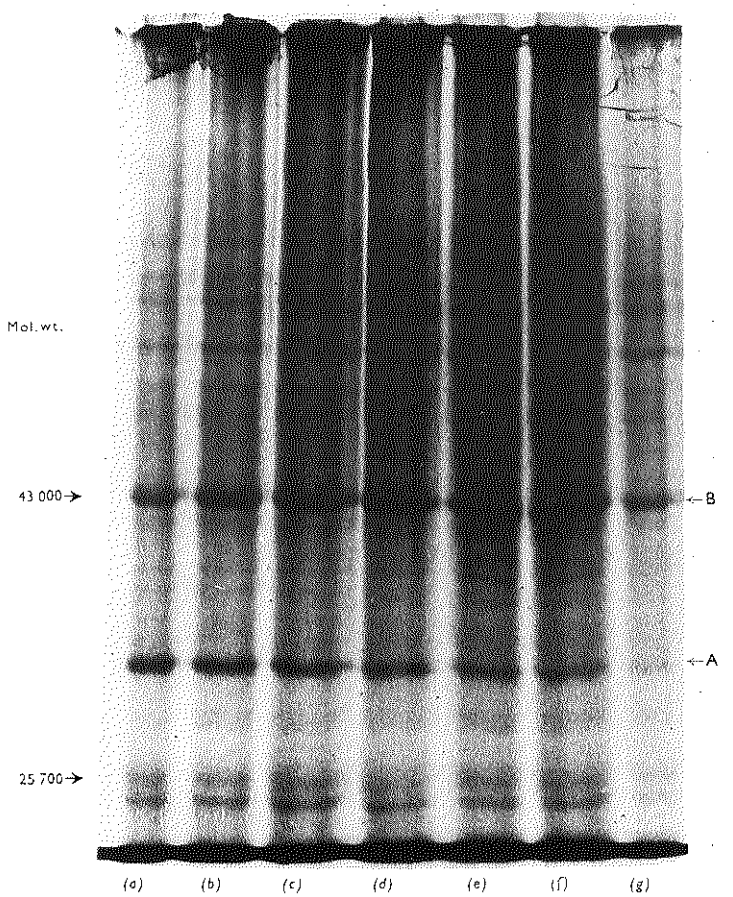


EXPLANATION OF PLATE 1

Radioautogram of [^{35}S]methionine-labelled proteins in Leydig cells incubated with or without luteinizing hormone for different time-periods

Leydig cells were incubated without luteinizing hormone for 0 min (*a*, *b*), 60 min (*e*, *f*), 120 min (*i*, *j*) and 180 min (*m*, *n*) and with luteinizing hormone (100 ng/ml) for 0 min (*c*, *d*), 60 min (*g*, *h*), 120 min (*k*, *l*) and 180 min (*o*, *p*), followed by incubation for 30 min with [^{35}S]methionine (65 μCi/ml). Incubations (*b*), (*d*), (*f*), (*h*), (*j*), (*l*), (*n*) and (*p*) were continued for an additional 30 min in the presence of added cycloheximide (25 μg/ml). The incubations were stopped by addition of 2 ml of Krebs Ringer buffer, pH 7.4, at 0 C and cells were sedimented by centrifugation for 30 min at 100 *g*. Total protein was extracted and electrophoresed on a discontinuous sodium dodecyl sulphate/polyacrylamide (10–15% w/v) gel. Before exposure of the gel to the X-ray film, the gel was impregnated with 2,5-diphenyloxazole. The gel was exposed to the X-ray film for 1 week.

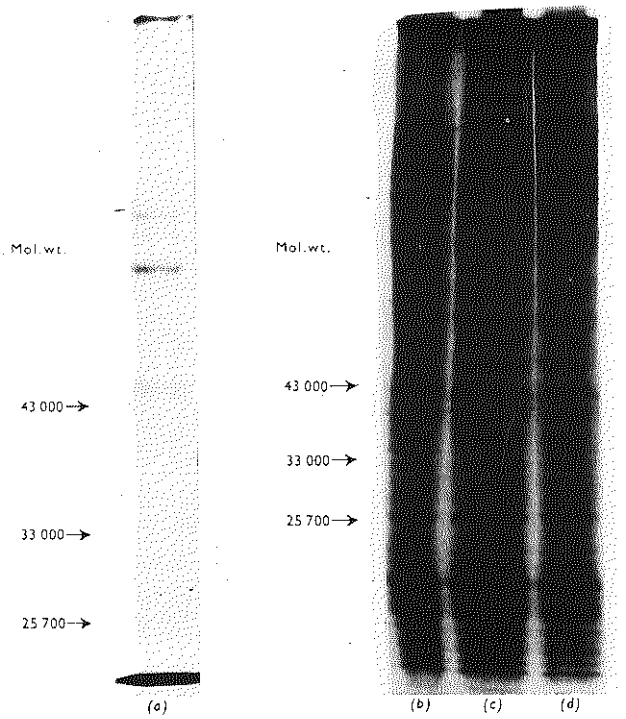
(facing p. 344)



EXPLANATION OF PLATE 2

Influence of cycloheximide on proteins labelled with [^{35}S]*methionine*

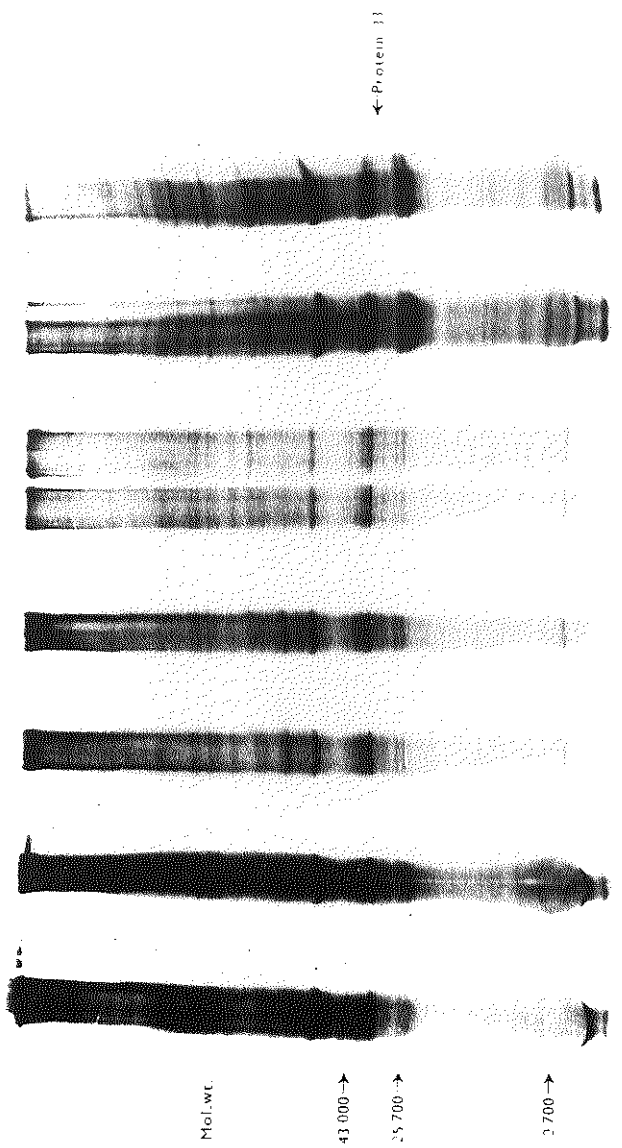
Leydig cells were first incubated with luteinizing hormone (100 ng/ml) for 30 min, followed by incubation with [^{35}S]-methionine for 30 min. After this incubation period, cycloheximide (25 μg/ml) was added for 0 (*a*), 5 (*b*), 10 (*c*), 15 (*d*), 25 (*e*), 35 (*f*) and 45 min (*g*). Proteins were separated on a sodium dodecyl sulphate/polyacrylamide (10% w/v) gel. After scanning of the radioautogram, the ratio of the activities in bands A and B was calculated. In a semi-logarithmic plot of this ratio against time, a straight line was obtained with a correlation coefficient (r) of 0.99, and a relative half-life of 11.7 min was calculated for band A. In a control experiment cells were incubated in the absence of luteinizing hormone, and a relative half-life of 10.9 min for band A was calculated.



EXPLANATION OF PLATE 3

Cellular localization of protein 33

Rat blood cells (*a*) or seminiferous tubules (*b*, *c*, *d*), obtained by wet dissection of rat testis, were incubated with [^{35}S]-methionine for 30min, followed by incubation with cycloheximide for 0min (*a*, *b*), 15min (*c*) and 30min (*d*), and the proteins were separated on a 10% (w/v) gel (*a*) or a 10–15% discontinuous (w/v) polyacrylamide gel (*b*, *c*, *d*).



EXPLANATION OF PLATE 4

Subcellular localization of protein 33

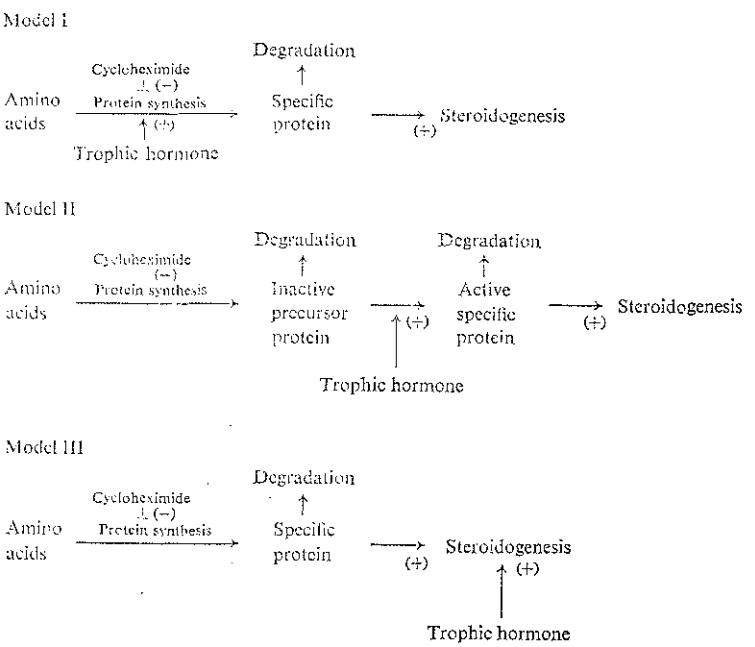
Leydig cells were incubated with [^{35}S]methionine for 30 min, followed by incubation with cycloheximide for 0 min (*a*, *c*, *e*, *g*) or 30 min (*b*, *d*, *f*, *h*). After incubation, the cells were sedimented and resuspended in 0.25 M-sucrose. The cell suspensions (3×10^7 cells/ml) were homogenized in an all-glass Potter–Elvehjem homogenizer by hand with ten strokes. The homogenates were first centrifuged for 10 min at 100 *g* (sediments *a* and *b*), the supernatant was centrifuged further for 10 min at 500 *g* (sediment *c*, *d*) and this supernatant was centrifuged for 10 min at 15000 *g* (sediments *e* and *f*, and supernatants *g* and *h*). For characterization of the subcellular fractions, DNA, monoamine oxidase, carboxyl esterase and lactate dehydrogenase were used as markers (see Table 1).

pellet and 10 min/15000*g* pellet, which indicates that the protein is localized in the particulate fraction of the cell (Plate 4). However, a more specific localization was difficult, because in different experiments variable amounts of protein 33 were found in different fractions, probably because of a variation in the extent of homogenization of the cells in different experiments.

Discussion

The aim of the present study was to investigate the possible induction and involvement of newly synthesized protein(s) in the action of luteinizing hormone on testosterone production by testis Leydig cells. Two proteins were detected which may be involved in this action: one is a cycloheximide-sensitive protein (protein 33) with a half-life of 11 min and the other a protein which appeared in Leydig cells 2h after addition of luteinizing hormone (protein 21). Protein 33 was relatively highly labelled with [^{35}S]methionine and could be detected easily among other labelled protein bands. This may be explained partly because of its short half-life (11 min); about 90% of the protein pool was labelled during a 30 min incubation period with [^{35}S]methionine. By contrast, protein 21 contained a much smaller amount of [^{35}S]methionine and could be detected only after impregnation of the polyacrylamide gels with 2,5-diphenyloxazole to increase the detection efficiency of the ^{35}S label and exposure of the gel to the X-ray film for 1–2 weeks. By that time part of the film became almost completely black (Fig. 2). When such small amounts of labelled proteins would appear in the upper part of the gel, they will be easily overlooked.

The detection of the two proteins in the present study raises the question of the role of protein synthesis in the mechanism of action of luteinizing hormone on steroidogenesis in the Leydig cell. In Scheme 1 a number of possible models are given. In model 1, which was originally proposed by Garren *et al.* (1965) for the adrenal gland, the regulation of steroidogenesis by trophic hormones is mediated by the synthesis of a regulatory protein with a short half-life. We did not observe the induction by luteinizing hormone of a protein with short half-life in rat testis Leydig cells, and therefore the present results



Scheme 1. *Hypothetical models for the role of protein synthesis in the regulation of steroidogenesis by trophic hormone*
−, Inhibition; +, stimulation.

do not support this model for the testis. In adrenal cells, Schulster *et al.* (1974) and Lowry & McMartin (1974) also rejected model 1 on basis of the rapid corticosterone response, which was observed less than 24s after addition of corticotropin or cyclic AMP. This appeared too fast for new protein synthesis. Although in testis Leydig cells the time between luteinizing hormone addition and testosterone response *in vitro* has been reported to be rather longer, in the order of 20min (Moyle & Ramachandran, 1973; Dufau & Catt, 1975; Janszen *et al.*, 1976), results of recent experiments in our laboratory indicate that stimulation of testosterone production may well occur within 5min after addition of luteinizing hormone (Cooke *et al.*, 1977). In the present study an increase of the synthesis of a specific protein (protein 21) was only observed 2h after addition of luteinizing hormone to Leydig cells, which is a long time after the initial stimulation (within 5min) of testosterone production by luteinizing hormone. For this reason it appears unlikely that protein 21 plays a role in the short-term stimulation of testosterone production by trophic hormones. However, this does not exclude a possible role of protein 21 in long-term effects of trophic hormones on Leydig cells. Such long-term effects have been described by Purvis *et al.* (1973) for rat testis.

On the basis of the present results it also cannot be excluded that the synthesis of protein 21 is only indirectly influenced by luteinizing hormone, i.e. via testosterone production. The mechanism of induction of protein 21 and its possible role in Leydig-cell function therefore requires further investigation.

The protein with short half-life (protein 33) detected in the present study could also play a role in the production of testosterone and its regulation by luteinizing hormone. It has properties of the regulator protein, which might be expected from previous studies, especially those involving protein-synthesis inhibitors, i.e. a short half-life (11 min) was estimated for protein 33, which is comparable with the half-life (13min) calculated from inhibition studies with cycloheximide on superfused Leydig-cell preparations (Cooke *et al.*, 1975). Further, in the testis, protein 33 is specifically located in the Leydig cells. These results are in better agreement with the characteristics of the regulatory protein depicted in model II of Scheme 1. This model is based on the proposition by Schulster *et al.* (1974) and Lowry & Martin (1974) that an inactive precursor protein with a short half-life is activated in the presence of trophic hormone. The latter does not have any direct effect on the synthesis *de novo* of the precursor protein, but cycloheximide would inhibit its synthesis. However, until now it has not been possible to detect an effect of luteinizing hormone on any of the properties of protein 33 studied, i.e. molecular weight, half-life and subcellular localization of the protein. So, at present, even a third possibility (model III in Scheme 1) cannot be excluded, i.e. that a protein with a short half-life is involved without being affected itself by the trophic hormone.

We are grateful to the National Institutes of Health, Endocrinology Study Section, Bethesda, MD, U.S.A., for gifts of sheep luteinizing hormone. This work was financially supported in part by the Dutch Foundation for Medical Research (FUNGO). The technical assistance of Miss M. J. A. van Driel is gratefully acknowledged.

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Biochemical Journal 1977 (accepted for publication)

REGULATION OF THE SYNTHESIS OF LUTROPIN-INDUCED PROTEIN IN RAT TESTIS LEYDIG CELLS

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SYNOPSIS

The mechanism of action of lutropin on the stimulation of the synthesis of a specific lutropin-induced protein in rat testis Leydig cells was investigated. Lutropin-induced protein has a mol. wt. of approx. 21000 and is detected by labelling the Leydig cell proteins with ^{35}S-methionine, followed by separation by polyacrylamide gel electrophoresis and radioautography of the dried gel. The incorporation of ^{35}S into lutropin-induced protein was used as an estimate for the synthesis of the protein. Incubation of Leydig cells with dibutyryl cyclic AMP or cholera toxin also resulted in the stimulation of synthesis of the protein. Synthesis of lutropin-induced protein, when maximally stimulated with 100 ng of lutropin/ml, could not be stimulated further by addition of dibutyryl cyclic AMP. Addition of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor, further increased synthesis of the protein in the presence of a submaximal dose of lutropin (10 ng/ml) but not in the absence of lutropin or with maximal amounts of lutropin (100 and 1000 ng/ml). Actinomycin D prevented the effect of lutropin on the stimulation of lutropin-induced protein synthesis

when added immediately or 1 h after the start of the incubation, but not when added after 5-6 h. This is interpreted as reflecting that after induction of mRNA coding for lutropin-induced protein, lutropin had no influence on the synthesis of the protein in the presence of actinomycin D. Synthesis of the protein was also stimulated in vivo by injection of choriogonadotropin into rats 1 day after hypophysectomy and the time course of this stimulation of lutropin-induced protein synthesis in vivo was similar to that obtained by incubating Leydig cells in vitro with lutropin. From these results it is concluded that stimulation of lutropin-induced protein synthesis by lutropin is most probably mediated by cyclic AMP and involves synthesis of mRNA.

INTRODUCTION

It has been shown that protein synthesis is involved in stimulation by lutropin of testosterone production in rat testis Leydig cells (Hall & Eik-Nes, 1962; Cooke et al., 1975b; Mendelson et al., 1975). After maximal stimulation of testosterone synthesis in rat testis Leydig cells by lutropin, addition of cycloheximide decreases testosterone production to control values. This decrease follows first order kinetics with a half-time of 13 min, indicating that continuous synthesis of a protein(s) with short half-life is necessary (Cooke et al., 1975b). Inhibitors of RNA synthesis also cause at least a partial inhibition of lutropin stimulated production of testosterone (Cooke et al., 1975a; Mendelson et al., 1975). These results may indicate that the synthesis of mRNA coding for this protein(s) may also be involved in lutropin regulation of testosterone production. From previous studies in our laboratory it is known, that addition of lutropin to Leydig cells stimulates the synthesis of a specific protein (lutropin-induced protein) with a mol. wt. of approx. 21000 (Janszen et al., 1976b, 1977). Lutropin-induced protein synthesis was not influenced by addition of testosterone or follitropin to the Leydig cells

(Janszen et al., 1976b). Addition of actinomycin D to Leydig cells prevented the stimulation of the synthesis of this protein (Janszen et al., 1976b). The half-life of this protein, however, was found to be longer than 30 min (Janszen et al., 1977) and increased synthesis of lutropin-induced protein could be observed only 2 h after addition of lutropin to Leydig cells, which is a long time after stimulation of testosterone production by lutropin can be detected (5-30 min; Cooke et al., 1977).

To obtain more information about the mechanism involved in the control of the synthesis of lutropin-induced protein, we have now studied the effects of dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine and actinomycin D on synthesis of the protein, because it is known that these compounds can influence testosterone production in Leydig cells (Catt et al., 1972; Cooke et al., 1975a). In addition the production of lutropin-induced protein in Leydig cells after stimulation with choriogonadotropin in vivo has been investigated. The results obtained are discussed in relation to the possible role of lutropin-induced protein in the regulation of steroidogenesis.

MATERIALS AND METHODS

Cholera toxin was a gift from R.S. Northrup National Institute of Allergy and Infectious Diseases, Bethesda, MD, U.S.A., and was prepared by the procedure of Finkelstein & Lospalluto (1970) under contract for the National Institute of Allergy and Infectious Diseases (NIAID) by Dr. R.A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, TX, U.S.A.

Sheep lutropin (NIH-LH-S18) was a gift from the Endocrinology Study Section, National Institute of Health, Bethesda, MD, U.S.A.

^{35}S-Methionine (280 Ci/mmol) was purchased from The Radiochemical Centre, Amersham, U.K. and 3-isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co.,

Milwaukee, WI, U.S.A. Human choriogonadotropin was obtained from Organon, Oss, The Netherlands. Leydig cell suspensions from adult rat testes were prepared and purified by centrifugation through Ficoll and Dextran solutions as described previously (Janszen et al., 1976a), except that testes were incubated in the collagenase solution (1 mg/ml) for 30 min instead of 18 min.

Leydig cells were incubated and proteins were labelled with ^{35}S-methionine, extracted, separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and detected by radioautography as described previously (Janszen et al., 1976b), except that proteins were labelled with ^{35}S-methionine for 60 min instead of 30 min. Quantification of radioactivity in the lutropin-induced protein band was performed as described previously (Janszen et al., 1977). The ratio of the density of the lutropin-induced protein band divided by the density of a band with a mol. wt. of approx. 23000 (which was shown to be unaffected by the different conditions used) was calculated. This ratio was used for a quantitative evaluation of the lutropin-induced protein. For comparison between different experiments the amount of radioactivity in lutropin-induced protein, measured as described above, was expressed as a percentage of the amount of radioactivity in lutropin-induced protein when maximally stimulated with 100 ng of lutropin/ml.

To investigate if steroidogenesis was stimulated by the different reagents used, Leydig cells were incubated in parallel experiments for 2 h in the presence of these various reagents. After the incubation period testosterone was extracted and determined as described by Verjans et al. (1973).

For statistical analysis the Student t-test for correlated data was used. Differences were considered significant when $P < 0.025$ (one-tailed).

Role of cyclic AMP in stimulation of LH-IP synthesis by lutropin

This was investigated by studying the effect of dibutyryl cyclic AMP, 3-isobutyl-1-methylxanthine and cholera toxin.

The effect of dibutyryl cyclic AMP on synthesis of lutropin-induced protein and testosterone was investigated in the presence and absence of maximum amounts of lutropin. The results in Table 1 show that the effect of 100 ng of lutropin/ml or of 1 mM dibutyryl cyclic AMP on stimulation of testosterone production and lutropin-induced protein synthesis are not significantly different from each other and no further stimulation of synthesis of the protein was observed when these two substances were added together.

TABLE 1

Effect of lutropin (100 ng/ml) and dibutyryl cyclic AMP (1 mM) on synthesis of testosterone and lutropin-induced protein in testis Leydig cells. Results are expressed as percentage of synthesis obtained in the presence of lutropin.

	Additions			
	None	Lutropin	Dibutyryl cyclic AMP	Lutropin plus dibutyryl cyclic AMP
Testosterone production (ng/2 h per 10^6 cells) (mean ± s.e.m.)	5±1 (12)	114± 16 (11)	126±28 (7)	n.d.
Lutropin-induced protein synthesis (mean ± s.d.)	22±24 (4)°	100 (4)°	100±23 (4)+	100±23 (4)+

number of experiments are given in parentheses

°$P < 0.005$

+not significant ($P > 0.025$)

n.d.: not determined

To estimate the effect of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine on lutropin-induced protein synthesis, the Leydig cells were first incubated for 3 h in the presence of different doses of lutropin without or with 3-isobutyl-1-methylxanthine; then ^{35}S-methionine was added and the incubation was continued for another 1 h. As shown in Table 2, lutropin-induced protein synthesis is significantly increased in the presence of 3-isobutyl-1-methylxanthine with a submaximal dose of lutropin, but not in the absence of lutropin or with a maximal dose (100 and 1000 ng/ml). In these experiments testosterone production in the presence of 1 ng of lutropin/ml increased from 6 ± 2 ng/2 h per 10^6 cells in the absence of 3-isobutyl-1-methylxanthine to 26 ± 16 ng/2 h per 10^6 cells in its present (mean ± s.d., n=4, $P < 0.025$).

TABLE 2

Effect of 0.25 mM 3-isobutyl-1-methylxanthine on lutropin-induced protein synthesis in Leydig cells in the presence of different doses of lutropin. Results are expressed as percentage of lutropin-induced protein synthesis, in the presence of 100 ng of lutropin, and are means ± s.d. of the numbers of experiments in parentheses.

	lutropin-induced protein synthesis		
lutropin ng/ml	- inhibitor	+ inhibitor	Effect of inhibitor
0	14±22	15±10 (4)	N.S.
1	14±22	34±26 (4)	N.S.
10	35±23	80±16 (3)	P < 0.005
100	100	89±12 (3)	N.S.
1000	95± 9	89±23 (3)	N.S.

number of experiments in brackets

N.S.: not significant (P > 0.025)

The Leydig cells were incubated in the presence of cholera toxin, which has previously been shown to stimulate production of both cyclic AMP and testosterone in Leydig cells in a parallel fashion (Cooke et al., 1977). Leydig cells were incubated for 3 h without or with lutropin or with 40 µg of cholera toxin/ml, followed by incubation with ^{35}S-methionine for 1 h. For measurement of testosterone production, Leydig cells were incubated for 2 h. Table 3 shows that in the pre-

TABLE 3

Effect of cholera toxin on testosterone production and lutropin-induced protein synthesis in Leydig cells. Lutropin-induced protein synthesis is expressed as a percentage of that in the presence of lutropin (100 ng/ml).

	control	+ lutropin	+ cholera toxin
testosterone production [x] (ng/2 h per 10^6 cells)	4±1	141±35	45±14 [x]
lutropin-induced protein synthesis [o]	4±5	100	80±18 [oo]

[x] mean ± s.d. of 4 different experiments

[o] mean ± s.d. of 6 different observations in 4 different experiments

[oo] $p < 0.005$ compared with control

sence of cholera toxin both testosterone production and lutropin-induced protein synthesis were increased, although a lower stimulation was found compared with the effect of lutropin. It has been previously demonstrated that stimulation of production of cyclic AMP and testosterone is delayed in the presence of cholera toxin compared with the stimulation obtained in the presence of lutropin (Cooke et al., 1977). To investigate if there was also a delay in the stimulation of lutropin-induced protein synthesis by cholera toxin, the time course of this stimulation was investigated. With cholera toxin as well as with lutropin, the first stimulation of synthesis of the protein was observed 2 h after

the start of the incubation, and synthesis was maximal after about 4 h (Fig. 1).

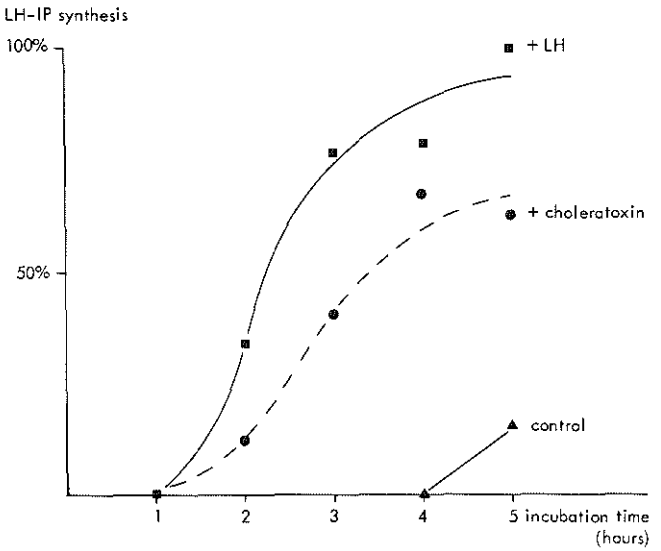

Figure 1 Time course of lutropin-induced protein synthesis in presence of lutropin or choleratoxin

Leydig cells were incubated for different time periods in the absence (▲) or presence of lutropin (■) or choleratoxin (●). ^{35}S-Methionine was then added and the incubation was continued for 1 h. Total cell protein was extracted and separated by electrophoresis and the amount of radioactivity in the LH-IP band was measured as described in the Materials and Methods section. Lutropin-induced protein synthesis is expressed as percentage of that 5 h after addition of lutropin

Role of mRNA synthesis in the stimulation of LH-IP synthesis by lutropin

The effect of actinomycin D on the stimulation by lutropin of lutropin-induced protein synthesis was investigated at different times after the start of the incubation. Stimulation of the synthesis of this protein was prevented completely when actinomycin D was added immediately or 1 h after the start of the incubation. Inhibition became progressively less when actinomycin D was added at later times until no inhibition was detectable when actinomycin D was added 4-5 h after the start of the incubation with lutropin (Fig. 2). The stability of mRNA was investigated in Leydig cells which were incubated with dibutyryl cyclic AMP for 3 h to stimulate lutropin-induced protein synthesis.

The synthesis of lutropin-induced protein in the control cells was 5± 6% of that in the dibutyryl cyclic AMP stimulated Leydig cells (=100%) (mean ± s.d. of 5 experiments). The dibutyryl cyclic AMP was then removed from the cells by washing with the incubation buffer followed by centrifugation (twice) and the incubation was then continued for 3 h in the presence of actinomycin D without or with lutropin. After this period ^{35}S-methionine was added and the incubation was continued for another hour. In this case the production of lutropin-induced protein in the Leydig cells that were incubated during the last 4 hours without or with lutropin was 91 ± 25% and 106 ± 38% respectively of the synthesis of lutropin-induced protein in Leydig cells only incubated for 4 h with dibutyryl cyclic AMP (mean ± s.d. of 5 experiments). In separate experiments it was demonstrated that with this washing procedure testosterone production in Leydig cells preincubated for 3 h in the presence of dibutyryl cyclic AMP fell to basal values after removal of dibutyryl cyclic AMP (7 ± 6 and 6 ± 3 ng of testosterone/2 h per 10^6 cells respectively for washed cells and control cells, mean ± s.d., n=3).

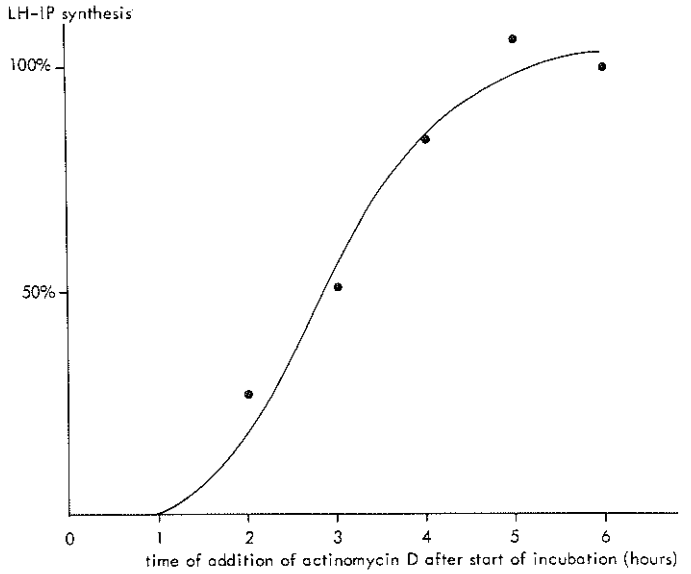

Figure 2 Effect of actinomycin D on lutropin-induced protein synthesis

Actinomycin D was added at different times after the start of incubation in the presence of lutropin. Leydig cells were incubated with lutropin (100 ng/ml) for different time periods, then actinomycin D (46 µM) was added and the incubations were continued. The total incubation time was 8 h. ^{35}S-Methionine was then added and the incubation was continued for another 1 h. Lutropin-induced protein synthesis is expressed as percentage of that in Leydig cells incubated for 9 h with lutropin without actinomycin D

Lutropin-induced protein synthesis in Leydig cells stimulated in vivo with choriogonadotropin

To investigate if lutropin-induced protein synthesis could be stimulated also in vivo, choriogonadotropin (100 I.U.) was injected into the tail vein of rats 1 day after hypophysectomy. From previous work it was known that this amount of choriogonadotropin would stimulate testosterone secretion in vivo (De Jong et al., 1973). Control rats

were injected with 0.9% NaCl 3 h before decapitation. After decapitation Leydig cells were prepared and incubated in vitro for 3 h without or with lutropin in the absence or presence of actinomycin D. ^{35}S-Methionine was then added and the incubation was continued for another 1 h.

Testosterone production in the absence or presence of lutropin was measured in parallel incubations (Table 4).

TABLE 4

Effect of choriogonadotropin on testosterone production in Leydig cells from 1 day hypophysectomized rats. Isolated cells were incubated for 2 h with or without 100 ng of lutropin/ml. "Control rats" were injected 3 h before decapitation with 0.9% NaCl solution and "HCG rats" with 100 I.U. of choriogonadotropin.

	testosterone production (ng/2 h per 10^6 cells	
	- lutropin	+ lutropin
control rats	6±3	263±65
HCG rats	57±17	53±7

Results are mean ± s.d. of three experiments.

In control rats lutropin-induced protein synthesis increased in the presence of lutropin, and this increase was prevented by addition of actinomycin D to the cells (Table 5 and Fig. 3). From Leydig cells of choriogonadotropin-injected rats a prominent ^{35}S-labelled protein with the same molecular weight as lutropin-induced protein was isolated (Fig. 3). Incorporation of ^{35}S into this protein was not affected by either lutropin or actinomycin D added during incubation of the cells (Table 5).

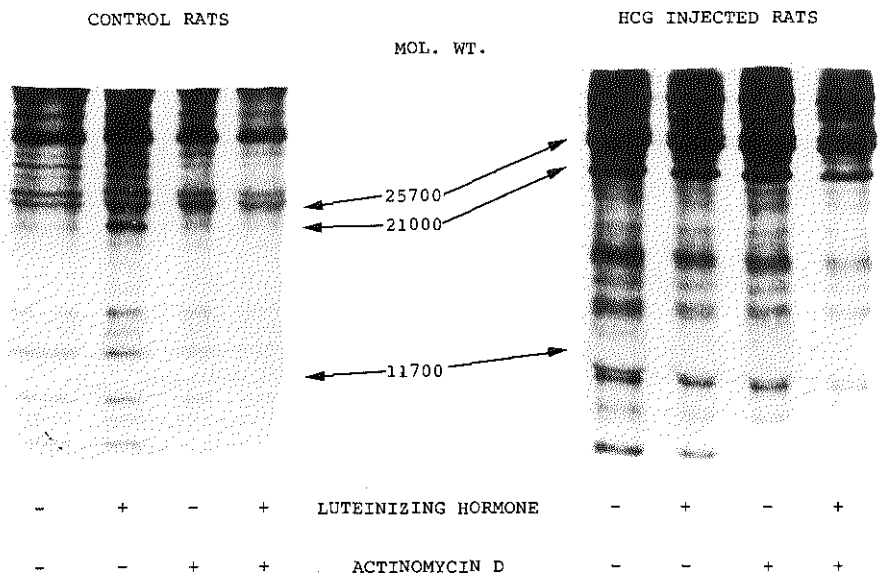

Figure 3 Effect of injection of human choriogonadotropin on lutropin-induced protein synthesis by Leydig cells in vitro

Rats, 1 day after hypophysectomy, were injected with 100 I.U. of human choriogonadotropin (b) or with 0.9% NaCl (control, a) 3 h before decapitation. Leydig cells were prepared and incubated for 3 h in the absence or presence of lutropin (100 ng/ml) and in the absence or presence of actinomycin D. Then ^{35}S-methionine was added and the incubation was continued for 1 h. After extraction total cell protein was separated on a 10-15% discontinuous sodium dodecyl sulphate polyacrylamide gel. Only the radioautogramme of the 15% part of the gel is shown

TABLE 5

Effect of choriogonadotropin on lutropin-induced protein synthesis in Leydig cells from 1 day hypophysectomized rats. Rats were injected 3 h before decapitation with 0.9% NaCl (control) or with 100 I.U. choriogonadotropin (HCG rats). Synthesis of lutropin-induced protein is expressed as percentage of synthesis in cells incubated in vitro with lutropin (100 ng/ml), without actinomycin D. Results are given as mean ± s.d. for three experiments.

	- lutropin		+ lutropin	
	Actinomycin D		Actinomycin D	
	-	+	-	+
control rats	14±12	7±7	100	10±12
HCG rats	91±23	98±34	100	106±6

In other experiments, rats that had been hypophysectomized for 1 day were injected with 100 I.U. of choriogonadotropin at various times before decapitation. Leydig cells were prepared and then incubated in vitro as described above. In the cells isolated 3 h after injection of choriogonadotropin again maximal ^{35}S-incorporation was obtained in a protein band of mol. wt. 21000. This was observed in incubations with and without added lutropin and was unaffected by the presence of actinomycin D. When rats were injected with choriogonadotropin 1 and 2 h before decapitation, addition of actinomycin D to the isolated cells resulted in a decrease of ^{35}S-incorporation into this protein band (Table 6).

TABLE 6

Lutropin-induced protein synthesis in isolated Leydig cells from rats injected with human choriogonadotropin at different times before decapitation.
Synthesis of lutropin-induced protein in the presence of lutropin was taken as 100% and the synthesis of lutropin-induced protein in the presence of actinomycin D was expressed as percentage of synthesis in the presence of lutropin.

rat injected with	hours before decapitation	synthesis of lutropin-induced protein
0.9% NaCl	3	11±3*
choriogonadotropin	1	26±10
choriogonadotropin	2	58±2
choriogonadotropin	3	91±19

*mean ± range of two determinations.

DISCUSSION

In the present study it has been demonstrated that dibutyryl cyclic AMP, cholera toxin and 3-isobutyl-1-methylxanthine can stimulate the synthesis of lutropin-induced protein in testis Leydig cells. These results may support a possible role of cyclic AMP in the stimulation of synthesis of this protein, because it has been demonstrated that cyclic AMP production in Leydig cells is stimulated by lutropin (see reviews by Rommerts et al., 1974; Catt & Dufau, 1976; Marsh, 1976) and by cholera toxin (Cooke et al., 1977). Phosphodiesterase inhibitors, such as 3-isobutyl-1-methylxanthine or theophylline, have been shown to potentiate lutropin action most probably by inhibition of the degradation of cyclic AMP by phosphodiesterase (Catt et al., 1974). Previously it has been shown that addition of actinomycin D to Leydig cells at the start of the incubation prevents stimulation of lutropin-induced protein synthesis

by lutropin (Janszen et al., 1976b).

From the lack of effect of actinomycin D on the lutropin-stimulated synthesis of lutropin-induced protein, when added to the Leydig cells 5 h after the start of the incubation (Fig. 2), it may be concluded that actinomycin D does not interfere with the synthesis of this protein as such. Hence, the inhibitory effect of actinomycin D on lutropin stimulation of the synthesis of this protein, when added earlier, may be explained by prevention of the accumulation of mRNA coding for lutropin-induced protein. This accumulation of mRNA may result either from stabilization of existing mRNA in the presence of lutropin or from increased synthesis of mRNA. The present results demonstrate that after stimulation with dibutyryl cyclic AMP the synthesis of lutropin-induced protein remains at an elevated level for several hours after removal of the dibutyryl cyclic AMP. This could indicate that under these conditions no marked degradation of mRNA occurs. However, these experiments were performed in the presence of actinomycin D in order to prevent new mRNA synthesis and secondary effects of this inhibitor on the synthesis of lutropin-induced protein cannot be excluded. Therefore, more research is needed to determine the stability of the mRNA in the presence and in the absence of the hormone.

When hypophysectomized rats were injected with choriogonadotropin 3 h before decapitation no subsequent inhibition of lutropin-induced protein synthesis occurred in the presence of actinomycin D. This indicates that in this situation synthesis of this protein was already maximally stimulated before addition of the actinomycin D. Testosterone production of Leydig cells from choriogonadotropin treated rats could not be stimulated further by addition of lutropin to the isolated cells in vitro. However, stimulation of Leydig cells in vitro with dibutyryl cyclic AMP and subsequent removal of the dibutyryl cyclic AMP resulted in a low basal testosterone production. These results may be explained by assuming that choriogonadotropin, injected in vivo,

remains bound to its receptor after isolation of the Leydig cells. Survival of the lutropin-receptor complex after washing Leydig cells by centrifugation has been described by Moyle and Ramachandran (1973).

Hsueh et al. (1976) demonstrated occupancy of receptors by endogenous lutropin after preparation of Leydig cells. To study the time course of induction of lutropin-induced protein synthesis in vivo, actinomycin D was added to the Leydig cells from in vivo stimulated animals to prevent further stimulation of lutropin-induced protein. Under these conditions only a small stimulation of lutropin-induced protein synthesis was found in Leydig cells from rats injected with choriogonadotropin 1 h before decapitation (the total time of stimulation was 2.5 h; 1 h in vivo and 1.5 h during cell preparation). Maximum stimulation of lutropin-induced protein synthesis was observed when the Leydig cells were exposed to choriogonadotropin for 4-5 h. It may be concluded therefore that the time course of stimulation of lutropin-induced protein synthesis in vivo and in vitro are very similar.

To summarize, the present evidence indicates that the synthesis of both lutropin-induced protein and testosterone is regulated by similar mechanisms involving cyclic AMP and mRNA under the control of lutropin. However, it is still difficult to conclude that lutropin-induced protein is obligatory involved in lutropin stimulation of testosterone biosynthesis, because a long time (2 h) is needed after lutropin addition before increased synthesis of the protein is detectable compared with 5-20 min for stimulation of testosterone production. Thus maximum testosterone synthesis can occur in the presence of low lutropin-induced protein synthesis.

Therefore, if lutropin-induced protein is involved in steroid production, it may not be the only lutropin-induced factor, theoretically the protein could even be an inhibitor of steroidogenesis. It is also possible that stimulation of lutropin-induced protein synthesis is unrelated to steroidogenesis and that it is only one of the first trophic effects of lutropin on Leydig cells irrespective of steroid produc-

tion. These questions cannot be answered at the moment and must await further studies with purified lutropin-induced protein.

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Biochemical Journal (submitted for publication)

THE EFFECT OF LUTROPIN ON SPECIFIC PROTEIN SYNTHESIS IN TUMOUR LEYDIG CELLS AND IN LEYDIG CELLS FROM IMMATURE RATS

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SYNOPSIS

The effect of lutropin on the synthesis of specific proteins in tumour Leydig cells and in Leydig cells from immature rats has been investigated. The amount of ^{35}S-incorporated into the various proteins after separation by electrophoresis on sodium dodecyl sulphate polyacrylamide gel was used as an estimate of their synthesis. Incubation of tumour Leydig cells with lutropin for 5 hours resulted in increased synthesis of proteins with apparent mol. wt.'s of 27000 and 29000. The shortest incubation time needed for a significant increase in the synthesis of these proteins was approximately 3 hours.

Incubation of Leydig cells from immature rats with lutropin (100 ng/ml) resulted in increased synthesis of proteins with apparent mol. wt's of 11000, 21000, 27000 and 29000. At higher concentrations of lutropin ($\geq$ 100 ng/ml) there was a decrease in the synthesis of proteins with an apparent mol. wt. of 13000. The stimulation of specific protein synthesis was observed approximately 2 h after the addition of lutropin to the cells.

The amount of lutropin required for the stimulation of

protein synthesis in both types of Leydig cells was similar to the amount of lutropin needed for stimulation of steroidogenesis. Lutropin-stimulated specific protein synthesis was not due, however, to increased levels of testosterone, because 1) addition of testosterone to the cells had no effect on the synthesis of the proteins, and 2) inhibition of steroidogenesis with eliptenphosphate (an inhibitor of the cholesterol side chain cleavage enzyme complex) did not abolish the effect of lutropin. The stimulation of specific protein synthesis was also not due to contaminating follitropin in the lutropin preparation, because follitropin itself had no effect on protein synthesis. Addition of actinomycin D to the cells at the start of the incubation prevented the effect of lutropin on specific protein synthesis, indicating that mRNA synthesis may be needed for the effect of lutropin on specific protein synthesis. Incubation of the cells with cycloheximide for 30 min after labelling of the proteins did not result in a detectable decrease in the amounts of the lutropin induced proteins indicating that their half lifes are longer than 30 min.

INTRODUCTION

Stimulation of steroidogenesis in rat testis Leydig cells by lutropin involves synthesis of protein and RNA (Cooke et al., 1975a; Mendelson et al., 1975) and it has been postulated that the continuous synthesis of a protein with short half life ($\lesssim$ 13 min) is necessary for the stimulation of steroidogenesis (Cooke et al., 1975b).

Recently it has been shown that lutropin stimulates in rat testis Leydig cells the synthesis of a protein with an apparent mol. wt. of 21000 (referred to as LH-IP). The half life of this protein is longer than 30 min (Janszen et al., 1976b, 1977a). The lutropin stimulation of the synthesis of this protein is mediated by cyclic AMP and probably involves mRNA synthesis (Janszen et al., 1977b). In the present study the effect of lutropin on the synthesis of lutropin-induced

protein (LH-IP) and other proteins has been investigated in two other types of Leydig cells; i.e. Leydig cells from immature rats and from a Leydig cell tumour, by first labelling of the proteins with ^{35}S-methionine followed by separation of the proteins on sodium dodecyl sulphate polyacrylamide gels, and fluorography of the dried gels, impregnated with 2,5-diphenyloxazole.

MATERIALS AND METHODS

Ovine FSH (NIH-FSH-S12) and ovine LH (NIH-LH-S19) were gifts from the Endocrinology Study Section of the National Institute of Health, Bethesda, Maryland, U.S.A. ^{35}S-Methionine (280 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, England. Eliptenphosphate was a gift from Ciba, Basal, Switzerland. The Leydig cell tumour was obtained from the Mason Research Institute Tumour Bank, Worcester, Massachusetts, U.S.A. and originated from R. Huseby, American Medical Centre at Denver, Spivak, Colorado, U.S.A. The tumour originally arose spontaneously in Fischer male rats. The tumour was grown and sustained in castrated 90 days old male Fischer rats. A cell suspension of the tumour was prepared by incubation of small pieces of the tumour in 7 ml Krebs Ringer bicarbonate buffer pH 7.4 containing 0.2% glucose and 1 mg/ml collagenase (Worthington) for 50 min at 37^{o}C in a 40 ml plastic tube under an atmosphere of O_2/CO_2 (95/5 v/v) under continuous shaking. After incubation, 25 ml 0.9% NaCl was added and the tube was inverted 10 times. The tubes were left to stand for 10 min at room temperature to sediment cell clumps. The supernatant was syphoned off and filtered through a nylon gauze (60 µm). The cells were then sedimented by centrifugation for 10 min at 100 g. The sedimented cells were washed once by centrifugation for 10 min at 100 g in Krebs Ringer bicarbonate buffer pH 7.4 containing 0.2% glucose and 0.1% albumin fraction V. Histochemical detection of 3β-hydroxy steroid dehydrogenase activity (Janszen et al., 1976) showed that almost all isolated cells formed formazan

precipitates. Leydig cell suspensions from immature rats were prepared from testes of Wistar rats substrain R-Amsterdam, 21-25 days old, as previously described (Janszen et al., 1976a). The Leydig cell suspensions were purified by centrifugation through a Ficoll solution (Janszen et al., 1976a). The cell suspension was washed once by centrifugation for 5 min at 100 g through a Krebs Ringer bicarbonate solution pH 7.4 containing 0.2% glucose and 0.1% albumin fraction V. The cells were incubated in a Krebs Ringer bicarbonate buffer pH 7.4 containing 0.2% glucose and 0.1% albumin fraction V and an amino acid mixture lacking in methionine under an atmosphere of O_2/CO_2 (95/5 v/v). Proteins were labelled, extracted, separated by SDS-PAGE and detected by fluorography as previously described (Janszen et al., 1977a). Mol. wt.'s of proteins were estimated by electrophoresis on a 10-20% continuous gradient SDS polyacrylamide gel with as mol. wt. markers phosphorylase a (93000), catalase (60000), ovalbumin (43000), chymotrypsinogen (25700) and cytochrome C (11700). When testosterone production was measured, the isolated cells were incubated as described above for 2 h in the presence or absence of added lutropin and testosterone was extracted and determined as described by Verjans et al. (1973). For statistical analysis the Student t-test for correlated data was used. Differences were considered significant when $P < 0.05$ (one-tailed).

RESULTS

Tumour Leydig cells

Incubation of tumour Leydig cells with lutropin (100 ng/ml) for 4 h followed by incubation for 1 h in the presence of ^{35}S-methionine to label the proteins resulted in increased ^{35}S incorporation in the two proteins with apparent mol. wt.'s of 27000 and 29000 (referred to as protein 27000 and protein 29000 respectively (Fig. 1b)). The increased ^{35}S incorporation into these proteins as compared to the control (incubated

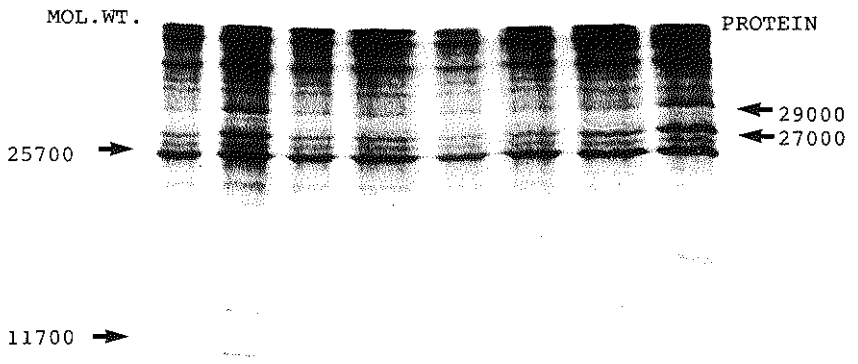

Figure 1 ^{35}S incorporation into proteins of tumour Leydig cells, incubated in the presence of various hormones

Tumour Leydig cells were incubated for 4 h in the presence of the following compounds: a) none, b) lutropin (100 ng/ml), c) testosterone (100 ng/10^6 cells), d) oestradiol-17β (100 ng/10^6 cells), e) pregnenolone (100 ng/10^6 cells), f) follitropin (100 ng/ml), g) none and h) lutropin (100 ng/ml), followed by incubation for 1 h in the presence of ^{35}S-methionine.

Incubation g and h were continued for 30 min in the presence of cycloheximide (25 µg/ml). The proteins were separated by electrophoresis on sodium dodecyl sulphate 10-15% discontinuous polyacrylamide gel. Only the 15% part of the gel is shown

Leydig cells without added lutropin) was statistically significant 2-3 h after the addition of lutropin to the cells (Fig. 2a,b) and during a 4 h incubation period maximum stimulation was not observed. The lowest dose of lutropin needed for a significant stimulation of testosterone production

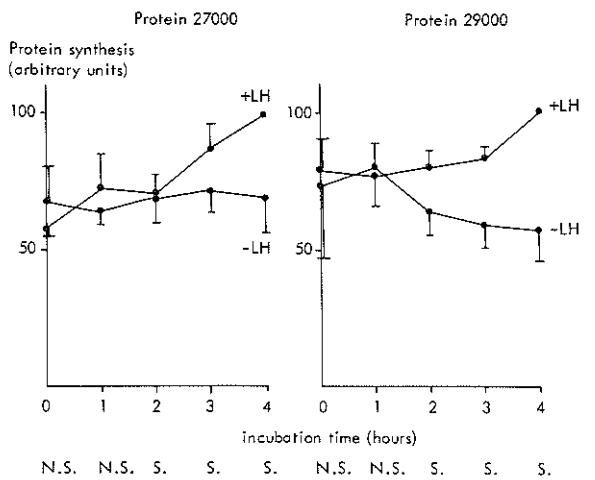

Figure 2 Time course of lutropin stimulation of the synthesis of protein 27000 and protein 29000 in tumour Leydig cells

Tumour Leydig cells were incubated with or without added lutropin (100 ng/ml) for different time periods followed by incubations of the cells with ^{35}S-methionine for 1 h. Specific protein synthesis is expressed as % of specific protein synthesis after 4 h stimulation with lutropin (mean ± s.d., n=3). N.S. = no significant difference between stimulated and control protein synthesis. S = significant difference between stimulated and control protein synthesis

was 100 ng/ml and a maximum testosterone response was obtained with approximately 1000 ng/ml (Table 1) (testosterone production increased from 1.0 ± 0.7 ng/10^6cells/2 h in control cells to 4.6 ± 1.1 ng/10^6 cells/2 h in the presence of lutropin 10^3 ng/ml, mean ± s.d., n=4). The lowest dose of lutropin to give a significant response in ^{35}S incorporation in proteins 27000 and 29000 was also 100 ng/ml, a maximum response was obtained with 1000 ng/ml (Table 1).

Table 1

Effect of different doses of LH on the synthesis of testosterone and the ^{35}S-incorporation into protein 27000 and protein 29000 in tumour Leydig cells. Results are expressed as % of the value obtained in the presence of 100 ng/ml LH. Means ± s.d. is given with the number of experiments between brackets.

Dose of LH (ng/ml)	testosterone production		protein 27000		protein 29000	
	%	effect of LH	%	effect of LH	%	effect of LH
0	26±14 (4)		42±27 (4)		31±21 (3)	
1	27±10 (4)	N.S.	36±11 (4)	N.S.	21±24 (4)	N.S.
10	54±26 (4)	N.S.	60±18 (3)	N.S.	76±50 (3)	N.S.
10^2	100 (4)	P<0.005	100 (4)	P<0.005	100	P<0.005
10^3	126±50 (4)	P<0.01	127±24 (4)	P<0.01	163±50 (4)	P<0.05
10^4	145±41 (3)	P<0.01	128±21 (4)	P<0.005	143±29 (4)	P<0.025

To investigate whether the increase in ^{35}S incorporation in protein 27000 and 29000 was due to increased levels of steroids in the lutropin-stimulated Leydig cells, testosterone, oestradiol-17β and pregnenolone (100 ng/10^6 cells) were added separately to the cell preparations instead of lutropin and incubated for 4 h followed by incubation with ^{35}S-methionine for 1 h. No change in ^{35}S incorporation into proteins 27000 or 29000 was obtained (Fig. 1c-e) when compared with the controls. In other experiments eliptenphosphate (300 µg/ml) was added to lutropin-stimulated tumour cells to prevent steroid synthesis (El Safoury & Bartke, 1974). The lutropin-stimulated ^{35}S labelling of the proteins was not inhibited under these conditions. To investigate whether the increase in ^{35}S incorporation was due to contamination of the lutropin preparation with follitropin, tumour cells were incubated with follitropin (100 ng/ml) instead of lutropin; no increase in ^{35}S incorporation into protein 27000 and 29000 was observed (Fig. 1f).

Addition of actinomycin D (46 µM) to the tumour Leydig cells at the start of the incubation, prevented the lutropin stimulation of ^{35}S incorporation into both proteins. Previously it has been shown that addition of this amount of actinomycin D to Leydig cells inhibited 89-93% of RNA synthesis (Cooke et al., 1975a).

In other experiments cycloheximide (25 µg/ml) was added to the cells after labelling of the proteins with ^{35}S-methionine and the incubation was continued for 30 min. No effect of this procedure was observed on the amount of ^{35}S incorporated into these two proteins (Fig. 1g,h) indicating that their half lifes are longer than 30 min.

Leydig cells from immature rats

Incubation of Leydig cells from immature rats with lutropin (100 ng/ml) for 4 h followed by incubation of the cells with ^{35}S-methionine for 1 h resulted in an increase in ^{35}S incorporation in proteins with apparent mol. wt.'s of respectively 11000, 21000, 27000 and 29000 (referred to as

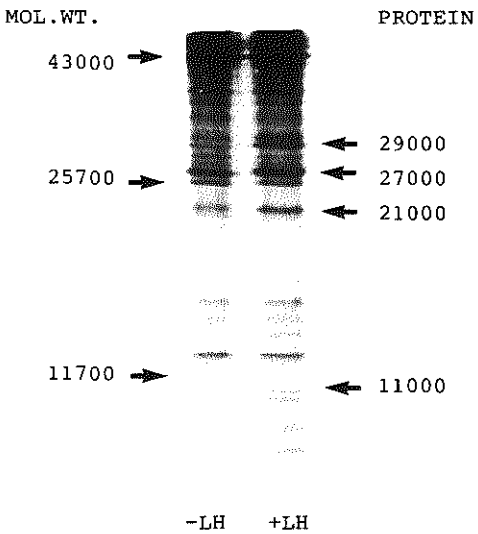

Figure 3 ^{35}S incorporation into proteins of Leydig cells from immature rats, incubated in the presence or absence of added lutropin

Leydig cells from immature rats were incubated with or without lutropin (100 ng/ml) for 4 h followed by incubation with ^{35}S-methionine for 1 h; the proteins were separated on 10-15% discontinuous sodium dodecyl sulphate polyacrylamide gel. Only the 15% part of the gel is shown.

protein 11000, protein 21000, protein 27000 and protein 29000) (Fig. 3). The first increases in ^{35}S incorporation into these proteins could be observed 1-2 h after the addition of lutropin to the cells (Fig. 4). At the start of the incubation period, the synthesis of protein 27000 was high in both control and lutropin treated cells. In the control cells the synthesis of this protein decreased while in the

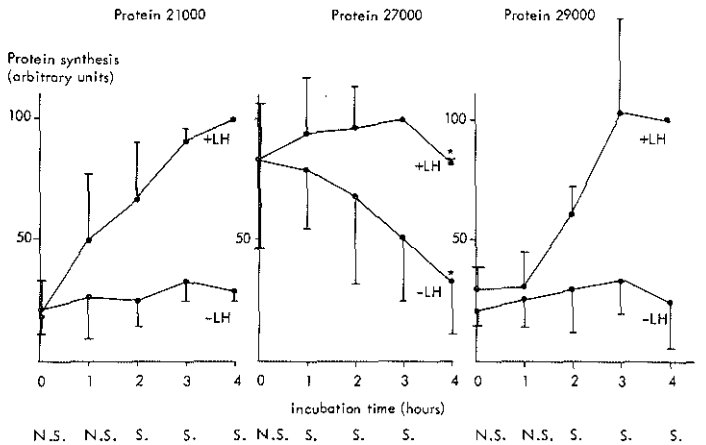

Figure 4 Time course of lutropin stimulation of the synthesis of protein 21000, protein 27000 and protein 29000 in Leydig cells from immature rats

Leydig cells from immature rats were incubated in the absence or presence of lutropin (100 ng/ml) for different time periods followed by incubation with ^{35}S-methionine for 1 h. Specific protein synthesis is expressed as % of specific protein synthesis stimulated with lutropin for 4 h (mean ± s.d., n=3; *mean ± range, n=2). N.S. = no significant difference between stimulated and control specific protein synthesis; S = significant difference between stimulated and control specific protein synthesis

lutropin-treated cells the synthesis of protein 27000 remained at an elevated level. The lowest amount of lutropin that resulted in a significant increase in ^{35}S incorporation was 1 ng/ml for protein 21000 and 10 ng/ml for protein 29000 (Table 2).

Table 2

Effect of different doses of LH on the ^{35}S incorporation into protein 21000, protein 27000 and protein 29000 in Leydig cells of immature rats. Results are expressed as % of ^{35}S incorporation in the proteins in the presence of 100 ng/ml LH. Mean ± s.d. is given with the number of experiments in brackets.

dose of LH (ng/ml)	protein 21000		protein 27000		protein 29000	
	%	effect of LH	%	effect of LH	%	effect of LH
0	24±19 (3)		40±28 (2)		29±4 (3)	
1	38±22 (3)	N.S.	46±21 (2)		49±13 (3)	P<0.025
10	88±37 (3)	P<0.05	75±22 (2)		77±29 (3)	P<0.025
10^2	100 (3)	P<0.025	100 (2)		100 (3)	P<0.005
10^3	99±13 (3)	P<0.025	92±2 (2)		97±8 (3)	P<0.005
10^4	103±3 (2)		102±8 (2)		113±20 (2)	

In the case of 2 experiments the means ± ranges are given.

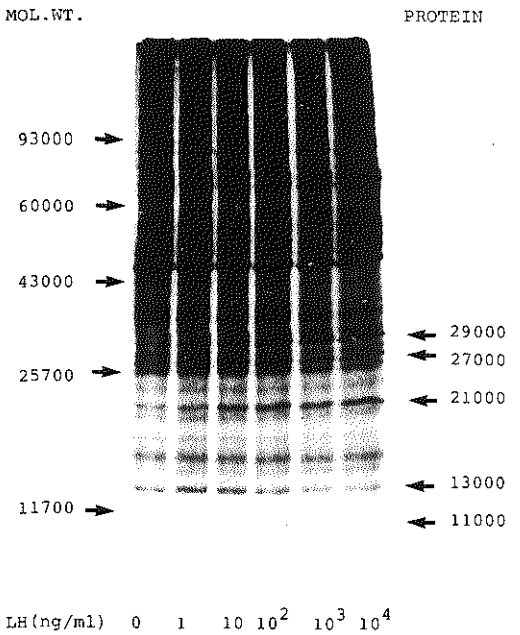

Figure 5 ^{35}S incorporation into proteins of Leydig cells from immature rats, incubated in the presence of different doses of lutropin

Leydig cells were incubated in the presence of different doses of lutropin for 4 h followed by incubation with ^{35}S-methionine for 1 h. Proteins were separated on a 10-20% continuous sodium dodecyl sulphate polyacrylamide gel.

With 10 ng/ml lutropin an increase in ^{35}S incorporation into protein 11000 was always observed (Fig. 5). Decreased ^{35}S incorporation into a protein with an apparent mol. wt. of 13000 was found with 100-10,000 ng/ml lutropin (Fig 5, Table 3). In contrast with this observation there was an increase in ^{35}S incorporation into protein 13000 with a low dose of lutropin (Table 3). No change in specific protein synthesis was obtained when the Leydig cells were incubated in the presence of follitropin (100 ng/ml) or with testosterone (100 ng/10^6 cells) instead of lutropin. Prevention of steroidogenesis by addition of eliptenphosphate (300 µg/ml)

Table 3

Effect of different doses of lutropin on the ^{35}S incorporation into protein 13000 in Leydig cells of immature rats. Results are expressed as % of ^{35}S incorporation in protein 13000 in the presence of 100 ng/ml lutropin (mean ± s.d.).

dose of LH (ng/ml)	%	effect of LH
0	122±10 (3)	
1	143±10 (3)	P < 0.05 ↑
10	145±24 (3)	N.S.
10^2	100	P < 0.025 ↓
10^3	89±23 (3)	P < 0.05 ↓
10^4	61,85+	

+individual results of 2 experiments

to the lutropin-treated Leydig cells did not prevent the lutropin stimulation of ^{35}S incorporation in the 4 proteins. Addition of actinomycin D (46 µM) to the Leydig cells from immature rats at the start of the incubation, prevented the lutropin stimulation of ^{35}S incorporation into all 4 proteins.

Incubation of the cells in the presence of cycloheximide (25 µg/ml) for 30 min after labelling of the proteins with

^{35}S-methionine did not result in a decrease in the amount of incorporated ^{35}S into any of the 4 proteins, indicating that their half lifes are probably longer than 30 min.

DISCUSSION

From the present results it may be concluded that lutropin stimulates the synthesis of proteins with apparent mol. wt.'s of 27000, and 29000 in tumour Leydig cells and proteins with apparent mol. wt.'s of 11000, 21000, 27000 and 29000 in Leydig cells from immature rats. The stimulatory effect of lutropin on the synthesis of these proteins is specific for lutropin and is not due to increased steroid production or to contaminating amounts of follitropin in the lutropin preparation. In the tumour Leydig cells stimulation of specific protein synthesis was obtained with the same amount of lutropin needed for the stimulation of steroidogenesis. The amount of lutropin needed for stimulation of steroidogenesis in Leydig cells from immature rats is approximately the same (van Beurden et al., 1976) as the dose needed for maximal stimulation of specific protein synthesis observed in the present study. From the results obtained in the experiments with cycloheximide it may be concluded that none of the specific proteins have short half lifes in the order of that proposed for the postulated regulator protein, which has been shown to play a role in the stimulation of adrenal steroidogenesis (Garren et al., 1965).

In a previous study with Leydig cells from mature rats it was demonstrated that lutropin stimulates the synthesis of a protein with an apparent mol. wt. of 21000 (lutropin-induced protein; LH-IP) (Janszen et al., 1976b, 1977a). Table 4 summarizes data obtained about the effect of lutropin on the synthesis of specific protein synthesis in Leydig cells from immature and mature rats and in rat tumour Leydig cells. The agreement in mol. wt.'s of lutropin-stimulated proteins in Leydig cells from different sources, may reflect that these proteins are identical, but conclusive evidence can only be

Table 4

Effect of lutropin on the synthesis of specific proteins in Leydig cells from immature rats, mature rats and in tumour Leydig cells.
+ reflects stimulation of the synthesis, 0 reflects no effect of lutropin on the synthesis.

	Protein 11000	Protein 21000	Protein 27000	Protein 29000
Leydig cells from immature rats	+	+	+	+
Leydig cells from mature rats	0	+	0	0
Tumour Leydig cells	0	0	+	+

obtained by further characterization of these proteins. In tumour Leydig cells the synthesis of a protein with an apparent mol. wt. of 21000 was observed. However, contrary to the observations with Leydig cells from immature and mature rats, synthesis of this 21000 mol. wt. protein in tumour Leydig cells was not affected by the presence of lutropin. Only further characterization of this protein may reveal whether or not it is identical to the 21000 mol. wt. protein from normal Leydig cells.

None of the lutropin sensitive proteins detected in this study have mol. wt.'s identical to other proteins synthesized or secreted by other steroid producing tissues. For example the 4 proteins reported by Rubin et al. (1974) and Laychock and Rubin (1974), which are secreted by the cat

adrenal in the presence of corticotropin, have apparent mol. wt.'s of respectively 70000, 58000, 48000 and 12500 on SDS-PAGE. Grower and Bransome (1970) reported that in the supernatant of rat adrenal, corticotropin increased the ^{3}H-leucine incorporation into one protein while at the same time the ^{3}H-leucine incorporation into another protein was decreased. The effects observed by these authors occurred within 30 min after addition of corticotropin and disappeared after approximately 60 min. However, no further characteristics of the 2 corticotropin sensitive proteins have been reported. In the Leydig cells from immature rats with increasing doses of lutropin the synthesis of protein 13000 first increased and then decreased. This decrease may be caused by a posttranslational modification, e.g. splitting off of part of the molecule, so that protein 11000 could be the ultimate product after modification of protein 13000. The difference in effects of lutropin on the stimulation of specific proteins in the Leydig cells obtained from different sources may reflect differences in the lutropin regulatory mechanisms in these Leydig cells. Several other differences between Leydig cells from immature and mature rats have been reported e.g. in steroid metabolism (van Beurden et al., 1976; van der Molen et al., 1975; Sowel et al., 1974; Wiebe, 1976) and in the effect of hypophysectomy on lutropin-stimulated steroid synthesis (van Beurden et al., 1976). Tumour Leydig cells were also different from mature Leydig cells; e.g. in the present study, testosterone only accounted for less than 10% of the total steroid production by the tumour Leydig cells (unpublished results). Such differences between the different Leydig cells make it difficult to extrapolate results, also for the effect of lutropin on specific proteins, from one type of Leydig cell to another.

ACKNOWLEDGEMENTS

We are grateful to the National Institutes of Health, Endocrinology Study Section, Bethesda, Maryland, U.S.A., for gifts of sheep lutropin and follitropin. This work was financially supported in part by the Dutch Foundation for Medical Research (FUNGO).

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