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Specific Protein Synthesis in Isolated Rat Testis Leydig Cells

INFLUENCE OF LUTEINIZING HORMONE AND CYCLOHEXIMIDE

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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 1h. However, after a 2h 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 30min 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 11min. 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 13min) (Cooke *et al.*, 1975). These results indicate that the continuous synthesis of protein with a short half-life equal to or less than 13min 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 1 mm 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×1 mm) 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 60 min with and without added luteinizing hormone (100 ng/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 30 min. 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 10 min), no significant difference between the radioactive patterns of control and

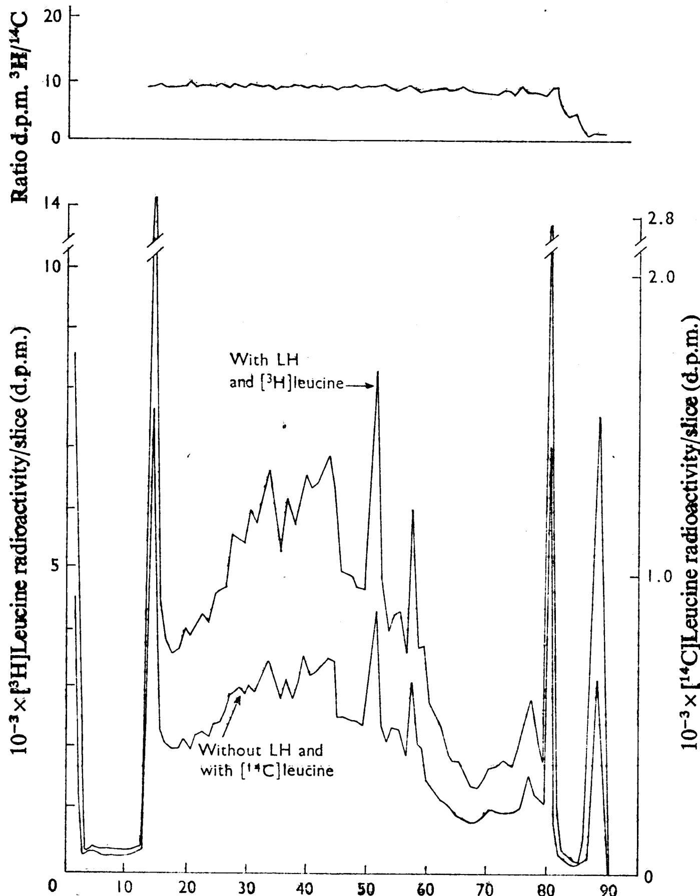


Fig. 1. *Synthesis of Leydig-cell proteins in the absence or presence of luteinizing hormone, and their separation by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis*

Leydig cells were incubated for 60 min with or without added luteinizing hormone (100 ng/ml) followed by incubation with [^{3}H]leucine (100 μCi/ml) and [^{14}C]leucine (10 μCi/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 ($\pm$S.D.) value of the ratio for slices numbered 13–82 was 9.51 $\pm$ 0.41. This ratio did not exceed the mean $\pm$ 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.

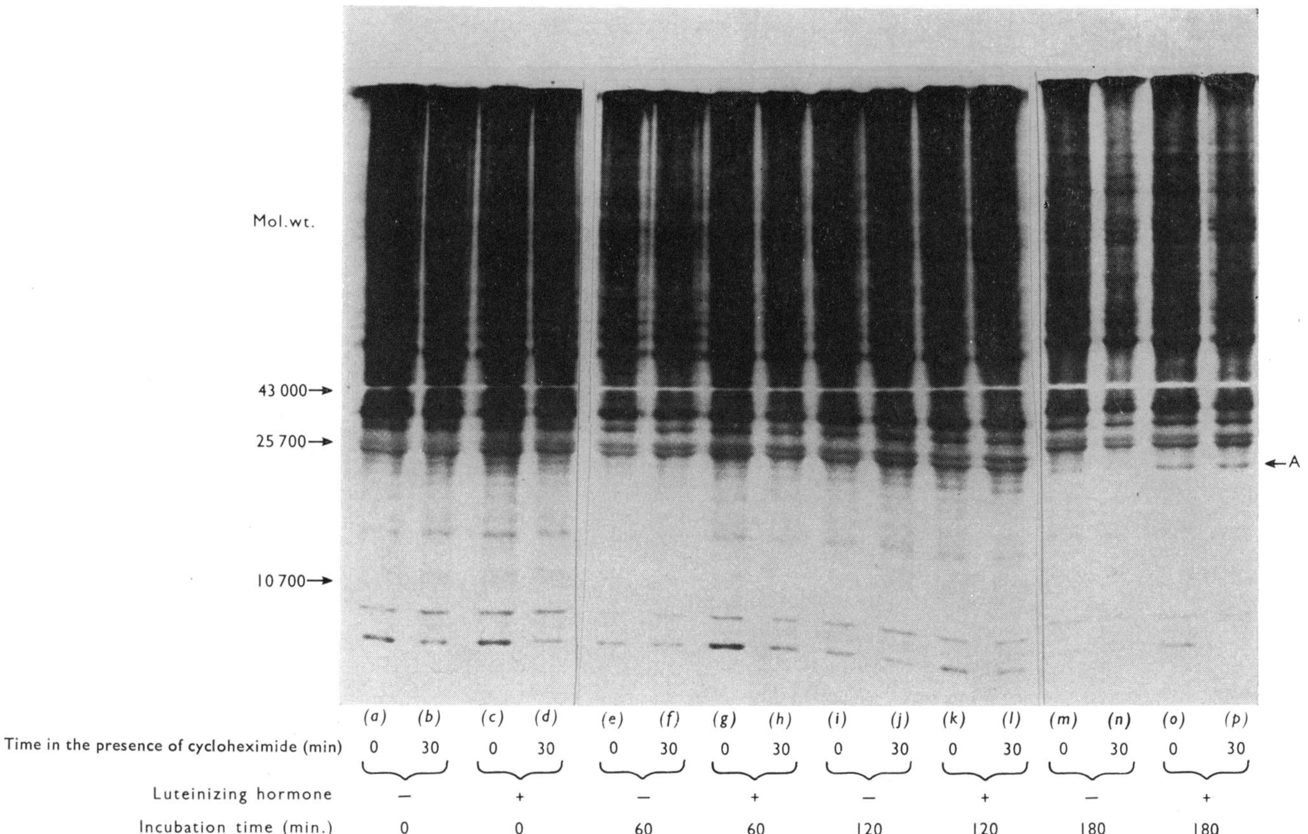
Subcellular localization of protein 33

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).

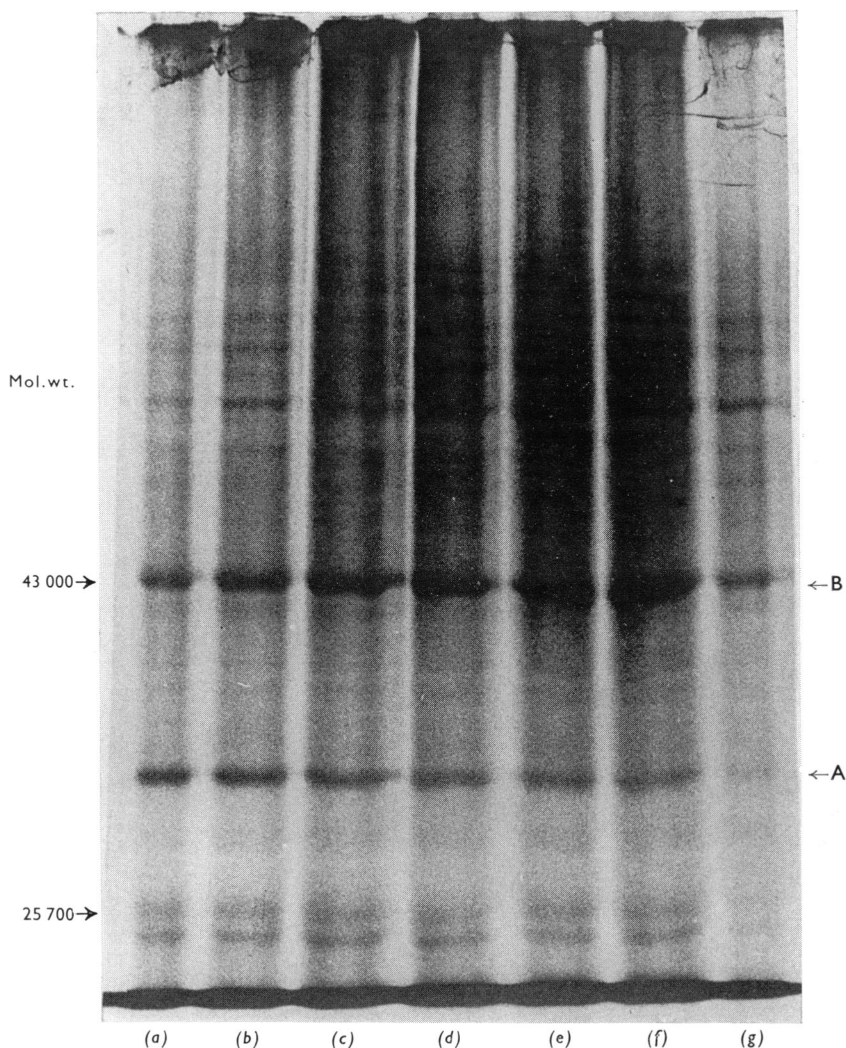
	Relative specific activity			
	Sediment			Supernatant
Markers	10min, 100*g*	10min, 500*g*	10min, 1500*g*	10min, 15000*g*
DNA	3.07	5.30	0.60	0.25
Monoamine oxidase	2.03	2.63	2.70	—
Carboxyl esterase	0.24	0.72	1.16	1.17
Lactate dehydrogenase	0.46	0.46	0.38	1.41
Protein	17%	4%	21%	58%



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 0min (*a*, *b*), 60min (*e*, *f*), 120min (*i*, *j*) and 180min (*m*, *n*) and with luteinizing hormone (100ng/ml) for 0min (*c*, *d*), 60min (*g*, *h*), 120min (*k*, *l*) and 180min (*o*, *p*), followed by incubation for 30min with [^{35}S]methionine (65μCi/ml). Incubations (*b*), (*d*), (*f*), (*h*), (*j*), (*l*), (*n*) and (*p*) were continued for an additional 30min in the presence of added cycloheximide (25μg/ml). The incubations were stopped by addition of 2ml of Krebs–Ringer buffer, pH7.4, at 0°C and cells were sedimented by centrifugation for 10min 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.



EXPLANATION OF PLATE 2

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

Leydig cells were first incubated with luteinizing hormone (100ng/ml) for 30min, followed by incubation with [^{35}S]-methionine for 30min. 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 11.7min was calculated for band A. In a control experiment cells were incubated in the absence of luteinizing hormone, and a relative half-life of 10.9min for band A was calculated.

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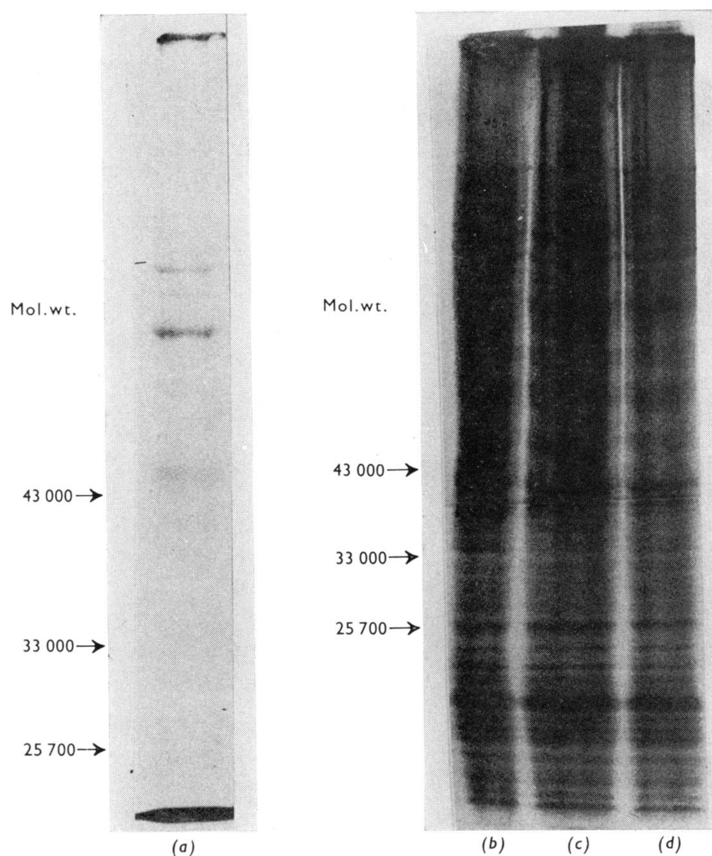
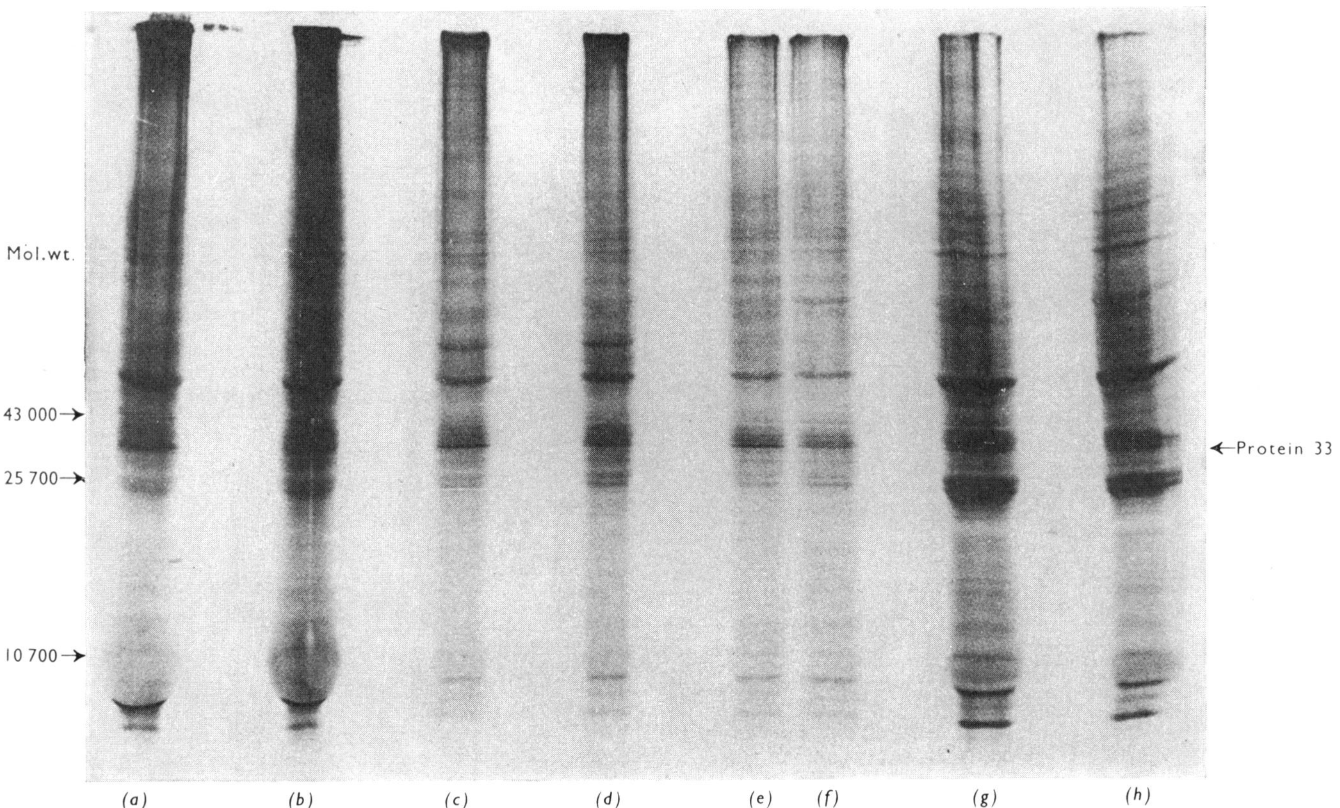


EXPLANATION OF PLATE 3

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 30min, followed by incubation with cycloheximide for 0min (*a*, *c*, *e*, *g*) or 30min (*b*, *d*, *f*, *h*). After incubation, the cells were sedimented and resuspended in 0.25M-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 10min at 100 ***g*** (sediments *a* and *b*), the supernatant was centrifuged further for 10min at 500 ***g*** (sediment *c*, *d*) and this supernatant was centrifuged for 10min 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).

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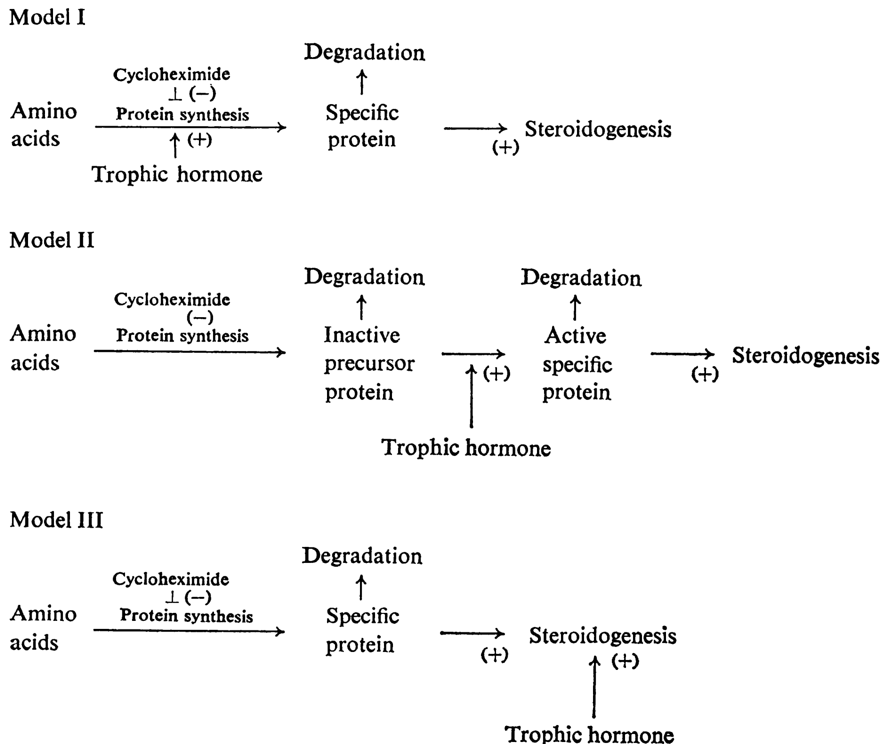
pellet and 10min/15000g pellet, which indicates that the protein is localized in the particulate fraction of the cell (Plate 4). However, a more specific localization was difficult, because in different experiments variable amounts of protein 33 were found in different fractions, probably because of 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 30min 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 (11min) 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.

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References

Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88.
Cooke, B. A. & Van der Kemp, J. W. C. M. (1976) *Biochem. J.* **154**, 371–378
Cooke, B. A., Janszen, F. H. A., Clotscher, W. F. & van der Molen, H. J. (1975) *Biochem. J.* **150**, 413–418
Cooke, B. A., Lindh, M. & Janszen, F. H. A. (1976) *Biochem. J.* **160**, 439–446
Cooke, B. A., Lindh, M. & Janszen, F. H. A. (1977) *FEBS Lett.* in the press
Dufau, M. & Catt, K. (1975) *Methods Enzymol.* **39**, 252–271
Ferguson, J. J. (1963) *J. Biol. Chem.* **238**, 2754–2759
Garren, L. D., Mey, R. L. & Davies, W. W. (1965) *Proc. Natl. Acad. Sci.* **53**, 1443–1450
Hall, P. F. & Eik-Nes, K. B. (1962) *Biochim. Biophys. Acta* **63**, 411–422
Hermier, C., Combarnous, Y. & Jutisz, M. (1971) *Biochim. Biophys. Acta* **244**, 625–633
Janszen, F. H. A., Cooke, B. A., van Driel, M. J. A. & van der Molen, H. J. (1976) *J. Endocrinol.* **70**, 345–359
Laemli, U. K. (1970) *Nature (London)* **227**, 680–685
Lowry, P. J. & McMartin, C. (1974) *Biochem. J.* **142**, 287–294
Mendelson, C., Dufau, M. & Catt, K. (1975) *Biochim. Biophys. Acta* **411**, 222–230
Moyle, W. R. & Ramachandran, J. (1973) *Endocrinology* **93**, 127–134
Moyle, W. R., Moudgal, M. R. & Greep, R. O. (1971) *J. Biol. Chem.* **246**, 4978–4982
Purvis, J. L., Canick, J. A., Rosenbaum, J. H., Hologgitas, J. & Latif, S. A. (1973) *Arch. Biochem. Biophys.* **159**, 32–38
Rommerts, F. F. G., van Doorn, L. G., Galjaard, H., Cooke, B. A. & van der Molen, H. J. (1973) *J. Histochem. Cytochem.* **21**, 572–579
Rommerts, F. F. G., Cooke, B. A. & van der Molen, H. J. (1974) *J. Steroid Biochem.* **5**, 279–285
Rubin, R. P., Jaanus, S. D., Carchman, R. A. & Puig, M. (1973) *Endocrinology* **93**, 575–580
Schulster, D., Tait, S. A. S., Tait, J. F. & Mrotek, J. (1970) *Endocrinology* **86**, 487–502
Schulster, D., Richardson, M. C. & Palfreyman, J. W. (1974) *Mol. Cell. Endocrinol.* **2**, 17–29
Shin, S.-I. (1967) *Endocrinology* **81**, 440–448
Tsafriri, A., Lieberman, M. E., Barnea, A., Bauminger, S. & Lindner, H. S. (1973) *Endocrinology* **93**, 1378–1386
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1964) in *Manometric Techniques and Tissue Metabolism*, 4th edn., p. 132, Burgess Publishing Co., Minneapolis
Younglai, E. V. (1975) *Endocrinology* **96**, 468–474