Effect of administration of luteinizing hormone (LH) and deprival of LH on the proteins of smooth endoplasmic reticulum of immature and adult rat Leydig cells

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Analysis of proteins of smooth endoplasmic reticulum (SER) of Leydig cells from immature and adult rats by two-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) revealed the presence of several new proteins in the adult rats. Administration of human chorionic gonadotropin to immature rats for ten days also resulted in a significant increase as well as the appearance of several new proteins. The general pattern of SDS-PAGE analysis of the SER proteins of Leydig cells resembled that of the adult rat. SDS-PAGE analysis of the SER proteins of Leydig cells from adult rats following deprivation of endogenous luteinizing hormone by administration of antiserum to ovine luteinizing hormone resulted in a pattern which to certain extent resembled that of an immature rat. Western Blot analysis of luteinizing hormone antiserum treated rat Leydig cell proteins revealed a decrease in the 17-α-hydroxylase compared to the control. These results provide biochemical evidence for the suggestion that one of the main functions of luteinizing hormone is the control of biogenesis and/or turnover SER of Leydig cells in the rat.

THE regulation of steroidogenesis in the Leydig cell has been reviewed extensively[1-3] and several of these reviews have dealt with the acute effect of luteinizing hormone (LH) on testosterone production by Leydig cells. However, it is known that LH also exerts trophic effects on Leydig cell growth and differentiation[4,5]. In this connection it is pertinent to note that several studies have demonstrated that the Leydig cell ultrastructure in general, and smooth endoplasmic reticulum in particular, is specifically altered after hypophysectomy or gonadotropin treatment[5-8]. It has been suggested that alteration in the amount or chemical characteristics of Leydig cell cytoplasmic organelle membranes might influence profoundly the activity of the steroidogenic enzymes present in the organelle[9]. In support of such a suggestion are the observations of Ewing and Zirkin[5] who have shown that the main function of LH is to maintain the integrity of the membranes of smooth endoplasmic reticulum (SER), which houses the important enzymes involved in the conversion of pregnenolone to testosterone. In addition, Wing *et al.*[10] have demonstrated that Leydig cell SER and testosterone secretion decreases as much as 80% and 93%, respectively, in rats by 10 days of LH withdrawal. In another study again Wing *et al.*[11] have established that the restoration of Leydig cell SER and testicular capacity to synthesize testosterone from pregnenolone was completed only after 8 days of continuous LH treatment, while there was no temporal association between the recovery of the cholesterol side-chain cleavage activity and the surface area of inner mitochondrial membrane surface area.

Based on these observations, it has been suggested that one of the most striking trophic effects of LH on the Leydig cell is the control of biogenesis and/or turnover of SER. Klinefelter *et al.*[12] have clearly established that following LH withdrawal there is a drastic decrease in the P 450-C-17-hydoxylase-C-17-20 lyase, an enzyme of SER in the Leydig cells. However, it should be noted that the SER is composed of several other proteins which may be directly or indirectly involved in testosterone production in addition to the specific enzymes involved in steroidogenesis. In addition, all the conclusions on the role of LH in regulation of SER of Leydig cells are based either on stereological study of SER or on monitoring the enzymes involved in testosterone production in total microsomes of Leydig cells. However, no studies are available wherein the effects of specific LH deprival on SER proteins of Leydig cells have been examined. The objective of the present study is to examine the role of LH in regulation of SER proteins of the Leydig cells of the rat using model systems, wherein LH effects are minimal. Accordingly, in the present study we have analysed by two-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) the proteins of the SER from Leydig cells of immature 21-day-old rat, which produce minimal

testosterone, and compared it with an adult rat SER. In addition, we have also examined the effect of administration of human chorionic gonadotropin (hCG) to immature rats and neutralization of endogenous LH in adult rats on SER proteins of Leydig cells.

Materials and methods

All routine chemicals used were of analytical grade and were procured locally. Biolyte Ampholites (*pH 3.3–9.3*) and Percoll were purchased from Pharmacia Ltd., Uppsala, Sweden. Collagenase was obtained from Worthing Corporation, Piscataway, New Jersey. Highly purified hCG (CR 123, 12,000 IU/mg) was a gift from NIH Study Section NIAMD, Bethesda, USA.

Production of antiserum to LH

Antiserum to highly purified ovine LH (gift from Dr. M. R. Sairam, Clinical Research Institute of Montreal, Montreal, Canada) was raised in adult rabbits and characterized as described earlier[13]. The antiserum was highly specific and did not show any cross-reactions with FSH as assessed by its ability to bind 125 IFSH (data not shown). The ability of this antiserum to neutralize endogenous LH in the rat has been established by monitoring the decrease in testicular weight and serum testosterone concentration following antiserum treatment for 10 days. The batch of antiserum used in the study was capable of decreasing testicular weight and serum testosterone in adult male rats by 55% and 60%, respectively, when given at a daily dose of 0.1 ml/rat/day for 10 days.

Animals

Immature rats (21 days old and 30–40 g body weight) and adult rats (120 days old, 150 g body weight) were used in the study. These were housed in groups or individually in polypropylene cages under regulated light conditions (14 h light and 10 h darkness). The rats were provided pelleted feed from Lipton India Ltd. and water was available *ad libitum*. hCG (1.3 IU/rat/day) was injected (S.C.) to immature rats from day 10 onwards for 10 days. Antiserum (0.1 ml/day/rat for 10 days) was administered (S.C.) to adult rats and animals treated with normal rabbit serum (NRS) served as controls. Animals were sacrificed 24 h after the last injection, their testicular weights were recorded and their testes processed for isolation of Leydig cells. Blood was collected and processed for serum testosterone by specific radioimmunoassay.

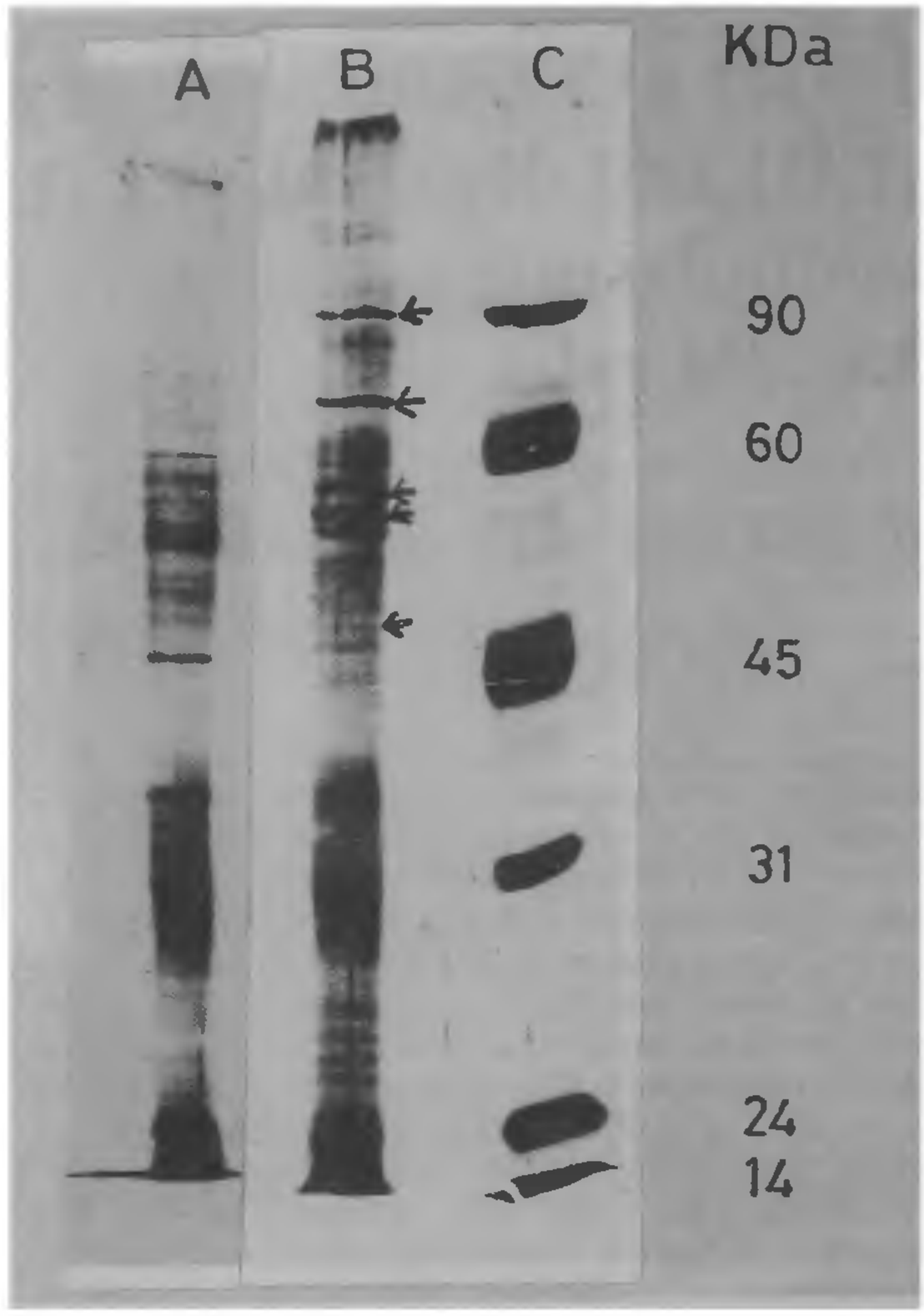


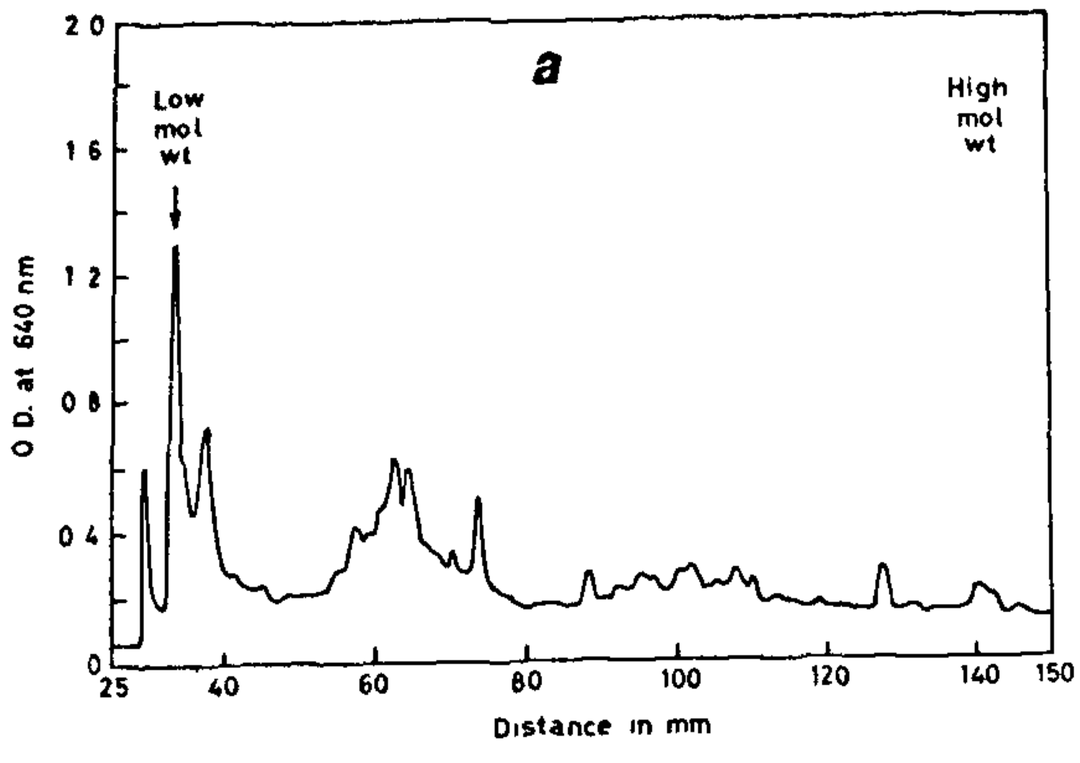
Figure 1. SDS-PAGE analysis of SER proteins of Leydig cells from immature (*a*) and adult rats (*b*) *c*, molecular weight markers: phosphorylase D (90 kD), bovine serum albumin (60 kD), ovalbumin (45 kD), carbonic anhydrase (31 kD), soya bean trypsin inhibitor (24 kD), lysozyme (14 kD).

Isolation of Leydig cells

Interstitial cells from treated or control rats were isolated from the decapsulated testis as described earlier[14]. From the crude interstitial cells, Leydig cells were purified by fractionation of these cells on linear Percoll gradient[15]. Leydig cells from a separate group of treated or control rats were incubated in the presence or absence of hCG to monitor the responsiveness to hCG as judged by increase in testosterone in the medium. Testosterone in the medium was estimated by radioimmunoassay[16] and all samples were analysed in a single assay. The intra- and inter-assay coefficients of variation were, respectively, 11% and 12%.

Isolation of smooth endoplasmic reticulum

Smooth endoplasmic reticulum was isolated[17] from Leydig cells. Leydig cells were homogenized in 0.25 M sucrose using a motor-driven homogenizer with a teflon pestle and the homogenate was centrifuged at 10000 *g*



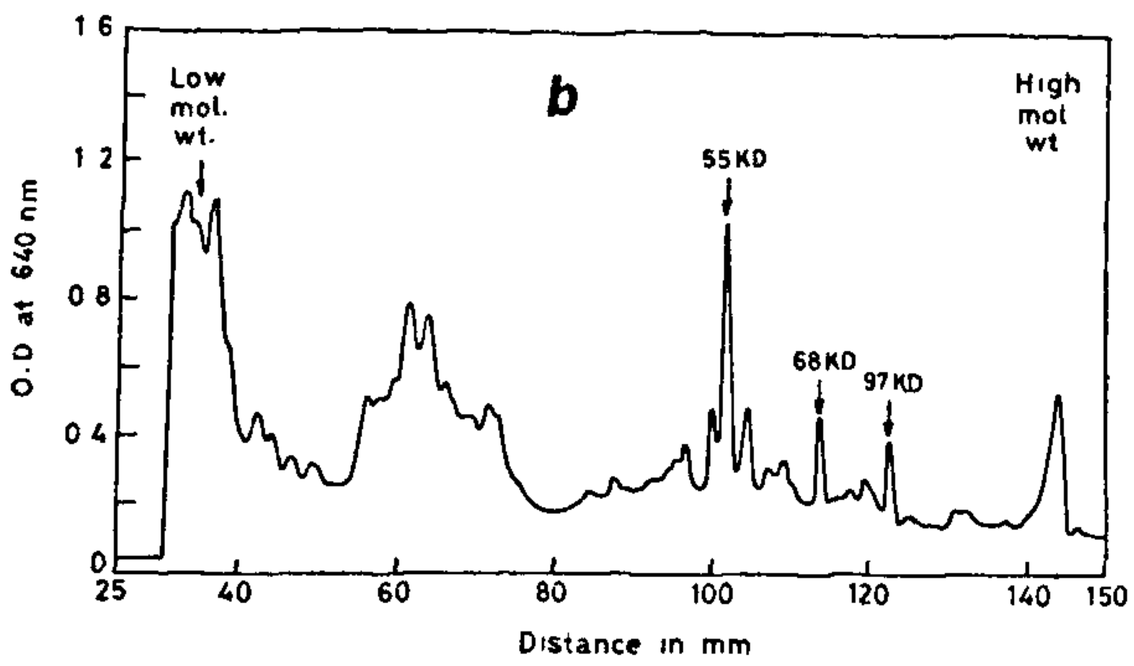


Figure 2*a*, *b*. Laser beam scan of the SDS-PAGE analysis of the SER proteins presented in Figure 1 *a* and *b*, respectively

for 20 min at 4°C in Beckman J2E21 centrifuge. The supernatant was saved and mixed with one molar CSCl to give a final concentration of 15 mM. Separation of rough and smooth microsomes was achieved by layering 4.5 ml of 10,000 × *g* supernatant containing 15 mM CSCl over 2 ml of 1.3 M sucrose containing 15 mM CSCl and centrifuging at 100,000 × *g* for 90 min in a Beckman ultracentrifuge. The clear upper phase was discarded and the entire fluffy layer at the interphase was collected and centrifuged at 100,000 × *g* for 90 min after adding 0.25 M sucrose and the pellet was collected. The purity of the SER was established by monitoring the RNA/protein ratio as well as by determining the specific activity of HMG CoA reductase, which is a marker enzyme for SER.

Electrophoretic analysis of SER proteins

Equivalent quantities (30 µg) of the protein from immature or adult or treated rat Leydig cell SER was initially subjected to SDS polyacrylamide gel (12%) electrophoresis[18] and the gels were scanned in LKB laser beam scanner (Model 12001). The protein was estimated according to the method of Lowry *et al.*[19], using bovine serum albumin as a standard. In addition, the proteins were subjected to isoelectric focusing on a pH gradient of 3.3–9.3 at 400 V for 12 h and at 800 V for 1 h, followed by SDS-PAGE electrophoresis in a second dimension on 12% gels[20]. The proteins were visualized by silver staining[21]. The results presented here are from a representative experiment in which all the procedures, viz. determination of the weight of the testis, serum testosterone, response to hCG, isolation of Leydig cells and analysis of SER proteins, were carried out and each experiment was repeated at least thrice.

Western blot analysis of Leydig cell proteins

In a separate experiment immature male rats were treated with LH antiserum or NRS for 10 days as described earlier and Leydig cells were isolated. The cells were homogenized in 0.1 M phosphate buffer (pH 7.4) and an equal quantity of the protein from each group was analysed by SDS-PAGE[18]. The proteins were transferred to nylon membrane according to the procedure of Towbin *et al.*[22]. The membranes were probed with rabbit antiserum to 17-α-hydroxylase (from Oxygene, Dallas, Texas), followed by treatment with horse radish peroxidase conjugated with anti-rabbit γ-globulin. Peroxidase activity was monitored using ECL kit from Amersham International (UK) and the membrane was processed as described by the supplier.

Results

Effect of neutralization of endogenous LH

As can be expected following neutralization of endogenous LH, there was a significant decrease in testicular weight, serum testosterone levels and response to added LH, indicating the efficacy of the antiserum used. It should also be noted that following the treatment with hCG there was an increase in testicular weight in addition to an increase in the response of the Leydig cells (data not shown).

SDS-PAGE analysis of the SER fraction from immature and adult rats revealed significant difference in the qualitative and quantitative pattern (Figure 1). Prominent among these is the absence of two high-molecular-weight proteins (indicated by arrows) in the immature rat SER. In addition, the intensity of several proteins (indicated by arrows) is also much more in the adult rats. These differences are also evident from the scan of the gels (Figure 2) (arrows indicate the proteins different from immature rat SER). It may be noted that there is a significant decrease in low-molecular-weight proteins in the adult SER and also the appearance of an

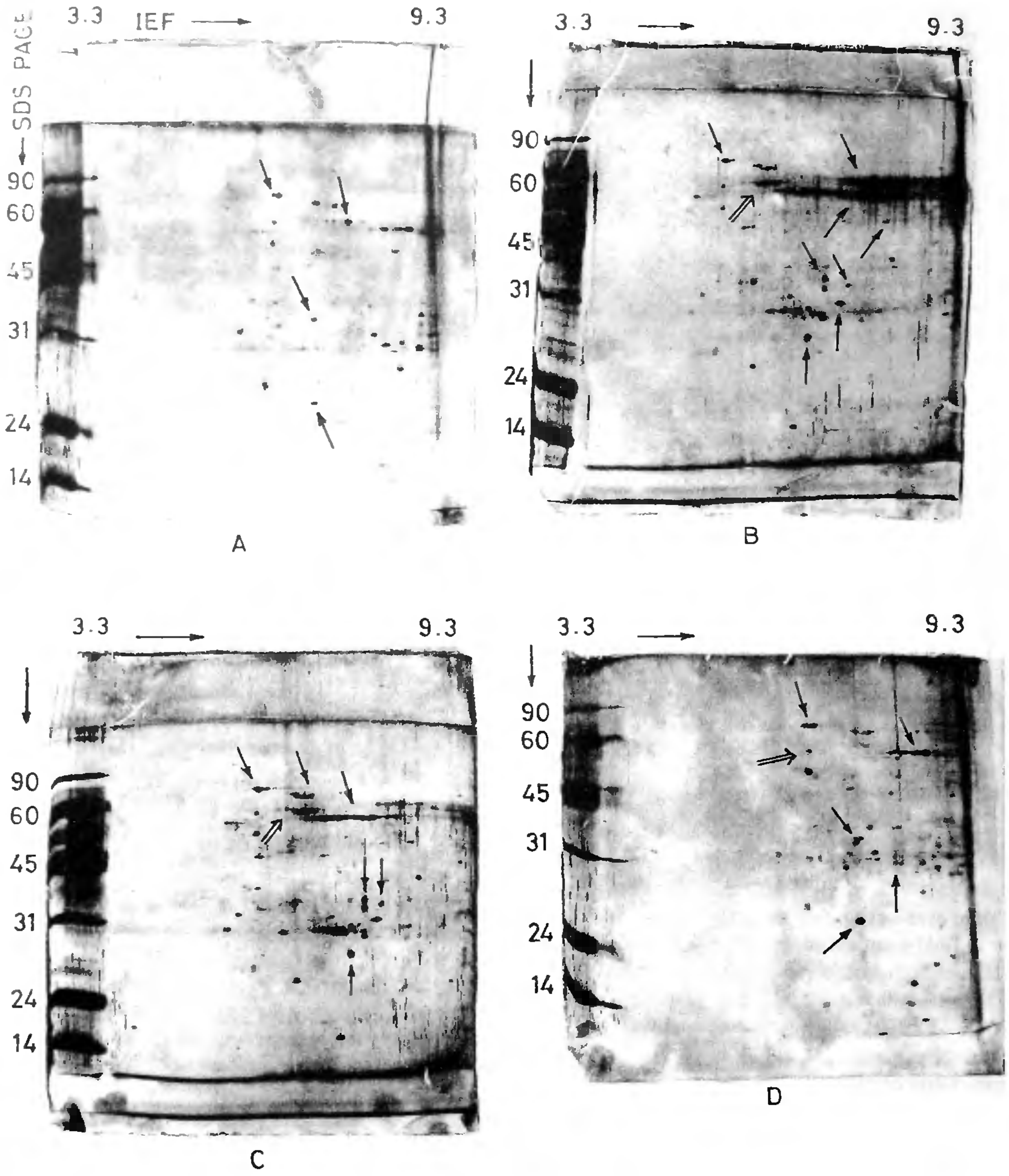


Figure 3. Two-dimensional SDS-PAGE analysis of the SER proteins of immature rat (*a*), hCG-treated immature rat (*b*), normal-rabbit-serum-treated adult rat (*c*) and LH-antiserum-treated adult rat (*d*) Molecular weight markers are as in the case of Figure 1 *c*.

approximately 55 kD protein in the adult rat SER which is totally absent in the immature rat SER.

Two-dimensional SDS-PAGE analysis of SER proteins

The pattern of the total proteins of SER isolated from immature, adult and hCG- and LH-antiserum-treated rat Leydig cells was investigated by two-dimensional polyacrylamide gel electrophoresis. An equal quantity of SER protein was fractionated on separate gels and silver-stained. It can be seen that a majority of the proteins migrate and cluster in the basic region and are in the range of 31 to 97 kD. This is true of normal, immature (Figure 3 *a*) or adult or hCG-treated immature rat (Figure 3 *b*) or normal-rabbit-serum-treated adult rat

(Figure 3*c*) or LH-antiserum-treated (Figure 3*d*) adult rat Leydig cell SER. While the overall pattern of the proteins between immature and adult rat Leydig cell SER appears to be similar, significant differences in the intensity and location of several proteins can be seen. Thus, while only 7–8 proteins can be identified in the mol wt range of 55–90 kD in the immature rat Leydig cell SER, several new proteins are seen in the adult rats with much higher intensity (indicated by arrows). Of special significance is a protein with a mol wt of 55 kD in the adult rat SER (indicated by arrow in Figure 3 *c*). Similarly, several new proteins are seen in the mol wt range of 31–24 kD and the isoelectric pH of most of these proteins is in the range of 6–9 (Figure 3 *b*, *c*). Treatment of immature rats with hCG resulted in a very significant increase in the number and quantity of proteins, particularly in the range of 55–90 kD (Figure 3 *b*). In contrast, the general pattern of proteins of SER following treatment of adult rats with antiserum to LH resembles to some extent that observed in the case of immature rat (Figure 3 *d*). In addition to the decrease in the intensity of several proteins, one prominent effect is the significant decrease in the proteins with a pH range of 6–9 and mol wt of 55–90 kD, which are very prominent in the adult or hCG-treated immature rats.

Western blot analysis

It can be seen from the results presented in Figure 4 that the signal for 17-α-hydroxylase in the antiserum-treated group is very weak compared to the control.

Discussion

Studies by Ewing and Zirkin[5] have clearly established that one of the striking trophic effects of LH on the Leydig cells is the control biogenesis and/or turnover of SER. Studies have also revealed that testes from rats treated with implants filled with steroid, which inhibits LH release, secreted much less testosterone than testes from control rats[23]. Thus, loss in steroidogenic function was correlated closely with a striking diminution in the surface area of smooth endoplasmic reticulum in Leydig cell cytoplasm. It was also demonstrated that restoration of LH release by removal of the steroid-filled implants resulted in the recovery of both Leydig cell SER membranes and testosterone production. Evidence was also provided that these effects were not a result of direct action of testosterone but were mediated via LH action[2]. Based on the studies, it was concluded that inhibition of LH release results in the inhibition of one or several of the SER enzymes involved in the conversion of pregnenolone to testosterone. The results obtained in the present study provide additional evidence to the role of LH in maintaining the integrity of SER as judged by the effect of hCG treatment on SER proteins of Leydig cells from immature rats. In this connection it is pertinent to note that following treatment with hCG there is a very significant increase in the proteins in the mol wt range of 55–90 kD. These proteins may represent specific enzymes involved in conversion of pregnenolone to testosterone or associated factors as well as structural proteins of SER. The results of studies using antiserum to LH also provide additional support for such a conclusion. The pattern of proteins, particularly in the range of 55–90 kD, following antiserum treatment resembles to some extent that of immature rat. It is pertinent to recall that studies of Klinefelter *et al.*[12] using highly purified Leydig cells have shown that depriving rats of LH causes Leydig cells to lose SER and diminish their P 450-C-17-hydroxylase-C-17-20 lyase activity. LH administration to hypophysectomized rats prevents these changes. It was also reported that when Leydig cell microsomal pellet isolated from LH-deprived rats was analysed by SDS-PAGE (12%) and subjected to immuno-blotting using a specific antibody to pig microsomal P 450-C-17-hydroxylase-C-17-20 lyase, there was loss of protein of 52,000 molecular weight, which migrated as a single band[12]. In this connection it is interesting to note that even in our studies one of the important changes we noticed is with reference to a protein of mol wt around 55,000, both in single-dimensional and two-dimensional analysis, which is very prominent in adult rat. It is pertinent to note that the molecular weight of the enzyme P 450-C-17-hydroxylase-C-17-20 lyase has been reported to be around 52,000 (ref. 2). It should be noted that one of the prominent differences in the proteins of SER from adult and immature rat is the appearance of protein around 55 kD, which can be seen as a distinct peak. Although Western blot analysis of a two-dimensional gel would have been most appropriate, due to practical problems such as sensitivity of detection we have subjected only the purified Leydig cell homogenate from LH-antiserum-treated animals to Western blotting. This revealed that there is a significant decrease in the 17-α-hydroxylase, thus providing evidence, though indirect, that the 55 kD protein may be 17-α-hydroxylase. It is

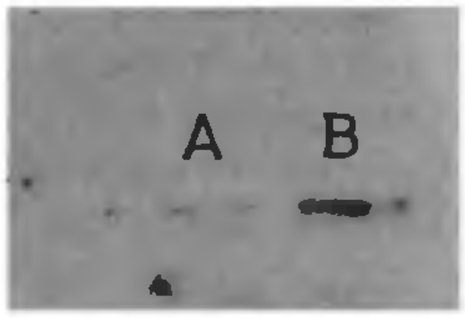


Figure 4. Western blot analysis of Leydig cell proteins from immature rats treated with normal rabbit serum or LH antiserum. 135 µg of proteins was loaded from each group and probed with antiserum of pig testis microsomal 17-α-hydroxylase (Oxygene, Dallas, USA), which was used at a dilution of 1:500. *a*, LH-antiserum-treated; *b*, NRS-treated.

well established that LH exerts both acute and trophic effects on the growth and functional differentiation of Leydig cells. The acute effects of LH include stimulation of synthesis/activity of enzymes involved in steroidogenesis, while trophic effects consist of stimulation of Leydig cell division and maintenance and biogenesis of SER. The results of the present study on the effect of LH deprivation on SER proteins of Leydig cells and on 17-α-hydroxylase activity provide biochemical evidence for such a conclusion; in addition, it may be suggested that the proteins which are found only in the SER of adult and hCG-treated immature rats may be representing some other enzymes or associated factors as well as protein involved in the biogenesis and maintenance of SER in Leydig cells.

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Use of androgens for contraception in men

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Androgens have been used for treating hypogonadism for several years. Experimental evidence suggests that in normal men intramuscular injections of androgens suppress sperm production while maintaining normal libido and accessory reproductive organ functions. The findings strongly support the view that the development of potent orally active androgens could provide an effective antifertility agent for men.

AN effective, safe and reversible antifertility drug for men is yet to be developed. The development of a good chemical contraceptive for men would add a new option for regulating family size and population growth. The mode of control of fertility in men could be through chemicals which can directly affect the testis and disrupt spermatogenesis and sperm formation, or through hormonal interference of testicular spermatogenesis. Though some chemicals like gossypol[1] showed promise in blocking the process of spermatogenesis, due to its adverse side effects, the use of this drug in the control of fertility appears to be remote. Similarly, agents which may cause selective interference with maturation of spermatozoa have not yet been found. Among the hormonal methods for the control of fertility, selective interference with the circulating levels of follicle stimulating hormone by specific antibodies[2] or by blockage of gonadotropic hormones of the pituitary