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EFFECT OF SERUM AND SERUM LIPOPROTEINS ON TESTOSTERONE PRODUCTION BY ADULT RAT LEYDIG CELLS *IN VITRO*

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Summary—The effect of serum factors other than luteinizing hormone on Leydig cell testosterone secretion was examined using an *in vitro* bioassay system based on the stimulation of purified adult rat Leydig cells during a 20 h incubation in the presence of a maximal dose of human chorionic gonadotrophin (hCG). Charcoal-extracted serum and testicular interstitial fluid (IF) from normal adult male rats were separated into lipoprotein and lipoprotein-deficient fractions by density ultracentrifugation. Stimulatory bioactivity was found in the lipoprotein fraction of both serum and IF, although the levels of lipoprotein and corresponding bioactivity recovered from IF were significantly lower (25%) than those of serum. There was no difference between the effects of serum lipoproteins on Leydig cell testosterone production stimulated by either hCG or dibutyryl cAMP. In time-course studies, the serum lipoprotein fraction had no effect on hCG-stimulated testosterone production *in vitro* at 3.0 or 6.0 h, but partially prevented the normal decline in hCG-stimulated testosterone production after 6.0 h. In contrast, unfractionated serum was stimulatory at all time-points. In the absence of hCG, the lipoprotein fraction was stimulatory at both 6.0 and 20 h, although not at 3.0 h. The lipoprotein-deficient protein fraction of serum had no effect on hCG-stimulated testosterone production alone, but significantly enhanced the bioactivity of the lipoprotein fraction, and caused a dose-dependent stimulation of testosterone production in the presence of a constant concentration of serum lipoproteins. Both a stimulatory peak of activity (apparent M_w 40–80 kDa), and a large M_w (> 100 kDa) inhibitor of testosterone production were identified in serum after fractionation by gel filtration (Sephadex G-100). The data indicate that (i) the stimulatory effect of serum on short-term hCG-stimulated Leydig cell testosterone production *in vitro* is predominantly due to the serum lipoprotein fraction, possibly by providing additional precursors for testosterone synthesis, (ii) the biological activity of the lipoproteins is influenced by both stimulatory and inhibitory serum proteins in addition to luteinizing hormone, and (iii) that serum lipoproteins may be involved in supporting Leydig cell steroidogenesis *in vivo*.

INTRODUCTION

Studies on the effect of human, rat and bovine serum on Leydig cells have indicated that serum factors other than luteinizing hormone (LH) influence Leydig cell testosterone production *in vitro* with both stimulatory and inhibitory effects being reported [1–5]. In our previous studies on testicular steroidogenesis-stimulating activity (SSA), charcoal-extracted serum stimulated short-term (< 20 h) testosterone secretion by adult rat Leydig cells, even in the presence of an excess of LH or human chorionic gonadotrophin (hCG) [6]. Although rat serum albumin has recently been shown to increase LH-stimulated steroidogenesis by Leydig cells *in vitro* [7], albumin alone could not account for the serum activity [6]. However, normal rat serum also contains circulating high-density lipoproteins (HDL), as well as low levels of low-density lipoprotein (LDL) [8]. As rat Leydig cells can utilize both HDL and LDL as a source of substrates for testosterone synthesis *in vivo* and *in vitro* [9–11], these observations suggest that the stimulatory effect of serum observed may be attributable to lipoproteins.

Relatively little is known about the actions of serum lipoproteins on short-term steroidogenesis by primary cultures of normal adult rat Leydig cells. It has been reported that HDL and LDL at high doses have only marginal effects on

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purified adult rat Leydig cell testosterone production at 6 h of culture [12], while more recent data indicated significant effects on these cells at 24–72 h [11, 13]. These data suggest that normal rat Leydig cells do not utilize lipoprotein for testosterone production in the short-term, but will do so in longer-term cultures. This apparent change in responsiveness of normal adult rat Leydig cells to serum lipoproteins *in vitro* is consistent with a study on primary cultures of Leydig cells from hypophysectomized adult rats which showed that these cells were unresponsive at 5 h, but had become responsive by 48 h in culture [10]. However, it is in contrast with results obtained using normal adult mouse Leydig cells, the MA-10 Leydig tumour cell line, or Leydig cells from hCG-desensitized adult rats where both short (4–8 hr) and longer-term (>24 h) effects were observed [12, 14–18].

The effects of serum lipoproteins and other serum factors on Leydig cell function have important implications for the measurement of both LH and testicular SSA in serum and testicular fluids in *in vitro* bioassays. Consequently, the short-term effects of serum and the serum lipoprotein fraction on isolated adult rat Leydig cell testosterone secretion were examined in the following study.

EXPERIMENTAL

Animals

Adult (90 to 150-day-old) male Sprague–Dawley rats, provided with GR2 + rat chow (3.0% total lipids; Clark King, Pakenham, Australia) *ad libitum*, were used throughout the study.

Collection of samples and preparation of lipoproteins

Animals were killed by decapitation and serum was collected from trunk blood allowed to clot (4 h, 4°C). Testes were dissected out and perfused via cannulation of the testicular artery with Dulbecco's phosphate-buffered saline, pH 7.4 (DPBS) to remove blood from the testicular vascular bed. Interstitial fluid (IF) was collected (16 h, 4°C) from a small incision in the caudal end of the testicular capsule [2, 19].

Total serum lipoproteins were collected by density ultracentrifugation using standard methods [8, 20]. Samples of testicular IF were diluted with DPBS to provide sufficient volume, as required. Serum and diluted IF samples were adjusted to a density of 1.21 with KBr in ultracentrifuge tubes (6 ml final volume), with a 4 ml overlayer of DPBS at the same density, and centrifuged (105,000 ***g***, 30 h). The resulting total lipoprotein (upper) and lipoprotein-deficient (lower) fractions were collected and dialysed against DPBS.

Serum and IF samples were extracted twice with purified charcoal (10 mg/ml, 4°C, 20 min), unless otherwise noted, to remove free steroids [2, 21]. In some experiments, the serum lipoprotein and lipoprotein-deficient fractions were also charcoal-extracted. All samples were immediately frozen and stored at −20°C prior to assay. Proteins were determined by Coomassie blue protein microassay using bovine serum albumin (BSA) as standard [22].

Gel filtration

Serum was fractionated at 4°C by gel filtration on a Sephadex G-100 (Pharmacia, Uppsala, Sweden) column (1.5 × 96 cm) at a flow rate of 20 ml/h with 10 mM phosphate-buffered saline, pH 7.4 (PBS) as elution buffer. Fractions (3.7 ml) were collected and stored at 4°C prior to bioassay.

Leydig cell bioassay

The bioassay system has been described in detail, previously [6]. Briefly, Percoll gradient-purified adult rat Leydig cells were incubated (3.0–20 h, 32°C, 5% CO_2 in oxygen) with assay samples (150 μl diluted in PBS and 0.1% BSA) in the presence or absence of a maximal dose of hCG (10IU/ml) at a final assay volume of 275 μl. Testosterone secretion into the medium was measured by [^{125}I]testosterone radioimmunoassay [23]. It has previously been established that oxygen tension (1–95%) has no effect on testosterone production by these cells during this incubation period [6]. As changes in [Ca^{2+}] can affect receptor-binding of some lipoproteins [24], calcium levels in medium and samples were adjusted in all experiments in order for the final

Table 1. Effect of calcium ion concentration on hCG-stimulated testosterone production by adult rat Leydig cells over 20 h in the presence (+) or absence (−) of serum lipoproteins (0.65 mg/ml) *in vitro*

[Ca^{2+}] (mM)	Lipoprotein	Testosterone production (ng/10^6 cells/20 h)
2.2	+	1697 ± 281^a
1.8	+	1608 ± 67^a
1.5	+	$1441 \pm 231^{a,b}$
1.4	+	$1538 \pm 95^{a,b}$
1.3	+	1311 ± 207^b
1.2	+	1316 ± 80^b
1.2	−	971 ± 57^c

Values are mean ± SD, $n = 3$ replicates. Values with the same superscript are not significantly different ($P > 0.05$).

assay [Ca^{2+}] to fall between 1.4–2.2 mM (see Table 1).

At the end of the culture period, cells were examined by phase-contrast microscopy using a Leitz inverted microscope with phase-contrast optics. The following criteria were employed as evidence of sample toxicity: (i) detachment of Leydig cells from the culture plate, (ii) cytoplasmic granulation and loss of refractile appearance, and (iii) cell fragmentation. These criteria correlate well with estimates of loss of cell viability as determined by NADH exclusion/diaphorase histochemistry [25, 26].

Comparisons between individual response values were made by paired t test or by ANOVA and Peritz's multiple range test [27]. Relative bioactivity estimates and % bioactivity recoveries were obtained from comparisons of the sample log-linear transformed dose–response lines between 150 and 300% of control (hCG-stimulated) testosterone production, using standard parallel-line bioassay statistics when dose–response lines were parallel [28], and at the arbitrary level of 225% of control when the dose–response lines were non-parallel, as described previously [6].

RESULTS

Separation of serum lipoproteins

After fractionation, 1.26 ± 0.21 mg protein/ml serum (mean ± SD, $n = 5$ pooled serum samples) was obtained in the lipoprotein fraction of rat serum (71.8 ± 2.2 mg total protein/ml). The recovery of protein in the lipoprotein fraction of IF from DPBS-perfused testes of 5 individual rats was 0.32 ± 0.03 mg/ml (mean ± SD), indicating an IF/serum total lipoprotein concentration ratio of 0.25.

Effects of serum lipoproteins on testosterone production

The serum lipoprotein fraction stimulated a dose-dependent increase in maximal hCG-stimulated testosterone production over 20 h

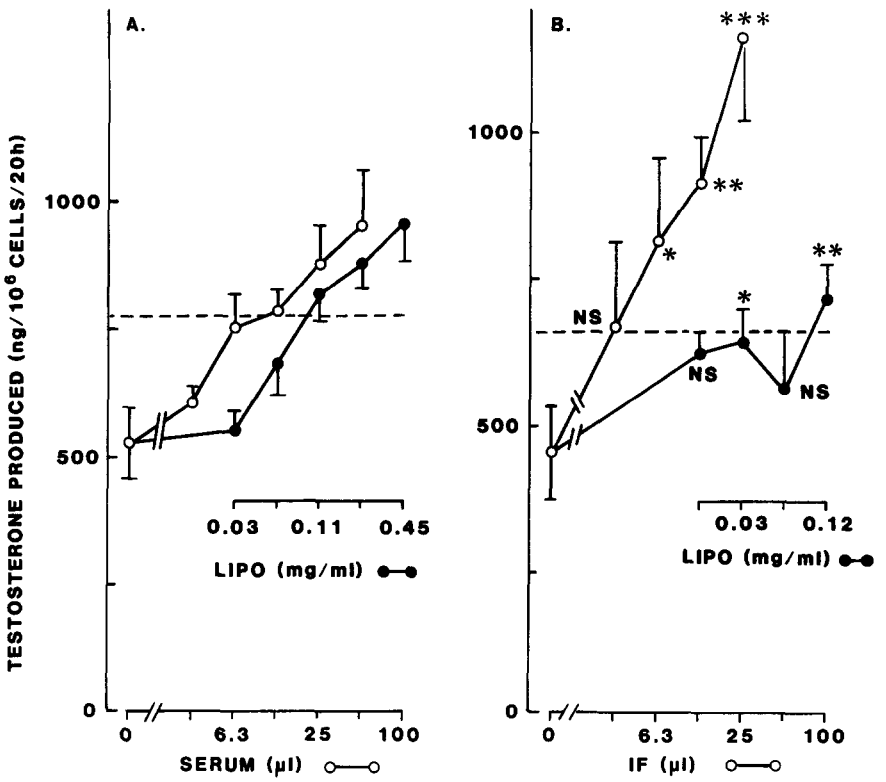

Fig. 1. Maximal hCG-stimulated testosterone production by adult rat Leydig cells over 20 h *in vitro*, in the presence of (A) rat serum (○—○) and the serum lipoprotein fraction (LIPO; ●—●), and (B) rat testicular IF (○—○) and the IF lipoprotein fraction (LIPO; ●—●). Dose axis scales for unfractionated fluids (serum and IF) and the corresponding lipoprotein fractions are directly comparable, based on the calculated protein recoveries after density ultracentrifugation. (– – –) Lower limit of bioassay response range (150% of hCG-stimulated testosterone response). All values are mean ± SD, $n = 3$ replicates in a single assay. Comparisons are with the control (hCG-stimulated only) response values. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ^{NS}not significantly different ($P > 0.05$).

Table 2. Effects of lipoprotein on hCG- and dibutyryl cAMP-stimulated testosterone production by adult rat Leydig cells over 20 h *in vitro*

	Testosterone production (ng/10^6 cells/20 h)	
	No lipoprotein	+ Lipoprotein (0.65 mg/ml)
hCG-stimulated	681 ± 15	1554 ± 241
Dibutyryl cAMP-stimulated	640 ± 60^{NS}	1714 ± 114^{NS}

Values are mean ± SD, $n = 3$ replicates. Comparisons are between the hCG- and dibutyryl cAMP-stimulated values obtained in the absence or presence of lipoprotein. ^{NS}Not significantly different ($P > 0.05$).

in vitro, similar to that observed with unfractionated serum, although the dose–response lines were not consistently parallel [Fig. 1(A)]. Bioactivity recovered in the lipoprotein fractions was 41.3 ± 11.8% compared with the unfractionated serum pools (mean ± SEM, $n = 3$ experiments). The minimum detectable dose of serum lipoproteins was 0.03 ± 0.02 mg lipoprotein/ml (mean ± SEM, $n = 5$ experiments), defined as the estimated lipoprotein dose required to stimulate testosterone production equivalent to the mean + 2SD of the control (hCG-stimulated alone) testosterone level in each experiment.

Changes in [Ca^{2+}] had no significant effect on the stimulatory effect of serum lipoproteins over the range of calcium concentrations employed in this study, although there was a significant decline in activity at [Ca^{2+}] below 1.4 mM (Table 1). There was no difference between the stimulatory effect of the serum lipoprotein fraction on testosterone production in the presence of either an excess of hCG or a comparable dose of dibutyryl cAMP (Table 2). There was no loss of bioactivity in the isolated serum lipoprotein fractions after charcoal extraction (102 ± 21% recovery of activity; mean ± SEM, $n = 5$ experiments).

The lipoprotein fraction of pooled testicular IF displayed minimum detectable doses of 0.02 and 0.01 mg lipoprotein/ml in two separate experiments [Fig. 1(B)], similar to the minimum detectable doses of the serum lipoprotein fractions. However, the lipoprotein fractions collected from IF were considerably less active than the serum lipoprotein fractions and could account for <5% of the total IF bioactivity [Fig. 1(B)], reflecting the lower concentration of lipoprotein in IF compared with serum.

Time-course studies

Serum lipoprotein at a dose (0.65 mg/ml lipoprotein) that was 20-times the minimum dose required to cause a significant increase in hCG-stimulated testosterone production at an incubation time of 20 h, had no effect on hCG-stimulated testosterone production at 3.0 or 6.0 h [Fig. 2(A)]. Consequently, the hCG-stimulated testosterone production rate between 6.0 and 20 h fell by 81.9 ± 11.2% (mean ± SEM, $n = 3$ experiments) of the initial (0–6.0 h) rate in the absence of lipoproteins (92.4 ± 4.7 down to 16.5 ± 10.1 ng/10^6 cells/h), but by only 31.6 ± 14.1% of the initial rate if serum lipoproteins (0.65 mg/ml) were present (104 ± 8.3 down to 62.4 ± 9.8 ng/10^6 cells/h). Lipoproteins had no effect on basal testosterone production over 3.0 h, but a significant effect was detectable at both 6.0 and 20 h [Fig. 2(B)]. The lack of effect of lipoprotein at 3.0 and 6.0 h was in contrast to the effect

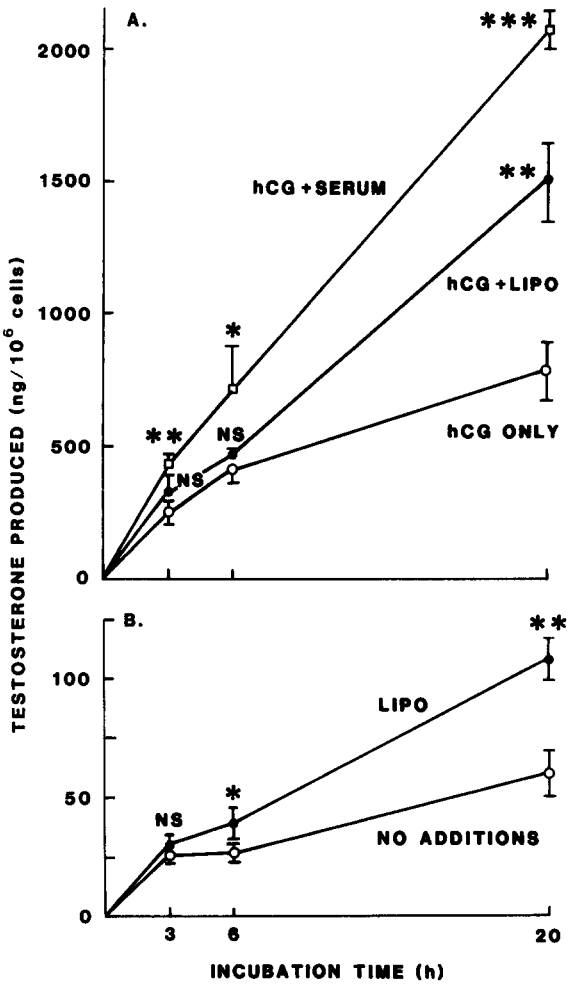

Fig. 2. Time-course of maximal hCG-stimulated testosterone production (A) and basal testosterone production (B) by adult rat Leydig cells *in vitro*, alone (○—○) and in the presence of either rat serum (150 μl: □—□) or the serum lipoprotein fraction (LIPO, 0.65 mg/ml; ●—●). All values are mean ± SD, $n = 3$ replicates in a single assay. Comparisons are with the respective testosterone response values in the absence of either serum or lipoprotein at each incubation time-point. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ^{NS}not significantly different ($P > 0.05$).

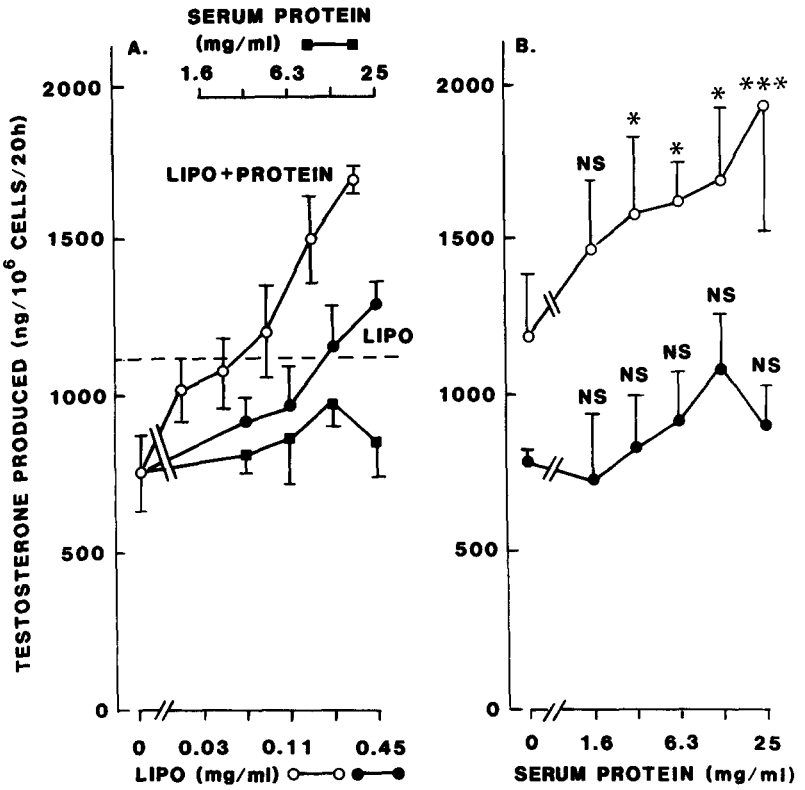

Fig. 3. Maximal hCG-stimulated testosterone production by adult rat Leydig cells over 20 h *in vitro*, in the presence of the lipoprotein-deficient rat serum protein fraction (PROTEIN) and serum lipoprotein fraction (LIPO). (A)Serum lipoprotein fraction (●—●) alone, serum protein fraction (■—■) alone, and re-combined serum lipoprotein and protein fractions (○—○). Dose axis scales are directly comparable, based on the calculated lipoprotein and protein recoveries after fractionation of serum by density ultracentrifugation. (B)Serum protein fraction in the presence (○—○) and absence (●—●) of a constant concentration of serum lipoproteins (0.075 mg/ml). (---) Lower limit of bioassay response range (150% of hCG-stimulated testosterone response). All values are mean ± SD, $n = 3$ replicates in a single assay. Comparisons are with the respective hCG-stimulated (± lipoprotein) control response values. $^{***}P < 0.001$; $^{*}P < 0.05$; ^{NS}not significantly different ($P > 0.05$).

of the comparable dose of serum (150 μl/well), which increased hCG-stimulated testosterone production at all three time points [Fig. 2(A)].

Effects of serum proteins on testosterone production

The lipoprotein-deficient protein fraction of serum had no significant effect on hCG-stimulated testosterone production, although an apparent upward trend, followed by a decline at the highest concentration, was consistently observed [Fig. 3(A and B)]. However, restoring the lipoprotein-deficient fraction to the serum lipoprotein fraction caused a significant increase (384 ± 62%; mean ± SEM, $n = 3$ experiments) in the bioactivity of the serum lipoprotein fraction [Fig. 3(A)]. Moreover, the lipoprotein-deficient serum protein fraction did cause a dose-dependent increase in hCG-stimulated testosterone production in the presence of a constant amount of serum lipoproteins [Fig. 3(B)].

These data suggested that there was a synergistic interaction between the serum lipoproteins and serum protein fraction. Following fractionation of serum by gel filtration on Sephadex G-100, stimulatory activity was found in fractions corresponding to the elution position of serum albumin (apparent M_w 40–80 kDa, Peak B), while fractions in the void volume (apparent M_w >100 kDa, Peak A) were inhibitory, when assayed in the presence of a constant amount of hCG and lipoprotein/assay well (Fig. 4). An additional minor peak was also observed in the total volume (V_t) fractions of the gel filtration profile, accounting for 28 ± 19% ($n = 3$ profiles) of the total stimulatory activity recovered after gel filtration. Similar gel filtration bioactivity profiles were obtained for charcoal-extracted serum, and serum which had not been charcoal-extracted prior to fractionation (data not shown).

The Peak A fractions were able to suppress Leydig cell testosterone production, in the

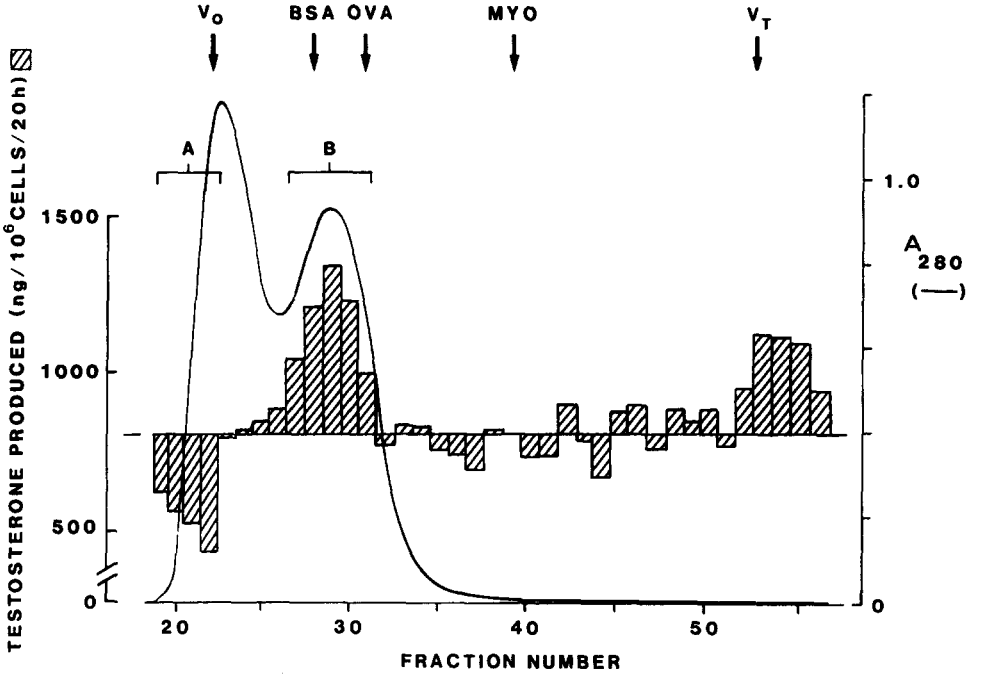

Fig. 4. Maximal hCG-stimulated testosterone production by adult rat Leydig cells over 20 h in the presence of a constant amount of lipoprotein/assay well (0.075 mg/ml) *in vitro*, and gel filtration (Sephadex G-100) fractions of rat serum. Values are testosterone production (■) above or below the control (hCG + lipoprotein-stimulated) level (solid horizontal line). Two major peaks of activity (A and B) are indicated. V_o, void volume; BSA, bovine serum albumin (67 kDa); OVA, ovalbumin (43 kDa); MYO, myoglobin (17 kDa); V_T, total volume.

presence of both hCG and lipoprotein, below the maximal hCG-stimulated level [Fig. 5(A)]. There was no evidence of cell toxicity in wells containing peak A fractions. Peak B stimulated hCG-stimulated testosterone production in both the presence and absence of a constant amount of lipoprotein/assay well, but the stimulatory activity of peak B was significantly enhanced in the presence of both hCG and lipoprotein, when compared with hCG alone [Fig. 5(B)].

DISCUSSION

The data in this study indicated that the stimulatory effect of charcoal-extracted serum on short-term LH/hCG-stimulated testosterone production by isolated adult rat Leydig cells *in vitro* was principally due to serum lipoproteins. However, there were at least two additional large molecular weight serum factors which influenced testosterone production *in vitro*, a stimulatory activity (40–80 kDa), and an inhibitor (> 100 kDa), and the overall activity of serum appeared to be due to a complex interaction between several serum species. Testicular IF also contained measureable bioactive lipoprotein activity, but these levels were approx. 4-times lower than those present in serum, indicating that the concentration of lipoprotein in testicular IF are comparable to those in IFs from other tissues [29]. Although lipoproteins cannot account for the majority of steroidogenesis-stimulating activity in testicular IF [2, 6], these data indicate that they have access to the testicular interstitial space which is a pre-requisite for their playing a role in the maintenance of Leydig cell steroidogenesis *in vivo*.

The importance of lipoprotein-derived cholesterol as a substrate for testosterone synthesis by normal Leydig cells remains to be established. In adult rats fed variable cholesterol diets containing radiolabelled cholesterol, Morris and Chaikoff [30] found that the majority of the cholesterol present in the testis *in vivo* was due to endogenous synthesis (70–90%). Moreover, in rats depleted of lipoproteins by treatment with 4-aminopyrazolo[3,4-*d*]pyrimidine (4-APP), there was no change in testis cholesterol levels, and sterol synthesis was unaffected [9]. Although serum testosterone levels fell in 4-APP-treated rats, this may have been a secondary effect due to reduced serum LH levels as a result of inhibition of pituitary release by 4-APP [9, 31].

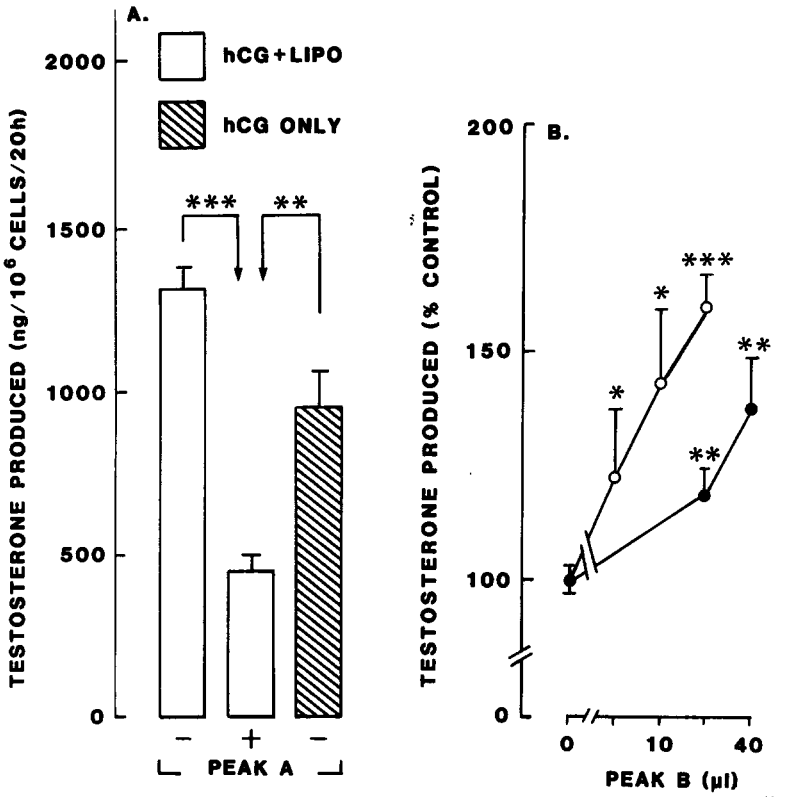

Fig. 5. Effect of rat serum gel filtration (Sephadex G-100) peaks A and B fractions on maximal hCG-stimulated testosterone production over 20 h by adult rat Leydig cells *in vitro*. (A) hCG alone (■); hCG with serum lipoproteins (LIPO; 0.075 mg/ml) alone, and in the presence of a G-100 Peak A fraction pool (67 µl/assay well) (□). (B) G-100 Peak B fraction pool in the presence (○—○) and absence (●—●) of a constant concentration of serum lipoproteins (0.075 mg/ml). Note that testosterone production is expressed relative to the respective control (hCG-stimulated ± lipoprotein) response values, and comparisons are also with the respective control response values. G-100 peak A and peak B doses are expressed as the equivalent volume of serum (µl) in the fraction pools, based on the calculated recoveries after gel filtration. All values are mean ± SD, $n = 3$ replicates in a single assay. ***$P < 0.001$; **$P < 0.001$; *$P < 0.05$.

However, Morris and Chaikoff [30] did report a direct relationship between the dietary cholesterol content and the proportion of plasma-derived cholesterol in the testis, while infusion of HDL into 4-APP-treated rats stimulated testis cholesterol levels and inhibited sterol synthesis [9], providing *in vivo* evidence for a contribution of lipoprotein-derived cholesterol to the intratesticular cholesterol pool. The numerous *in vitro* studies on steroid production by murine tumour Leydig cells, and by primary Leydig cell cultures from normal, cholesterol-depleted and LH/hCG-treated rats or mice, suggest that lipoproteins provide a minor proportion of cholesterol for testicular steroid synthesis under normal conditions, but this source becomes more important when endogenous cholesterol stores are low, or *de novo* synthesis is inadequate [12, 14, 16–18, 31].

Consequently, the observations of the present study indicated that serum lipoproteins have no effect on hCG-stimulated testosterone production by rat Leydig cells in short term culture (0–6 h), presumably when endogenous cholesterol stores and *de novo* synthesis were adequate, but caused a significant stimulation during later culture periods (6–20 h), possibly as cellular cholesterol sources became depleted. As serum lipoproteins also enhanced testosterone production even under non-stimulated conditions, lipoproteins may also play a direct role in stimulating Leydig cell steroidogenesis, and the delay in the effect of lipoproteins observed may have been due to the time required for lipoprotein uptake and metabolism to occur [10, 15]. In either case, under maximal hCG-stimulated conditions, the net effect of serum lipoproteins was to partially maintain testosterone production after 6.0 h, suggesting that the reduced sensitivity of normal adult rat Leydig cells to stimulation by LH/hCG or cAMP analogues between 6.0 and 20 h *in vitro* was largely due to steroid

precursor limitation. It is well known that treatment of Leydig cells with LH/hCG or cAMP analogues causes desensitization and down-regulation of the Leydig cell due to LH receptor loss, adenylate cyclase dissociation, and down-regulation and oxygen-mediated damage of the cytochrome *P*-450 enzymes, cholesterol side-chain cleavage and 17α-hydroxylase/C_{17-20} lyase [32–34]. The data in the present study supports previous reports that substrate limitation is also an important factor in Leydig cell down-regulation [12, 16, 17, 31], at least in the short term, and this is probably the major factor responsible for decreasing hCG-stimulated testosterone production with time in our culture system.

In addition to the lipoprotein fraction activity, interactions between serum lipoproteins and proteins in serum, particularly a large molecular weight stimulator and an inhibitor, were observed. Gel filtration fractionation of serum resolved a peak of stimulatory activity (40–80 kDa), corresponding to the elution position of serum albumin, which previously has been shown to stimulate Leydig cell testosterone production *in vitro*, even after charcoal-extraction to remove bound fatty-acids and sterols [6, 7]. While the identity of the serum factor with serum albumin will require confirmation by purification, the serum factor displayed a synergistic interaction with the lipoprotein fraction, suggesting either (i) that it enhances the utilization of lipoproteins, or (ii) that it requires additional substrate support to maintain an effect. In addition to the large molecular weight activity, a small, but significant, peak of stimulatory activity in the column total volume was observed. Since this small molecular weight peak was observed in gel filtration profiles of both charcoal-treated and non-treated serum samples, it may have been due to dissociation of minor amounts of free fatty acids and other steroid precursor molecules from the lipoprotein fraction during serum storage and/or fractionation.

The large molecular weight serum inhibitor suppressed Leydig cell testosterone production *in vitro* below hCG-stimulated levels, indicating that this activity was not simply interfering with lipoprotein uptake. There have been several recent reports of protein inhibitors of LH action in serum, but these proteins have yet to be identified and their biological significance assessed [4, 5]. However, it was interesting to note that the gel filtration void volume fractions of serum containing both the inhibitor and serum lipoproteins, but not smaller molecular weight serum components, were inhibitory overall, further reinforcing the complex nature of the interaction between the various serum factors involved in the total activity of serum on testosterone production *in vitro*.

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