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Effects of differentiation state and post-castration time lapse on the epididymal response of the lizard to testosterone *in vitro:* changes in specific protein and mRNA levels

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Summary

The epididymis of the viviparous lizard secretes large amounts of proteins among which L-proteins are prominent components. It undergoes great morphological and physiological modifications during its testosterone-controlled annual cycle. The effects of testosterone on L-proteins synthesis and L-mRNA concentrations were studied in cultures of organs regressed after castration. Of three tested serum supplements (2% Ultroser, 10% fetal calf serum, 10% calf serum) calf serum was shown to be essential for androgen-specific control of L-proteins synthesis. The duration of castration governed the in-vitro response to testosterone principally at the level of L-proteins synthesis. The onset of synthesis was delayed in 2-month post-castration explants, compared with 1-month post-castration explants, and was dissociated from appearance of the mRNA. This suggests that there is translational control of secretory proteins in the regressed epididymis. Conversely, the response to testosterone at the mRNA level was delayed in explants from animals castrated during a non-secretory state, compared with explants from animals castrated at the onset of secretion. These results, together with other data, suggest that expression of the L-proteins is under multifactorial control and that the influence of multiple controlling elements varies with the stage of differentiation.

Keywords: testosterone, castration, epididymis, specific protein, androgen-dependent mRNAs.

Introduction

The accessory organs of the male genital tract are known to be controlled by androgenic hormones (Price & Williams-Ashman, 1961). However, the mechanisms involved in the response to the withdrawal and the restoration of androgens are still not well understood. The epididymis is one of the most important of these organs because spermatozoa mature there (Bedford, 1975; Hamilton, 1975; Depeiges & Dacheux, 1985). This maturation could occur in part through specific proteins and glycoproteins secreted by epithelial cells under the influence of testosterone (Cameo & Blaquier, 1976; Lea, Petrusz & French, 1978; Orgebin-Crist & Jahad,

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1978; Brooks & Higgins, 1980; Jones, Van Glos & Brown, 1981). These specific proteins have been used to characterize the effects of testosterone on the epididymis *in vitro* (Orgebin-Crist & Menezo, 1980; Klinefelter & Hamilton, 1984).

The viviparous lizard has a seasonal reproductive cycle with important changes in the morphology and function of the epididymis. After post-mating degeneration of the epididymis, the epithelium is first reconstituted by non-secretory small cubic cells (stage 1). After several divisions, these cells get larger and begin to secrete (stage 3) before they become hypersecretory in stage 6. This progression is correlated with tissue testosterone levels (Dufaure *et al.*, 1986). During the mating period (stage 6), the epithelial cells hypertrophy and secrete about fifteen proteins that mix with spermatozoa (Depeiges, Force & Dufaure, 1987). A major protein has been identified, the L-protein (MW 19000), which attaches to the head of the sperm (Depeiges & Dufaure, 1983). This protein was described recently to consist of a group of at least 10 antigenically related proteins (Depeiges, Morel & Dufaure, 1988). Two different cDNA clones (Lv123 and Lv132) were found to code for these related proteins (Courty, Morel & Dufaure, 1986; and unpublished data) and were used in the present work.

The effects of castration in morphology, L-proteins synthesis and L-mRNA concentration depends on the time interval following castration and on the state of epididymal differentiation at castration (Morel *et al.*, 1986). In this report, using an organotypic culture system (Dufaure & Gigon, 1975; Gigon, 1975), we show that these same parameters influence the restoration of atrophied organs by testosterone treatment *in vitro*.

Materials and methods

Animals

Adult males of the viviparous lizard, (*Lacerta vivipara* Jacquin), were collected in the Massif Central (France) during two different periods of the sexual cycle (stages 1 and 3). Castration was performed via the abdominal route under ether anaesthesia.

Organ culture

One, two and four months after surgery, animals were killed by decapitation. After removal of the adrenals, the epididymides were cut into three fragments. The explants were cultured as described by Lasnitzki (1970), using Medium 199 (Institut Pasteur, Paris, France) supplemented with 10% calf serum, 10% fetal calf serum or 2% Ultroser G (IBF-LKB), respectively. The fetal calf serum was treated for 15 min with activated charcoal (1 mg/mg protein) then purified by two centrifugations (2 min at 5000 × *g*). Testosterone (Sigma, St Louis, MO, U.S.A.) dissolved in propylene glycol was added to the medium at a concentration of 150 nM. Cultures were maintained at 30°C under 5% CO_2 in air. The medium was renewed after the first 24 h and every 2 days thereafter.

Incubation and preparation of soluble proteins

At the end of the culture period, the organs were incubated in 300 µl Eagle's medium with 40 µCi/ml ^{35}S-methionine (specific activity > 1000 Ci/mmol) for 4 h

at 30°C under 5% CO_2 in air. After rinsing with 0.12 M Tris-HCl buffer, pH 7.2 at 4°C, homogenization in the same buffer and centrifugation at 12 000 × ***g*** for 10 min, supernatants were collected and stored at −80°C. Total soluble proteins were measured by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

Measurement of radioactivity and electrophoresis

A solution of 5 µl soluble protein was spotted onto a square (1 × 1 cm) of 3 mm Whatman paper impregnated previously with 10% trichloracetic acid (TCA). After three washes with 5% TCA (one 5-min wash at 90°C and two other 5-min washes on ice) and two washes with ethanol, the radioactivity incorporated into the precipitated proteins was determined by scintillation counting.

Proteins (50 000 d.p.m. acid-precipitable radioactivity) were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on a discontinuous gradient (12.5% – 18%) (Laemmli, 1970). The gels were impregnated with 2, 5-diphenyl-oxazole (PPO) for fluorography (Laskey & Mills, 1975). Dried gels were exposed to Kodak X-Omat S films for 8 days at −80°C, then fluorograms were scanned on an LKB laser densitometer (Ultroscan XL).

RNA extraction

After tissue homogenization of eight epididymides in 560 µl 50 mM Tris HCl, pH 7.5, 10 mM EDTA, and 5 M guanidium thiocyanate, RNAs were precipitated with 3.5 M LiCl over 36 h at 4°C. After two centrifugations and washings with 3 M LiCl, total RNAs were extracted according to Cathala *et al.* (1983) and precipitated by ethanol and sodium acetate at −20°C.

Probes

Two clones were used: Lv123 and Lv132. They were pBr322 plasmids containing a cDNA insert corresponding to the L-proteins mRNA (for clone Lv132 see Courty *et al.*, 1986). With these two clones all the mRNAs that correspond to the L-proteins can be detected. Plasmid DNA was labelled by nick translation with α^{32}p-dATP (Amersham, Amersham, U.K.) according to Rigby *et al.* (1977) (specific activity = 10^8 d.p.m./µg DNA).

RNA dot blot hybridization

RNA pellets were dissolved in 10% formaldehyde, 10 × standard saline citrate (SSC) (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7). Samples were heated for 15 min at 50°C and chilled immediately on ice for denaturation. RNA was spotted onto a nitrocellulose sheet previously equilibrated in 10 × SSC. Following sample application, nitrocellulose was dried for 2 h at 80°C. Filters were hybridized for 24 h at 42°C in 20 mM sodium phosphate (pH 6.5), 5 × SSC, 1 × denhart (0.02% Ficoll, 0.02% polyvinyl pyrolidone, 0.02% BSA), 50% formamide, 6% dextran sulphate, 0.1% SDS, with 5 × 10^5 c.p.m. of nick-translated plasmid probe per ml. After three washings in 2 × SSC and three washings in 0.5 × SSC, filters were exposed to Kodak X-Omat S films at −80°C for autoradiography. Changes in L-mRNA amounts were assessed by scanning the films on an LBK laser densitometer (Ultroscan XL, Bromma, Sweden).

Microscopy
Before culture, one epididymis was fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% osmium tetroxide in the same buffer and then embedded in Epon. Semi-thin (1-μm) sections were stained with toluidine blue and photographed under a light microscope.

Statistical methods
The significance of differences between means was calculated using Student's *t*-test and analysis of variance.

Results
Early studies on the regulation of the lizard epididymis have shown that testosterone (150 nM) is the most efficient androgen stimulating in-vitro regressed explants (Gigon, 1975; Depeiges, Betail & Dufaure, 1981). In this work the ability of regressed explants to respond to 150 nM testosterone was examined by measuring the mRNA concentration for the L-proteins and their synthesis. Initially, the effects of various serum supplements on the explant responses were checked.

Effects of various serums on proteins synthesis in vitro
Epididymides were obtained from animals 4 months after castration at stage 3. The profiles of ^{35}S-methionine incorporation were similar, regardless of the supplement added to the medium (Fig. 1). The incorporation rate peaked after 2 days. Thereafter, the rate decreased slowly and 5 days later reached a level close to that measured at the beginning of the culture experiments. Testosterone had no effect on the incorporation rate in the presence of calf serum (Fig. 1c) or fetal serum (Fig. 1b). Conversely, in the presence of Ultroser (Fig. 1a), testosterone resulted in an incorporation rate 50% higher than that of cultures maintained in the absence of this hormone. After 7 days of culture in the presence or absence of 150 nM testosterone, the explants were incubated for 4 h with (^{35}S)-methionine. In the presence of calf serum, testosterone induced the incorporation of ^{35}S-methionine into L-proteins (Fig. 2g), an effect that was not observed with Ultroser (Fig. 2c). In the presence of charcoal-stripped fetal calf serum, L-proteins synthesis occurred in the absence of testosterone (Fig. 2d), but when testosterone (150 nM) was added, the rate of L-proteins synthesis was increased by a further 50% (Fig. 2e).

Effects of stage of castration and of androgen withdrawal time
Animals were castrated during stages 1 or 3. At stage 1 (Fig. 3a), the epithelium consisted of a single layer of cubic, non-secretory cells. One month after castration (Fig. 3b), the epithelium had thickened, causing a pseudostratified appearance, and the cell nuclei were irregular in shape. Cell debris was noted in the lumen, indicating the onset of degeneration. Two months after castration (Fig. 3c), the signs of degeneration were more noticeable; possible lysozomal vacuoles were observed. The lumen was totally obstructed and the thickness of the epithelium had decreased. At stage 3 (Fig. 3d), the epithelial cells had begun to hypertrophy and secretion granules were visible in some cells. One month after castration (Fig. 3e),

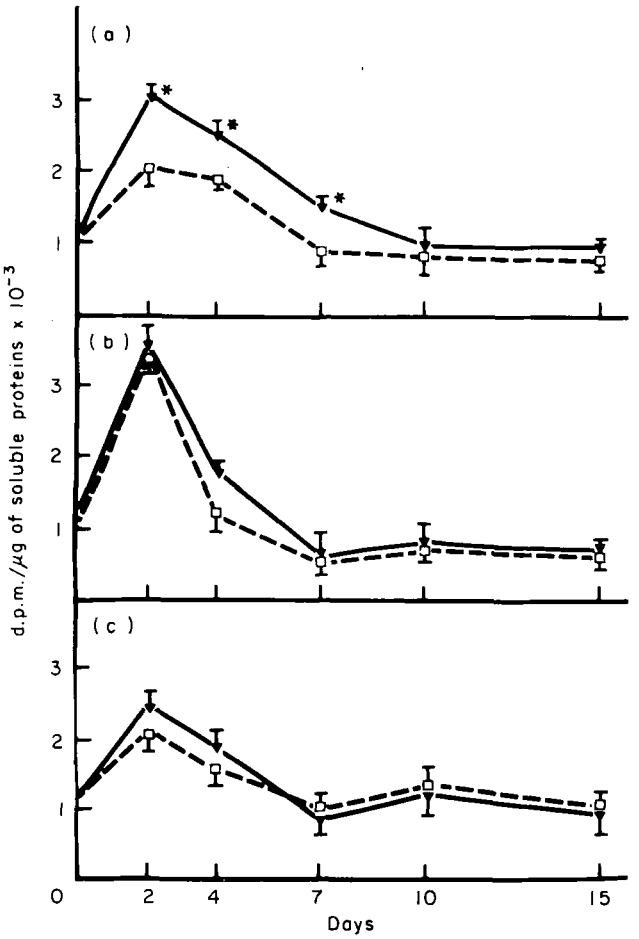

Fig. 1. Effects of various serum supplements on overall protein synthesis by explants of lizard epididymis. Three regressed explants from lizards castrated 4 months earlier (stage 3) were cultured for 2, 4, 7, 10 and 15 days in the presence (——) or absence (– – –) of 150 nM testosterone. The explants were cultured in medium 199 containing 2% Ultroser (a), 10% fetal calf serum (b) or 10% calf serum (c). Overall protein synthesis was measured after labelling for 4 h in the presence of ^{35}S-methionine by counting the radioactivity incorporated in the soluble proteins (means ± SEM). $^{*}P < 0.05$, compared with control without testosterone (. . . .) (Student's *t*-test).

the epithelium was disorganized, a lumen was absent and the cells were filled with lysosomal vacuoles. Two months after castration (Fig. 3f), the epithelium was in a phase of reorganization. Cells were arranged in a single layer around the lumen. Lysosomal vacuoles were still present in some cells. One and two months after surgery, the explants were cultured for 2, 5 or 10 days in the presence or absence of 150 nM testosterone (medium 199 + 10% calf serum). The amount of soluble protein (Table 1) did not change during the 10 days of culture, regardless of the time period (1 or 2 months) following castration. However, the amount of soluble proteins was significantly different between the experiments performed after castration at stages 1 and 3, suggesting a stage effect. Incorporation of radioactivity

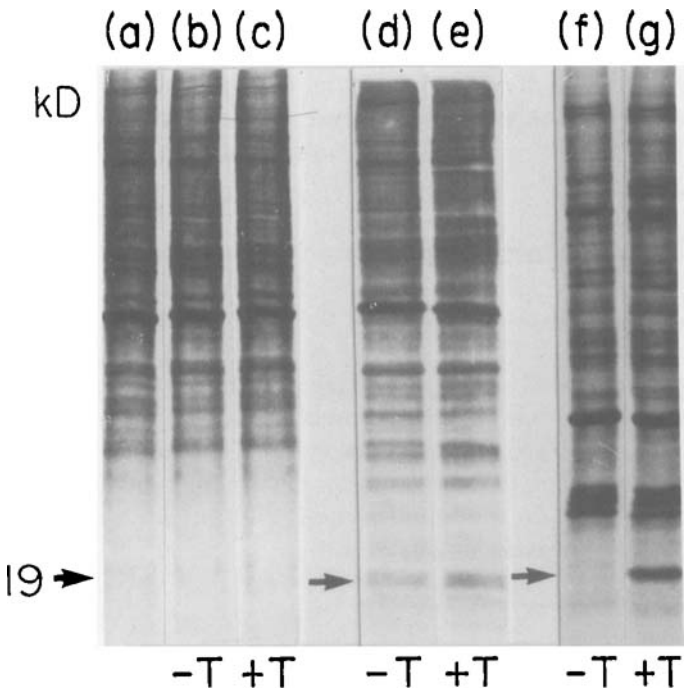

Fig. 2. Effects of various serum supplements on the synthesis of L-proteins. Labelled proteins (50 000 d.p.m. per lane) obtained as described in Fig. 1 were submitted to one-dimensional SDS-PAGE (12.5–18%) and revealed by fluorography. (a) Fluorographic pattern of labelled epididymal proteins from lizards castrated 4 months earlier (stage 3); other lanes show the patterns obtained after 7 days of culture in the presence of: (b) and (c) 2% Ultroser, (d) and (e) 10% fetal calf serum, or (f) and (g) 10% calf serum. The explants were cultured with (+T) or without (−T) 150 nM testosterone. Arrows indicate the L-protein band.

did not change during the culture period (Table 1). Incorporation was greater in explants cultured for 1 month following castration at stage 3 than in those cultured for 1 month following castration at stage 1 ($P < 0.05$). Addition of testosterone significantly enhanced the amount of soluble protein in explants cultured at stage 3, but had no effect on the rate of protein synthesis, thus suggesting an effect on protein turnover.

Culture response obtained 1 month after castration at stages 1 or 3. In explants from lizards castrated at stage 1, the Lv123-mRNAs were significantly present only 5 days after the culture onset. Their concentration increased two-fold between day 5 and day 10 (Fig. 4a) while L-protein synthesis increased three-fold (Fig. 4c). No difference between the treated and untreated explants was found using the Lv132-mRNA. In explants from lizards castrated at stage 3 both the Lv123 and Lv132 mRNAs were already detected 2 days after the culture onset (Fig. 4b). The Lv123 increased seven-fold up to day 10, while the Lv132 increased two-fold. L-protein synthesis increased seven-fold during the same time interval.

Culture responses obtained 2 months after castration at stages 1 or 3. The appearance of the L-mRNAs over time (Fig. 5a and b) was identical to that of the 1-month post-castration cultures (Fig. 4a and b), and the concentrations at the end of culture (day 10) were about the same as in the first experiment. However, the response in

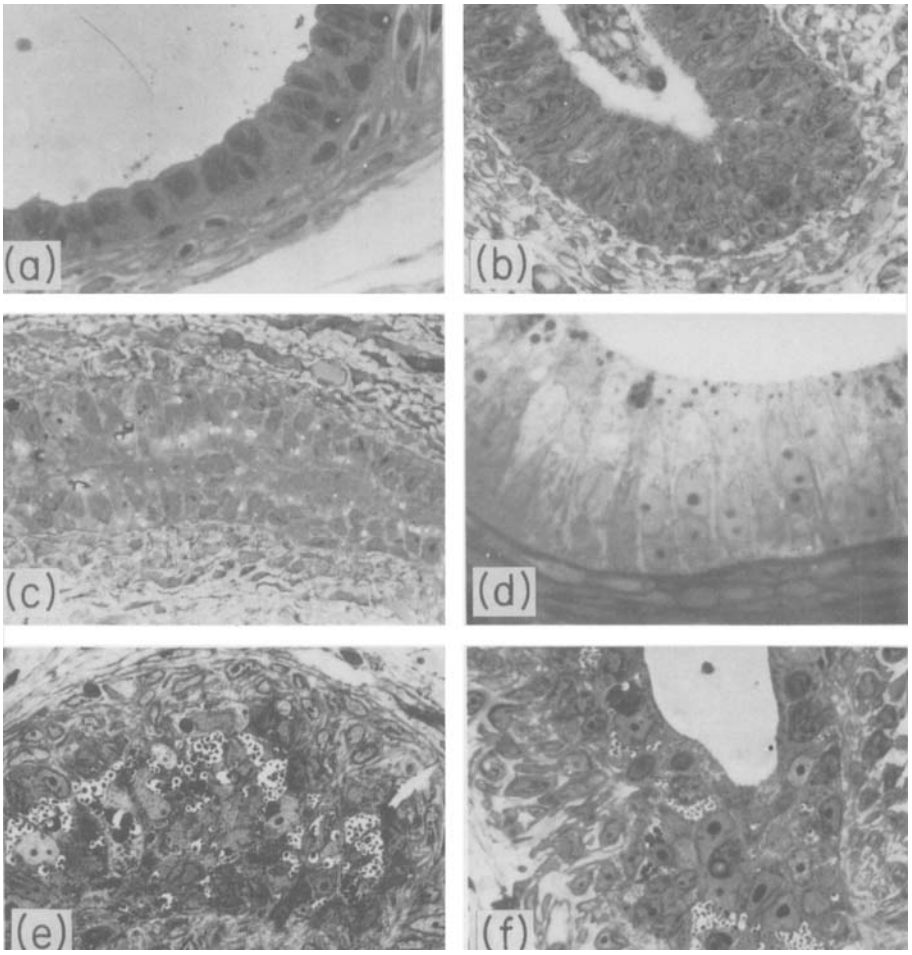

Fig. 3. Histological appearance of the epididymis before and after castration. (a–c) Castration performed at stage 1: (a) control at stage 1, (b) 1 month after castration, (c) 2 months after castration; (d–f) castration performed at stage 3: (d) control at stage 3, (e) 1 month after castration, (f) 2 months after castration. × 600.

terms of L-protein synthesis was delayed until day 10 for stage 1 (Fig. 5c) and until day 5 for stage 3 (Fig. 5d). Furthermore, incorporation rates in the L-band on day 10 were two-fold lower in this experiment (Fig. 5) than in the former (Fig. 4).

Discussion

Androgens are known to enhance overall protein synthesis and to induce specific proteins (Mainwarring, 1977). In the lizard, as in the rat (Orgebin-Crist & Menezo, 1980), the rate of protein synthesis *in vitro* is biphasic, regardless of the conditions of supplements. The inclusion of androgens in the culture medium provoked different responses depending on the culture conditions. Thus, androgen-dependency of overall protein synthesis was observed only in the presence of Ultroser, while in the presence of calf serum, testosterone enhanced the amount of

Table 1. Effects of stage, time since castration and testosterone on the protein synthesis and amounts

Time after castration	Culture time (days)	Added testosterone (150 μM)	Soluble proteins (μg/organ)		^{35}S-methionine incorporation (pM/organ)	
			Stage 1	Stage 3	Stage 1	Stage 3
1 month	2	−	314 ± 13	367 ± 12	0.20 ± 0.01	0.58 ± 0.11
		+	339 ± 6	386 ± 18	0.22 ± 0.02	0.51 ± 0.06
	5	−	ND	277 ± 26	0.16 ± 0.02	0.52 ± 0.07
		+	ND	310 ± 18	0.24 ± 0.08	0.62 ± 0.18
	10	−	281 ± 28	316 ± 18	0.26 ± 0.03	0.47 ± 0.06
		+	307 ± 10	360 ± 15	0.30 ± 0.06	0.63 ± 0.09
2 months	2	−	225 ± 28	313 ± 18	0.27 ± 0.04	0.31 ± 0.01
		+	228 ± 35	350 ± 10	0.27 ± 0.05	0.45 ± 0.08
	5	−	237 ± 13	351 ± 17	0.34 ± 0.03	0.30 ± 0.02
		+	280 ± 20	360 ± 12	0.41 ± 0.04	0.40 ± 0.03
	10	−	195 ± 16	388 ± 41	0.23 ± 0.06	0.21 ± 0.04
		+	212 ± 19	460 ± 59	0.30 ± 0.08	0.26 ± 0.03

Values are means ± SEM.

One or two months after castration during stages 1 or 3, explants were cultured for 2, 5 and 10 days with or without 150 nM testosterone. Soluble protein concentration was determined by the method of Lowry *et al.* (1951). ^{35}S-methionine incorporation was assessed by TCA precipitation and scintillation counting (see Materials and methods section). ND = Not determined.

The significances of the effects were determined using an analysis of variance. The amount of proteins was significantly higher ($P < 0.05$) in stage 3 than in stage 1 experiments and in testosterone-treated explants when compared with untreated samples (stage 3). At 1 month after castration, the incorporation of radioactivity was significantly higher ($P < 0.05$) at stage 3 than at stage 1.

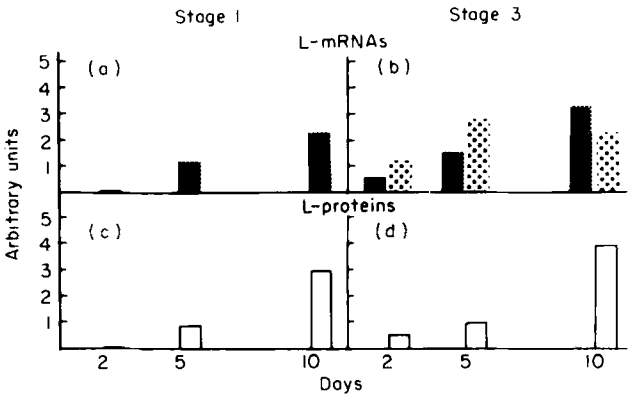

Fig. 4. Effects of testosterone on the L-mRNAs concentrations and L-proteins synthesis in explants cultured 1 month after castrations at stages 1 and 3. Regressed explants from lizard castrated 1 month earlier (stages 1 and 3) were cultured using medium 199 + 10% calf serum in the presence or absence of 150 nM testosterone. Total RNA was extracted 2, 5 and 10 days after the culture onset and spotted onto nitrocellulose sheets. The dots were hybridized to nick-translated plasmid DNA from Lv 123 (■) and Lv 132 (▨) clones. The autoradiographs were scanned and the absorbance values expressed as arbitrary units. The differences between the values from testosterone-treated (a) and untreated (b) explants were plotted 2, 5 and 10 days after the culture onset, the proteins were labelled for 4 h in the presence of (^{35}S)-methionine. Labelled soluble proteins (50 000 d.p.m.) were electrophoresed and revealed by fluorography. The L-proteins band was scanned and the differences in absorbance values between control (c) and treated (d) explants were expressed. Each histogram represents the mean of two experiments.

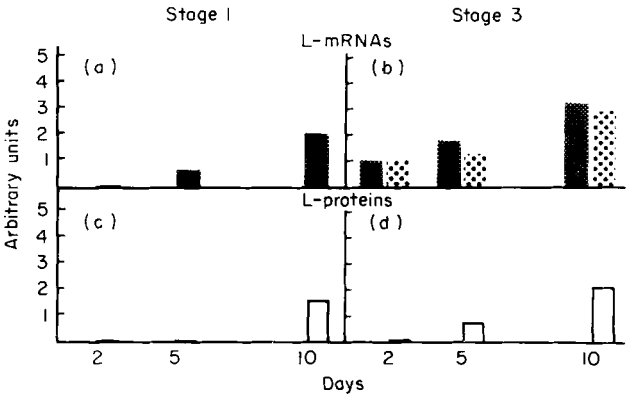

Fig. 5. Effects of testosterone on the L-mRNAs concentrations and L-proteins synthesis in explants cultured 2 months after castrations at stages 1 and 3. Regressed explants from lizard castrated 2 months earlier (stages 1 and 3) were cultured using medium 199 + 10% calf serum in the presence or absence of 150 nM testosterone. Total RNA was extracted 2, 5 and 10 days after the culture onset and spotted onto nitrocellulose sheets. The dots were hybridized to nick-translated plasmid DNA from Lv 123 (■) and Lv 132 (▨) clones. The autoradiographs were scanned and the absorbance values expressed as arbitrary units. The differences between the values from testosterone-treated (a) and untreated (b) explants were plotted. 2, 5 and 10 days after the culture onset, the proteins were labelled for 4 h in the presence of (^{35}S)-methionine. Labelled soluble proteins (50 000 d.p.m.) were electrophoresed and revealed by fluorography. The L-proteins band was scanned and the differences in absorbance values between control (c) and treated (d) explants were expressed. Each histogram represents the mean of two experiments.

soluble proteins without an effect on overall protein synthesis. These data show that, depending on the supplement conditions, testosterone can regulate the synthesis or turnover of proteins overall (Brooks, 1981). Testosterone binding-proteins in calf serum could decrease the available testosterone concentration below the threshold required for induction of overall protein synthesis, as addition of 10% fetal calf serum to the culture medium decreased testosterone retention in prostate explants by 40% and reduced the organ response (Lasnitzki & Franklin, 1972, 1975; Blaquier, 1973). Such an effect could explain the results of serum and Ultroser, and also suggests a difference in the androgen threshold for protein synthesis and turnover. The requirement of serum for L-protein synthesis suggests that factors other than testosterone are needed for the expression of specific proteins. Furthermore, use of fetal calf serum could add factors that are themselves able to stimulate specific protein synthesis, thus masking the testosterone effect. It is unlikely that these factors are steroids since the fetal calf serum was extracted with activated charcoal before use. A growth factor present in fetal calf serum could induce specific stimulation of L-protein synthesis, since serum proteins have been reported to activate cell proliferation *in vitro* in the epididymal tail of the rat (Majumder & Turkington, 1976).

The present study describes the use of two cDNA clones that are able to bind to all of the identified mRNAs coding for the L-proteins (Courty *et al.*, unpublished data). It appears that testosterone regulates the concentration of specific mRNAs in the lizard epididymis, as has been observed recently in the rat (Brooks *et al.*, 1986). It corroborates previous results obtained in the prostate (Mpanias, Hiremath & Wang, 1985; Parker, Hurst & Page, 1985), seminal vesicles (Moore *et al.*, 1984)

and kidney (Berger *et al.*, 1986) of several mammals. The rate of L-proteins synthesis in organs maintained with testosterone followed approximately the change of the L-mRNA levels, suggesting that it is in part controlled by the concentration of mRNA. However, a discrepancy existed in cultures made 2 months after castration. Although alterations of gene expression at various control levels (mRNA splicing or transport; Darnell, 1982) could explain such discrepancies, it has generally been interpreted as a clue to translational regulation (Kistler, Ostrowski & Kistler, 1980; Brooks *et al.*, 1986; Morel *et al.*, 1986). These discrepancies were observed 2 months after castration and seem to be related to the degree of cellular atrophy. This translational control could result from the presence of factors preventing translation (Brooks *et al.*, 1986) or from an absence of elements necessary for the translation of such mRNAs. The membranes of the rough endoplasmic reticulum (RER) and associated factors are necessary for the translation of mRNA coding for secretory proteins (Garoff, 1985). In the lizard epididymis, contrary to what is seen in rat seminal vesicles (Fawell & Higgins, 1984), a delay between accumulation of specific mRNA and development of the RER (determined by microscopy, unpublished data from this laboratory) exist at stage 1 of the annual cycle. At this stage, no L-protein was synthesized while the mRNA was translatable *in vitro* (Courty *et al.*, unpublished data). Two levels of control could be involved in this temporal discrepancy, the synthesis of the RER proteins and associated factors or their assemblage. It will be necessary to evaluate the importance of these levels of control in the regulation of L-proteins synthesis during the early phases of testosterone stimulation in the lizard.

The mRNA responses to testosterone were delayed in tissues from animals castrated at stage 1, compared with tissues from animals castrated at stage 3. This indicated the existence of differences in organ testosterone responsiveness between these two groups of castrated animals. The differences could either affect the initial steps of androgen action (binding to receptor, rate of 5α reduction, or receptor-binding to nuclear acceptors; O'Malley, 1984) or later steps (mRNA turnover; Cato, 1983). Viviparous lizards have a seasonal reproductive cycle during which important changes in the morphology and function of the epididymis are observed (Dufaure *et al.*, 1986). In this study castration was performed at two different stages of the sexual cycle. The period following castration at stage 1 corresponded to the pre-hibernation period in the wild, whereas the period following castration at stage 3 corresponded to the pre-mating period (Dufaure *et al.*, 1986). The discrepancies between cultures from stages 1 and 3 could be due either to the maintenance of some pre-existing differences during castration, or to divergent cell changes after castration. In animals with seasonal cycles, components other than androgens are known to change during the cycle, particularly under the influence of environmental conditions (Reiter, 1980). Thus, the physiological status prevailing during the post-castration period was markedly different between the two cases. It is possible that plasma components other than those of testicular origin may influence some epididymal functions. We are presently testing the effects of other steroid hormones on L-proteins gene(s) expression. Our current results emphasize the importance of the stage of differentiation of the epithelial cells for the expression of these genes.

Acknowledgments

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