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Characterization of hormonally regulated secretory proteins from the caput epididymidis of the rabbit

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1. The incorporation of [^{35}S]methionine into protein was investigated in tissue minces from different regions of the rabbit epididymis incubated *in vitro*. Rates of synthesis were in the order: epididymal regions 2–5 > region 7 > region 6 > region 1 > region 8 > ductus deferens > ductuli efferentes. 2. Separation of labelled proteins on polyacrylamide gels containing sodium dodecyl sulphate followed by fluorography revealed that one protein (mol.wt. approx. 90000) was characteristic of region 1, four proteins (one of mol.wt. 54000 and three of mol.wt. 20000) were synthesized principally in regions 2–5, and one protein (mol.wt. 22500) was produced mainly in regions 6, 7 and 8. 3. Castration for 14 days decreased incorporation of [^{35}S]methionine into total protein to less than 10% of that in controls in all regions of the epididymis. However, testosterone treatment for a further period of 14 days restored protein synthesis to normal values in regions 6, 7 and 8, but not in region 1 or regions 2–5. In regions 2–5 the synthesis of three proteins of mol.wt. 20000 declined after castration, but was not stimulated by exogenous testosterone. Since the 20000-mol.wt. proteins were major tissue proteins, accounting for 16–25% of the total synthesized, they were used as markers for investigating hormone action in the epididymis. 4. Castration followed immediately by testosterone treatment or ligation of the ductuli efferentes resulted in a decrease in their synthesis, suggesting that they are partially dependent on factors in testicular fluid. Purification and characterization showed them to be acidic glycoproteins with a number of biochemical and immunological properties in common. 5. It is suggested that there is a synergistic action between blood androgens and factors in testicular fluid in regulating protein synthesis in the proximal regions of the rabbit epididymis.

The growth and secretory activity of the male accessory sex glands (prostate, seminal vesicles, coagulating glands, bulbo-urethral glands, ampullae and epididymides) are dependent primarily on testicular androgens (Mann, 1964; Brandes, 1974; Wilson, 1978). In recent years the identification of specific and highly sensitive biochemical markers for measuring the stimulatory effects of testosterone on these tissues has provided much new information on the early molecular events during steroid-hormone action (reviewed by Higgins & Gehring, 1978). In rat ventral prostate an analysis of the hybridization kinetics of poly(A)-containing RNA with complementary DNA probes revealed that testosterone regulated preferentially the synthesis of a class of highly abundant poly(A)-containing RNA that coded for major secretory proteins (Parker & Mainwaring, 1977; Parker *et al.*, 1978). Higgins *et al.* (1976, 1978) also identified several androgen-dependent proteins in rat seminal vesicles, but in their case the synthesis of the poly(A)-containing RNA proceeded apace with general protein synthesis.

We have examined protein synthesis in the rat epididymis (Jones *et al.*, 1980*a*) and identified and characterized several proteins whose synthesis was stimulated by circulating androgens or by factors in testicular fluid. Cell-free translation and nucleic acid-hybridization techniques indicated that testosterone controlled preferentially the transcription of new mRNA coding for these proteins

Abbreviation used: SDS, sodium dodecyl sulphate.

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(D'Agostino *et al.*, 1980). However, they were not major tissue proteins, and this precluded further investigations into the molecular events governing their synthesis. In the present paper we describe three major proteins which are synthesized in a specific region of the caput epididymidis of the rabbit and which are potentially useful markers for studying the action of testosterone and testicular fluid on this organ.

Materials and methods

Materials

All chemicals and radiolabelled chemicals were of the highest purity available commercially and were obtained from sources described previously (Jones *et al.*, 1980*a*).

Animals

Male New Zealand White rabbits each weighing 3.5–4.5 kg were used in these experiments. Animals were anaesthetized with an intravenous injection of sodium pentabarbitone (Nembutal; Abbott Laboratories, Queenborough, Kent, U.K.), the testes removed through a scrotal incision and the epididymides replaced in the scrotum. In other experiments the entry of testicular fluid into the epididymis was blocked by placing a ligature (sterile silk, Grade W595; Ethicon Ltd., Sighthill, Edinburgh, Scotland, U.K.) at the junction of the ductuli efferentes and ductus epididymidis. Care was taken to preserve asepsis during all surgical procedures. Testosterone was solubilized in arachis oil and administered at dosages of 0.25, 5.0, 25.0 or 250.0 mg/rabbit per day. Animals were killed by cervical dislocation and excised tissues were placed immediately in phosphate-buffered saline (0.13 M-NaCl, 7 mM-Na_2HPO_4, 2.8 mM-NaH_2PO_4), pH 7.2, chilled on ice.

Incorporation of [^{35}S]methionine into protein

Each epididymis was trimmed free of fat and overlying connective tissue and sub-divided into separate regions as shown in Fig. 1. These regions approximate closely to the different morphological areas defined by Nicander (Nicander, 1957; Nicander & Ploen, 1979) and Jones *et al.* (1979*a*). Tissue minces were prepared and incubated with [^{35}S]-methionine as described by Jones *et al.* (1980*a*).

Analytical electrophoresis

Electrophoresis of proteins on non-denaturing gels or on denaturing polyacrylamide gels containing SDS and detection of labelled proteins by fluorography were carried out by methods described by Jones *et al.* (1980*a*).

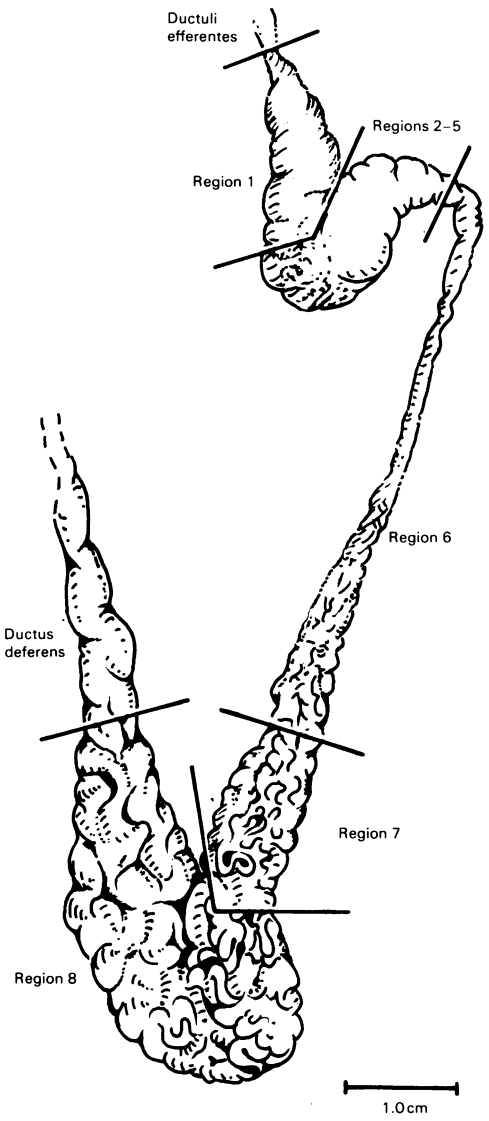

Fig. 1. *Gross morphology of normal rabbit epididymis* Sketch of rabbit epididymis (testis removed) to illustrate the sub-division of the organ used in this investigation. This sub-division is based on the original terminology of Nicander (1957) and also that of Jones *et al.* (1979*a*).

Isoelectric focusing

Purified proteins were focused on a Bio-Rad vertical slab-electrophoresis system (Model 220) using an Ampholine of range pH 3–10 by the method of O'Farrell (1975).

Collection of epididymal secretions and purification of acidic proteins

Luminal contents were collected from the cauda

epididymidis of normal rabbits by retrograde injection via the ductus deferens of phosphate-buffered saline, pH 7.2, preceded by a bubble of air or mineral oil. All manipulations were performed under a ×10 stereo microscope. Approx. 50–200μl of luminal contents was collected from each rabbit, transferred to glass capillary tubes and centrifuged at 5000**g** for 5min in a Hawksley Microhaematocrit centrifuge. The supernatant plasma, containing 30–40mg of protein/ml, was separated from the pelleted spermatozoa and stored frozen at −20°C pending analysis.

All purification procedures were carried out at 4°C. Epididymal plasma was pooled from selected animals (see below), dialysed overnight against 60mM-NaCl in 50mM-Tris/HCl, pH 7.9, and centrifuged at 15000**g** for 15min. The clear supernatant was applied to a column (6.0cm × 1.0cm^2) of DEAE-Sephadex A-25 equilibrated in the same buffer and adsorbed proteins were eluted with a linear gradient of 60–200mM-NaCl in the Tris buffer. Fractions from selected peaks were analysed on denaturing and non-denaturing polyacrylamide slab gels and those fractions containing the proteins under investigation were pooled, dialysed against distilled water and freeze-dried. Contaminating proteins were removed by gel filtration on a column (90cm × 2cm^2) of Sephadex G-75 equilibrated in 20mM-Tris/HCl, pH 8.2, containing 150mM-NaCl. Peaks containing highly purified proteins were dialysed exhaustively against distilled water and freeze-dried. Molecular-weight standards for gel filtration were bovine serum albumin (mol.wt. 66000), ovalbumin (mol.wt. 45000), soya-bean trypsin inhibitor (mol.wt. 22000) and cytochrome *c* (mol.wt. 12300).

Neuraminidase digestion of purified secretory proteins

Purified proteins were incubated at a concentration of approx. 0.3mg/ml with 200 units of neuraminidase (proteinase-free)/ml in 100mM-Tris/maleate/merthiolate buffer, pH 5.7 (Boursnell *et al.*, 1970) (one unit of neuraminidase will liberate 1μmol of *N*-acetylneuraminic acid/min at 37°C, pH 5.5, with α-1-acid glycoprotein as substrate). The incubations were performed at 20°C and samples were withdrawn at 2, 8 and 12 days for electrophoresis on denaturing and non-denaturing polyacrylamide gels.

Preparation of antisera

Samples (10 or 50μg) of purified proteins were emulsified in 5ml of phosphate-buffered saline and 5ml of Freund's Complete Adjuvant (Difco Laboratories, P.O. Box 14B, West Molesey, Surrey, KT8 0SE, U.K.) and injected subcutaneously at multiple sites into two sheep. A sample (50ml) of pre-immune serum was collected at the time of the first immunization. At 3 and 5 weeks later animals were given booster injections of 10μg or 50μg of protein, and blood was collected at the same time for testing on Ouchterlony double-diffusion agar plates (Clausen, 1969). When a satisfactory antibody titre was reached, 200ml of blood was collected and the serum stored frozen at −20°C. An immune reaction was obtained in both sheep, so serum from the animal that had received the lower dose of antigen was used in all subsequent experiments.

Affinity chromatography

Samples (100μl) of epididymal plasma pooled from selected animals were dialysed overnight at 4°C against 10mM-Tris/HCl, pH 8.0, and applied to a column (7cm × 20cm^2) of agarose-linked concanavalin A (*Canavalia ensiformis* lectin; Pharmacia, Hounslow, Middx. TW3 1NE, U.K.) equilibrated in the same buffer. Unbound protein was washed from the column with 10mM-Tris/HCl and bound protein was desorbed with 0.3M-α-methyl D-mannoside in the same buffer. Eluted proteins were precipitated with 90% (v/v) ethanol, dissolved in 50μl of 62.5mM-Tris/HCl buffer, pH 7.4, containing 1% (w/v) SDS and 1% (v/v) β-mercaptoethanol and analysed by electrophoresis on denaturing polyacrylamide gels.

Other procedures

Total carbohydrate was measured by the orcinol reaction (Vasseur, 1948), and protein by the Hartree (1972) modification of the Lowry method, with bovine serum albumin as standard. [^{35}S]Methionine incorporation into specific proteins was quantified by cutting out the appropriate areas of the gels, and digesting with 1ml of 30% (v/v) H_2O_2 at 50–60°C for 48h. Radioactivity was counted with a Nuclear–Chicago liquid-scintillation counter in a scintillation 'cocktail' consisting of 6g of butyl-PBD [5-(biphenyl-4-yl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba ARL, Duxford, Cambs., U.K.) in 250ml of Triton X-100, 45ml of water and 705ml of toluene. Efficiency for ^{35}S was about 30%.

Results

Protein synthesis in normal rabbit epididymis

As a prelude to examining the hormonal regulation of protein synthesis, we investigated if there were significant differences in the type of proteins synthesized in different morphological regions of the normal rabbit epididymis. Tissue minces were prepared from five separate areas shown in Fig. 1, incubated with [^{35}S]methionine, and labelled proteins detected by fluorography after separation on SDS/polyacrylamide gels. Preliminary experiments showed that the rate of incorporation of

radioactivity into acid-insoluble protein was linear for at least 4h at 33°C for all regions of the epididymis (results not shown). Rates were in the order regions 2–5 > region > 7 > region 6 > region 1 > region 8 > ductus deferens > ductuli efferentes. Fluorography revealed that some proteins were synthesized in certain regions and not in others (Fig. 2); one protein (mol.wt. approx. 90000) was found principally in region 1, one protein (mol.wt. 54000) mainly in regions 2–5, a third protein (mol.wt. 22500) was common to regions 2–5, 6, 7 and 8, and a fourth protein (mol.wt. 20000) was synthesized predominantly in regions 2–5, with lesser amounts produced in regions 1, 6 and 7.

In later experiments we found that the labelled protein which was synthesized predominantly in regions 2–5 and which migrated with a mol.wt. of 20000 actually consisted of several proteins which differed in charge. This was shown by electrophoresis of labelled proteins from regions 2–5 on non-denaturing polyacrylamide gels at pH 8.9, which revealed three major acidic proteins, α, β and γ (Fig. 2, track vi). When separated on SDS/polyacrylamide gels in the second dimension, all three proteins migrated with a mol.wt. of 20000.

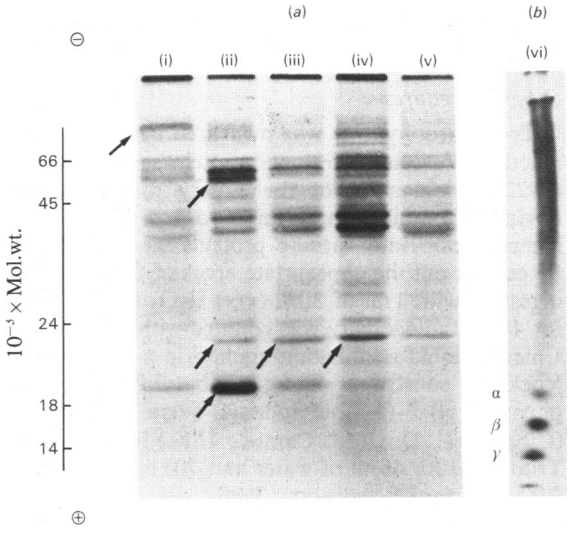

Fig. 2. *Fluorograph of [^{35}S]methionine-labelled proteins synthesized in different regions of the normal rabbit epididymis*

Tissue minces were incubated with [^{35}S]methionine and the 1000*g*-postnuclear-supernatant fractions analysed on denaturing polyacrylamide gels containing SDS (*a*) or on non-denaturing polyacrylamide gels (*b*). Radioactive proteins were detected by fluorography. The material applied to the gels was from: region 1 (i); regions 2–5 (ii and vi); region 6 (iii); region 7 (iv); region 8 (v). Each track contained 40000 c.p.m. The three proteins labelled α, β and γ have the same mobility as those in Fig. 6, track (v).

Hormonal regulation of protein synthesis in the epididymis

After castration, the incorporation of [^{35}S]-methionine into acid-insoluble protein decreased progressively in tissue minces from all regions of the epididymis until after 12–14 days it was 5–10% of that in normal animals (Fig. 3). Treatment of 14-day-castrated animals with testosterone (5mg/rabbit per day) for a further 14 days stimulated protein synthesis to normal or above normal values in regions 6, 7 and 8, but it had only a partial restorative effect in regions 1 and 2–5 (Fig. 3). Increasing the dose of testosterone to 25 or 250mg/rabbit per day did not improve the response.

Analysis of labelled proteins on SDS/polyacrylamide gels followed by fluorography revealed that, in addition to its effects on general protein synthesis, testosterone also regulated specifically the synthesis of a number of proteins. In regions 6, 7 and 8, the incorporation of [^{35}S]methionine into a protein of mol.wt. 22500 decreased after castration to less than 10% of that in normal animals, but was restored to original values by testosterone (results not shown). However, the most striking result was obtained in regions 2–5 (Fig. 4*a*). In this area the synthesis of

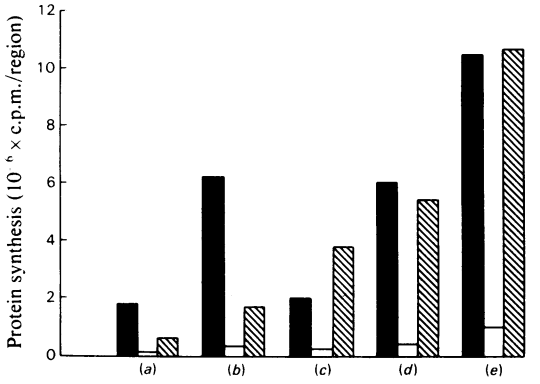

Fig. 3. *Effects of castration and testosterone replacement on epididymal protein synthesis*

Approx. 35mg of minced epididymal tissue from groups of three normal rabbits (■), three rabbits castrated for 14 days (□), and three rabbits castrated for 14 days but given 5mg of testosterone/day for 14 days (▨), was incubated with 25μCi of [^{35}S]methionine for 2h at 33°C. Total protein synthesis/region was determined by precipitation of post-nuclear supernatants with ice-cold 5% (w/v) trichloroacetic acid. (*a*) Region 1; (*b*) regions 2–5; (*c*) region 6; (*d*) region 7; (*e*) region 8. Each value represents the mean of three individual determinations.

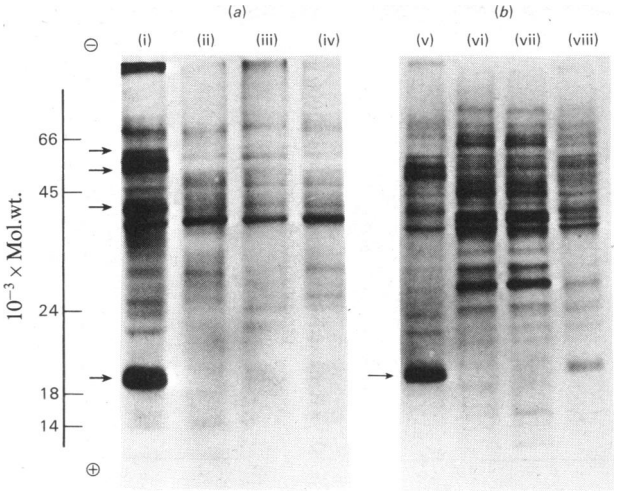

Fig. 4. *Fluorography of [^{35}S]methionine-labelled proteins synthesized in regions 2–5 from rabbits of different hormonal status*

Postnuclear (1000*g*) supernatants pooled from groups of three animals were analysed on 15% (w/v) polyacrylamide gels containing SDS and radiolabelled proteins detected by fluorography. Each track contained approx. 40000c.p.m. (*a*) Effects of castration and testosterone replacement: (i) normal animals; (ii) animals castrated for 14 days; (iii) animals castrated for 14 days but given 25mg of testosterone/day for 14 days; (iv) animals castrated for 14 days, but given 250mg of testosterone/day for 14 days. (*b*) Effects of castration followed immediately by testosterone supplementation: (v) normal animals; (vi) animals castrated for 14 days; (vii) animals castrated for 14 days, but given 0.25mg of testosterone/day for 14 days; (viii) animals castrated for 14 days, but given 25mg of testosterone/day for 14 days.

proteins of mol.wts. 60000, 54000, 43000 and 20000 was decreased by castration to approx. 5% of normal, but, unlike the 22500-mol.wt. protein in regions 6, 7 and 8, testosterone replacement did not restore their synthesis. Exceptionally high doses of testosterone (250mg/rabbit per day for 14 days) were also without effect. This result was shown most clearly with the 20000-mol.wt. proteins, where incorporation of [^{35}S]methionine was 11800, 210, 360 and 395c.p.m. in normal rabbits or, 14-day-castrated animals with no, 5 or 250mg of testosterone/rabbit per day respectively. Separation of labelled post-nuclear supernatants on non-denaturing polyacrylamide gels confirmed these results and showed that all three acidic proteins were affected equally and simultaneously. Because the 20000-mol.wt. proteins were major tissue proteins, accounting for about 25% of the total [^{35}S]-methionine incorporated, they proved to be the best markers for studying the effects of different hormonal treatments, and hence in subsequent experiments we have concentrated on these proteins.

The failure of exogenous testosterone to restore the synthesis of the 20000-mol.wt. proteins after castration suggests that factors in testicular fluid may be important in stimulating the activity of regions 2–5. Testicular fluid contains very high concentrations of testosterone (35–40 times that in blood; Cooper & Waites, 1974; Bartke *et al.*, 1975), in addition to other unusual substances such as androgen-binding protein and inositol (Setchell, 1970). We have shown previously in the rat that if testicular fluid is prevented from entering the epididymis by ligating the ductuli efferentes, then the synthesis of protein in the initial segment region is completely abolished (Jones *et al.*, 1980*a*,*b*). After ligation of the ductuli efferentes in the rabbit, there was a progressive decline in the incorporation of [^{35}S]methionine into total protein to 20–25% of that in normal animals after 12–14 days (Fig. 5*a*). There was also a decrease in the synthesis of the 20000-mol.wt. proteins (Fig. 5*b*) when analysed on denaturing or non-denaturing polyacrylamide gels. Unlike castration, however, ligation of the ductuli efferentes did not abolish their synthesis completely, but decreased the specific incorporation of [^{35}S]-methionine to 30–40% of that of controls after 12–14 days.

It was not possible to obtain a reliable estimate of cell number from DNA measurements, because of the presence of varying numbers of spermatozoa in the tissue. However, it is unlikely that these results could be caused solely by a decrease in the number of epithelial cells. First, castration or ligation of the

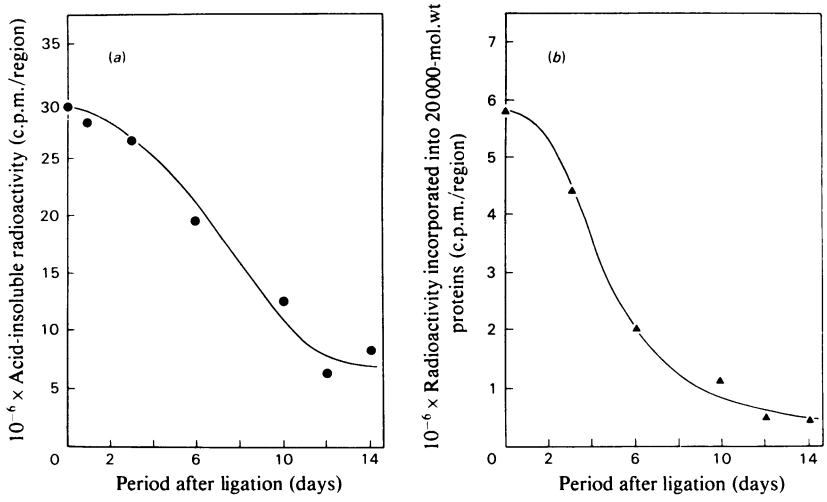

Fig. 5. *Effects of ligation of the ductuli efferentes on epididymal protein synthesis*

Approx. 35 mg of minced tissue from regions 2–5 was incubated with 25 μCi of [^{35}S]methionine and (*a*) total protein synthesis/region was measured by precipitation with 5% (w/v) trichloroacetic acid, (*b*) [^{35}S]methionine incorporation into the 20000-mol.wt. proteins was measured after analysis of postnuclear supernatants on 15% (w/v) polyacrylamide gels containing SDS. After fluorography the appropriate area of gels was located and counted for radioactivity. Each point represents the mean of duplicate determinations.

ductuli efferentes affected the synthesis of certain proteins specifically, and not others. Second, histological studies have shown that, although the morphology of region 1 is adversely affected by the absence of testicular fluid, the epithelium in regions 2–5 remains essentially intact, since less than 10% of the principal cells show signs of degeneration (L. Nicander, personal communication). Therefore the results are indicative of a real decrease in protein synthesis per epithelial cell.

Further evidence of a role for testicular fluid was shown in experiments where we investigated if testosterone, given immediately after castration, would prevent involution of the caput and the subsequent loss of synthesis of the 20000-mol.wt. proteins. In earlier work we found that 0.25 mg of testosterone/rabbit per day was the minimum dose required to maintain the activity of the cauda epididymidis by using the concentration of Na^+ ions in the luminal plasma as a marker for androgen action (Jones, 1977). However, this amount of testosterone was not sufficient to maintain the synthesis of the 20000-mol.wt. proteins (Fig. 4*b*). Increasing the dose to 25 mg/rabbit per day had some preservative effect in that the specific incorporation of [^{35}S]methionine was maintained at 36% of control values. Although not measured directly, it is likely that this large amount of testosterone would have created hyperphysiological concentrations of androgens in the blood. Danzo & Eller (1980) administered approx. 8 mg of testosterone/day for 14 days to castrated rabbits and found that the amount of testosterone in peripheral blood plasma was 5–7 times higher than in normal animals.

Finally, we have examined the tissue specificity of the 20000-mol.wt. proteins. A survey of proteins synthesized in the anterior prostate, posterior prostate, seminal vesicles, bulbo-urethral glands, ampullae and kidney showed that they were only found in the epididymis.

Purification and characterization of acidic secretory proteins from the epididymis

On the basis of their hormonal regulation several proteins were purified from the secreted luminal plasma collected from the cauda epididymidis. When luminal plasma was analysed on non-denaturing polyacrylamide gels at pH 8.9 in the first dimension, the three most acidic proteins (α, β and γ; Figs. 6*a* and 6*b*) had the same mobility as the three proteins originating from regions 2–5 and whose synthesis was affected by castration and ligation of the ductuli efferentes. When separated on SDS/polyacrylamide gels in the second dimension, proteins α, β and γ all migrated with a mol.wt. of 20000. Initially we analysed luminal plasma which had been pooled from several animals, but subsequently we examined plasma from separate rabbits and found that all three proteins were never present in any one

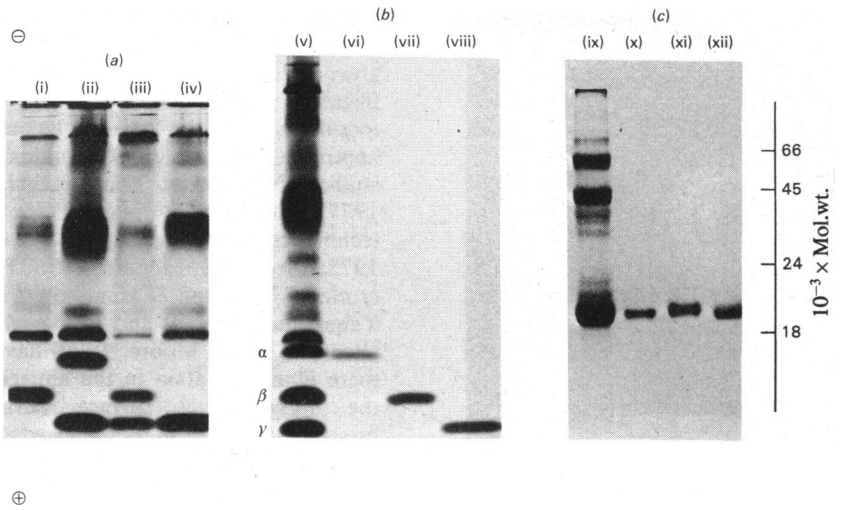

Fig. 6. *Purification of epididymal secretory proteins*

(*a*) Electrophoresis of epididymal plasma from individual rabbits on non-denaturing 7% (w/v) polyacrylamide gels to ilustrate the variation between animals. Some 12% of rabbits had the pattern shown in track (i), 22% as in track (ii), 18% as in track (iii), and 36% as in track (iv). A total of 46 rabbits was analysed. (*b*) and (*c*), Electrophoresis of pooled epididymal plasma [tracks (v) and (ix)], purified protein α [tracks (vi) and (x)], purified protein β [tracks (vii) and (xi)] and purified protein γ [tracks (viii) and (xii)] on non-denaturing (*b*) and denaturing (*c*) polyacrylamide gels. The protein migrating immediately behind protein α in track (v) has the same mobility as serum albumin. Tracks (i)–(v) and (ix) contained approx. 80 μg of protein, the remainder approx. 15 μg.

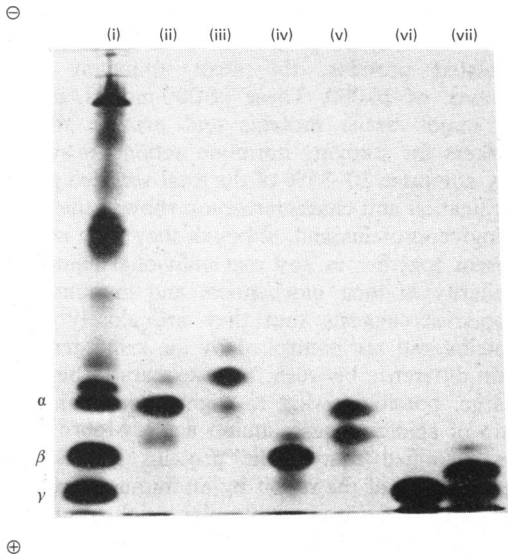

Fig. 7. *Neuraminidase digestion of purified epididymal secretory proteins*

Purified proteins α, β and γ at a concentration of approx. 0.3 mg/ml were incubated at 20°C for 8 days in the presence or absence of 200 units of neuraminidase (proteinase-free, Behringwerke)/ml. The proteins were then analysed on 7% (w/v) non-denaturing polyacrylamide gels. Track (i), whole cauda-epididymidal plasma; tracks (ii), (iv) and (vi), control incubations of proteins α, β and γ respectively; tracks (iii), (v) and (vii), neuraminidase digestion of proteins α, β and γ respectively.

individual (Fig. 6*a*). About 36% of animals secreted only protein γ, 12% protein β, 22% α and γ, and 18% β and γ. The remaining 12% consisted of proteins α and β together and α alone.

Proteins α, β and γ were separated from basic proteins and each other by chromatography on DEAE-Sephadex A25 and pH 7.9. Final purification was achieved on Sephadex G-75 to greater than 90% homogeneity (Figs. 6*b* and 6*c*). All three proteins migrated with a mol.wt. of approx. 20000 on SDS/polyacrylamide gels and 22000 on gel filtration, suggesting that they are monomeric. The pI values of proteins α, β and γ were 4.65, 4.25 and 4.15 respectively.

The likelihood that proteins α, β and γ are glycoproteins was suggested by the following. (*a*) All three proteins gave a positive reaction for carbohydrate in the orcinol test, with values of 4–7%, with mannose as standard. However, orcinol does not react with amino sugars or sialic acid, and hence the above values are likely to be underestimates. (*b*) Removal of terminal sialic residues by digestion with neuraminidase for 8 days at 20°C altered the charge on each protein to the extent that they became less anionic and migrated as two or three separate proteins (Fig. 7). (*c*) All three proteins were bound to a concanavalin A lectin column. Other proteins in the epididymal plasma that were bound to concanavalin A were those with mol.wts. 28000, 43000, 54000, 60000 and approx. 78000.

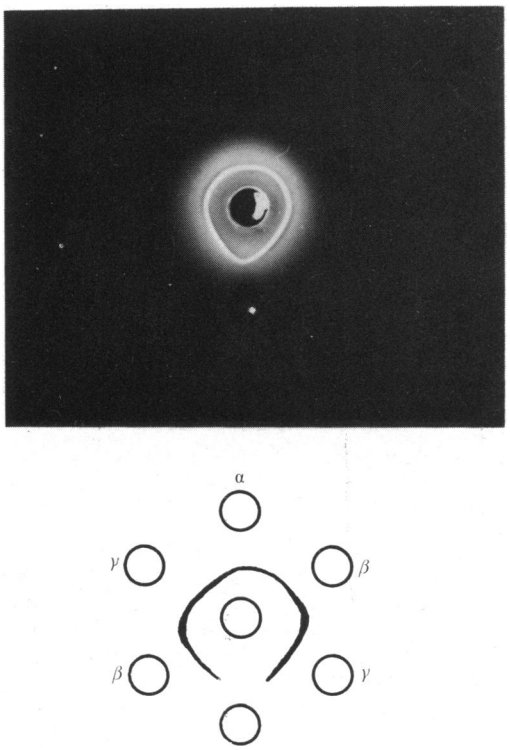

Fig. 8. *Immunological properties of epididymal secretory proteins α, β and γ*

Ouchterlony double-diffusion agar gel of proteins α, β and γ tested against a sheep antiserum raised to protein γ. The outer wells contained 15–20μg of protein in the positions indicated and the centre well 10μl of immune serum. Pre-immune serum did not give a reaction.

A further indication of the close similarity between all three 20000-mol.wt. proteins was shown by the finding that antibodies raised against protein γ cross-reacted with proteins α and β, with no evidence for spur formation when tested on Ouchterlony double-diffusion agar gels (Fig. 8). Confluence of precipitation arcs (the so-called 'reaction of complete identity'; Clausen, 1969) indicates that antigens have a number of immunogenic determinants in common, whereas intersection of precipitation arcs (the 'reaction of non-identity') is evidence for little or no serological relationship.

Discussion

The mechanisms involved in the acquisition of forward motility and fertilizing capacity by spermatozoa as they pass through the proximal regions of the epididymis remain largely unsolved (reviewed by Bedford, 1975), but current evidence indicates that one important aspect is a re-structuring of the plasma membrane, especially with regard to surface glycoproteins. Since the initial events during fertilization involve membrane fusion between spermatozoan and egg, these glycoproteins may be important in cellular recognition. Lectin-binding studies (Nicolson & Yanagimachi, 1972; Millette, 1977; Nicolson *et al.*, 1977), surface-labelling techniques (Bedford *et al.*, 1972; Yanagimachi *et al.*, 1972; Fléchon & Morstin, 1975; Fournier-Delpech *et al.*, 1977; Olson & Hamilton, 1978; Nicolson & Yanagimachi, 1979) and whole-cell electrofocusing (Bedford, 1963; Moore, 1979) have suggested that there is an alteration in the antigenic properties of the spermatozoal surface during maturation, brought about either by direct adsorption of glycoproteins from the epididymal secretion or by the action of glycosidases or glycosyltransferases. These processes are androgen-dependent, since removal of the testes causes an immediate cessation of spermatozoal maturation (Orgebin-Crist, 1973). Therefore the identification of proteins synthesized and secreted by the epididymis together with a knowledge of their hormonal regulation is an important initial step towards investigating their interaction with the spermatozoal membrane. In the present work we have shown that different proteins are synthesized in different morphological areas of the normal rabbit epididymis. The most active area consists of regions 2–5, which synthesizes a number of hormonally regulated proteins, the most abundant having mol.wts. of 20000. These 20000-mol.wt. proteins are major tissue proteins and provide the best markers for studying hormone action. In addition, they comprise 30–35% of the total secreted protein. Purification and characterization showed them to be sialoglycoproteins and, although they were never all present together in any one individual animal, the similarity in their biochemical and immunological properties suggests that they are closely related proteins and are controlled by the same gene. The main difference between them appears to be one of charge, possibly owing to slight alterations in the ratio of acidic to basic amino acids. Moore (1980) has identified four acidic proteins in the caput epididymidis of the rabbit by an immunoperoxidase technique, but since no molecular weights were given it is not clear from that work if they are the same or different from the ones reported here.

These secretory proteins originate from the epididymal epithelium, which in the rabbit is composed mainly of two cell types, principal cells and basal cells. As their name implies, principal cells constitute over 85% of the total epithelium, and on morphological grounds are the most likely cell type to be involved in secretory activity (Nicander & Malmqvist, 1977). They possess a well-developed endoplasmic reticulum, apical secretory vesicles and one of the largest Golgi complexes of any cell in the

animal body. Furthermore, they involute rapidly after castration (Jones *et al.*, 1979*b*). Basal cells, on the other hand, contain very sparse endoplasmic reticulum, a small Golgi apparatus and lie between the principal cells and the basal lamina; they do not have contact with the luminal border.

Our observation that the synthesis of the 20000-mol.wt. proteins was abolished by castration, but could not be reversed by exogenous testosterone, suggests either that the caput epididymidis loses its capacity to respond to androgens or that other factors, possibly in testicular fluid, are involved.

Considering the first possibility, evidence from work on other androgen-responsive tissues does not suggest that castration causes a complete loss of cytoplasmic receptor proteins for 5α-dihydrotestosterone. In a detailed study of androgen receptors in rat ventral prostrate, Sullivan & Strott (1973) found that, after an initial decline, receptor numbers rose again in long-term castrated animals and that these changes were independent of adrenal or pituitary hormones. They concluded that there was both an androgen-dependent and an androgen-independent mechanism for regulating receptor number. Calandra *et al.* (1975) reported initially that rat epididymal androgen receptors disappeared after long-term (30 days) castration, but later they concluded that this was due to the insensitivity of the assay technique and proteolysis in the tissue extracts (Calandra *et al.*, 1977). Danzo & Eller (1976, 1978) have demonstrated an 8S cytoplasmic androgen receptor in the caput epididymidis of 3-day-castrated rabbits, but long-term castration with or without androgen replacement was not investigated. The same authors have also shown that rabbit epididymal 5α-reductase is androgen-dependent (Danzo & Eller, 1980), but the decrease in activity after castration (14 days) was not greater than in the rat epididymis (Robaire *et al.*, 1977), which responds very rapidly to exogenous testosterone (Jones *et al.*, 1980*a*). In addition, unusually high concentrations of 5α-reduced androgens are present in the blood of male rabbits (Schanbacher & Ewing, 1975). Our recent experiments suggest that these androgens are derived mainly from the metabolism of testosterone in peripheral tissues such as muscle and skin (Booth & Jones, 1980). Therefore, on the basis of the above evidence it would not seem likely that castration would cause a total loss of receptors from the epididymis or eliminate the availability of 5α-dihydrotestosterone. Nonetheless, the inability of exogenous testosterone to restore the synthesis of the 20000-mol.wt. proteins in the caput epididymidis after a period of castration was pronounced and unmistakable. This suggests that changes, irreversible by testosterone alone, must have taken place and that factors other than androgens may be involved in regulating the synthesis of these proteins.

The decrease in general and specific protein synthesis in regions 2–5 after ligation of the ductuli efferentes favours the hypothesis that testicular fluid is important in maintaining the functional activity of the caput epididymidis. This is commensurate with our previous work on the rat (Jones *et al.*, 1980*a*,*b*) and the morphological findings of Danzo *et al.* (1977), Moniem *et al.* (1978) and Fawcett & Hoffer (1979), who showed that the presence of testicular fluid was necessary to prevent involution of the epithelium in the initial segment. Testicular fluid contains a wide range of different proteins (Koskimies & Kormano, 1975), one of which, androgen-binding protein, transports large amounts of testosterone into the epididymis. Androgen-binding protein is secreted by Sertoli cells in the testis and is later absorbed and degraded within principal cells in the caput epididymidis (reviewed by Hansson *et al.*, 1975). It is noteworthy that in the rabbit testicular fluid is absorbed mainly in regions 2–5 (Nicander & Ploen, 1979; Jones *et al.*, 1979*a*) and that this is the area which contains the highest concentration of androgen-binding protein (Ritzen & French, 1974). However, other components of testicular fluid may also be involved; for example, a potent growth factor has recently been reported in the testis and testicular fluid from mice and rams (Brown *et al.*, 1981; Feig *et al.*, 1980). It is difficult to say whether these components exert their influence at a transcriptional or post-transcriptional level, but for the time being we can exclude spermatozoa from having a direct stimulatory effect, since the 20000-mol.wt. proteins can be detected in the caput epididymidis of pre-pubertal rabbits before spematozoa have left the testis (R. Jones, unpublished work). Thus, it may be that there is synergism between blood-borne androgens and factors in testicular fluid in regulating protein synthesis in the caput epididymidis, whereas in the cauda epididymidis androgens alone are fully effective.

Although the results reported in the present paper are preliminary, they should facilitate future studies on the molecular events controlling hormone action in the epididymis and also provide a basis for investigating the interaction between epididymal secretory proteins and spermatozoa during their maturation.

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