Hormonal Regulation of Protein Synthesis in the Rat Epididymis

CHARACTERIZATION OF ANDROGEN-DEPENDENT AND TESTICULAR FLUID-DEPENDENT PROTEINS

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1. Protein synthesis has been investigated in different regions of the rat epididymis by measuring incorporation of [³⁵S]methionine in tissue minces incubated *in vitro* followed by analysis of labelled proteins on polyacrylamide gels containing sodium dodecyl sulphate. Rates of synthesis were highest in the proximal cauda > distal cauda > initial segment > ductuli efferentes > corpus > distal caput > proximal caput. One protein (mol.wt. 23 000) characterized the initial segment, three proteins (mol.wts. 18 500, 19 000 and 32 000) the caput and one protein (mol.wt. 47 000) the cauda. 2. After castration, [³⁵S]methionine incorporation in all regions of the epididymis was reduced to <10% of that in normal animals but could be restored to control levels within 5 days by testosterone treatment. Other steroids (corticosterone, oestrogen or progesterone) were ineffective. 3. The synthesis of the 18 500, 19 000, and 32 000 mol.wt. proteins in the caput and the 47 000 mol.wt. protein in the cauda were preferentially regulated by androgens, whilst the synthesis of 23 000 and approx. 80 000 mol.wt. proteins in the initial segment was dependent upon factors present in testicular fluid. 4. The androgen-dependent and testicular fluid-dependent proteins were major components of epididymal secretion. Purification and characterization of the 18 500, 19 000, 23 000 and 32 000 mol.wt. proteins showed them to be acidic glycoproteins with a carbohydrate content of 7.6–13.2%. The 47 000 mol.wt. protein, on the other hand, is highly basic. 5. A possible role for these proteins in the acquisition of motility, fertilizing capacity and storage of spermatozoa in the epididymis is discussed.

The mammalian epididymis constitutes part of the male accessory glands of reproduction and is an important target organ for androgens (Mann, 1964). Its functions are three-fold: first, it absorbs the relatively large volume of fluid emanating from the testis [1–2 ml/h per 100 g of testis (Setchell, 1970)], a role confined principally to the initial segment and caput epididymidis; second, it produces a specialized milieu for the development of motility and fertilizing capacity of spermatozoa (Bedford, 1966); third, it stores spermatozoa in a quiescent state before they are ejaculated (Jones & Glover, 1975). Androgens are required for both the differentiation of the gland from the mesonephros and for the maintenance of its secretory activity in the adult animal (Mann, 1964; Wilson, 1978). However, unlike the prostate and seminal vesicles, the epididymis receives an addi-

tional supply of androgens directly from the testis via testicular fluid as well as from the normal blood and lymphatic supply. This testicular fluid testosterone, which in the rat is 40-fold higher than in blood plasma (Bartke *et al.*, 1975), is thought to be important for maintaining the full secretory and absorptive activity of the initial segment and proximal caput epididymidis (Fawcett & Hoffer, 1979).

To elucidate the mechanisms involved in sperm maturation and storage several investigators have studied the protein composition of epididymal secretion (Huang & Johnson, 1975; Koskimies & Kormano, 1975). Although epididymal secretion is rich in proteins (30–60 mg/ml) some of these originate in the testis, e.g. androgen-binding protein (French & Ritzen, 1973). Recent evidence suggests that spermatozoa acquire a coating of a glycoprotein during their transit through the caput

Abbreviation used: SDS, sodium dodecyl sulphate.

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epididymidis and that this process is involved in the acquisition of fertilizing capacity (Fournier-Delpech *et al.*, 1977; Lea *et al.*, 1978). Hoskins and co-workers have also isolated a protein from bovine seminal plasma and have shown that it stimulates forward motility in immature spermatozoa from the caput epididymidis of a number of different species (Acott & Hoskins, 1978; Acott *et al.*, 1979). This protein, designated forward motility protein, originates in the epididymis and has many properties in common with the sperm-coating protein mentioned earlier.

Therefore, current evidence indicates that proteins secreted in the epididymis have an important physiological role in the development of motility and fertilizing capacity by spermatozoa after they leave the testis. As a first step to investigating the interaction between spermatozoa and epididymal secretion we have identified those proteins that are regulated by steroid hormones or factors in testicular fluid. This paper describes the regulation by testosterone of proteins synthesized in different regions of the rat epididymis together with their purification and characterization.

Materials and Methods

Materials

All chemicals were of the highest purity available commercially. Acrylamide, *NN'*-methylenebisacrylamide and sodium dodecyl sulphate were purchased from BDH, Poole, Dorset, U.K. Sigma (London) Chemical Co. Poole, Dorset, U.K., supplied β -mercaptoethanol, molecular weight markers [dalton mark VI vial containing bovine serum albumin (mol.wt. 66 000), ovalbumin (mol.wt. 45 000), pepsin (mol.wt. 34 700), trypsinogen (mol.wt. 24 000), β -lactoglobulin (mol.wt. 18 400) and lysozyme (mol.wt. 14 300)], testosterone, oestradiol-17 β , progesterone and corticosterone. Cyproterone acetate was a gift from Dr. E. Berger, Shering A.G., Berlin. Sephadex and DEAE-Sephadex were obtained from Pharmacia (G.B.), London W5 5SS, U.K. and L-[³⁵S]methionine (900–1200 Ci/mmol) from The Radiochemical Centre, Amersham, Bucks., U.K. Neuraminidase (Behringwerke) from *Cholera vibrio* was supplied by Hoechst, Hounslow TW4 6JH, U.K.

Animals

Adult male Wistar rats each weighing 300–350 g were used throughout these experiments. Animals were anaesthetized with diethyl ether, the testes were removed through a scrotal incision, and the epididymides returned to the scrotum. In some experiments, the ductuli efferentes were ligated close to the extra-testicular rete testis with sterile braided silk (Ethicon, 00) and the testis and epididymis replaced

in the scrotum. Steroids were solubilized in arachis oil and 0.1 ml was injected subcutaneously in the flank region. Cyproterone acetate was dissolved in benzyl benzoate/castor oil (1:5, v/v). Doses administered were 1 mg/day per rat for testosterone, corticosterone, progesterone and oestradiol, and 20 mg/day per rat for cyproterone acetate. Animals were killed by cervical dislocation and excised tissues were placed immediately in phosphate-buffered saline chilled on ice.

Incorporation of [³⁵S]methionine into protein

Each epididymis was divided into ductuli efferentes, initial segment, proximal caput, distal caput, corpus, proximal cauda and distal cauda (Hamilton, 1975) and segments from three animals were pooled. After weighing, the tissues were minced in sterile phosphate-buffered saline, washed twice to remove excess spermatozoa, and 30 mg portions of wet tissue were transferred to sterile glass vials containing 1 ml of Dulbecco's modified Eagle's medium (without methionine; Dulbecco & Freeman, 1959) and 15 μ Ci of L-[³⁵S]methionine (specific activity 900–1300 Ci/mol). Vials were gassed for 2 min with air/CO₂ (19:1) and incubated with shaking at 33°C. This temperature was selected to correspond with that of the testis and epididymis *in vivo*, which is 3–4°C below body temperature. After 2 h an equal volume of ice-cold sucrose medium (0.7 M-sucrose containing 6 mM-MgCl₂, 0.50 M-Tris/HCl pH 7.5, 50 mM-KCl and 0.2% Triton X-100) was added, the tissue was homogenized with a motor-driven Teflon homogenizer at 500 rev./min. for 30 s, and centrifuged at 1000 *g* for 15 min. Incorporation of labelled methionine into total protein was measured by mixing portions of the post-nuclear supernatant with ice-cold 5% (w/v) trichloroacetic acid and collecting the precipitated protein on glass fibre filters (GF/C, Whatman Biochemicals, Maidstone, Kent, U.K.). Preliminary experiments showed that [³⁵S]methionine incorporation was linear in tissue minces from all regions of the epididymis for at least 4 h at 33°C.

Analytical electrophoresis

Denaturing gels. Electrophoresis was carried out on slab gels (10 cm $\times$ 16 cm $\times$ 0.2 cm) consisting of 15% (w/v) polyacrylamide, 0.1% (w/v) *NN'*-methylenebisacrylamide and 0.3% (w/v) sodium dodecyl sulphate with a 5% stacking gel (1 cm $\times$ 16 cm $\times$ 0.2 cm) (Studier, 1970). Electrophoresis buffer contained 1% (w/v) sodium dodecyl sulphate in 62 mM-Tris/glycine, pH 8.2. Gels were stained with 0.05% (w/v) Coomassie Brilliant Blue or Naphthalene Black in an aqueous solution containing 40% (v/v) methanol and 7% (v/v) acetic acid for 1 h at room temperature and destained overnight in the same solvent without dye.

Non-denaturing gels. Samples were dialysed overnight at 4°C against electrophoresis buffer (10 mM-Tris/glycine, pH 8.3) and applied to slab gels (10 cm × 16 cm × 0.2 cm) containing 7% (w/v) polyacrylamide, 0.18% (w/v) *NN'*-methylenebisacrylamide, 0.03% (v/v) *NNN'*-tetramethylethylenediamine and 0.7 mg of ammonium persulphate/ml in 373 mM-Tris/HCl buffer, pH 8.9 (Davis, 1964). Stacking gel (1 cm × 16 cm × 0.2 cm) consisted of 2.5% (w/v) polyacrylamide, 0.625% (w/v) *NN'*-methylenebisacrylamide, 0.06% (v/v) *NNN'*-tetramethylethylenediamine and 0.005% (w/v) riboflavin in 62.5 mM-Tris/HCl, pH 6.7. Gels were photopolymerized by exposing them to u.v. light at room temperature. Proteins were stained as described above.

Detection of radioactive proteins. Portions (1 ml) of the post-nuclear supernatants were dialysed overnight against 6.25 mM-Tris/glycine, pH 7.5, containing 0.1% (w/v) sodium dodecyl sulphate, freeze dried, and solubilized in 0.1 ml of distilled water containing 1% (v/v) β -mercaptoethanol. Samples were boiled for 2 min, and a portion (40 000 c.p.m.) was applied to gels. After electrophoresis, proteins were stained with Coomassie Blue and gels were impregnated with 2,5-diphenoxazole as described by Bonner & Laskey (1974). Fluorographs were prepared by placing the dried gel in contact with Kodak X-Omat H film at -70°C for up to 1 week. Labelled proteins were quantified by cutting out the appropriate region of the gel, digesting it in 1.0 ml 30% (v/v) hydrogen peroxide at 55° for 48 h, and measuring its radioactivity in 5 ml of scintillation fluid (Cocktail N, Fisons Scientific, Loughborough, Leics. U.K.).

Isoelectric focusing. Purified proteins were focused on 0.5 × 5.5 cm cylindrical polyacrylamide gels containing 7% (w/v) acrylamide, 0.2% (w/v) *NN'*-methylenebisacrylamide, 5% (v/v) glycerol, 0.05% (v/v) *NNN'*-tetramethylethylenediamine, 0.2% (w/v) persulphate and 2% Ampholine, pH range 3.5–10. Current was set at 2 mA/gel.

Collection of secreted proteins and purification of androgen-dependent proteins

Luminal contents were collected from the cauda epididymidis of normal rats by retrograde injection of phosphate-buffered saline via the vas deferens. A small bubble of mineral oil stained with Sudan Black B preceded the saline and served to prevent undue dilution of the luminal contents. All manipulations were performed under a ×10 stereomicroscope. Between 10–20 μ l of luminal contents was diluted with 300 μ l of phosphate-buffered saline, mixed thoroughly to disperse spermatozoa, and centrifuged at 10 000 *g* for 5 min in a Beckman Microfuge. The supernatant contained 2–3 mg of protein/ml.

All purification procedures were carried out at 4°C. The diluted cauda epididymal plasma was dialysed overnight against 0.05 M-Tris/HCl, pH 7.9, and centrifuged at 15 000 *g* for 15 min. The clear supernatant, containing 15–20 mg of protein, was loaded onto a column (14 cm × 1 cm²) of DEAE-Sephadex A25 equilibrated in the same buffer and absorbed proteins were eluted with a gradient of 0–0.3 M-NaCl. Fractions from selected peaks were analysed on denaturing and non-denaturing polyacrylamide slab gels as described above. The fractions containing highly purified protein were pooled, dialysed exhaustively against distilled water, and freeze dried. In some cases further purification was obtained on a second DEAE-Sephadex A25 column (6 cm × 0.8 cm²) using a 0–0.2 M-NaCl gradient and the purified proteins were processed as described above.

Gel filtration was performed on a column (90 cm × 2 cm²) of Sephadex G-75 equilibrated in 20 mM-Tris/HCl, pH 8.2, containing 150 mM-NaCl. Standards were bovine serum albumin (mol.wt. 67 000), ovalbumin (mol.wt. 45 000), soya bean trypsin inhibitor (mol.wt. 22 000) and cytochrome c (mol.wt. 12 300).

Neuraminidase digestion of androgen-dependent proteins

Approximately 80 μ g of each purified protein was mixed with 0.15 ml of 93 mM-Tris/maleate/methiolate buffer (Bourns *et al.*, 1970) and 30 μ l of neuraminidase solution (500 units/ml: 1 unit will liberate 1.0 μ mol of *N*-acetylneuraminic acid/min at 37°C, pH 5.5, using human α -1-acid glycoprotein as substrate). The mixtures were incubated at 25°C and portions were withdrawn at 6, 18 and 48 h for electrophoresis on denaturing and non-denaturing polyacrylamide gels.

Amino-acid analysis of proteins

Purified proteins were freeze dried in hydrolysis tubes and treated with performic acid (Moore, 1963) to convert cysteine and methionine to cysteic acid and methionine sulphone. Samples were then hydrolysed at 110°C for 18 h in 0.25 ml of 5.7 M-HCl containing 5 μ l of butanedithiol. Amino acids were analysed on a Durrum D-500 automated analyser.

Histology

Epididymides were fixed in Bouin's fluid for 24 h, embedded in wax and processed by routine histological procedures.

Other procedures

Total protein was measured by the Hartree (1972) modification of the Lowry method using bovine serum albumin as standard. Total carbohydrate was assayed by the orcinol reaction (Vasseur, 1948).

Radioactivity was measured with a Nuclear Chicago liquid-scintillation counter using a scintillation cocktail consisting of 6 g of butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] (Ciba ARL, Duxford, Cambs., U.K.) in 250 ml of Triton X-100, 45 ml of water and 705 ml of toluene. Efficiency for ^{35}S was 35%. DNA was measured at 260 nm after extraction of tissue in phenol/chloroform followed by alkaline hydrolysis (Penman *et al.*, 1968).

Results

Protein synthesis in rat epididymis

Initial experiments were designed to identify specific proteins synthesized by different regions of the normal epididymis and to investigate if their synthesis was regulated by androgens or factors present in testicular fluid. The proteins synthesized in seven regions of the epididymis, namely, ductuli efferentes, initial segment, proximal caput, distal caput, corpus, proximal cauda and distal cauda, were identified by fluorography on SDS/polyacrylamide gels. The rate of incorporation of [^{35}S]methionine into protein was highest in the proximal cauda > distal cauda > initial segment > ductuli efferentes > corpus > distal caput > proximal

caput. Several proteins were confined to specific regions of the epididymis (Fig. 1); one protein (mol.wt. 23 000) was characteristic of the initial segment, two proteins (mol.wts. 19 000 and 32 000) the caput and one protein (mol.wt. 47 000) the cauda. In most subsequent experiments in which the hormonal regulation of these proteins was investigated, tissue from the initial segment, proximal caput and distal caput was pooled, as was tissue from the proximal cauda and distal cauda.

Effects of castration and testosterone replacement on protein synthesis

Total incorporation of [^{35}S]methionine into protein in all regions of the epididymis decreased sharply after castration and after eight days had declined to about 10% of that in tissue from normal animals (Fig. 2). Castration for 10–14 days did not cause a further significant decrease and hence 8-day castrated animals were used in all following experiments. Testosterone replacement reversed these changes very rapidly; [^{35}S]methionine incorporation into protein was stimulated 2–3-fold above levels in castrated animals 12 h after the first injection, reaching 7–10-fold after 1–2 days. There was also a tendency for an overshoot phenomenon to occur; that is, after an initial increase, protein synthesis declined slightly on the third and fourth days after testosterone treatment but then recovered slowly by the fifth day. The possibility that the early restorative action of testosterone on protein synthesis was caused by a stimulation of cell proliferation leading to an increase in cell number is unlikely for two reasons. First, the levels of DNA in the caput epididymidis of 8-day castrated animals given testosterone for 0, 1, 2 and 4 days were 0.27, 0.24, 0.25 and 0.29 mg/caput respectively. Histological sections of the caput epididymidis confirmed that there were no spermatozoa in the caput region 8 days post castration, and therefore the above values represent only tissue DNA. The results indicate that cell division had not been initiated until 3–4 days after androgen therapy began. Second, Niemi & Tuohimaa (1971) have shown that the mitotic index in the epididymis after castration and testosterone treatment is much lower than in either the ventral prostate or seminal vesicles in which cell proliferation does not begin until 36–48 h after testosterone replacement (Rennie *et al.*, 1975; Higgins *et al.*, 1976a). It was also noticeable that the wet weight of each region did not increase significantly over the 5-day period of testosterone treatment.

Analysis of labelled proteins on SDS/polyacrylamide gels by fluorography revealed that testosterone regulated the synthesis of specific proteins in addition to its effects on general protein synthesis (Fig. 3). Thus, in the caput, the synthesis of proteins with mol.wts., of 32 000 and 19 000, and

Fig. 1 Fluorograph of [^{35}S]methionine-labelled proteins from different regions of the normal rat epididymis. Minced tissue was incubated with [^{35}S]methionine and 1000 g postnuclear supernatant fractions were analysed on SDS/polyacrylamide gels as described in the Materials and Methods section. Each track contained 40 000 c.p.m.; radioactive proteins were detected by fluorography. Material loaded on the gel was from: (1) ductuli efferentes; (2) initial segment; (3) proximal caput; (4) distal caput; (5) corpus; (6) proximal cauda; (7) distal cauda. This subdivision of the epididymis is based on the classification by Hamilton (1975).

Fig. 2. Effect of castration and testosterone replacement on epididymal protein synthesis

Epididymides were removed from groups of three rats at different periods after castration and testosterone replacement and were divided into initial segment, proximal + distal caput, corpus and proximal + distal cauda. Tissue within each region was pooled and weighed. Portions (approx. 30mg) of minced tissue were incubated with $15\mu\text{Ci}$ of [^{35}S]methionine for 2h at 33°C and total protein synthesis in each region was measured after precipitation with 5% trichloroacetic acid. Each value represents the mean of duplicate determinations. ●, [^{35}S]methionine incorporation; ○, wet weight. (a) initial segment; (b) caput; (c) corpus; (d) cauda. Inserts show the incorporation of [^{35}S]methionine into the 18500 + 19000 mol.wt. proteins in the caput and the 47000 mol.wt. protein in the cauda after analysis of labelled proteins on SDS/polyacrylamide gels followed by counting the appropriate region of the gel. Each track contained 60000 c.p.m. (i) Normal rat; (ii) 8-day castrated rats; (iii), (iv) and (v) 8-day castrated rats receiving testosterone for 12, 24 and 48h respectively.

in the cauda a protein of mol.wt. 47000, was decreased by castration and restored by testosterone. Quantification of the amounts of [^{35}S]methionine in the proteins of mol.wt. 19000 and 47000 indicated that the fraction of total protein synthesized falls from approximately 4 and 6% respectively in normal animals to approximately 0.5 and 1% respectively 8 days after castration (Fig. 2b and d, inserts). Testosterone restores the synthesis of these proteins to about normal levels, an increase being apparent 12h after the first injection.

Examination of diluted cauda epididymal plasma on SDS/polyacrylamide gels (Fig. 5a, track 1) indicates that all of these androgen-dependent proteins are secreted and together comprise over 50% of the protein in epididymal plasma. In fact subsequent purification and characterization of the

19000-mol.wt. protein showed that it consists of two closely migrating proteins with mol.wts. of about 18500 and 19000 on SDS/polyacrylamide gels (see below).

Only testosterone restored the synthesis of these proteins in that treatment of 8-day castrated animals with oestradiol- 17β , progesterone, or corticosterone was ineffective. Interestingly, a high dose of the anti-androgen cyproterone acetate (20mg/day per rat) given in combination with testosterone, did not decrease the synthesis of any of the androgen-dependent proteins in the caput or cauda epididymidis by more than 15%, although under identical experimental conditions to those described here it has been shown to be highly effective in inhibiting the synthesis of the ventral prostate and seminal vesicle androgen-dependent proteins (Higgins *et al.*,

Fig. 3. Fluorograph of [^{35}S]methionine-labelled proteins from (a) *caput epididymis* and (b) *cauda epididymis*, taken from animals of different hormonal status

Minced epididymal tissue was incubated with [^{35}S]methionine and fluorographs were prepared as described in the Materials and Methods section. Each track contained 40000 c.p.m. Labelled proteins were from: (1) normal rats; (2) rats castrated for 8 days; (3), (4) and (5) rats castrated for 8 days and treated with testosterone for 12, 24 and 48 h respectively. Arrows indicate position of androgen-dependent proteins.

1976b; Parker *et al.*, 1978). This anomalous response of the epididymis to cyproterone acetate has been noted earlier (Jones, 1977).

We also investigated the synthesis of the epididymal androgen-dependent proteins in relation to post-natal development. The proteins of mol.wts. 18500, and 19000 were produced in the *caput epididymidis* from rats as early as 10 days *post partum*, but the 47000-mol.wt. protein was not synthesized by the *cauda epididymidis* until animals were at least 20 days old. Finally, we have examined the tissue specificity of these proteins. A preliminary survey of accessory sex glands indicates that the mobilities of the androgen-dependent proteins in the epididymis are different from those in other tissues, supporting the notion that they are specific to distinct regions of the epididymis.

Effects of ligating the ductuli efferentes on protein synthesis in the caput epididymidis

Evidence suggests that substances in testicular fluid are necessary to maintain the activity of the initial segment and proximal *caput epididymidis* (Elliott, 1965; Brooks, 1979; Fawcett & Hoffer, 1979). Therefore, it was of interest to investigate if the synthesis of the *caput*-specific androgen-dependent proteins was affected by ligation of the ductuli efferentes. The incorporation of [^{35}S]methionine into the 18500 and 19000 mol.wt. proteins was hardly altered by ligation of ductuli for 14 days (Fig. 4) or 28 days (R. Jones, unpublished work). However, synthesis of a protein of mol.wt. approx. 80000 was

Fig. 4. Fluorograph of [^{35}S]methionine-labelled tissue proteins from initial segment and proximal *caput epididymidis* after castration and after ligation of the ductuli efferentes

Minced tissue from the initial segment and proximal *caput epididymidis* was incubated with [^{35}S]methionine and fluorographs were prepared as described in Materials and Methods section. Each track contained 40000 c.p.m. and represents: (1) normal rats; (2) rats 14 days after ligation of the ductuli efferentes; (3) rats 8 days after castration; (4) rats 8 days after castration + testosterone replacement for 4 days. The small arrow indicates the position of the androgen-dependent proteins and the large arrows indicate the position of the proteins whose synthesis is stimulated by testicular fluid.

decreased to about 50% of control levels, and incorporation of label into the 23 000 mol.wt. protein that is specific to the initial segment was abolished altogether. Further evidence that the synthesis of the 23 000 and approx. 80 000 mol.wt. proteins is dependent upon factors present in testicular fluid is provided by the finding that they were not restored by administering testosterone to castrated animals.

Purification and characterization of specific epididymal proteins

On the basis of their tissue specificity and hormonal regulation, several proteins in secreted plasma collected from the cauda epididymidis were purified by ion-exchange chromatography. These were (a) two androgen-dependent proteins with mol.wts. 18 500 and 19 000 synthesized in the caput region and that together comprised about 35% of secreted protein; (b) one protein with mol.wt. 23 000 whose synthesis was abolished by ligation of the ductuli efferentes; and (c) a diffuse-staining protein, mol.wt. 32 000, synthesized in the caput epididymidis. The cauda-specific androgen-dependent protein (mol.wt. 47 000) was also present in secreted plasma, but to date has not been purified.

The purification of the proteins from diluted cauda epididymal plasma was monitored on both denaturing and non-denaturing polyacrylamide gels, as non-denaturing gels at pH 8.3 revealed a simpler pattern of proteins, consisting of five major bands versus approx. 20 on denaturing gels (Fig. 5). After

one chromatographic step on DEAE-Sephadex at pH 7.9, bound proteins were eluted as four peaks, designated I–IV (Fig. 6). Peaks I and IV contained purified proteins of mol.wt. 23 000 and 32 000 respectively and peaks II and III the 18 500 and 19 000 mol.wt. proteins respectively. To achieve better separation of peaks II and III, the appropriate fractions were pooled, reapplied to a second DEAE column and eluted with a shallower gradient. When analysed on the two gel systems it was found that the proteins with mol.wts. 23 000, 18 500, 19 000 and 32 000 in peaks I, II, III and IV respectively were purified to >95% homogeneity (Fig. 5). Although the 32 000 mol.wt. protein gave a single band on SDS gels, on non-denaturing gels it consisted of two polypeptides that to date have not been separated.

All of the purified proteins were present as single bands on SDS/polyacrylamide gels, with or without prior reduction of disulphide bonds by β -mercaptoethanol. Moreover, their mobility was unaltered in the absence of β -mercaptoethanol, suggesting that they are monomeric, a conclusion confirmed by molecular weight estimates on Sephadex G-75 (Table 1). All the proteins gave a positive reaction in the orcinol test, with carbohydrate contents ranging from 7.6 to 13.2% using glucose as standard. With galactose or mannose as standard, values were 12–14% higher. Glucosamine and β -N-acetylglucosamine do not react with orcinol reagent, and since most glycoproteins contain

Fig. 5. Electrophoresis of purified epididymal proteins

Epididymal plasma proteins, purified by chromatography on DEAE-Sephadex A25 (Fig. 6), were analysed by electrophoresis (a) on denaturing gels containing SDS and (b) on non-denaturing gels. Proteins were stained with Coomassie Blue. Tracks (1), (6) and (11) represent unfractionated diluted cauda epididymal plasma; (2) and (7) represent peak I; (3) and (8) represent peak II; (4) and (9) represent peak III; (5) and (10) represent peak IV; the amounts of protein applied to (a) were 100 μ g of diluted cauda epididymal plasma and 10 μ g of purified proteins and onto (b) 100 μ g of diluted cauda epididymal plasma and 20 μ g of purified proteins. Arrows indicate the position of androgen-dependent proteins and the asterisk the testicular-fluid-dependent protein.

Fig. 6. *Chromatography of diluted cauda epididymal plasma proteins on DEAE-Sephadex A25*
The 15 000 g supernatant solution obtained after dialysis of diluted cauda epididymal plasma against 0.05 M-Tris/HCl, pH 7.9, was loaded onto a column (14 cm $\times$ 2 cm²) of DEAE-Sephadex A25 in the same buffer and proteins were eluted with a gradient of 0–0.3 M-NaCl. Fractions (1.2 ml) were collected and their protein concentration was monitored at 280 nm.

Table 1. *Summary of some properties of the epididymal-specific proteins*
Figures in parentheses refer to the elution pattern on DEAE-Sephadex (Fig. 6).

Mol.wt. on		Carbohydrate content (%)	pI	Region where synthesized
SDS/polyacrylamide gels	Sephadex G-75			
18 500 (II)	21 000	13.2	5.9	Caput
19 000 (III)	23 000	7.6	5.7	Caput
23 000 (I)	23 500	11.1	5.4	Initial segment
32 000 (IV)	32 000	9.6	4.2	Caput
47 000	—	—	>7.9	Cauda
~80 000	—	—	—	Caput

Table 2. *Amino-acid composition of purified epididymal-specific proteins*

Epididymal plasma proteins, purified by chromatography on DEAE-Sephadex A25 (Figs. 5 and 6) were hydrolysed and the amino acids were analysed as described in the Materials and Methods section. Values for aspartic acid and glutamic acid include asparagine and glutamine respectively.

Amino-acid composition (mol/100 mol recovered)

Amino acid	Protein mol.wt. (by SDS/ polyacrylamide gels) ...				
		23 000	18 500	19 000	32 000
Aspartic acid		11.2	9.9	8.8	13.8
Threonine		4.4	7.3	7.7	5.4
Serine		8.9	7.2	6.7	7.6
Glutamic acid		11.8	11.8	11.5	11.8
Proline		7.6	4.3	2.9	8.8
Glycine		10.5	7.4	7.7	7.6
Alanine		6.1	7.4	8.7	4.8
Valine		7.7	6.9	7.5	7.2
Methionine		1.3	2.4	2.2	1.3
Isoleucine		2.5	3.7	3.5	2.3
Leucine		8.7	9.4	9.8	8.0
Tyrosine		3.1	4.0	4.4	5.2
Phenylalanine		3.0	4.2	4.2	3.4
Histidine		3.2	3.2	3.1	3.1
Lysine		6.5	7.6	7.9	5.7
Arginine		3.5	3.3	3.0	3.8

significant quantities of these two sugars (Marshall & Neuberger, 1972), the above values may be underestimates. Incubation of the purified proteins with neuraminidase at 25°C for 6–48 h did not alter their mobility on polyacrylamide gels, suggesting that they have a low content of sialic acid. The activity of the neuraminidase was checked against Cowper's-gland mucin (Boursnell *et al.*, 1970).

Isoelectric focusing on 7% polyacrylamide gels indicated that the proteins are acidic (Table 1) as expected from their elution pattern on DEAE-Sephadex at pH 7.9. Since the 47 000 mol.wt. protein was not bound to the column under these conditions it is assumed that its isoelectric point is greater than 7.9.

Amino acid analysis of the purified proteins (Table 2) showed that in each case aspartic acid and glutamic acid amounted to more than 20% of the total. Presumably, the proportion of asparagine and glutamine residues must be low to account for the acidic nature of the proteins. Proline residues were unusually abundant in the 23 000 and 32 000 mol.wt. proteins.

Discussion

The identification and characterization of proteins in epididymal secretion is essential to investigate their role in the development of motility and fertilizing capacity by spermatozoa after they leave the testis. We have found that the synthesis of all of the major acidic proteins revealed on Ornstein-Davis gels are regulated either by testosterone or by factors present in testicular fluid and we estimate that they comprise over 50% of the proteins in epididymal secretion. Of the six polypeptides whose synthesis is hormonally regulated (Table 1), four have been purified and characterized biochemically. Three, synthesized specifically in the caput epididymidis, are induced by testosterone whilst the fourth, synthesized in the initial segment region, is controlled by factors in testicular fluid.

The kinetics of [³⁵S]methionine incorporation into protein after induction by testosterone indicate that the effects of androgens on the epididymis are rapid and that they stimulate the synthesis *de novo* of proteins rather than their formation from inactive precursors. Of the proteins whose synthesis is stimulated by testosterone, the polypeptides with mol.wts. 18 500 and 19 000 in the caput and mol.wt. 47 000 in the cauda provide the best markers for androgen action. Since these proteins are affected to a much greater extent by androgen manipulation than other proteins, it is evident that the hormone is acting differentially. This resembles the situation in the ventral prostate (Parker *et al.*, 1978) rather than in the seminal vesicle (Higgins *et al.*, 1976a,b) in

which testosterone appears to exert only a generalized response.

The inability of exogenous testosterone to restore the synthesis of the 23 000 mol.wt. protein is probably related to the dependence of the initial segment region on testicular fluid (Fawcett & Hoffer, 1979). Although we have not classified this protein as androgen-dependent, this may not be strictly accurate since its synthesis may require a high level of intraluminal testosterone. In the rat, the combined levels of testosterone and 5 α -dihydro-testosterone in testicular fluid entering the initial segment amount to 51.4 ng/ml as opposed to 1.3 ng/ml in peripheral blood plasma (Bartke *et al.*, 1975). In addition, testicular fluid contains androgen-binding protein (mol.wt. 90 000–95 000; Weddington *et al.*, 1975), a specific secretory product of Sertoli cells in the testis. Androgen-binding protein is absorbed in the initial segment and caput epididymidis and presumably transports a high concentration of androgen into the secretory epithelium. The subsequent degradation of the binding protein-androgen complex would release large amounts of testosterone and 5 α -dihydro-testosterone intracellularly and thereby stimulate the synthesis of the 23 000 mol.wt. protein. Whether or not the androgens regulate its synthesis, we have to postulate that the mechanism of action is different from that controlling the 18 500 and 19 000 mol.wt. proteins which presumably are regulated via the androgen-receptor complex (Danzo & Eller, 1978). Other components of testicular fluid, e.g. spermatozoa or inhibin (Main *et al.*, 1979) may also be involved.

Previous investigators have shown the presence of a few bulk proteins in whole epididymal cytosol (Cameo & Blaquier, 1976). Our analysis of secreted protein confirms the presence of five major acidic proteins. Most interest has centred on the 32 000 mol.wt. protein as a protein with similar molecular weight has been detected on the plasma membrane of spermatozoa from the cauda, but not from the caput epididymidis (Fournier-Delpech *et al.* 1973, 1977; Olson & Hamilton, 1978; Lea *et al.*, 1978). Lea *et al.* (1978) and Garberi *et al.* (1979) have purified this protein and our results are in good agreement with theirs. It remains to be demonstrated if there is a significant interaction between the 18 500 and 19 000 mol.wt. proteins and spermatozoa and if these proteins play a role in the acquisition of fertilizing capacity.

We also discovered an androgen-dependent protein synthesized solely in the cauda epididymidis. This protein (mol.wt. 47 000) is highly basic but since it is not a major secreted protein its isolation has not yet been accomplished. As spermatozoa are fully fertile by the time they arrive in the cauda epididymidis this protein may have a role in sperm

storage rather than in sperm maturation. Further experiments are needed to achieve its purification and characterization.

The results presented in this communication, therefore, should provide the basis for further studies on hormone action in this accessory gland and for investigating the physiological role of androgen-dependent proteins.

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