Androgen-Dependent Synthesis of Basic Secretory Proteins by the Rat Seminal Vesicle

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1. Two basic proteins were purified from secretions of rat seminal vesicles by using Sephadex G-200 chromatography and polyacrylamide-gel electrophoresis under denaturing conditions. 2. It is not certain that these two proteins are distinct species and not sub-units of a larger protein, but their properties are similar. Highly basic ($pI = 9.7$), they migrate to the cathode at high pH and their amino acid composition shows them to be rich in basic residues and serine. Threonine and hydrophobic residues are few. Both proteins are glycoproteins and have mol.wts. of 17000 and 18500. 3. Together these two proteins account for 25–30% of the protein synthesized by the vesicles, but they are absent from other tissues. 4. Changes in androgen status of the animal markedly affect these proteins. After castration, a progressive decrease in the basic proteins is observed and the synthesis of the two proteins as measured by [35 S]methionine incorporation *in vitro* is decreased. Testosterone administration *in vivo* rapidly restores their rates of synthesis. 5. These effects on specific protein synthesis are also observed for total cellular protein, and it is suggested that testosterone acts generally on the total protein-synthetic capacity of the cell and not specifically on individual proteins. Proliferative responses in the secretory epithelium may also be involved. 6. The extreme steroid specificity of the induction process suggests that the synthesis of these basic proteins is mediated by the androgen-receptor system. 7. The biological function of these proteins is not clear, but they do not appear to be involved in the formation of the copulatory plug.

Steroid hormones control many aspects of cellular development, differentiation and metabolic activity [for a detailed review, see Litwack (1975)]. In recent years considerable progress has been made towards an understanding of the nature of steroid-hormone action at the molecular level [for a detailed review, see King & Mainwaring (1974)]. The principal plasma androgen, testosterone, is first converted into the active steroid, 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstan-3-one), by a 5 α -reductase present in the androgen-dependent accessory sexual tissue (Bruchovsky & Wilson, 1968; Anderson & Liao, 1968). This steroid activation is followed by binding of dihydrotestosterone with high affinity to cytoplasmic receptor proteins (Fang *et al.*, 1969; Mainwaring, 1969; Unhjem *et al.*, 1969). Conformational changes in the receptor-steroid complex then occur (Mainwaring & Irving, 1973), and the complex becomes associated with chromatin in the nucleus (Mangan *et al.*, 1968; Fang & Liao, 1971; Mainwaring & Peterken, 1971). It is thought that the complex acts as a genetic regulatory element, directly or indirectly altering the transcription of specific parts of the genome to produce, ultimately, the observed biological response.

Control of the synthesis of specific proteins by steroids has been extensively studied in relatively few systems (Tomkins, 1974; Rosen & O'Malley, 1975; Schimke *et al.*, 1975; Schutz *et al.*, 1975), but it appears that these steroid-mediated effects can be ascribed to changes in the cellular concentration of active messenger ribonucleic acids for the specific proteins (Means *et al.*, 1972; Scott *et al.*, 1972; Rhoads *et al.*, 1973; Schutz *et al.*, 1973; Steinberg *et al.*, 1975).

Although progress has been impressive for some classes of steroid hormones, the mechanism of action of androgenic steroids is less clearly understood, principally because no suitable system involving androgen induction of specific proteins has been described. Androgen-elicited increases in rat prostatic aldolase (Mainwaring *et al.*, 1974) are unfortunately too small to be of use in this respect, and further, this enzyme is not tissue-specific. Significant progress in this field requires a system in which androgens evoke substantial induction of a specific protein accounting for a major part of the protein output of the responsive cells.

The seminal vesicles of the rat elaborate a major part of the protein-rich seminal plasma (Mann, 1964).

In common with other male accessory sexual organs, their functional and structural integrity depends on the continuous presence of testosterone (Moore *et al.*, 1930; Price & Williams-Ashman, 1961). In the present paper we describe the isolation and purification of characteristic basic secretory proteins from rat seminal vesicles and demonstrate that their synthesis is androgen-dependent. We suggest that further investigations of this system will advance our understanding of the molecular mode of action of the androgens.

Materials and Methods

Animals

Adult male Sprague-Dawley rats (200–250 g) from this Institution were used. Bilateral orchidectomy was performed by the scrotal route under Fluothane anaesthesia. Steroids (in arachis oil) were injected subcutaneously in the flank region. Doses were corticosterone and testosterone, 1.25 mg, oestradiol, 10 μ g, and cyproterone (17-acetoxy-6 α -chloro-1 β ,2 α -methylene-pregna-4,6-diene-3,20-dione) acetate, 25 mg per animal. Controls received injections of oil alone. Animals were killed by cervical dislocation.

Purification of basic proteins from vesicular secretion

Seminal vesicles of intact (not orchidectomized) animals were clamped off and removed after coagulating glands and connective tissue had been dissected away. Secretion was expressed into about 10 vol. of 0.15 M-NaCl/3 M-urea at 0°C and mixed thoroughly. Insoluble material was removed by centrifugation at 20000g for 15 min and the NaCl/urea-soluble fraction was loaded on a column (100 cm $\times$ 2.5 cm diam.) of Sephadex G-200 equilibrated with 3 M-urea/0.05 M-potassium phosphate buffer, pH 7.5, at 4°C. Elution was with the same buffer; 6.8 ml fractions were collected. Those containing protein (shown by E_{280}) were combined, dialysed against 3 mM-NH₄HCO₃ at 4°C, freeze-dried, redissolved in 6 M-urea (at 10–20 mg/ml) and stored at –20°C until required. Large-scale denaturing polyacrylamide-gel electrophoresis was used to separate the basic proteins further. A water-cooled electrophoresis apparatus (Shandon) was used at 4°C. The cylindrical gel system consisted of a 12 cm separating gel with a 2 cm stacking gel each, containing 0.1% sodium dodecyl sulphate and 3 M-urea (see below). Up to 6 mg of protein prepared from the Sephadex G-200 column above was made up in 1% sodium dodecyl sulphate/6 M-urea/0.14 M-mercapto-ethanol together with 5 μ g of Bromophenol Blue. The gel was run at 35 mA/200 V until the tracking dye was 1–2 cm from the end of the separating gel. The gel was removed and stored at 0°C while a thin longitudinal slice (0.2 cm thick) was stained with 1% (w/v)

Naphthalene Black to locate the protein bands. The appropriate regions were cut from the main portion of the gel, chopped finely with scissors in cold 3 M-urea/0.05 M-phosphate, pH 7.5 (30 ml), and then disrupted with a blender (Silverson, slow speed), avoiding foaming. The polyacrylamide was filtered off and re-extracted. The combined extracts were dialysed at 4°C against 3 mM-NH₄HCO₃, freeze-dried and dissolved in 6 M-urea (at 1 mg/ml).

Extraction of basic protein from seminal vesicles

The vesicles plus any contained secretion were cut up finely with scissors in homogenization buffer (2 ml per gland) consisting of 0.02 M-Tricine [*N*-tris-(hydroxymethyl)methylglycine], pH 7.6, 8 M-urea, 0.001 M-MgCl₂ and 0.002 M-CaCl₂ at 0°C and then disrupted with a laboratory blender (Silverson Machines Ltd., Chesham, Bucks., U.K.), for 1 min at slow speed and 30 s at full speed. Silicone antifoam emulsion [Sigma (London) Chemical Co., Kingston-on-Thames, Surrey, U.K.; 0.1%, v/v] controlled foaming of the secretion.

Insoluble material was removed at 30000g for 20 min and re-extracted with an equal volume of homogenization buffer. The combined urea-soluble portions were treated with 2.5 vol. of ethanol plus 0.1 vol. of 0.5 M-potassium acetate buffer, pH 5. Precipitated material was collected by centrifugation (20000g for 10 min), washed with ethanol (70%, v/v) and dissolved overnight at 37°C in 1% (w/v) sodium dodecyl sulphate plus 8 M-urea (5 vol.). The insoluble material remaining was removed by centrifugation (20000g for 10 min), and the supernatant analysed for basic proteins (see below).

In our hands the extraction procedure of Manyai (1964), involving precipitation of total vesicular proteins with HClO₄ followed by extraction of the basic proteins in 1.67 M-NaCl at 100°C, was unsatisfactory, resulting in only partial recovery of basic protein (25–30%).

Incorporation of radioactive amino acids

Seminal vesicles were removed from the animals, care being taken to avoid contamination with coagulating-gland products. Any vesicle secretions were expressed and the glands chopped finely with scissors in 5 ml of Dulbecco's modified Eagle's medium (Dulbecco & Freeman, 1959) (without methionine or serum) at 37°C. The tissue and medium were transferred to stoppered 25 ml conical flasks, 0.1 ml of L-[³⁵S]methionine (50–100 μ Ci; 200–500 Ci/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.) was added, the flasks were flushed with O₂ + CO₂ (95:5) and incubated at 37°C with shaking for 45 min. Cold homogenization medium (6 ml) was then added and the flasks were transferred to an ice

bath. The tissue and medium were processed for basic proteins as described above. Separate experiments showed that under these conditions incorporation was linear for at least 1 h.

Radioactivity incorporated into basic proteins was determined by using acid/urea or denaturing gels (as below). Purified basic protein (20 μ g) was added so that the appropriate regions of the gels could be located after staining with Naphthalene Black. These regions were cut out, the polyacrylamide was digested with H_2O_2 (0.4 ml) at 50–60°C for 1 h and radioactivity determined. Incorporation of radioactivity into total protein was measured by precipitating samples with 5% (w/v) trichloroacetic acid at 0°C and collecting the precipitated protein on membrane filters (Millipore, code HAWP 02500).

Analytical electrophoresis

Polyacrylamide gels (0.5 cm diam.) consisted of a separating gel (7 cm long) and a stacking gel (2 cm); 3 M-urea was present throughout.

(a) *Acid/urea gels.* The method of Reisfeld *et al.* (1962), involving 15% (w/v) polyacrylamide gels, was used, but the tray buffer was diluted 1:10 with 3 M-urea before use. Protein samples (up to 100 μ g in 10–100 μ l) in 6 M-urea were run at 4°C towards the cathode at 2 mA/tube (about 100 V) until the tracking dye (Pyronine Y) reached the end of the separating gel. Gels were stained with Naphthalene Black 10B (Gurr Division, Searle Diagnostic, High Wycombe, Bucks., U.K.; 1%, w/v) in acetic acid/methanol/water (1:4:5, by vol.) for 1–2 h at room temperature (15–25°C). Background stain was removed by using the same solvent without the dye.

(b) *Denaturing gels (Laemmli, 1970).* The 15% polyacrylamide gels contained sodium dodecyl sulphate (0.1%) and urea (3 M). Protein samples were made up in 1% sodium dodecyl sulphate/6 M-urea/0.14 M-mercaptoethanol and heated at 100°C for 90 s. Electrophoresis (2 mA/tube, 150 V) was towards the anode at room temperature until the tracking dye, Bromophenol Blue (5 μ l, 1%), reached the end of the separating gel. Protein bands were fixed and detected with Naphthalene Black 10B as in (a).

(c) *Isoelectric-focusing polyacrylamide gels.* Equal volumes of Ampholines (LKB, Croydon, Surrey), pH ranges 5–8, 8–9.5 and 9–11, were mixed and 0.6 ml of the mixture was added to 10 ml of a mixture of 7% (w/v) acrylamide, 0.8% methylenebisacrylamide, 0.5% *NNN'*-tetramethylethylenediamine, riboflavin (5 μ g/ml) and 3 M-urea. Gels were then photopolymerized (at 340 nm for 30 min), and basic protein (50 μ g) plus 25 μ l of the Ampholine mixture in 6 M-urea was layered on the gel surface. A further 25 μ l of Ampholines in 6 M-urea was placed on top of the protein sample, followed by the cathode buffer (0.1 M-NaOH). Anode buffer was 0.01 M-acetic acid and the

electrophoresis was at 50 V (1 mA/gel) at 4°C until the current decreased almost to zero (about 18 h). The gels were then removed from the tubes, placed on a glass plate cooled on ice and the pH gradient along their length was determined by using an antimony contact electrode and pH-meter (Pye-Unicam model 290). The Ampholines were then dialysed out of the gels with several changes of methanol/acetic acid/water and finally stained for protein with Naphthalene Black.

(d) *Cellulose acetate electrophoresis.* Cellulose acetate strips (Cellogel; Reeve Angel Ltd., London E.C.4, U.K.; 17 cm $\times$ 2.5 cm) were immersed in 3 M-urea/0.05 M-sodium barbitone/HCl buffer, pH 8.5, for 2 h at room temperature. The strips were blotted dry and the protein sample (up to 25 μ g in 25 μ l) was loaded at the centre point. Strips were subjected to 300 V (1 mA/strip) at 4°C for 5 h. Proteins were fixed and detected with Naphthalene Black 10B as in (a).

Determination of protein in polyacrylamide gels

After background stain had been removed, the absorption of the protein-bound dye was determined by scanning the gel at 500 nm in a Pye-Unicam SP.500 spectrophotometer fitted with a gel-scanning attachment and recorder (Gilford Instruments, Oberlin, OH, U.S.A.). The areas under the peaks were computed, and related to basic protein by using a calibration curve. Separate experiments verified that the dye absorption was linearly related to protein content up to at least 15 μ g.

Detection of carbohydrate in polyacrylamide gels

The procedure of Glossmann & Neville (1971), based on periodic acid/Schiff's reagent, was used to detect glycoproteins on denaturing polyacrylamide gels.

Amino acid analysis of proteins

Purified basic protein (20 μ g) in 6 M-urea was precipitated in heat-annealed hydrolysis tubes (4 cm $\times$ 0.5 cm diam.) with trichloroacetic acid (5%, w/v) at 0°C, washed three times with 0.25 ml of trichloroacetic acid, three times with ether and dried *in vacuo*. Before acid hydrolysis, cysteine and methionine were converted into cysteic acid and methionine sulphone respectively with performic acid (Moore, 1963). For acid hydrolysis the tubes, containing 0.25 ml of constant-boiling HCl (5.7 M) and 5 μ l of butanedithiol, were sealed and incubated at 110°C for 16, 24 or 48 h. Amino acids were separated and determined on a single-column automated analyser (Durrum; model D-500), the maker's operating instructions being used.

Other determinations

Radioactivity was measured with a Nuclear-Chicago II liquid-scintillation counter by using scintillation fluid consisting of 6g of butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole; Ciba ARL, Duxford, Cambridge, U.K.] in 250ml of Triton X-100, 45ml of water, 705ml of toluene. Efficiency for ^{35}S was 48–50%. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as standard. By comparing their mobilities on denaturing polyacrylamide gels with those of ten purified proteins (mol.wts. 40000–13000) (Weber *et al.*, 1972), the apparent molecular weights of the basic proteins were determined. Carbohydrate composition of proteins was determined by the method of Bhatti *et al.* (1970), by converting the monosaccharides into their trimethylsilyl ether derivatives and then separating these by g.l.c.

Proteinase activity was determined at 37°C in two ways: (a) by measuring the extent to which an indicator dye was released from the insoluble protein substrate, Azocoll (for details see Calbiochem technical bulletin); (b) by using the synthetic substrate, α -*N*-benzoyl-DL-arginine *p*-nitroanilide hydrochloride (Sigma) (Notides *et al.*, 1973). Ribonuclease was assayed by measuring the extent to which *Torula* yeast total RNA (Sigma) was rendered soluble in HClO_4 /uranyl acetate (Kalnitsky *et al.*, 1959). Deoxyribonuclease (exonuclease) was assayed by using radioactive *Escherichia coli* DNA prepared by the method of Rennie *et al.* (1975). Incubations included 10–20 μg of [^3H]DNA (40000–80000 d.p.m.), 5 μmol of MgCl_2 , 50 μmol of Tris/HCl buffer, pH 7.4, and seminal-vesicle basic protein (1 mg) in 0.5 ml. After 10 min at 37°C, 0.5 ml of cold 0.5M- HClO_4 together with 50 μg of bovine serum albumin were added. Precipitated DNA was collected on filters (Millipore code HAWP 02500), the filters were dried and the retained radioactivity was measured.

Chemicals

All chemicals were of the highest purity available commercially. Acrylamide was from BDH (Poole, Dorset, U.K.), and Eastman-Kodak (Rochester, NY, U.S.A.) supplied methylenebisacrylamide. Sodium dodecyl sulphate was obtained from Serva (Heidelberg, West Germany). Sigma (London) supplied testosterone and corticosterone. Cyproterone acetate was a gift from Schering A.G. (Berlin, Germany). Oestradiol-17 β was from Searle (High Wycombe, Bucks., U.K.). A.R.-grade urea (Fisons, Loughborough, Leics., U.K.) was passed through a column (30cm $\times$ 5cm diam.) of mixed-bed ion-exchange resin (Bio-Rad; AG501-X8) before use.

Results

Proteins of vesicular secretion

Vesicular secretion, a surprisingly concentrated protein solution (250–300mg/ml), is highly unstable even in the absence of enzymes from the closely associated coagulating glands. Removed directly from the glands, rat vesicular secretion gels rapidly, urea being necessary for stable solutions at 0–25°C (compare the findings of Manyai, 1964). In the presence of 3M-urea about 75–80% of vesicular protein is soluble; decreasing the urea concentration leads to protein precipitation (results not shown).

Electrophoresis of the NaCl/urea-soluble fraction of vesicular secretion on cellulose acetate-gel strips revealed three major protein components, all migrating to the cathode (Plate 1a). No acidic proteins or minor components were identified. Analysed on acid/urea/polyacrylamide gels, the most obvious component of the secretory proteins is a rapidly migrating (R_f 0.69) basic protein, seminal-vesicle basic protein, which accounts for about 25–30% of the total secretory protein (Plate 1b). A number of more slowly moving basic proteins, both major and minor, are also present, together with material remaining at the top of the gel. Similarly, on denaturing gels, a number of protein bands of different sizes can be resolved. Two major bands, seminal-vesicle basic protein S (SV basic protein S) and seminal-vesicle basic protein F (SV basic protein F), run very close to each other (Plate 1b), near the ion front (R_f 0.77 and 0.80 respectively). These together correspond to the SV basic protein band observed on acid/urea gels (see below).

Purification of SV basic protein

Previous workers have used ion-exchange chromatography to separate vesicular proteins (Ballard & Williams-Ashman, 1964; Manyai, 1964). In the present study DEAE-cellulose (Whatman DE-23), CM-Sephadex (Pharmacia CM50) and phosphocellulose (Whatman P11) eluted with salt or pH gradients failed to fractionate NaCl/urea-soluble vesicular proteins. Further, incomplete recovery of the applied proteins was a common occurrence, owing to protein precipitation on the column matrix, even in the presence of 3M-urea. This also was the experience of Orsini & Shulman (1971).

By using Sephadex G-200, three protein fractions, accounting for 88% of the applied protein, were separated from NaCl/urea-soluble vesicular proteins (Fig. 1). Fraction I (42% of the applied protein) was eluted in the void volume, whereas the minor fraction II (3%) and fraction III (43%) were included. Each fraction ran as a single component when re-chromatographed on Sephadex G-200 and had a u.v. spectrum of a typical protein. On acid/urea gels, SV

EXPLANATION OF PLATE 1(a)

Cellulose acetate electrophoresis of seminal-vesicle secretory proteins

NaCl/urea-soluble protein (15 μ g) was applied at the point indicated by the arrow. Electrophoresis was at pH 8.5, and proteins were stained with Naphthalene Black.

EXPLANATION OF PLATES 1(b), 1(c) AND 1(d)

Electrophoresis of seminal-vesicle secretory proteins

(b) Polyacrylamide-gel electrophoresis. NaCl/urea-soluble proteins (25 μ g) were analysed on acid/urea gel (left) and denaturing gel (right). 'S' and 'F' indicate the positions of SV basic proteins S and F respectively (see the text). SVBP, seminal-vesicle basic protein. (c) and (d) Separation on Sephadex G-200. The NaCl/urea-soluble secretion was fractionated on Sephadex G-200. Fractions were analysed on acid/urea polyacrylamide gels (c) and denaturing gels (d), together with the starting material. In each case gels represent, from left to right, 25 μ g of starting material, 10 μ g of fraction I, 2 μ g of fraction II and 10 μ g of fraction III (see Fig. 1 and the text). Stacking gels are at the top, and electrophoresis is from top to bottom. Naphthalene Black staining was used.

EXPLANATION OF PLATE 2

Purification of seminal-vesicle basic proteins S and F and evidence that they are glycoproteins

(a) Purification of SV basic proteins S and F. Proteins were separated on preparative denaturing gels (see the Materials and Methods section). Gels are, from left to right, SV basic protein (20 μ g) before fractionation, SV basic proteins S (12 μ g) and F (8 μ g). The stacking gels are at the top, and electrophoresis is from top to bottom. Naphthalene Black staining was used. (b) Evidence that SV basic proteins S and F are glycoproteins: SV basic protein (50 μ g) was separated into SV basic proteins S and F on denaturing gels. Carbohydrate-containing material was stained with periodic acid/Schiff reagent (see the Materials and Methods section). Background stain was high with this reagent.

Fig. 1. Chromatography of seminal-vesicle proteins on Sephadex G-200

The NaCl/urea-soluble fraction of seminal-vesicle secretion (400mg of protein; 15 ml) was loaded on a column (100cm × 2.5cm diam.) of Sephadex G-200 and eluted with 0.05M-phosphate buffer, pH 7.5, containing 3M-urea. Fractions (6.8 ml) were collected and their E_{280} was read.

basic protein was clearly confined to fraction III, whereas fractions I and II contained the more slowly migrating species of the starting protein mixture (Plate 1c). These conclusions were confirmed with denaturing gels where SV basic protein was seen to consist of two protein species designated 'SV basic protein S' and 'SV-basic protein F' (Plate 1d). No other protein species could be detected in concentrations greater than 1% of the total protein of fraction III. Preparative denaturing gels were used to separate the two species (see the Materials and Methods section and Plate 2a), such that cross-contamination was less than 5%. That this difference in mobility of the two species is not due solely to subtle modifications of the same parent protein, e.g. phosphorylation, acetylation etc., can be discounted by the significant differences in their amino acid compositions (see Table 2). However, at this point it is not clear whether SV basic protein is a single protein composed of subunits (S and F) or that the two subunits are themselves distinct proteins that fail to separate on acid/urea gels (even containing as much as 8M-urea).

Properties of SV basic proteins

SV basic protein accounts for about 25–30% of total vesicular protein and the secretion of intact (non-orchidectomized) male rats contains SV basic

Table 1. Carbohydrate composition of SV basic protein

SV basic protein (1.2mg) in 6M-urea was dialysed extensively against 3mM-NH₄HCO₃ at 4°C and freeze-dried. Carbohydrate residues in duplicate samples (170, 246 μg) of the freeze-dried protein were measured as their trimethylsilyl ether derivatives by g.l.c. N.D., not detectable.

Sugar	Content (g/100g)
D-Mannose	0.31
D-Galactose	0.37
N-Acetylneuraminic acid	N.D.
N-Acetyl-D-glucosamine	0.53
N-Acetyl-D-galactosamine	N.D.

proteins S and F in the ratio approx. 2:1 (w/w). SV basic protein had an isoelectric point of about pH9.7 (note that these isoelectric-focusing gels fail to separate SV basic proteins S and F).

Direct analysis (Table 1) of purified SV basic protein showed that it contains 1.2% (by wt.) of carbohydrate, which is comparable with many other glycoproteins (Winzler, 1968). Contamination of SV basic protein by a glycoprotein containing a much higher proportion of carbohydrate is unlikely, since denaturing gels showed that SV proteins S and F both stain positively for carbohydrate (Plate 2b).

Table 2. *Amino acid composition of SV basic proteins S and F*

The proteins were separated on preparative denaturing polyacrylamide gels, treated with performic acid and hydrolysed with 6M-HCl for 16, 24 and 48 h at 110°C. Amino acids in the hydrolysate were separated and measured by using an automated analyser (see the Materials and Methods section for details). Values for aspartic acid and glutamic acid include asparagine and glutamine respectively. The values below are those for 24 h of hydrolysis. Estimates of the decomposition of threonine and serine and the incomplete release of valine and isoleucine during this time-period were made by reference to the hydrolysis values at 16 h and 48 h. ND, not determined.

Residue	Composition (nmol/100nmol recovered)	
	SV basic protein S	SV basic protein F
Cys	0.42	1.05
Asp	5.99	9.72
Thr	1.76	3.50
Ser	20.47	14.67
Glu	17.67	15.15
Pro	3.09	3.49
Gly	5.21	8.13
Ala	5.56	6.00
Val	4.57	3.45
Met	1.75	3.43
Ile	3.21	3.31
Leu	3.38	3.36
Tyr	2.75	0.88
Phe	3.63	4.80
His	1.46	1.43
Lys	10.49	9.26
Arg	8.61	8.37
Trp	ND	ND

By using denaturing gels, the mol.wts. of SV basic proteins S and F were estimated to be approx. 18 500 and 17 000 respectively. Although glycoproteins containing more than 10% (by wt.) of carbohydrate behave anomalously on denaturing gels (Segrest & Jackson, 1972), the proportion of carbohydrate in SV basic protein is much lower (1.2%) than this, and such anomalous behaviour is likely to be negligible.

Amino acid analyses of the separated SV proteins S and F are shown in Table 2. Lysine and arginine are present in approximately equal amounts and, together with histidine, these basic amino acids constitute a relatively high proportion of the total amino acids of SV basic proteins S (20.5%) and F (19.1%). Aspartic acid and glutamic acid together account for about 24% of the total amino acids, but presumably a considerable proportion of these residues are normally present as asparagine and glutamine, to account for the extremely basic nature of these proteins. Also noteworthy are the low contents

of hydrophobic amino acids (valine, isoleucine, leucine and methionine) and threonine, and the high serine content compared with many globular proteins (Reeck, 1968). There are about twice as many methionine residues in SV basic protein F as in SV basic protein S.

A survey of many tissues showed that these basic proteins, characteristic of the seminal vesicle, could not be detected in liver, spleen or testis, and were also absent from the other androgen-dependent accessory sexual tissues, including ventral prostate, preputial gland, epididymis (caput and cauda) and coagulating gland (results not shown).

Synthesis of SV basic protein is androgen-dependent

Testosterone is necessary for maintaining the structure and function of the seminal vesicle (Moore *et al.*, 1930; Price & Williams-Ashman, 1961). Not unexpectedly, therefore, bilateral orchidectomy had a dramatic effect on the production of SV basic protein. Pronounced atrophy of the vesicles (Fig. 2a) was followed after a lag period of about 1 day by a fall in amounts of SV basic proteins S and F recovered from the glands (Fig. 2b). By 2–3 days after orchidectomy, the amounts of both proteins were decreased by half, and after 10 days, less than 1% of that in intact animals could be detected. This progressive disappearance of SV basic protein appears to be accounted for, at least in part, by a decrease in the rate at which it is synthesized (Fig. 2c). In the intact animal, the rates of incorporation of [³⁵S]methionine into SV basic proteins S and F *in vitro* are similar, consistent with the finding that there is twice as much SV basic protein S as SV basic protein F (Fig. 2b), and that the methionine content of the former is half that of the latter (Table 2). After orchidectomy, the rates of incorporation of methionine into both protein species *in vitro* declined steadily, reaching about half those of the intact animal within 2–3 days and ultimately (after 10–12 days) falling to 5% and 25% respectively. Interestingly, however, the rate of incorporation into SV basic protein S appears to be more sensitive to orchidectomy, so that the ratio of incorporation rates into SV basic proteins S and F decreased from approx. 0.85 to 0.17 by 10–12 days. Besides this specific de-inductive effect on SV basic protein, orchidectomy also greatly decreased general protein synthesis as measured by [³⁵S]methionine incorporation into total cellular protein *in vitro* (Fig. 2d). In fact it appears that orchidectomy affects general protein synthesis and the specific synthesis of SV basic proteins S and F to similar extents, the proportion of total protein synthesis devoted to SV basic protein being maintained at about 20–25% (Fig. 2e).

These negative effects of orchidectomy can be reversed readily by the administration of testosterone

Fig. 2. Effect of orchidectomy on synthesis of SV basic protein by seminal vesicle

Animals were orchidectomized. At each time-point, groups of four were killed and the seminal vesicles removed and weighed. One half-gland from each animal was pooled and used to measure SV basic proteins S and F by denaturing polyacrylamide-gel electrophoresis. The other halves were pooled and incubated with [35 S]methionine *in vitro* for 45 min. The radioactivity incorporated into SV basic proteins S and F was measured by denaturing gel electrophoresis, and incorporation into total protein was measured by acid precipitation. (a) Wet weight of seminal vesicles (average and range of four animals per group). (b) Content of SV basic protein S (□), SV basic protein F (○) and SV basic protein (F+S) (▲). (c) Incorporation of [35 S]methionine into SV basic-protein S (□), SV basic protein F (○) and SV basic protein (S+F) (▲). (d) Incorporation of [35 S]methionine into total acid-precipitable protein (■). (e) Incorporation of [35 S]methionine into SV basic proteins (S+F) (▲) and total protein (■), and tissue wet wt. (●).

Fig. 3. Induction of synthesis of seminal-vesicle basic protein by testosterone

Animals orchidectomized 14 days previously were given daily injections of testosterone and killed in groups of four at the times indicated. Details of the determination of tissue wet weights and incorporation of [35 S]methionine into SV-basic proteins S and F and total protein are given in the Materials and Methods section and the legend to Fig. 2. (a) Tissue wet weight, average and range of four animals per group. (b) Incorporation of [35 S]methionine into SV basic protein S ($\square$), SV basic protein F ($\circ$) and SV basic proteins (S+F) ($\blacktriangle$). Values $\square$, $\circ$ and $\blacktriangle$ are from intact (non-orchidectomized) animals. (c) Amount of SV basic protein S ($\square$), SV basic protein F ($\circ$) and SV basic protein (S+F) ($\blacktriangle$) per gland. (d) Incorporation of [35 S]methionine into total acid-precipitable protein ($\blacksquare$). (e) Incorporation of [35 S]methionine into SV basic protein (S+F) ($\blacktriangle$) and total protein ($\blacksquare$), and tissue wet weight ($\bullet$).

Table 3. Steroid specificity of SV basic protein induction

Orchidectomized (14 days) animals were injected daily with the steroid(s) indicated; 3 days after the first injection they were killed and the seminal vesicles removed. One half gland from each animal (four animals per group) served for determination of SV basic protein, and the other portions were combined and used to measure incorporation of [35 S]methionine into SV basic protein (see the Materials and Methods section). Results of two experiments (I and II) are presented. ND, not determined.

Steroid	Incorporation of [35 S]methionine into SV basic protein (d.p.m./45 min per gland)		SV basic protein (mg/gland)	
	I	II	I	II
None	509 000	280 000	0	0
Testosterone	4 563 000	2 934 000	2.80	1.54
Cyproterone	ND	ND	0	ND
Testosterone+cyproterone	1 900 000	1 200 000	0.79	0.54
Oestradiol	560 000	358 000	0	0
Testosterone+oestradiol	ND	ND	ND	1.57
Corticosterone	648 000	385 000	0	0
Testosterone+corticosterone	ND	ND	2.61	1.15

in vivo. Tissue weight of the seminal vesicles was increased towards normal (Fig. 3a), and the ability of the tissue to incorporate methionine into SV basic proteins S and F *in vitro* increased progressively to reach (after 3 days) a value similar to, or even greater than, that in intact animals (Fig. 3b). As a result, the amounts of these proteins in the vesicles were greatly increased, commencing about 18 h after the start of testosterone therapy (Fig. 3c). Even after 3 days, however, these concentrations were still only about 10% of those found in normal animals, and further, it was clear that the ratio of SV basic protein S to SV basic protein F characteristic of the intact animal had not been restored (compare Figs. 2b and 3c); note that the measured amounts of basic proteins include secretions within the glands as well as intracellular protein. Even though the rates of synthesis of the basic proteins are restored to normal within 3 days, it may well take much longer to accumulate stored secretion to the concentration found in intact animals. Besides these specific effects, it was found that testosterone treatment also influenced the rate of general protein synthesis, restoring this parameter to normal within 2–3 days (Fig. 3d). Thus in orchidectomized animals, testosterone affects general protein synthesis and the specific synthesis of SV basic proteins S and F to similar extents (Fig. 3e).

Steroid specificity of induction

This facility of testosterone to reverse the effects of orchidectomy is not shared by the oestrogen, oestradiol-17 β , or by the principal rat glucocorticoid, corticosterone (Table 3). At physiological doses, the effects of these two steroids on amino acid incorporation into SV basic proteins S and F *in vitro* and on vesicular contents of these basic proteins were less than 4% of those observed with testosterone under

similar conditions. Further, oestradiol and corticosterone did not inhibit the inductive effects of testosterone, even at very high doses. However, the synthetic steroid, cyproterone acetate, which has been shown to be a potent anti-androgen in a number of systems (Neumann & von Berswordt-Wallrabe, 1966; Geller *et al.*, 1969), and which inhibits 5 α -dihydrotestosterone binding to the specific androgen receptor in rat ventral prostate (Fang & Liao, 1969; Stern & Eisenfeld, 1969; Belham & Neal, 1971), inhibited the inductive effects of the androgenic steroid on basic-protein synthesis (Table 3).

Function of SV basic protein

At present it is not clear what precise physiological role this secretory protein has. It is well established that when brought into contact with secretions of the coagulating glands at ejaculation, rodent seminal-vesicle proteins coagulate to form the post-coital vaginal plug, presumably aiding fertilization (Camus & Gley, 1896; Blandau, 1945; Price & Williams-Ashman, 1961; Joshi *et al.*, 1972).

Williams-Ashman and his group (Ballard & Williams-Ashman, 1964; Notides & Williams-Ashman, 1967), and Manyai (1964) both reported investigations of certain basic-protein fractions from rodent seminal vesicles. They showed that after seminal-vesicle secretions were coagulated by soluble extracts of coagulating glands, the precipitated protein consisted mainly of their basic proteins and that these proteins were undetectable among the proteins resisting this enzymic precipitation.

We used the experimental procedure of Williams-Ashman and his colleagues (Gotterer *et al.*, 1955) to coagulate seminal-vesicle proteins. The precipitated protein was removed by centrifugation and dissolved in 1% sodium dodecyl sulphate/8M-urea. Proteins

Table 4. *Effect of coagulating-gland extract on solubility of SV basic protein*

The procedure of Gotterer *et al.* (1955) was used. Seminal-vesicle secretory proteins soluble in 0.15M-NaCl at 0°C were precipitated at 67% (v/v) acetone and the acetone-dried powder was extracted with 0.15M-NaCl at 25°C. Soluble proteins were treated with extract of fresh rat coagulating glands (prepared by homogenizing the tissue in 5 vol. of 0.15M-NaCl at 0°C and removing debris by centrifugation at 10000g for 10 min). Each cuvette contained, in 1.2 ml, 23.6 mg of seminal-vesicle protein, 30 µmol of Tris/HCl, pH 7.4, 5 µmol of MnCl₂. Reaction was started with coagulating-gland extract (50 µl, 1.4 mg of protein) and the *E*₆₆₀ was measured at 25°C against a reagent blank. In control cuvettes, water replaced coagulating-gland extracts. When the precipitation reaction reached equilibrium (no further effect of added coagulating-gland extract), precipitated protein was removed by centrifugation and dissolved in 1% sodium dodecyl sulphate/8M-urea (1 ml), and soluble proteins were precipitated with trichloroacetic acid (5%, w/v, at 0°C) and likewise dissolved in sodium dodecyl sulphate/urea. Denaturing gels were used to measure SV basic protein in both fractions; total protein was determined by the method of Lowry *et al.* (1951). Results (means and individual values) of two experiments are presented.

Coagulating-gland extract	Soluble fraction		Coagulated fraction	
	Total (mg)	SV basic protein* (mg)	Total (mg)	SV basic protein* (mg)
+	17.62 (17.05, 18.20)	7.90 (7.55, 8.25)	4.03 (4.05, 4.01)	1.45 (1.40, 1.50)
—	22.85 (22.61, 23.09)	9.85 (9.60, 10.10)	—	—

* Represents SV basic proteins S and F present in ratio 2:1 in all fractions.

not coagulated were precipitated from the soluble fraction with 5% (w/v) trichloroacetic acid at 0°C and likewise dissolved in sodium dodecyl sulphate/urea. Both soluble- and coagulated-protein preparations were analysed on denaturing polyacrylamide gels.

Although both SV basic proteins S and F were detected in the coagulated proteins and in the same ratio as in the original vesicular secretion, their specific activities (relative to total protein) were not significantly different from that of the SV basic protein remaining in the soluble fraction (Table 4). Thus it is clear that the coagulum does not consist mainly or exclusively of SV basic protein and it is perhaps more reasonable to conclude that the protein may be no more than fortuitously trapped within the coagulum. Nevertheless it cannot be discounted that SV basic protein has some active role in the process.

It is known that rodent seminal plasma contains nucleases and proteinases (Mann, 1964). Although this may be so, direct determinations (see the Materials and Methods section) failed to demonstrate proteinase, ribonuclease or deoxyribonuclease activities in SV-basic-protein preparations.

Discussion

Other basic proteins from rat and guinea-pig seminal vesicles have been described, but these differ from SV basic proteins S and F. That from guinea pigs, described by Williams-Ashman (Ballard & Williams-Ashman, 1964; Notides & Williams-Ashman, 1967), is notable in being devoid of tyrosine, proline and cysteine, all of which are present in significant

amounts in both SV basic proteins (S and F), whereas the lysine/arginine ratio of this protein, nearly 2:1, exceeds that of SV basic proteins S and F (1:1). Further, both guinea-pig and rat basic proteins were precipitated completely by extracts of coagulating glands, whereas SV basic protein failed to precipitate selectively under the same conditions. Vesiculase also precipitated the rat seminal vesicle basic protein described by Manyai (1964). The amino acid analysis of this basic protein revealed a lysine/arginine ratio of 4:1 and that proline and cysteine were absent.

It is clear that changes in the concentration of circulating testosterone have a pronounced effect on the incorporation of [³⁵S]methionine into the basic proteins, suggesting that the hormone increases protein synthesis *de novo* rather than their formation from inactive precursor proteins or the secretory process itself.

If the incorporation of [³⁵S]methionine into protein over 45 min is used as an index of the absolute rate of protein synthesis (assuming that during this short time the effects of protein turnover are negligible), it appears that plasma testosterone concentrations control the absolute rates of synthesis of SV basic proteins S and F. After orchidectomy it was noted that SV basic protein F almost completely disappeared from the vesicles (to less than 1% of normal content), yet its rate of synthesis was decreased to a much lesser degree (to 25% of the normal rate). This was also true to a lesser extent for SV basic protein S. Thus it cannot be discounted that testosterone could also affect additional parameters determining the amount of basic secretory proteins in the vesicles.

In other systems sensitive to steroid hormones, such as the chick oviduct (Palmiter, 1975) and cultured rat hepatoma cells (Baxter *et al.*, 1972), the effects of the hormone appear to be confined to modulation of the synthesis of a restricted number of specific proteins. The hormonal response is not characterized by a general change in synthesis of all cellular proteins, nor does it involve changes in the proliferation rate of the hormone-responsive cells. Such is not the case with the rat seminal vesicle. During de-induction, incorporation of [35 S]methionine into total cellular protein was decreased to the same extent as into the basic secretory proteins. Likewise, during induction by testosterone, increases in the synthetic rates for the basic proteins and total cellular protein occurred in parallel. Thus in the seminal vesicle, the steroid hormone may act by increasing protein synthesis generally and not by any specific effect on the rate of synthesis of an individual protein. It is noteworthy in this context that in the closely related androgen-dependent ventral prostate, among the earliest subcellular events after hormone stimulation are increases in rRNA, nucleolar RNA polymerase, ribosome density and polyribosome size (Liao & Williams-Ashman, 1962; Liao & Lin, 1967; Mangan *et al.*, 1967; Mainwaring & Wilce, 1972, 1973; Mangan & Mainwaring, 1972).

In the seminal vesicle, the columnar epithelium is principally affected by changes in the androgenic status of the animal (Moore *et al.*, 1930; Price & Williams-Ashman, 1961; Deane, 1963). These are reported to include proliferative as well as trophic changes. Hence, besides changing the capacity of individual cells to synthesize proteins, androgens could also control the number of cells involved. Several investigators have studied cell proliferation in the seminal vesicle (Burkhart, 1942; Rabinovitch *et al.*, 1951; Cavazos & Melampy, 1954; Allen, 1958; Kassenaar *et al.*, 1962; Kochakian, 1963; Ritter, 1969; Morley *et al.*, 1973; Alison *et al.*, 1974, 1976). Unfortunately species other than the rat have often been used, and the time-course of changes in androgen status has not always been directly comparable, but death of epithelial cells could be a significant factor in the de-induction process. Reversal of the effects of orchidectomy by testosterone involves hyperplasia, but significant changes in the mitotic index of seminal-vesicle epithelium do not occur until testosterone has been present for 36–48 h (Burkhart, 1942; Cavazos & Melampy, 1954; Allen, 1958; Morley *et al.*, 1973). Hence at least the increases in protein synthesis seen immediately after orchidectomized animals were given testosterone presumably do not involve significant proliferative effects. Hyperplasia in itself cannot account for the induction of SV basic proteins S and F, since oestradiol failed to replace testosterone, yet it is reported to induce epithelial hyperplasia in rat seminal vesicle (Allen, 1956).

As in the much more extensively studied rat ventral prostate (King & Mainwaring, 1974), the action of testosterone in the seminal vesicle is thought to involve conversion of the hormone into 5 α -dihydrotestosterone by NADPH-dependent 5 α -reductase, with subsequent binding of 5 α -dihydrotestosterone to cytoplasmic androgen-receptor proteins present in this tissue (Mainwaring & Mangan, 1973). In the seminal vesicle, oestradiol and corticosterone are ineffective inducers, and cyproterone acetate markedly inhibits testosterone action. This is consistent with androgen action proceeding via the specific receptor, since at physiological doses, oestradiol and corticosterone do not bind to this macromolecule, whereas cyproterone acetate competes with 5 α -dihydrotestosterone for androgen-specific binding sites (Fang & Liao, 1969; Stern & Eisenfeld, 1969; Belham & Neal, 1971).

The results described in the present paper, though preliminary, suggest that this system contains the potential for obtaining further insight into the molecular mechanism of androgenic steroids.

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