Functional development of sex accessory organs of the male rat

Use of oestradiol benzoate to identify the neonatal period as critical for development of normal protein-synthetic and secretory capabilities

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Functional development of the sex accessory tissues was studied in the male rat. Three potentially crucial developmental periods (neonatal, prepubertal and pubertal) were examined, and then the functional integrity of the accessory tissues was investigated in the adult, when the animals would have been expected to display normal function. Four accessory tissues (the seminal vesicles, ventral prostate and caput and cauda epididymides) were used because of their different embryological origins and responses to androgens in the adult. Synthesis and secretion of previously characterized tissue-specific androgen-dependent proteins were taken as indicators of normal function. Development was perturbed by using oestradiol benzoate, since this was known to affect gross development of the seminal vesicles and ventral prostate when given to neonatal rats. Treatment during the first 5 days after birth severely restricted development of the seminal vesicles and ventral prostate. Protein secreted by the former was only 1% of the normal amount, and in many cases several major secretory proteins were essentially missing. Prostatic protein secretion was less than 20% of normal, but all the major proteins were detectable. In both tissues overall protein synthesis per cell was quantitatively normal, but the proportion devoted to specific major secretory proteins was markedly depressed, i.e. the response is differential. In contrast, treatment during the prepubertal period was without noticeable effects. Development of the seminal vesicles and prostate was somewhat inhibited by treatment at puberty, but these changes were minor compared with those after neonatal exposure to oestradiol benzoate. No effects on epididymal protein synthesis or secretory proteins were observed, and epididymal weight and DNA content were only moderately decreased regardless of when oestradiol benzoate was administered during sexual maturation. Hence the neonatal period is not so critical for epididymal development. The substantial changes elicited by oestrogen treatment during neonatal life in seminal-vesicle and prostatic protein synthesis and secretion were compared with those evoked in sexually mature males by either oestrogen treatment or castration. Both these latter treatments resulted in a general decrease in seminal-vesicle protein synthesis and secretion, but the marked differential effects on major proteins after neonatal exposure were absent. Castration did, however, evoke a differential prostatic response, but this was not seen after oestrogen treatment of adults.

Functional development of the accessory sexual tissues of the male rat occurs in a number of stages and is critically dependent on a number of hormones, including the androgens (Ohno, 1979;

Abbreviations used: SDS, sodium dodecyl sulphate; KRP solution, Krebs–Ringer phosphate solution.

Wilson *et al.*, 1980). Late in foetal life, testosterone produced by the foetal testes (Warren *et al.*, 1973; Feldman & Block, 1978; Weisz & Ward, 1980) promotes differentiation of the seminal vesicles and epididymides from the Wolffian-duct system, and dihydrotestosterone stimulates development of the

prostate from the urogenital sinus (Price, 1936; Flickinger, 1969a, 1970; Wilson *et al.*, 1980). At puberty, when plasma androgens increase substantially (Podesta & Rivarola, 1974; Dohler & Wuttke, 1975; Lee *et al.*, 1975; Moger, 1977), spectacular and rapid growth of the differentiated tissues occurs, leading to pronounced synthesis of secretory products (Callow & Deanesly, 1935; Desjardins *et al.*, 1969; Brooks, 1976b). However, other periods between late foetal life and puberty may also be crucial for acquisition of functional activity of the male accessory sexual tissues in the adult.

We decided to investigate this possibility by first perturbing the normal developmental programme for a limited time, then allowing development to proceed and finally measuring functional activity in the animals when normal activity could be expected. There are probably many ways of perturbing the system, none of which is ideal. For instance, if we were to use ablative procedures such as castration or hypophysectomy, we would then have to institute and to maintain continuously the correct replacement therapy if the original perturbation is not to be indefinite. This we cannot do with certainty given the complex multi-hormonal nature of the developmental process. A more practicable alternative is to treat the animal with an antagonist to the developmental signal for the duration of the critical time and then discontinue treatment. In selecting such an antagonist, we were guided by previous work where it had been shown that treatment of neonatal rats with oestrogens led to decreased size of the seminal vesicles and ventral prostate in the adult (Kincl *et al.*, 1963, 1965; Harris, 1964; Harris & Levine, 1965; Simmons, 1971; Lamartiniere *et al.*, 1979). Oestradiol (oestra-1,3,5-triene-3,17 β -diol) 3-benzoate would therefore seem to be a useful probe with which to examine different developmental stages.

As indices of functional development and integrity, the synthesis and secretion of specific proteins by the seminal vesicles, ventral prostate and caput and cauda epididymides were chosen. These marker proteins have been described extensively and are synthesized in an androgen-dependent manner in the adult (Higgins *et al.*, 1976; Parker *et al.*, 1979; Brooks & Higgins, 1980). We chose these four tissues, since (a) in the adult each responds to androgens in a uniquely different fashion with respect to qualitative and quantitative effects on protein synthesis (Higgins *et al.*, 1976; Higgins & Burchell, 1978; Higgins & Parker, 1980; Parker *et al.*, 1979; Parker & Scrace, 1979; Brooks & Higgins, 1980), and (b) their different embryological origins and developmental responses to hormones, outlined above, might also be expected to involve differences in exact timing and extent of the critical periods in their functional development.

We show here that the neonatal period is critical for functional development of the seminal vesicles and ventral prostate, but probably not for the caput and cauda epididymides. In contrast, only minor functional changes occurred in the adult after exposure to oestradiol benzoate during the pubertal period, and none at all after treatment during the prepubertal period. Changes after neonatal oestrogen treatment were also compared with those elicited in the sexually mature adult by similar oestrogen treatment or by castration.

Materials and methods

Chemicals and media

BDH (Poole, Dorset, U.K.) supplied all chemicals ('AnalaR' where available) except: oestradiol benzoate (Cambrian Chemicals, Croydon, U.K.); acrylamide, amino acids, bovine serum albumin (fraction V), calf thymus DNA and Coomassie Brilliant Blue R250 (Sigma, Poole, Dorset, U.K.); *NN'*-methylenebisacrylamide (Eastman Kodak, Rochester, NY, U.S.A.); 2,5-diphenyloxazole (Fisons, Loughborough, Leics., U.K.), Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} (Calbiochem, Bishops Stortford, Herts., U.K.); L-[³⁵S]methionine (The Radiochemical Centre, Amersham, Bucks., U.K.; 750–1350 Ci/mmol), Fuji RX X-ray film (Fuji Photo Co., Tokyo, Japan). Krebs–Ringer phosphate solution was prepared as described by DeLuca (1972), but with decreased calcium. Final composition was 150 mM-Na⁺, 4.8 mM-K⁺, 1.3 mM-Ca²⁺, 1.2 mM-Mg²⁺, 129 mM-Cl⁻, 15.5 mM-PO₄³⁻, 1.2 mM-SO₄²⁻, 10 mM-D-glucose. Methionine-free amino acid mixture contained all 19 L-amino acids (final concentration of each in incubations = 0.1 mM) except methionine. For injection, oestradiol benzoate was first dissolved (4 mg/ml) in ethanol, mixed with arachis oil and the ethanol evaporated at room temperature under nitrogen.

Animals

Male and pregnant female (approx. day 15 of gestation) Sprague–Dawley rats were from Tuck (Bishops Stortford, Herts., U.K.) and were kept in standard laboratory cages at 22°C (12h-light/12h-dark). At birth, pups were sexed and the females removed. Half the males of each litter received five daily subcutaneous injections of oestradiol benzoate (50 µg) in oil (20 µl), while the controls received oil alone and had their ears and tails clipped for identification. Before being returned to their mothers, treated and control pups were left on tissue paper for 5 min to absorb any oil leaking from the injection site. After weaning (20–22 days) the rats were kept in random groups of four. Prepubertal rats (45–55 g; 22–25 days old), pubertal rats (140–150 g; 40–45 days old) and sexually

mature adult rats (400–500 g; 90–150 days old) received five daily subcutaneous injections of oestradiol benzoate (approx. 0.5 mg/100 g body wt.) in arachis oil. Age-matched controls received oil alone. The rats were first examined at 80–85 days of age, except for adult rats, which were used 24 h after their fifth injection. In one experiment further measurements of neonatally treated rats were made at 120 days of age.

Processing of tissues

Animals were killed by cervical dislocation. Epididymis (right side) and ventral prostate were dissected free of adipose tissue and weighed on a torsion balance. Caput and cauda epididymides were separated (Brooks, 1976a) and reweighed. Tissues were finely minced with a stainless-steel razor on a paraffin-wax block, transferred to a 50 ml Erlenmeyer flask containing 10 ml of KRP solution and shaken at 35°C for 10 min to extract secretion or luminal fluid. The medium was removed with a Pasteur pipette and 25 mg portions of tissues were first blotted on tissue paper moistened with KRP solution, then incubated with [³⁵S]methionine (see below). The rest of the tissue was returned to the extraction medium and kept on ice. In the case of the seminal vesicles, the coagulating glands were first removed and then each lobe was clamped off and removed complete with secretion. Secretion was expressed into 10 ml of KRP solution, mixed and frozen at –20°C. The tissue was weighed and minced as above. A portion (25 mg) was incubated with [³⁵S]methionine (see below), and the rest was shaken in 10 ml of KRP solution (as above) to complete the extraction of secretion. Suspensions of tissue in KRP solution were centrifuged (1000 g for 10 min) at 4°C and the supernatants containing the secretory proteins or luminal fluid were frozen at –20°C until assayed for protein and analysed on gels (see below). Meanwhile the tissue DNA was determined by incubating the pellet with 5 ml of 0.25 M-HClO₄ at 0°C (for 30 min), after which the pellet was recovered by centrifugation (3000 g for 10 min) at 4°C and digested twice at 70°C with 4 ml lots of 0.5 M-HClO₄. Solubilized DNA was assayed in the combined hot-HClO₄ supernatants by the diphenylamine procedure of Burton (1956), with calf thymus DNA as standard. In the case of the epididymis, spermatozoa in the gland could have contributed to the DNA, but previous experience (Borenfreund *et al.*, 1961) suggests that much more vigorous solubilization procedures would have been required for spermatozoal DNA.

Incorporation of [³⁵S]methionine

Tissue (25 mg) was incubated by shaking at 35°C in 1 ml of KRP solution containing methionine-free amino acids and [³⁵S]methionine (25 µCi). After 1 h

the tissue was disrupted (Brooks & Higgins, 1980) in 5 ml of 0.25 M-sucrose/0.02 M-Tricine (pH 7.4)/2 mM-CaCl₂/1 mM-MgCl₂/0.1% Triton X-100. The homogenate was centrifuged at 150 000 g for 30 min at 4°C. The pellet was assayed for DNA (see above) and the supernatant was frozen at –20°C after samples had been taken for determination of protein (see below) and methionine incorporation. The latter involved precipitating samples (25 µl) with cold trichloroacetic acid, collecting the protein on membrane filters and estimating retained radioactivity by scintillation counting (for details see Brooks & Higgins, 1980).

Analysis of proteins on polyacrylamide gels

Samples (approx. 200 µg of protein) were precipitated in cold trichloroacetic acid and redissolved in 100 µl of dispersion buffer [60 mM-Tris/HCl (pH 6.8)/20% (v/v) glycerol/2% (w/v) SDS] containing 5% (v/v) 2-mercaptoethanol and 0.004% Bromophenol Blue at 100°C (2–3 min) (Brooks & Higgins, 1980). Frozen seminal-vesicle secretion was made 10% in SDS, heated at 100°C for 2–3 min until solubilized and then 1–5 µl of solubilized secretion (containing 50–60 µg of protein) was mixed with dispersion buffer (100 µl) and processed as above. Samples were loaded on polyacrylamide gels (Brooks & Higgins, 1980). Separating gels consisted of a linear gradient of 10–20% acrylamide. SDS (0.1%) was present throughout and the buffer system of Laemmli (1970) was used. Molecular weights were calculated by comparing protein mobilities with those of standard proteins (Brooks & Higgins, 1980). Gels for analysis of secretory proteins were fixed and stained with Coomassie Brilliant Blue (Brooks & Higgins, 1980). Gels with radioactive samples were impregnated with 2,5-diphenyloxazole, and fluorographed at –70°C for 1–14 days (Bonner & Laskey, 1974).

Protein determination

This was done by the method of Lowry *et al.* (1951), with bovine serum albumin as standard and with SDS added to blanks and standards where appropriate.

Results

Protein composition of sex-accessory-gland secretions of the mature male

At 80–90 days of age the reproductive tract of the male rat is well developed (Table 1), with large paired testes active in spermatogenesis and mature spermatozoa present in large numbers in the epididymides. Prominent paired seminal vesicles are normally distended with 0.5–1.0 ml of a thick yellowish protein-rich (>300 mg/ml, Table 2) secretion. Electrophoresis under denaturing conditions

Table 1. *Effects of oestradiol benzoate administration at various developmental stages on body weight and on the development and DNA content of the male reproductive organs*

Male rats received five daily subcutaneous injections of oestradiol benzoate in oil at the following ages; neonatal = birth–5 days; prepubertal = 20–25 days; pubertal = 40–45 days, and adult = 90–150 days. Measurements for neonatal, prepubertal and pubertal groups were made at 80–90 days. Adults were examined 24 h after the last injection. Control group A received oil alone at birth; prepubertal and pubertal controls gave similar results and are omitted from the Table, but controls (group B) for the adult animals are included. Data are means $\pm$ S.E.M. for *n* animals. Means of treated and appropriate control groups were tested for significant difference by using Student's *t* test ($P < 0.001$ except; * $P > 0.5$; † $P < 0.5$; ‡ $P < 0.1$; § $P < 0.01$). Full details are given in the Materials and methods section.

	Period of oestradiol benzoate administration				Control A (<i>n</i> = 14)	Control B (<i>n</i> = 7)
	Neonatal (<i>n</i> = 16)	Prepubertal (<i>n</i> = 10)	Pubertal (<i>n</i> = 9)	Adult (<i>n</i> = 10)		
Body wt. (g)	438 $\pm$ 7‡	333 $\pm$ 10§	268 $\pm$ 14	245 $\pm$ 17*	398 $\pm$ 18	439 $\pm$ 20
Testes wt. (g)	2.18 $\pm$ 0.11	2.77 $\pm$ 0.06†	2.45 $\pm$ 0.14§	2.89 $\pm$ 0.2‡	2.94 $\pm$ 0.07	3.20 $\pm$ 0.09
Seminal vesicles						
Wt. (mg)	112 $\pm$ 10	450 $\pm$ 26*	2222 $\pm$ 41§	373 $\pm$ 22‡	431 $\pm$ 19	463 $\pm$ 28
DNA (μ g)	349 $\pm$ 31	731 $\pm$ 33*	467 $\pm$ 68§	1057 $\pm$ 127*	735 $\pm$ 45	1088 $\pm$ 111
Ventral prostate						
Wt. (mg)	108 $\pm$ 13	317 $\pm$ 20‡	203 $\pm$ 36	269 $\pm$ 20	389 $\pm$ 19	466 $\pm$ 49
DNA (μ g)	360 $\pm$ 20	541 $\pm$ 94*	242 $\pm$ 46	692 $\pm$ 80†	528 $\pm$ 33	939 $\pm$ 145
Epididymides						
Wt. (mg)	760 $\pm$ 28	785 $\pm$ 35	593 $\pm$ 54	874 $\pm$ 40	1032 $\pm$ 33	1286 $\pm$ 60
(a) Caput epididymides						
Wt. (mg)	250 $\pm$ 14	325 $\pm$ 16	304 $\pm$ 30	342 $\pm$ 7	448 $\pm$ 18	565 $\pm$ 45
DNA (μ g)	253 $\pm$ 10	417 $\pm$ 18‡	338 $\pm$ 45§	586 $\pm$ 62†	541 $\pm$ 38	783 $\pm$ 111
(b) Cauda epididymides						
Wt. (mg)	375 $\pm$ 9‡	319 $\pm$ 19§	188 $\pm$ 26	373 $\pm$ 20	426 $\pm$ 20	562 $\pm$ 22
DNA (μ g)	402 $\pm$ 44	553 $\pm$ 36‡	260 $\pm$ 17	753 $\pm$ 85‡	718 $\pm$ 74	1173 $\pm$ 155

shows that seminal-vesicle secretion consists predominantly of five major peptides (Fig. 1a; 86K, 48K, 39K, 15K and 11K) and a few minor peptides.

Prostatic and epididymal secretions contain far less protein (Table 2), but the pattern revealed by gel electrophoresis is more complex (Fig. 1). Major prostatic secretory proteins (Fig. 1b) include two glycoproteins (22K and 12K) plus two smaller peptides (10K and 8K). Proteins of the caput (Fig. 1c) and cauda (Fig. 1d) epididymides are very similar, but can be distinguished by differences in staining intensity of some minor bands (e.g. 26K). The band at 16K consists of two major secretory proteins originating in the caput epididymidis (Brooks & Higgins, 1980).

Effects of neonatal oestrogen treatment on size and DNA content of male reproductive tissues

Male rats exposed to high doses of oestradiol benzoate during the first 5 days of life show, at 80–90 days of age, several abnormalities in the development of the male reproductive system when compared with untreated controls of identical age (Table 1). Although slightly larger (10%) than the controls, treated males had much smaller testes (wet

weight lower by 26%). Similarly the epididymides were smaller (by 26%) than in the controls, with the caput apparently being affected to a greater extent than the cauda. In both regions, the DNA content was decreased to about half, suggesting that the smaller organ size was due mainly to fewer cells.

Neonatal oestrogen treatment had the greatest effect on the seminal vesicles and ventral prostate, each of which was only one-quarter the size of the control tissue (Table 1). In both cases the DNA content (Table 1) was decreased, but not to the same extent as the tissue weight, suggesting that oestrogen must have affected cell size as well as cell proliferation.

No histological examinations of the accessory glands were made, so it is not clear which, if any, of the various cell types are preferentially affected by oestrogens. Apart from their smaller size and absence of secretion (see below), no obvious gross morphological abnormalities were noted in any of the organs. Data in Table 1 refer to a series of 20 neonatal males treated with oestrogen, but four were excluded since they had grossly swollen necrotic testes on both sides (two animals) or one side (two animals).

Table 2. *Effects of oestradiol benzoate administered at different developmental stages on the content of secretory protein in the male reproductive organs*
Details of control and treated animals are given in Table 1. Secretion was collected and assayed for total protein (see the Materials and methods section). Data are means $\pm$ s.e.m. for *n* animals. Means of treated and appropriate control groups were tested for significant difference by using Student's *t* test ($P < 0.001$ except: * $P > 0.5$; † $P < 0.5$; ‡ $P < 0.1$; § $P < 0.01$).

	Period of oestradiol benzoate administration							
	Neonatal (<i>n</i> = 16)		Prepubertal (<i>n</i> = 10)		Pubertal (<i>n</i> = 9)		Adult (<i>n</i> = 10)	
	(mg)	(mg/mg of DNA)	(mg)	(mg/mg of DNA)	(mg)	(mg/mg of DNA)	(mg)	(mg/mg of DNA)
Seminal vesicles	2.9 $\pm$ 0.1	8.2 $\pm$ 0.8	245 $\pm$ 16*	335 $\pm$ 26*	149 $\pm$ 47†	319 $\pm$ 103*	120 $\pm$ 20	113 $\pm$ 28§
Ventral prostate	3.5 $\pm$ 0.4	6.5 $\pm$ 0.9	14.9 $\pm$ 1.5†	41.4 $\pm$ 8.4*	8.9 $\pm$ 2.0	36.8 $\pm$ 9.9*	19.4 $\pm$ 4.2†	28.0 $\pm$ 6.9*
Caput epididymides	6.1 $\pm$ 0.3§	24.1 $\pm$ 1.4§	6.5 $\pm$ 0.6*	15.6 $\pm$ 1.5*	7.5 $\pm$ 1.3†	22.2 $\pm$ 5.0†	9.2 $\pm$ 0.6§	15.7 $\pm$ 2.0†
Cauda epididymides	9.6 $\pm$ 0.2‡	23.8 $\pm$ 2.6	8.1 $\pm$ 1.2*	14.6 $\pm$ 2.4†	6.2 $\pm$ 1.4†	23.9 $\pm$ 5.7‡	8.8 $\pm$ 1.1§	11.7 $\pm$ 2.0†
							(mg)	(mg/mg of DNA)
							247 $\pm$ 13	336 $\pm$ 27
							20.0 $\pm$ 1.1	37.9 $\pm$ 3.2
							9.2 $\pm$ 0.9	17.0 $\pm$ 2.0
							8.5 $\pm$ 0.8	11.8 $\pm$ 1.6
							16.4 $\pm$ 1.9	14.0 $\pm$ 2.5

Effects of neonatal oestrogen treatment on proteins present in accessory-gland secretions

Secretion was noticeably absent (1% of normal) from the seminal vesicles of treated males, in contrast with the copious secretion of the controls (Table 2). Nonetheless, in some animals all the usual major secretory proteins (86K, 48K, 39K, S and F) could still be detected on denaturing gels, albeit as a much smaller proportion of the total, whereas in other animals some major proteins (86K, and 39K most commonly) appeared to be missing (Fig. 1a).

The effects of neonatal oestrogen treatment on other male accessory tissues were less dramatic. Although prostatic secretory protein was about one-sixth of the normal amount (Table 2), all the major secretory proteins (22K, 12K, 10K, 8K) could still be detected, but they accounted for a smaller proportion of the total protein than in controls (Fig. 1b). About two-thirds of the normal amount of protein was found in luminal fluid from the caput epididymidis, whereas that from the cauda region was essentially unchanged in amount (Table 2). For both caput and cauda epididymides, protein profiles of luminal fluids isolated from treated rats were indistinguishable from those of normal controls (Figs. 1c and 1d).

When the results were compared on a DNA basis (Table 2), it appeared that the decreased content of seminal-vesicle and prostatic secretory protein after neonatal oestrogen treatment was due to the lower activity of individual cells and not merely to fewer secretory cells. This point was further investigated by following the incorporation of radioactive methionine into protein.

Effects of neonatal oestrogen treatment on protein synthesis in male reproductive tissues

The pronounced decrease in amounts of protein present in secretions of the seminal vesicles and ventral prostate could be due to oestrogenic effects on either protein synthesis or some aspect of the secretory process. Analyses of the radioactive proteins synthesized *in vitro* by tissue pieces from seminal vesicles (Fig. 2a) and ventral prostate (Fig. 2b) show that these tissues make peptides with mobilities on denaturing gels identical with those of the secretory proteins. However, comparisons made by loading similar amounts (as c.p.m.) of radioactive protein from treated and control tissues on to gels reveal that oestradiol treatment results in a relative increase in the synthesis of some proteins and a decrease in others. Thus the marker secretory proteins of both prostate and seminal vesicles can still be detected, but their contributions to the total incorporation are substantially decreased.

Despite these profound differential effects on the proteins synthesized by seminal vesicles and ventral

Fig. 1. Effects of oestradiol benzoate administered at various stages of sexual maturation on proteins secreted by male sex-accessory tissues

Male rats received five daily subcutaneous injections of oestradiol benzoate in oil at the following ages: neonatal (N) = birth–5 days; prepubertal (PP) = 20–25 days; and pubertal (P) = 40–45 days. Measurements were made at 80–90 days. Adults (A) were 90–150 days old and were used immediately after treatment. Secretions from these treated rats (T) were collected and proteins (approx. 200 µg) were separated on SDS/polyacrylamide gels, stained with Coomassie Blue and compared with those of age-matched controls (C). The Figure is a composite consisting of material from the different treatment groups run on different occasions. Proteins mentioned in the text are indicated by their molecular masses in kilodaltons (K). For details see the Materials and methods section. (a) Seminal vesicles; (b) ventral prostate; (c) caput epididymidis; (d) cauda epididymidis.

Table 3. Effects of oestradiol benzoate administered at various developmental stages on protein-synthetic activity of male reproductive organs

Tissue pieces were incubated with [³⁵S]methionine for 1 h at 35°C. Total incorporation into soluble protein was calculated as c.p.m. incorporated/h per mg of DNA. Results (means ± s.e.m. for *n* determinations) are expressed as 100 × treated/control. The difference between this ‘%’ value and 100% was tested for significance by using Student’s *t* test for paired values (**P* > 0.5; †*P* < 0.5; ‡*P* < 0.1; §*P* < 0.01).

Period of oestradiol benzoate administration	Incorporation of [³⁵ S]methionine (%)			
	Seminal vesicles	Ventral prostate	Caput epididymidis	Cauda epididymidis
Neonatal	109 ± 18 (8)*	122 ± 34 (8)*	92 ± 24 (4)*	103 ± 16 (4)*
Prepubertal	105 ± 8 (3)*	99 ± 7 (3)*	72 ± 2 (3)§	78 ± 12 (3)†
Pubertal	83 ± 13 (5)†	117 ± 26 (6)*	145 ± 46 (4)†	147 ± 48 (4)†
Adult	70 ± 8 (6)‡	92 ± 35 (5)*	83 ± 35 (6)*	74 ± 15 (6)†

Fig. 2. Effects of oestradiol benzoate administered at various stages of sexual maturation on the proteins synthesized in male sex accessory tissues

Rats were treated as in Fig. 1, but tissue pieces were incubated *in vitro* with [35 S]methionine. Soluble proteins from treated (T) and matched control (C) rats were separated on SDS/polyacrylamide gels and detected by fluorography (see the Materials and methods section). The Figure is a composite consisting of material from the different treatment groups run on separate occasions. Abbreviations: N, neonatal; PP, prepubertal; P, pubertal; A, adult.

prostate, the overall rate of protein synthesis by individual cells (calculated on the basis of DNA) in these two accessory tissues does not seem to be affected by neonatal oestrogen treatment (Table 3). Such treatment may well have additional effects on some aspect of the secretory process in these tissues, but this possibility was not investigated further.

In contrast with what occurred in the seminal vesicles and prostate, neonatal oestrogen treatment did not alter the profiles of proteins synthesized by either the caput or cauda epididymidis (Fig. 2c), nor did it alter significantly their overall rates of protein synthesis (Table 3). This result is therefore consistent with the unaltered profile of proteins present in luminal fluids (Figs. 1c and 1d).

Effects of neonatal oestrogen treatment are only partially reversed with age

It is possible that the pronounced effects of neonatal oestrogen treatment on the seminal vesicles and ventral prostate arise because the oestrogen has merely delayed the onset of maturity, and that such animals 'catch up' with their control littermates somewhat later. In one experiment, therefore, a group of animals was examined at 81 and 120 days of age (Table 4). Some compensatory growth in the seminal vesicles certainly seemed to occur from 81 to 120 days after treatment, since DNA and to a lesser extent tissue weight increased to normal. However, no such compensatory growth occurred in the prostate, and even in the seminal vesicles full secretory activity was clearly not restored, there being only about 10% of the normal amount of secretory protein in control animals at 120 days. Furthermore, in both tissues, secretory-protein profiles and proteins synthesized in the presence of [35 S]methionine, although different from the controls, remained the same at 81 and 120 days of age (results not shown). Delayed maturity does not therefore seem a complete explanation for the effects of neonatal oestrogen treatment.

Effects of oestradiol benzoate given to prepubertal, pubertal and adult males

Having established that oestradiol benzoate given during the neonatal period causes profound changes in the secretory behaviour of the accessory tissues when the animals become adult, we investigated whether oestrogen treatment at other developmental

Proteins synthesized by rats castrated 7 days before the experiments (δ^0) are compared with those of normal controls (δ^+). Proteins mentioned in the text are indicated by their molecular masses in kilodaltons (K). (a) Seminal vesicles; (b) ventral prostate; (c) caput (Cpt) and cauda (Cda) epididymidis.

Table 4. *Effects of neonatal oestrogen treatment are not reversed with age*

Neonatal rats were treated at birth for 5 days with oestradiol benzoate. Measurements were made on some of the rats at 81 days of age and on the rest at 120 days of age. Data are means $\pm$ S.E.M. for n animals. Means of treated and control groups were tested for significant difference by using Student's t test ($P < 0.001$ except: $\dagger P < 0.5$; $\ddagger P < 0.1$; $\S P < 0.01$).

	Age = 81 days		Age = 120 days	
	Control ($n = 3$)	Treated ($n = 3$)	Control ($n = 2$)	Treated ($n = 3$)
Seminal vesicles				
Wt. (mg)	363 $\pm$ 17	150 $\pm$ 8	420 $\pm$ 30	332 $\pm$ 28 $\dagger$
DNA (μ g)	629 $\pm$ 6	360 $\pm$ 59 $\ddagger$	625 $\pm$ 32	687 $\pm$ 55 $\dagger$
Secretory protein (mg)	220 $\pm$ 28	3.3 $\pm$ 0.2 $\S$	313 $\pm$ 30	29.6 $\pm$ 8.6 $\S$
Ventral prostate				
Wt. (mg)	458 $\pm$ 39	150 $\pm$ 19 $\S$	595 $\pm$ 95	223 $\pm$ 18 $\ddagger$
DNA (μ g)	704 $\pm$ 55	440 $\pm$ 36 $\ddagger$	798 $\pm$ 18	613 $\pm$ 64 $\dagger$
Secretory protein (mg)	21.2 $\pm$ 1.6	4.6 $\pm$ 1.3 $\S$	32.5 $\pm$ 4.5	7.6 $\pm$ 0.5 $\ddagger$

stages and in the mature male might cause similar changes.

Prepubertal period. Rats treated for 5 days with oestradiol benzoate when aged 20–25 days (Table 1) were only slightly smaller (16%) than controls and had normal-sized testes. In marked contrast with rats treated in the neonatal period, tissue weights and DNA contents of the seminal vesicles and ventral prostate were essentially the same as in control animals. Both caput and cauda epididymides were smaller, with proportionately less DNA, indicating fewer cells, but this effect was no different from that noted in animals treated as newborns. In all accessory tissues the spectra of proteins synthesized (Fig. 2) and secreted (Fig. 1) were normal, with undiminished amounts (Table 2) of secretory proteins from the seminal vesicles and cauda epididymidis. Significantly less protein was isolated from ventral prostate (75% of normal) and caput epididymidis (71% of normal), but, on a DNA basis, it is clear that the cells of these tissues are probably still as active in secretion (Table 2) and synthesis (Table 3) as normal cells. Thus the developmental process appears to be insensitive to oestrogen treatment during the prepubertal period.

Pubertal period. Oestradiol benzoate inhibits development if administered at 40–45 days of age. Such rats were considerably smaller (33% decrease) and had slightly smaller (17% decrease) testes (Table 1). Under-development of seminal vesicles and ventral prostate (Table 1) was again apparent, but less marked than with neonatal treatment. DNA was proportionately decreased (Table 1), suggesting that the oestrogen had mainly affected cell proliferation rather than cell size. Although total amounts of secretory proteins present in these two tissues were about half of those in the controls (Table 2), individual cells (on a DNA basis) are probably just as active as normal. Proteins secreted

(Fig. 1*b*) and synthesized (Fig. 2*b*) by the ventral prostate are normal, but distinct differences were seen in the seminal vesicle (Fig. 1*a*). Thus, in some animals the higher-molecular-weight secretory proteins (86K, 48K, 39K) were absent and in a few the smaller proteins S (15K) and F (11K) were also noticeably decreased. In some cases an additional prominent band with a mobility similar to albumin (68K) was present. By overloading gels with 35 S-labelled proteins (Fig. 2*a*), differences could be seen in the higher-molecular-weight range which might account for these dissimilar profiles. There is no particularly obvious protein corresponding to the 68K albumin-like band, but, if it is albumin, it is unlikely to be synthesized *de novo* within the seminal vesicle.

During this developmental period, oestradiol has about the same effect on the epididymis as it did in the neonatal and prepubertal phases (Table 1). DNA is again proportionately decreased, suggesting fewer cells, but these cells are probably just as, or even more, active in protein secretion (Table 2) and synthesis (Table 3) than usual. No differences were detected in the types of protein secreted (Figs. 1*c* and 1*d*) or synthesized (results not shown) by either region.

Adult animals. Treatment of adults for 5 days with oestradiol benzoate caused a small decrease (19%) in seminal-vesicle weight, but no change in DNA content (Table 1). The amount of seminal-vesicle secretion was decreased by 64% (Table 2). This decrease can be attributed, at least in part, to a lower rate of protein synthesis (Table 3), but, in contrast with neonatal treatment, there were no differential effects on the classes of proteins synthesized (Fig. 2*a*). Prostatic weight was decreased by over 40%, but decreases in DNA and secretory protein were less significant (Tables 1 and 2). Although the rate of protein synthesis was lowered

by about 25% (Table 3), this was not accompanied by any obvious differential effects on the types of protein synthesized (Fig. 2*b*). Finally, the epididymis of adults was affected by oestrogen to about the same degree as at other maturational ages (Table 1), although the decrease in amount of protein in luminal fluid may have been greater (Table 2).

Discussion

Using oestradiol benzoate as a probe to identify periods crucial for the development of the male accessory glands, we have confirmed earlier reports (Kincl *et al.*, 1963, 1965; Harris & Levine, 1965; Simmons, 1971; Lamartiniere *et al.*, 1979) that the neonatal period is particularly critical for growth of the seminal vesicles and ventral prostate. However, we have extended these morphological observations and shown that the neonatal phase is also important for later functional activity in the adult. In both seminal vesicles and ventral prostate, neonatal oestrogen exposure caused a marked decrease in the amount of secretion produced by the glands in the adult, especially by the seminal vesicles (less than 1% of the normal amount). These effects on secretory proteins were not due to a generalized fall in overall rates of protein synthesis, but were markedly differential in that there was a pronounced shift away from synthesis of major secretory proteins characteristic of control animals. Neonatal oestrogen treatment does not merely delay development, but causes permanent functional abnormalities in the seminal vesicles and ventral prostate. Harris & Levine (1965) also found that morphological effects of neonatal oestrogen treatment on these tissues were not reversed even over 14 months.

Although the neonatal phase is clearly critical for the seminal vesicles and ventral prostate, other periods seem much less important. Thus oestradiol benzoate given during the prepubertal period was completely ineffective, and even at puberty its effects on these accessory tissues were minor compared with those after neonatal exposure. It could of course be argued that these periods are still critical for development, but that their developmental signals are not antagonized by oestradiol benzoate.

Interestingly, functional development of the epididymis does not appear to be critically dependent on the neonatal period. It is possible that the epididymis has a critical period at some time just before or just after the neonatal stage and is thus out of phase with the seminal vesicles and prostate. Alternatively, epididymal developmental signals may differ from those for the other tissues and are not antagonized by oestradiol benzoate. Nevertheless, it is clear that tissues of common embryological origin (seminal vesicles and epididymides) need not share

either a common critical developmental period or a common developmental signal. In fact there is extensive ultrastructural evidence (Flickinger, 1969*a,b*, 1970, 1971; Leeson & Leeson, 1964*a,b*; Sun & Flickinger, 1979) that these glands have different pre- and post-natal developmental patterns.

The neonatal phase has also been shown to be important for the development of non-reproductive tissues showing sexual dimorphism in the adult. Here, androgenic 'imprinting' or 'programming' must occur in neonatal life if male rather than female activity is to occur subsequently in the adult. Examples of this neonatal imprinting concern the brain (Harris, 1964; Young *et al.*, 1964; Harris & Levine, 1965) and the liver (DeMoor & Denef, 1968; Einarsson *et al.*, 1973; Chung, 1977). Differentiation of the accessory glands in the male, but not the female, obviously limits their response to neonatal developmental signals, and so, in this sense, the phenomenon that we report here is not perhaps directly analogous to these examples of imprinting. Nonetheless, it is comparable in so far as a critical neonatal period exists during which a stimulus must be received if subsequent development is to proceed normally. Furthermore, if this critical period is missed, later stimulation appears to be ineffective.

At present we are uncertain as to how oestradiol benzoate acts during the neonatal period to antagonize normal maturation. It could act directly via oestrogen receptors known to be present in immature and adult accessory tissues (van Beurden-Lamers *et al.*, 1974; Danzo *et al.*, 1977; Robinette *et al.*, 1978). If androgenic steroids are involved in the neonatal period, oestradiol benzoate may antagonize their action in the target tissue by competing for androgen receptors (Fang *et al.*, 1969; Rennie & Bruchovsky, 1973). However, if androgen receptors are involved, there is some evidence to suggest that the permanent effects of neonatal exposure to oestrogens are not due to continued absence of androgen receptors in the accessory glands (Simmons, 1971). Oestradiol benzoate may of course be acting indirectly via the pituitary to depress gonadotropin release and hence testosterone secretion by the testes (Verjans *et al.*, 1974; Fraser, 1979; Robaire *et al.*, 1979) or, by analogy with examples of imprinting (Harris, 1964; Young *et al.*, 1964; Harris & Levine, 1965; DeMoor & Denef, 1968; Einarsson *et al.*, 1973; Chung, 1977), it may programme the brain in some yet unrecognized manner. Alternatively, neonatal oestrogen may be acting via the testis, since there is evidence that oestrogen can have a direct effect on testicular steroidogenesis (Bartke *et al.*, 1977; Steinberger *et al.*, 1977; Sivelle *et al.*, 1978; Robaire *et al.*, 1979). Finally, in view of the importance of stromal-epithelial interactions in morphogenesis (Lasnitzki & Mizuno, 1979; Cunha *et al.*, 1980) and their possible

role in normal functioning of these glands in the adult (Franks *et al.*, 1970; Drews & Drews, 1975), it might even be that neonatal imprinting is mediated primarily by oestrogenic effects on the stroma of the accessory glands rather than the epithelium. Whatever the mechanism, Kincl *et al.* (1965) have shown the effect to be essentially oestrogen-specific, since progesterone, cortisol and other steroids are ineffective.

When we compare the effects of oestrogen treatment of neonatal rats with those of exposing sexually mature adults to the same hormone or of castrating adults, different patterns are seen for seminal vesicles and ventral prostate. Thus prostatic secretory-protein synthesis is affected in the same differential fashion by both neonatal oestrogen treatment and adult castration (Fig. 2*b*; Parker *et al.*, 1979; Parker & Scrace, 1979; Higgins & Parker, 1980). Such is not the case with the seminal vesicles, where castration results in a generalized decrease in protein synthesis (Higgins *et al.*, 1976; Higgins & Burchell, 1978; Higgins & Parker, 1980), as distinct from the differential effects of neonatal oestrogen treatment. In the adult, on the other hand, oestradiol does have the same generalized effect on seminal-vesicle protein synthesis as castration, which might have been expected from the similar ultrastructural degeneration after these two procedures (Thompson *et al.*, 1979). Ultrastructural studies (Groth & Brandes, 1960; Kjaerheim *et al.*, 1974) might also suggest that castration and oestrogen exposure in the adult would elicit similar responses from the prostate. Surprisingly, we found this did not happen; oestrogen evoked a generalized response. At the moment we have no explanation for this finding, but the comparisons discussed above emphasize the need for caution in interpreting ultrastructural changes in terms of biological function and underline the fact that closely related androgen-dependent accessory tissues nevertheless display quite different developmental patterns and hormonal responses.

Work with mice has shown that removal of the seminal vesicles, but not the ventral prostate, affects male fertility (Pang *et al.*, 1979). It is perhaps reasonable to conclude from this that the infertility reported for rats exposed to oestrogens during neonatal life is not due to the abnormal synthesis and secretion of prostatic proteins reported here. Unfortunately we cannot positively conclude that infertility is a direct consequence of abnormal seminal-vesicle function, since there may be many other contributory factors. Thus neonatal oestrogen treatment has been shown to decrease spermatogenesis (Kincl *et al.*, 1963; Maqueo & Kincl, 1964; Harris & Levine, 1965; Hendricks & Gerall, 1970; Lamartiniere *et al.*, 1979), to impair penile development (Feder, 1967; Zadina *et al.*, 1979) and to depress mating activity (Whalen, 1964; Harris &

Levine, 1965; Feder, 1967; Hendricks & Gerall, 1970; Brown-Grant *et al.*, 1975; Zadina *et al.*, 1979). A different experimental approach will be required to evaluate the relative importance of all these factors.

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References

- Bartke, A., Williams, K. I. H. & Dalterio, S. (1977) *Biol. Reprod.* **17**, 645–649
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Borenfreund, E., Fitt, E. & Bendich, A. (1961) *Nature (London)* **191**, 1375–1377
- Brooks, D. E. (1976*a*) *Biochem. J.* **156**, 527–537
- Brooks, D. E. (1976*b*) *J. Reprod. Fertil.* **46**, 31–38
- Brooks, D. E. & Higgins, S. J. (1980) *J. Reprod. Fertil.* **59**, 363–375
- Brown-Grant, K., Fink, G., Greig, F. & Murray, M. A. F. (1975) *J. Reprod. Fertil.* **44**, 25–42
- Burton, K. (1956) *Biochem. J.* **62**, 315–323
- Callow, R. R. & Deanesly, R. (1935) *Biochem. J.* **29**, 1424–1445
- Chung, L. W. K. (1977) *Biochem. Pharmacol.* **26**, 1979–1984
- Cunha, G. R., Chung, L. W. K., Shannon, J. M. & Reese, B. A. (1980) *Biol. Reprod.* **22**, 19–42
- Danzo, B. J., Wolfe, M. S. & Curry, J. B. (1977) *Mol. Cell. Endocrinol.* **6**, 271–279
- DeLuca, H. F. (1972) *Manometric and Biochemical Techniques* (Umbreit, W. W., Burris, R. H. & Stauffer, J. F., eds.), 5th edn., pp. 146–150, Burgess, Minneapolis
- DeMoor, P. & Denef, C. (1968) *Endocrinology* **82**, 480–491
- Desjardins, C., MacMillan, K. L. & Hafs, H. D. (1969) *Anat. Rec.* **161**, 17–22
- Dohler, K. D. & Wuttke, W. (1975) *Endocrinology* **97**, 898–907
- Drews, U. & Drews, U. (1975) *Cell* **6**, 475–479
- Einarsson, K., Gustafsson, J. A. & Stenberg, A. (1973) *J. Biol. Chem.* **248**, 4987–4997
- Fang, S., Anderson, K. M. & Liao, S. (1969) *J. Biol. Chem.* **244**, 6584–6595
- Feder, H. H. (1967) *Anat. Rec.* **157**, 79–86
- Feldman, S. C. & Block, E. (1978) *Endocrinology* **102**, 999–1007
- Flickinger, C. J. (1969*a*) *Z. Zellforsch.* **96**, 344–360
- Flickinger, C. J. (1969*b*) *J. Ultrastruct. Res.* **27**, 344–360
- Flickinger, C. J. (1970) *Z. Zellforsch.* **109**, 1–14
- Flickinger, C. J. (1971) *Z. Zellforsch.* **113**, 157–173
- Franks, L. M., Riddle, P. N., Carbonnel, A. W. & Gey, G. O. (1970) *J. Pathol.* **100**, 113–119
- Fraser, H. M. (1979) in *Mechanisms of Hormone Action* (Austin, C. R. & Short, R. V., eds.), vol. 7, pp. 1–52, Cambridge University Press, Cambridge
- Groth, D. P. & Brandes, D. (1960) *J. Ultrastruct. Res.* **4**, 166–181

- Harris, G. W. (1964) *Endocrinology* **75**, 627–647
- Harris, G. W. & Levine, S. (1965) *J. Physiol. (London)* **181**, 379–400
- Hendricks, S. E. & Gerall, A. A. (1970) *Endocrinology* **87**, 435–439
- Higgins, S. J. & Burchell, J. M. (1978) *Biochem. J.* **174**, 543–551
- Higgins, S. J. & Parker, M. G. (1980) *Biochem. Actions Horm.* **7**, 287–309
- Higgins, S. J., Burchell, J. M. & Mainwaring, W. I. P. (1976) *Biochem. J.* **158**, 271–282
- Kincl, F. A., Pi, A. F. & Lasso, L. H. (1963) *Endocrinology* **72**, 966–968
- Kincl, F. A., Pi, A. F., Maqueo, M., Lasso, L. H., Oriol, A. & Dorfman, R. I. (1965) *Acta Endocrinol. (Copenhagen)* **49**, 193–206
- Kjaerheim, A., Dahl, E. & Tveter, K. J. (1974) *Lab. Invest.* **31**, 391–397
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lamartiniere, C. A., Dieringer, C. S., Kita, E. & Lucier, G. W. (1979) *Biochem. J.* **180**, 313–318
- Lasnitzki, I. & Mizuno, T. (1979) *J. Endocrinol.* **82**, 171–178
- Lee, V. W. K., DeKretser, D. M., Hudson, B. & Wang, C. (1975) *J. Reprod. Fertil.* **42**, 121–126
- Leeson, C. R. & Leeson, T. R. (1964a) *Anat. Anz.* **114**, 159–167
- Leeson, T. R. & Leeson, C. R. (1964b) *Anat. Anz.* **114**, 168–180
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Maqueo, M. & Kincl, F. A. (1964) *Acta Endocrinol. (Copenhagen)* **46**, 25–30
- Moger, W. H. (1977) *Endocrinology* **100**, 1027–1032
- Ohno, S. (1979) *Monogr. Endocrinol.* **11**
- Pang, S. F., Chow, P. H. & Wong, T. M. (1979) *J. Reprod. Fertil.* **56**, 129–132
- Parker, M. G. & Scrace, G. T. (1979) *Eur. J. Biochem.* **85**, 399–406
- Parker, M. G., Scrace, G. T. & Mainwaring, W. I. P. (1979) *Biochem. J.* **170**, 115–121
- Podesta, E. J. & Rivarola, M. A. (1974) *Endocrinology* **95**, 455–461
- Price, D. (1936) *Am. J. Anat.* **60**, 79–123
- Rennie, P. & Bruchovsky, N. (1973) *J. Biol. Chem.* **248**, 3288–3297
- Robaire, B., Ewing, L. L., Irby, D. C. & Desjardins, C. (1979) *Biol. Reprod.* **21**, 455–463
- Robinette, C. L., McGraw, R. G., Cricco, R. P. & Mawhinney, M. G. (1978) *Arch. Biochem. Biophys.* **191**, 517–524
- Simmons, J. E. (1971) *Acta Endocrinol. (Copenhagen)* **67**, 535–543
- Siville, P. C., McNeilly, A. S. & Collins, P. M. (1978) *Biol. Reprod.* **18**, 878–885
- Steinberger, E., Chowdhury, M. & Tcholakian, R. (1977) *Andrologia* **9**, 307–312
- Sun, E. L. & Flickinger, C. J. (1979) *Am. J. Anat.* **154**, 27–56
- Thompson, S. A., Rowley, D. R. & Heidger, P. M. (1979) *Am. J. Anat.* **154**, 525–544
- van Beurden-Lamers, W. M. O., Brinkmann, A. O., Mulder, E. & van der Molen, H. J. (1974) *Biochem. J.* **140**, 495–502
- Verjans, H. L., de Jong, F. H., Cooke, B. A., van der Molen, H. J. & Eik-Nes, K. B. (1974) *Acta Endocrinol. (Copenhagen)* **77**, 636–642
- Warren, D. W., Haltmeyer, G. C. & Eik-Nes, K. B. (1973) *Biol. Reprod.* **8**, 560–565
- Weisz, J. & Ward, I. L. (1980) *Endocrinology* **106**, 306–315
- Whalen, R. E. (1964) *J. Comp. Physiol. Psychol.* **57**, 175–182
- Wilson, J. D., Griffi, J. E. & George, F. W. (1980) *Biol. Reprod.* **22**, 9–17
- Young, W. C., Goy, R. W. & Phoenix, C. H. (1964) *Science* **143**, 212–218
- Zadina, J. E., Dunlap, J. L. & Gerall, A. A. (1979) *J. Comp. Physiol. Psychol.* **93**, 314–322