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# Concentrations of Putrescine and Polyamines and their Enzymic Synthesis during Androgen-Induced Prostatic Growth

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1. Castration of adult rats resulted in marked decreases in the amounts of putrescine, spermidine and spermine in the ventral prostate gland. Spermidine concentrations decline rapidly over the first 11 days after androgen withdrawal, reaching a value of only 12% of normal controls. Spermine concentrations diminish more slowly, reaching 24% of normal within 11 days. The spermidine/spermine molar ratio falls from 0.9 to 0.46 under these conditions. Putrescine concentrations decrease by 70% at 7 days after castration and then remain constant for some days. 2. After daily injections of testosterone propionate to rats castrated 7 days previously, prostatic spermidine and putrescine concentrations increase significantly within 24h; normal or even greater values are observed within 8 and 4 days respectively. In contrast, the spermine concentration does not increase until 5 days after commencement of androgen treatment. 3. The activities of two enzymes involved in polyamine biosynthesis (ornithine decarboxylase and a putrescine-activated *S*-adenosyl-L-methionine decarboxylase system) were greatly decreased soon after castration: after 7 days the respective values were 15% of normal for ornithine decarboxylase and 7% of normal for putrescine-dependent decarboxylation of *S*-adenosyl-L-methionine. Injection of testosterone propionate into animals castrated 7 days previously induced a rapid increase in both enzymic activities: ornithine decarboxylase was doubled in 6h, and increased three- to four-fold within 48h, whereas the putrescine-dependent decarboxylation of *S*-adenosyl-L-methionine doubled in 3h and increased tenfold within 48h of commencement of daily androgen treatments. 4. The activity of these enzyme systems was very low in the ventral prostates of hypophysectomized rats and was increased by administration of testosterone in a manner similar to that found in castrated rats. 5. Alterations in the activity of two ventral-prostate enzymes involved in ornithine production (arginase) and utilization (ornithine–2-oxoglutarate transaminase) that result from changes in the androgenic status of rats are described. 6. The findings presented suggest that the activities of ornithine decarboxylase and the putrescine-dependent *S*-adenosyl-L-methionine decarboxylase system, rather than ornithine concentrations, are rate-limiting for the formation of putrescine and polyamines in rat ventral prostate. 7. The relation of polyamines to androgen-induced prostatic growth is discussed with particular reference to the biosynthesis of proteins and nucleic acids.

The polyamines spermidine and spermine, and their parent diamine putrescine are widely distributed among mammalian cells (Tabor, Tabor & Rosenthal, 1961; Jänne, Raina & Siimes, 1964; Tabor & Tabor, 1964). Several reviews have documented numerous effects of the polyamines on some enzymic processes and the ability of these substances to interact with and stabilize polynucleotides and various biological membranes (Tabor *et al.* 1961; Raina, 1963; Tabor & Tabor, 1964; Cohen & Raina, 1967; Jänne, 1967; Williams-Ashman, Pegg & Lockwood, 1969). However, most of these studies were in cell-free systems under

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rather unphysiological conditions. Such effects of spermidine and spermine *in vitro* may turn out to reflect their metabolic importance in living cells but at present the physiological significance of polyamines in mammalian tissues remains enigmatic.

Studies on the relation of polyamines to the growth of micro-organisms and animal tissues have concentrated on the possibility that spermidine, spermine or both may regulate the biosynthesis of nucleic acid and proteins. Direct addition of spermidine and spermine can enhance RNA synthesis by DNA-dependent RNA polymerases (Krakow, 1963; Fox & Weiss, 1964; Ballard & Williams-Ashman, 1966; Abraham, 1968; Walter, Zillig, Palm & Fuchs, 1967). The polyamines also form stabilizing complexes with DNA (Tabor & Tabor, 1964; Liquori *et al.* 1967) and ribosomes (Cohen & Lichtenstein, 1960; Colbourn, Witherspoon & Herbst, 1961; Silman, Artman & Engelberg, 1965; Norton, Erdmann & Herbst, 1968), and can stimulate protein biosynthesis in some cell-free ribosomal systems (Hershko, Amoz & Mager, 1961; Martin & Ames, 1962; Tanner, 1967). The possible importance of polyamines in living organisms was investigated by searching for correlations between polyamine concentrations and increases in protein and polynucleotide production during growth of bacteria (Cohen & Raina, 1967; Cohen, Hoffner, Jansen, Moore & Raina, 1967; Raina, Jansen & Cohen, 1967), chicken embryos (Raina, 1963; Caldarera, Barbiroli & Moruzzi, 1965; Moruzzi, Barbiroli & Caldarera, 1968), sea-urchin eggs (Barros & Guidice, 1968) and in rat liver after treatment with growth hormone (Kostyo, 1966; Jänne, 1967) or partial hepatectomy (Dykstra & Herbst, 1965; Raina, Jänne & Siimes, 1966; Jänne, 1967). It is surprising that the prostate gland was not examined previously, because in many mammals this organ is the richest source of polyamines in the body (Mann, 1964; Tabor & Tabor, 1964; Rhodes & Williams-Ashman, 1964), and its growth and functional activities are dependent on the circulating concentrations of androgenic steroids.

The ventral prostate gland of the rat has been widely employed as a test object for biochemical studies on androgen-induced tissue growth, and a considerable body of information is available on the metabolic characteristics of this organ (Price & Williams-Ashman, 1961, Williams-Ashman, 1965*a*,*b*; Williams-Ashman, Liao, Hancock, Jurkowitz & Silverman, 1964; Williams-Ashman & Shimazaki, 1967, 1968; Liao & Fang, 1969). Castration of adult rats leads to a rapid involution of the ventral prostate and prompt cessation of its secretory functions. The effects of androgens on the growth of this organ have often been studied in adults castrated a week or more previously, by which time the regression of the gland is very pronounced. Prostatic growth resulting from daily dosage of such animals with testosterone is a complex process involving hypertrophy of epithelial cells, retention of secretory products in the glandular acini, hyperplasia of various prostatic cell types and elaboration of new blood vessels. Castration leads to a marked decline in the endoplasmic reticulum membranes and ribosomes in prostatic epithelial cells; the nuclei become small and pyknotic, and there is a decline in oxygen consumption and in the mitochondrial population density (Price & Williams-Ashman, 1961; Williams-Ashman, 1965*a*,*b*; Williams-Ashman & Shimazaki, 1968). Androgen treatment reverses these changes and leads to the following chronological alterations in macromolecular biosynthetic activities. Incorporation of labelled amino acids into protein by isolated ribosomes (Liao & Williams-Ashman, 1962; Williams-Ashman *et al.* 1964) and mitochondria (Pegg & Williams-Ashman, 1968*a*) is increased within 48h of commencement of testosterone injections. The entry of labelled precursors into DNA, and DNA polymerase activity, show immense yet transitory increases at 2–5 days after the commencement of daily androgen treatments (Kosto, Calvin & Williams-Ashman, 1967; Coffey, Shimazaki & Williams-Ashman, 1968*b*). RNA synthesis and nuclear RNA polymerase activities (determined at low ionic strengths) increased much more rapidly; detectable increases in RNA polymerase activity were observed within 1h of testosterone administration (Williams-Ashman *et al.* 1964; Liao, Leininger, Sagher & Barton, 1965; Liao & Fang, 1969). This change in nuclear RNA polymerase activity and a pronounced but transient fall in the total ATP concentration in the gland (Ritter, 1966; Coffey, Ichinose, Shimazaki & Williams-Ashman, 1968*a*) are the earliest metabolic events known to occur in rat ventral prostate after administration of testosterone to castrated adult rats.

The wealth of such information on metabolic and cytological changes during early phases of androgen-induced prostatic growth and the exceptionally high concentrations of polyamines in the ventral prostate suggest that this organ should provide excellent material for investigation of the biosynthesis and functions of polyamines in mammalian tissues. Studies on the enzymic pathways for the formation of putrescine, spermidine and spermine in this organ were reported from this laboratory (Pegg & Williams-Ashman, 1968*b*, 1969; Pegg, 1969; Williams-Ashman *et al.* 1969). Caldarera, Moruzzi, Barbiroli & Moruzzi (1968) documented effects of androgens on polyamine concentrations in rat ventral prostate and the influence of spermine and spermidine *in vitro* on prostatic nuclear RNA

polymerase activities. They concluded that the androgen-induced alterations in RNA polymerase activity may be mediated via changes in polyamine concentrations in the prostate. From their measurements of changes in spermidine concentrations in rat uterus, prostate and seminal vesicle after gonadectomy and the subsequent administration of sex hormones, Moulton & Leonard (1969) concluded that 'steroid-induced changes in spermidine levels may play an important role in the synthesis of certain types of RNA, formation of ribosomes, and synthesis of protein in accessory sex organs'.

Polyamines, and particularly spermine, are present in seminal plasmas of various mammals and the seminal polyamines are largely if not exclusively derived from prostatic secretion (Price & Williams-Ashman, 1961; Mann, 1964; Tabor & Tabor, 1964). Whether the polyamines in semen are of functional significance in metabolism, survival or transport of spermatozoa in the male or female genital tracts is not known; little or no positive evidence exists in favour of such physiological actions of spermine and spermidine (Mann, 1964; Williams-Ashman, 1962). On the contrary, the appearance of polyamines in prostatic fluid (formed by apocrine or merocrine mechanisms) could simply represent a 'washing out' of these substances during the secretory process, and their primary functions related more to the dynamic biochemistry of macromolecular biosyntheses and active secretory mechanisms in prostatic epithelial cells, which elaborate large quantities of proteins for export into prostatic fluid (Williams-Ashman *et al.* 1969). It should be emphasized that in sexually mature animals the total concentrations of spermidine and spermine measured in the ventral prostate represent the sum of intracellular polyamines and the polyamines present in secretions retained in the lumina of the gland.

Putrescine is formed in the rat ventral prostate by direct decarboxylation of L-ornithine (Pegg & Williams-Ashman, 1968*b*). The importance of ornithine as a precursor of polyamines in mammalian tissues has only recently been realized. Ornithine can be formed in the prostate by action of arginase and is also removed by the action of ornithine transaminase (L-ornithine–2-oxo acid aminotransferase, EC 2.6.1.13) (Williams-Ashman *et al.* 1969). Spermidine is formed from putrescine and *S*-adenosyl-L-methionine (Tabor & Tabor, 1964; Pegg & Williams-Ashman, 1968*c*, 1969). However, it was not possible to separate the enzyme responsible for spermidine synthesis in the ventral prostate into two distinct fractions, one catalysing the decarboxylation of *S*-adenosyl-L-methionine and the other the transfer of the propylamine moiety from 'decarboxylated *S*-adenosyl-L-methionine' to putrescine forming spermidine, as has been shown for the enzymes that synthesize spermidine in *Escherichia coli* (Tabor, 1962*a*,*b*; Wickner, Tabor & Tabor, 1969). The decarboxylation of *S*-adenosyl-L-methionine by prostatic extracts was markedly and specifically stimulated by the addition of putrescine; moreover, spermidine, carbon dioxide and 5′-methylthioadenosine were formed in stoicheiometric amounts in the presence of putrescine (Pegg & Williams-Ashman, 1968*c*, 1969). The putrescine-dependent decarboxylation of *S*-adenosyl-L-methionine by prostatic extracts therefore represents a convenient assay of the ability of the extracts to form spermidine.

This paper describes changes in the concentrations of ornithine, putrescine, spermidine and spermine in the prostate gland after castration and androgen treatment, and details alterations in the activities of enzymes involved in polyamine biosynthesis under the same conditions. Possible sites for the regulation of polyamine synthesis in mammalian tissues are considered and the importance of polyamines in the androgen-induced growth of the prostate is discussed with particular reference to the role of polyamines in nucleic acid metabolism. Portions of this work were the subject of preliminary communications (Pegg & Williams-Ashman, 1968*d*,*e*; Williams-Ashman *et al.* 1969).

## EXPERIMENTAL

*Materials.* L-[1-$^{14}$C]Methionine (15.0mCi/mmol) was purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. DL-[1-$^{14}$C]Ornithine (2.3mCi/mmol), [1,4-$^{14}C_2$]putrescine dihydrochloride (5.22mCi/mmol), aminopropyl[1,4-$^{14}C_2$]tetramethylenediamine trihydrochloride (spermidine, 5.9mCi/mmol), and bis(aminopropyl)[1,4-$^{14}C_2$]tetramethylenediamine tetrahydrochloride (spermine, 4.25mCi/mmol) were obtained from the New England Nuclear Corp., Boston, Mass., U.S.A.

The hydrochloride salts of putrescine, spermidine and spermine and L-arginine, pyridoxal phosphate, 2-oxoglutaric acid (monopotassium salt), hydrindantin, tris, urease (urea aminohydrolase, EC 3.5.1.5), *S*-adenosyl-L-methionine, ATP and testosterone propionate were products of the Sigma Chemical Co., St Louis, Mo., U.S.A. L-Ornithine, L-methionine and ninhydrin were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. *o*-Aminobenzaldehyde was obtained from Fluka A.-G., Buchs SG, Switzerland.

*S*-Adenosyl-L-[carboxy-$^{14}$C]methionine was synthesized as described by Pegg & Williams-Ashman (1969) and stored at a concentration of 2$\mu$mol/ml at −20°C after adjustment of the solution to pH3.0 with M-HCl. Unlabelled commercial *S*-adenosyl-L-methionine was repurified and also stored in solution, pH3.0, at −20°C (Pegg & Williams-Ashman, 1968*c*, 1969).

*Animals.* Adult male rats of the Sprague–Dawley strain (300–400g body wt.) were purchased from Huntingdon Farms, West Conshohocken, Pa., U.S.A., and maintained on a diet of Purina Laboratory Chow and water *ad*

*libitum* under a constant 12h light–12h dark lighting schedule. Castration (orchiectomy) was performed via the scrotal route under ether anaesthesia. Testosterone propionate (1mg) was administered subcutaneously in 0.2ml of sesame oil. Animals were killed at the same time each day to minimize effects due to diurnal fluctuations. Male hypophysectomized rats (140–160g body wt.) were purchased from Hormone Assay Laboratories, Chicago, Ill., U.S.A. Rats were killed by cervical dislocation for experiments in which enzyme activities were measured, or by injection of 1ml of sodium pentobarbital soln. (65mg/ml) when polyamine concentrations were determined. The ventral prostate glands were freed of fat and connective tissue *in situ* and rapidly placed in the homogenizing medium.

*Preparation of tissue extracts for enzyme assays.* All operations were performed at 0–4°C. Ventral prostate tissue was weighed and homogenized in a glass apparatus fitted with a rotating pestle with 3vol. of homogenizing medium consisting of 0.25M-sucrose, 10mM-2-mercaptoethanol and 0.1mM-disodium EDTA at pH7.1 (adjusted with NaOH). The homogenate was centrifuged at 120*g* for 10min and the pellet discarded. The supernatant fluid was centrifuged at 15000*g* for 10min. The crude mitochondrial pellet was washed twice by resuspension in the homogenization medium followed by centrifugation at 15000*g* for 10min. The washed pellet was either frozen at −20°C for not longer than 3 days, or used directly for assay of ornithine aminotransferase activity. The supernatant fluid from the first centrifugation at 15000*g* was then spun at 45000rev./min for 90min in the no. 50 rotor of a Spinco model L-2 ultracentrifuge. The supernatant fluid was passed through a Bio-Gel P-2 column (20cm × 0.7cm) previously equilibrated with the homogenization medium. Portions (2ml) of the ultracentrifuged supernatant fraction were freed from materials of low molecular weight in this manner; all the protein present in the solution was collected in a total volume of 2.6ml. In some experiments all of the preparative solutions were made 0.1mM with respect to pyridoxal phosphate, but this had no significant effect on the activities of the enzymes under investigation and was discontinued.

*Assay of ornithine–2-oxo acid aminotransferase activity.* The reaction was followed by measuring the formation of glutamic $\gamma$-semialdehyde by the procedure of Peraino & Pitot (1963). The reaction mixture contained 125$\mu$mol of $KH_2PO_4$(adjusted to pH7.6 with KOH), 100$\mu$mol of L-ornithine, 20$\mu$mol of 2-oxoglutarate, 0.05$\mu$mol of pyridoxal phosphate, 0.05ml of 0.2M-*o*-aminobenzaldehyde (dissolved in 40% ethanol) and approx. 2mg of protein of the washed mitochondrial fraction from the prostatic homogenate, in a total volume of 1.0ml. The mixture was incubated at 37°C for 30min. The reaction was stopped by the addition of 2.0ml of 7.5% (w/v) trichloroacetic acid. The protein precipitate was removed by centrifugation and the extinction at 440nm measured. The amount of 1-pyrroline-5-carboxylic acid (the spontaneously cyclized form of glutamic $\gamma$-semialdehyde) present was calculated by using $\epsilon$ 2710 for the coloured complex produced with *o*-aminobenzaldehyde (Strecker, 1965).

*Assay of arginase activity.* Arginase activity was determined by determining the rate of ornithine formation. A 0.5ml portion of the desalted ultracentrifuged supernatant fraction from the prostatic homogenate was activated with $Mn^{2+}$ (Greenberg, 1960) by incubation at 37°C for 30min in the presence of 25$\mu$mol of tris–HCl buffer, pH7.3, and 3$\mu$mol of $MnCl_2$. The assay was then initiated by the addition of 75$\mu$mol of tris–NaOH medium, pH12.1, and 60$\mu$mol of L-arginine, pH9.4. The final pH of the assay mixture was 9.4 and the total volume 1.5ml. After incubation at 37°C for 30min the reaction was stopped by the addition of 10% (w/v) trichloroacetic acid and the protein precipitate removed by centrifugation. Ornithine in the supernatant solution was measured by the colorimetric method of Chinard (1952) as modified by Ratner (1962). The reagent was made by dissolving 250mg of ninhydrin and 37.6mg of hydrindantin in a mixture of 4.0ml of 6.0M-$H_3PO_4$ and 6.0ml of acetic acid. A brief period of warming was necessary for dissolving the ninhydrin. A 1ml portion of the reagent was added to 0.5ml of the trichloroacetic acid filtrate, 0.5ml of water and 1.5ml of acetic acid. This mixture was heated at 100°C for 30min, cooled to room temperature and the volume increased by the addition of 2.5ml of acetic acid. The extinction at 515nm was then measured and the ornithine present determined by reference to a standard curve constructed by treating known concentrations of ornithine in a similar manner.

*Measurement of the stoicheiometry between the formation of ornithine and urea from arginine by prostatic arginase.* In these experiments the arginase assay was carried out as described above except that the reaction was stopped by the addition of 0.3ml of 0.5M-$H_2SO_4$. The precipitate was removed by centrifugation and the pH of the supernatant adjusted to 7.2 with 100$\mu$mol of $KH_2PO_4$ solution (adjusted to pH7.2 with KOH) and M-NaOH as required. Water was added to bring the final volume to 3.0ml. Portions (0.5ml) of this solution were used for the determination of ornithine and urea. The amount of urea present was measured by the method of Conway (1947). The centre well of a modified Conway micro-diffusion dish (Obrink, 1955) contained 1.0ml of 0.01M-HCl and the outer chamber contained 0.5ml of jack-bean urease [0.2% (w/v) in 35% (v/v) glycerol]. The sample (0.5ml) containing urea was rapidly placed in the outer chamber and thoroughly mixed after the lid was replaced. After 15min, 1.0ml of saturated $K_2CO_3$ solution was added to the outer chamber and the dish rotated intermittently for the next 2h. The contents of the centre well were then removed and combined with water washings from the well to give a volume of 10ml. Then 1ml of Folin–Wu Nessler's Reagent (A. H. Thomas Inc., Philadelphia, Pa., U.S.A.) was added and the extinction at 480nm recorded. The amount of urea present was calculated by comparison with a standard curve constructed by treating known concentrations of urea in the same manner.

*Assay of ornithine decarboxylase activity.* Prostatic ornithine decarboxylase was measured by recording the production of $^{14}CO_2$ from [1-$^{14}$C]ornithine by the desalted ultracentrifuged prostatic extract (Pegg & Williams-Ashman, 1968*b*). Details of the procedure for trapping and counting $^{14}CO_2$ were given previously (Pegg & Williams-Ashman, 1968*b*,*c*, 1969). The reaction mixture contained 50$\mu$mol of tris–HCl buffer, pH7.2, 0.05$\mu$mol of pyridoxal phosphate, 2.5$\mu$mol of 2-mercaptoethanol, 2.1$\mu$mol of DL-[1-$^{14}$C]ornithine (0.29c.p.m./pmol) and the prostatic extract in a total volume of 1.0ml. The flasks were incubated at 37°C for 1h. The reaction was

measured with at least two different protein concentrations for each group of animals and the assay conditions were such that the rate of release of $^{14}CO_2$ was proportional to the amount of protein added over the range used (4–30mg) and to the time of incubation.

*Assay of putrescine-activated S-adenosyl-L-methionine decarboxylase activity.* The putrescine-dependent decarboxylation of *S*-adenosyl-L-methionine was measured by recording the production of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxy*-$^{14}$C]methionine by the desalted ultracentrifuged prostatic extract (Pegg & Williams-Ashman, 1968*c*, 1969). The reaction mixture contained 2.5$\mu$mol of putrescine, 100$\mu$mol of $NaH_2PO_4$ solution (adjusted to pH7.0 with 5M-NaOH), 0.2$\mu$mol of *S*-adenosyl-L-[*carboxy*-$^{14}$C]-methionine (1.0c.p.m./pmol) and the prostatic extract in a total volume of 1.0ml. The reaction proceeded for 30min at 37°C. The rate of release of $^{14}CO_2$ was proportional to the amount of prostatic protein added over the range used in these experiments (2–20mg) and a linear rate of reaction was obtained over the 30min incubation period. All preparations from rats with each hormonal treatment were assayed with at least two protein concentrations and the mean value was determined.

*Assay of spermidine synthesis by prostatic extracts.* The formation of spermidine by prostatic extracts was assayed by measuring the incorporation of [1,4-$^{14}C_2$]-putrescine into spermidine in the presence of *S*-adenosyl-L-methionine. The assay medium contained 50$\mu$mol of $NaH_2PO_4$ solution (adjusted to pH7.0 with NaOH), 0.1$\mu$mol of *S*-adenosyl-L-methionine, 1.25$\mu$mol of [1,4-$^{14}C_2$]putrescine (1.0c.p.m./pmol) and the prostatic extract in a total volume of 0.5ml. The tubes were incubated at 37°C for 30min, and the labelled spermidine produced was separated and its radioactivity measured as previously described (Pegg & Williams-Ashman, 1968*c*, 1969).

*Determination of the concentrations of putrescine and polyamines in the rat ventral prostate.* The amounts of putrescine, spermidine and spermine present in the prostate after castration and androgen treatment were determined after homogenization of the tissue in acid and extraction of the amines into butan-1-ol at alkaline pH by separation by paper electrophoresis and staining with ninhydrin. This procedure, which is based on that described by Raina (1963), is described in detail below. Losses during the extraction procedure were corrected for by adding a small amount of each of the amines labelled with $^{14}$C to the tissue before homogenization, and counting a portion of each of the final separated products after electrophoresis.

The prostates were removed as quickly as possible and placed in a pre-weighed homogenizer containing 2ml of 0.01M-HCl. The homogenizer and contents were weighed to determine accurately the wet weight of prostate tissue added, and at least 0.4g of tissue was used. Then 0.05ml of a solution containing 0.2$\mu$Ci of [1,4-$^{14}C_2$]putrescine (5.22mCi/mmol), 0.2$\mu$Ci of aminopropyl[1,4-$^{14}C_2$]tetramethylenediamine (5.9mCi/mmol) and 0.2$\mu$Ci of bis-(aminopropyl)[1,4-$^{14}C_2$]tetramethylenediamine (4.25mCi/mmol) in 0.1M-HCl was added. The tissue was homogenized by using 20 strokes of a rotating Teflon pestle, and 1ml of 10% (w/v) trichloroacetic acid was added. The tubes were shaken on a mechanical shaker for 10min and the protein precipitate removed by centrifugation. The precipitate was re-extracted by homogenization in 5% (w/v) trichloroacetic acid followed by shaking for 30min and the protein again removed by centrifugation. The combined supernatants were then extracted twice with 5ml volumes of diethyl ether to remove trichloroacetic acid, and the last traces of ether were evaporated in a stream of air. The solution was made alkaline by the addition of 0.1ml of 10M-NaOH and saturated with salt by the addition of 1.25g of a mixture of 31.25g of $Na_2SO_4$ and 4.5g of $Na_3PO_4$. Then 5ml of butan-1-ol was added and the mixture shaken vigorously for 30min. The phases were then separated by centrifugation and the butan-1-ol layer removed, acidified by the addition of 0.15ml of 4M-HCl and evaporated to dryness under reduced pressure in a rotary evaporator at 60°C. The residue contains appreciable quantities of salt as well as polyamines and it was not possible to carry out electrophoresis on this material (cf. Raina, 1963). It was therefore dissolved in 1.0ml of 0.1M-HCl and applied to a small column (3cm × 0.2cm) of Dowex 50($H^+$ form) [the resin was Dowex 50W(X2; 100–200 mesh) and was prepared by washing twice with 20vol. of 5M-HCl followed by sufficient water to raise the pH of the effluent to above 5.0]. The column was washed with 20ml of 0.2M-HCl and the polyamines were then eluted with 10ml of 5M-HCl. This solution was evaporated under reduced pressure at 60°C and the final residue dissolved in 0.1ml of 0.01M-HCl and stored at 4°C until required. Suitable portions of this solution were applied to strips of Whatman no. 1 paper (31cm × 4cm) and subjected to electrophoresis at 300V for 2h with 0.1M-sodium citrate buffer, pH4.3. The strips were dried at room temperature for 1h and stained with ninhydrin solution (1g of ninhydrin, 0.1g of cadmium acetate, 10ml of water, 5ml of acetic acid and 100ml of acetone). After drying for 10min at room temperature the strips were heated at 110°C for 30min to develop the colour. The strip was cut up, the regions corresponding to putrescine, spermidine and spermine were taken and the colour of each was eluted by soaking for 30min in 4ml of a solution containing 200mg of cadmium acetate in 10ml of water, 40ml of ethanol and 50ml of acetic acid. The paper was then removed and the extinction read at 505nm. Standard curves for each polyamine were constructed by using solutions of known concentration and subjecting portions of these to electrophoresis and staining. At least three determinations of each sample were made and the mean result was taken. For every sample, two strips to which 0.005ml of the solution had been applied were not stained, but were cut into pieces 0.5cm across; each piece was placed in 15ml of scintillation fluid and its radioactivity measured. The radioactivity present in the regions corresponding to putrescine, spermidine and spermine was compared with that added at the start of the extraction procedure, and the results for the polyamine concentrations were corrected according to the recovery factor obtained in this manner (the standard radioactive polyamine solution was also applied to paper strips of the same size as the samples to ensure a similar degree of quenching). The recoveries varied between 74% and 89% during these experiments. Allowance was made for the amount of putrescine added as a radioactive marker when this was more than 1% of the total found. The amounts of radioactive spermine and spermidine added were never more than 1% of the total found and were ignored.

During these experiments it was noticed that another compound reacting with ninhydrin was present in the butan-1-ol extracts of prostatic homogenates. This material ran between spermine and spermidine on the paper electrophoresis, and gave a greyish spot with the ninhydrin–cadmium reagent in contrast with the reddish-brown spot of the polyamines. It was not possible to identify this material, but it was not one of the common amino acids. Its concentration was not decreased in the extracts from castrated as compared with normal animals. In fact the amount appeared to increase somewhat after castration, but accurate measurement was not attempted in the absence of identification of the nature of this material.

*Determination of radioactivity.* The scintillation fluid used in these experiments was xylene–dioxan–ethanol (10:10:11, by vol.) containing 7.5% (w/v) of naphthalene, 0.45% (w/v) of 2,5-diphenyloxazole and 0.0045% (w/v) of 1,4-bis-(5-phenyloxazol-2-yl)benzene. Radioactive samples were placed in 15 ml of this solution in a glass counting vial and assayed with a Nuclear–Chicago no. 722 liquid-scintillation system. The counting efficiency for $^{14}C$ was 55% for samples from the enzyme assays and 52% for samples absorbed on paper. Suitable corrections were applied for background counts and for radioactivity present in zero-time control samples in all enzyme experiments (these values were less than 100 c.p.m.). Sufficient counts were recorded to obtain S.D. of the net count rate of less than $\pm 5\%$.

*Other methods.* Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with dry crystalline bovine serum albumin (Sigma) as a standard. All pH measurements were made with a glass electrode and refer strictly to the stated temperature. The significance of results was determined by applying Student's *t* test.

## RESULTS

The concentrations of putrescine, spermidine and spermine in the ventral prostate of adult rats at various times after castration and androgen treatment are shown in Table 1. It must be emphasized that these values represent the sum of both intracellular polyamines and the polyamines present extracellularly in the prostatic secretion. We were unable to obtain unequivocal values for the amounts of putrescine and polyamines in rat ventral prostate secretions. However, in some preliminary experiments, in which secretion was stimulated by administration of pilocarpine (Huggins, 1947) and collected by a capillary tube from the exposed gland in laparotomized animals anaesthetized with Nembutal, the concentrations of polyamines found in the 'secretion' were of the same order of magnitude as in the whole gland, whereas those of putrescine were lower. In these experiments any intact cells present in the secretions collected were removed by centrifugation before determination of putrescine, spermidine and spermine. However, in the absence of reliable methods for gauging the degree of cellular damage under these conditions, and also because the composition of 'resting' and 'active' prostatic secretions may differ (Huggins, 1947), we cannot draw any firm conclusions from these observations. Normal whole rat ventral prostate was found in the present studies to contain approximately equal amounts of spermine and spermidine (6–7 $\mu$mol/g

Table 1. *Influence of androgen administration on putrescine and polyamine concentrations in rat ventral prostate*

The animals used in this experiment weighed 370–410 g. The number of groups assayed for each hormonal treatment is shown in parenthesis. Each group contained at least three animals. Testosterone treatment consisted of subcutaneous injections of 1 mg of testosterone propionate in 0.2 ml of sesame oil given each day for the number of days shown in the table. The 9-day and 11-day castrated groups were given 0.2 ml of sesame oil alone for 2 and 4 days respectively. Putrescine and polyamines were determined as described in the Experimental section and the results ± S.E.M. are given.

| Animals | Days of testosterone treatment | Wt. of prostate (mg) | Putrescine content ($\mu$mol/g wet wt.) | Spermidine content ($\mu$mol/g wet wt.) | Spermine content ($\mu$mol/g wet wt.) | Spermidine/ spermine ratio |
|---|---|---|---|---|---|---|
| Normal (8) | — | 418 ± 21 | 0.36 ± 0.03 | 6.20 ± 0.14 | 6.94 ± 0.06 | 0.89 |
| Castrated 3 days (4) | — | 316 ± 20 | 0.35 ± 0.04 | 4.75 ± 0.04 | 6.12 ± 0.14 | 0.78 |
| Castrated 5 days (4) | — | 119 ± 7 | 0.29 ± 0.04 | 1.59 ± 0.13 | 3.17 ± 0.16 | 0.50 |
| Castrated 7 days (8) | — | 74 ± 3 | 0.11 ± 0.07 | 1.26 ± 0.16 | 2.58 ± 0.16 | 0.49 |
| Castrated 9 days (4) | — | 60 ± 4 | 0.11 ± 0.01 | 1.07 ± 0.10 | 2.14 ± 0.26 | 0.50 |
| Castrated 11 days (4) | — | 58 ± 6 | 0.10 ± 0.02 | 0.77 ± 0.04 | 1.66 ± 0.03 | 0.46 |
| Castrated 8 days (5) | 1 | 84 ± 7 | 0.16 ± 0.01 | 1.72 ± 0.16 | 2.83 ± 0.12 | 0.61 |
| Castrated 9 days (5) | 2 | 143 ± 9 | 0.28 ± 0.04 | 2.51 ± 0.18 | 2.45 ± 0.24 | 1.02 |
| Castrated 10 days (5) | 3 | 179 ± 7 | 0.28 ± 0.02 | 3.82 ± 0.23 | 2.94 ± 0.09 | 1.30 |
| Castrated 11 days (4) | 4 | 243 ± 10 | 0.53 ± 0.09 | 4.28 ± 0.22 | 2.83 ± 0.06 | 1.52 |
| Castrated 12 days (4) | 5 | 355 ± 22 | 0.45 ± 0.09 | 4.51 ± 0.20 | 2.54 ± 0.24 | 1.27 |
| Castrated 13 days (4) | 6 | 393 ± 24 | 0.41 ± 0.06 | 5.58 ± 0.20 | 3.81 ± 0.13 | 1.45 |
| Castrated 14 days (4) | 7 | 445 ± 26 | 0.45 ± 0.07 | 5.76 ± 0.24 | 3.62 ± 0.23 | 1.60 |
| Castrated 15 days (4) | 8 | 471 ± 42 | 0.39 ± 0.04 | 6.22 ± 0.11 | 4.36 ± 0.10 | 1.43 |

of tissue) and smaller quantities of putrescine (0.3–0.4 $\mu$mol/g). The values for spermidine and spermine agree well with reports by Tabor & Tabor (1964) and Rhodes & Williams-Ashman (1964), who used different analytical procedures. In a paper by Moulton & Leonard (1969) rather higher values for spermidine in normal rat ventral prostate are recorded. It is noteworthy that the concentrations of spermidine and spermine in rat ventral prostate are higher than those reported for most other mammalian tissues (Tabor & Tabor, 1964; Jänne *et al.* 1964). The concentration of putrescine in rat ventral prostate is also higher than that reported for rat liver (0.1–0.25 $\mu$mol/g) by Jänne (1967).

After castration there was a rapid decrease in the prostatic spermidine content, particularly, between 3 and 7 days after surgery. The spermine content shows a similar but considerably slower decrease. Putrescine concentration falls abruptly between 5 and 7 days after castration (Table 1). At 7 days after castration the concentrations of putrescine, spermidine and spermine are 0.11, 1.3 and 2.6 $\mu$mol/g of tissue respectively. The administration of 1 mg of testosterone propionate/day to rats castrated 7 days previously leads to an immediate increase in the concentrations of spermidine and putrescine. The increases shown in Table 1 are significant ($P<0.02$) at 24 h after androgen administration and highly significant ($P<0.001$) 48 h after the initial hormone injection. Within 4 days after the commencement of daily testosterone treatment, the putrescine concentration had attained a value somewhat in excess of the normal, and by 8 days both spermidine and putrescine concentrations had returned to normal. In contrast with the constant increase in the spermidine concentration found within 1 day of the beginning of androgen administration and reaching normal values after 8 days, there was no significant change in the spermine content for 4 days and only a slow increase in the spermine concentration between 4 and 8 days after commencement of hormonal treatment. Even after 8 days of testosterone administration the concentration of spermine had not returned to a normal value, being only 4.3 $\mu$mol/g of prostate (Table 1).

The effect of castration and androgen treatment on prostatic ornithine decarboxylase activity and the putrescine-dependent *S*-adenosyl-L-methionine decarboxylase system is shown in Table 2. At 7 days after castration there was a dramatic decrease in the activities of both enzymes. The prostatic ornithine decarboxylase had declined to about 15% of the control value and the putrescine-dependent *S*-adenosylmethionine decarboxylase activity to about 7% of normal. As previously reported (Pegg & Williams-Ashman, 1968*b*) the ornithine decarboxylase activity of centrifuged prostatic homogenates from normal rats was increased by dialysis, or by passage through a Bio-Gel P-2 column, suggesting the presence in crude homogenates of inhibitor(s) of low molecular weight. It is possible that these inhibitors are putrescine and polyamines, which are known to depress the enzymic decarboxylation of ornithine in rat ventral prostate (Pegg & Williams-Ashman, 1968*b*).

We indeed observed that the ornithine decarboxylase activity of homogenates of the prostate

Table 2. *Effect of castration and androgen administration on the activities of prostatic ornithine decarboxylase and* S-*adenosyl-*L-*methionine decarboxylase*

There were at least five animals in each group and the decarboxylase activities represent the mean of assays at at least two different protein concentrations. Animals were given subcutaneous injections of 1 mg of testosterone propionate in 0.2 ml of sesame all at the time before death shown in the table. Where times longer than 24 h are shown, injections were given every day at the start of each 24 h period. Results are given as nmol of $^{14}CO_2$ released/30 min per mg of protein present in the desalted ultracentrifuged extracts of the prostatic homogenates.

| Animals used | Time after testosterone treatment | Ornithine decarboxylase activity (nmol of $^{14}CO_2$/30 min per mg of protein) | *S*-Adenosyl-L-methionine decarboxylase activity (nmol of $^{14}CO_2$/30 min per mg of protein) |
|---|---|---|---|
| Normal controls | — | 0.171 ± 0.017 | 0.355 ± 0.018 |
| Castrated for 7 days | — | 0.031 ± 0.007 | 0.025 ± 0.007 |
| Castrated for 7 days | 3 h | 0.040 ± 0.009 | 0.049 ± 0.011 |
| Castrated for 7 days | 6 h | 0.067 ± 0.004 | 0.074 ± 0.013 |
| Castrated for 7 days | 12 h | 0.075 ± 0.011 | 0.101 ± 0.010 |
| Castrated for 8 days | 24 h | 0.087 ± 0.015 | 0.160 ± 0.015 |
| Castrated for 9 days | 2 days | 0.102 ± 0.023 | 0.227 ± 0.023 |
| Castrated for 11 days | 4 days | 0.108 ± 0.012 | 0.225 ± 0.018 |
| Castrated for 14 days | 7 days | 0.154 ± 0.021 | 0.330 ± 0.027 |
| Castrated for 21 days | 14 days | 0.180 ± 0.032 | 0.370 ± 0.035 |

extracts from castrated rats was increased by dialysis to a much smaller extent than was that of similar preparations from normal or androgen-treated animals. However, the results given in Tables 2 and 3 for ornithine decarboxylase activity relate to extracts that had been freed of low-molecular-weight material by gel filtration and that, when assayed in the standard assay medium, gave linear increases in carbon dioxide release with increases in the amount of prostatic-extract protein added.

Daily treatment with testosterone propionate of animals castrated 7 days previously led to a rapid increase in the activities of both ornithine and *S*-adenosylmethionine decarboxylase systems. Decarboxylation of ornithine by prostatic extracts was doubled 6h after androgen administration and increased three-to four-fold within 24–48h. Decarboxylation of *S*-adenosylmethionine in the presence of excess of putrescine was significantly increased within 3h after the administration of testosterone, and was increased sixfold and tenfold respectively at 24 and 48h after the first hormone injection (Table 2). Although there was a very rapid and large initial increase in the activities of both of these enzymes, complete restoration of normal activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase did not occur until at least 7 days after commencement of hormone treatment (Table 2). Injection of 1mg of testosterone propionate in sesame oil/day into normal rats for up to 7 days had no significant effect on the activity of either enzyme, although the ventral prostate glands were larger than in control animals.

Table 3 shows the activities of ornithine decarboxylase in putrescine-dependent *S*-adenosylmethionine decarboxylase in prostatic extracts from hypophysectomized rats and from hypophysectomized rats treated with androgen for 1 or 2 days. The activities of these enzymes are very low in the prostates of animals deprived of testosterone by removal of the pituitary. Administration of testosterone caused a large increase in enzymic activity similar to that induced by the administration of the androgen to castrated animals. This strongly suggests that alterations in the output of anterior-pituitary hormones resulting from the administration of the large doses of testosterone used in these experiments do not play a major role in the increased decarboxylase activity found in castrated animals after androgen treatment. The difference between the results shown for normal controls in Tables 2 and 3 may be due to the considerable difference in age and weight in the animals used in these experiments.

We have previously reported that the decarboxylation of *S*-adenosylmethionine catalysed by extracts of the rat ventral prostate and other mammalian tissues is markedly stimulated by putrescine, and that spermidine and 5′-methylthioadenosine are formed in stoicheiometric amounts with the carbon dioxide released (Pegg & Williams-Ashman, 1968*b*,*c*, 1969). Table 4 shows the results of experiments in which prostatic extracts were prepared from normal, castrated and androgen-treated castrated rats. Measurements were made of the decarboxylation of *S*-adenosylmethionine in the presence and absence of putrescine, and of the formation of spermidine from putrescine and *S*-adenosylmethionine. In all cases the decarboxylation of *S*-adenosylmethionine was greatly increased in the presence of putrescine, and, within the limits of experimental error, the same degree of stimulation by putrescine was achieved. Further, there was close agreement between the release of $^{14}CO_2$ from *S*-adenosyl-L-[*carboxy*-$^{14}$C]-methionine in the presence of putrescine and the formation of [$^{14}$C]spermidine from [1,4-$^{14}C_2$]-putrescine in the presence of *S*-adenosylmethionine by each of the prostatic preparations when assayed at similar concentrations of reagents. These results

Table 3. *Effect of testosterone treatment of ornithine decarboxylase and* S-*adenosyl*-L-*methionine decarboxylase of the ventral prostate of hypophysectomized rats*

The hypophysectomized rats weighed about 150g and the sham-operated controls about 160g. There were ten animals in each group. Testosterone treatment consisted of the subcutaneous injection of 1 mg of testosterone propionate in 0.2ml of sesame oil for the number of days before death shown.

| Animals used | Ornithine decarboxylase activity (nmol of $^{14}CO_2$ released/30min per mg of protein) | *S*-Adenosyl-L-methionine decarboxylase activity (nmol of $^{14}CO_2$ released/30min per mg of protein) |
|---|---|---|
| Sham-operated controls | 0.184 ± 0.023 | 0.275 ± 0.021 |
| Hypophysectomized | 0.014 ± 0.003 | 0.011 ± 0.002 |
| Hypophysectomized, treated for 1 day with testosterone | 0.036 ± 0.007 | 0.074 ± 0.016 |
| Hypophysectomized, treated for 2 days with testosterone | 0.106 ± 0.011 | 0.199 ± 0.023 |

Table 4. *Decarboxylation of* S*-adenosyl-*L*-methionine in the presence and absence of putrescine and spermidine formation by prostatic extracts from normal, castrated and androgen-treated rats*

The testosterone-treated animals received subcutaneous injections of 2mg of testosterone propionate in 0.2ml of sesame oil at 48h and 24h before death. Decarboxylation of *S*-adenosyl-L-methionine in the presence of putrescine and spermidine synthesis was measured as described in the Experimental section. Decarboxylation of *S*-adenosyl-L-methionine in the absence of putrescine was measured by incubating the prostatic extract with 100$\mu$mol of $NaH_2PO_4$ solution (adjusted to pH7.0 with NaOH) and 0.2$\mu$mol of *S*-adenosyl-L-[*carboxy*-$^{14}$C]methionine (5.0c.p.m./nmol) in a total volume of 1.0ml. All incubations were for 30min at 37°C.

| Treatment of animals | Decarboxylation of *S*-adenosylmethionine in the absence of putrescine (nmol of $^{14}CO_2$ released/30min per mg of protein) | Decarboxylation of *S*-adenosyl-L-methionine in the presence of putrescine (nmol of $^{14}CO_2$ released/30min per mg of protein) | Spermidine formed (nmol/30min per mg of protein) |
|---|---|---|---|
| Normal | 0.025 | 0.376 | 0.347 |
| Castrated 7 days | 0.002 | 0.021 | 0.031 |
| Castrated 9 days, testosterone for 2 days | 0.017 | 0.241 | 0.223 |

Table 5. *Properties of arginase in rat ventral prostate*

The 100000*g* supernatant was passed through a Bio-Gel P-2 column before being assayed for arginase activity at pH9.4 unless otherwise stated. All arginase activation steps were for 30min. Other details of the assay are discussed in the Experimental section. The 100% activity is equivalent to 51.3$\mu$mol of ornithine formed/30min per g wet wt. Arginase activities were proportional to the quantity of enzyme added to the reaction mixtures.

| Assay conditions | Incubation time (min) | % of activity of Complete System |
|---|---|---|
| Complete | 30 | 100 |
| Complete | 15 | 48 |
| $MnCl_2$ omitted | 30 | 18 |
| L-Arginine omitted | 30 | <1 |
| Complete; arginase activated at 55°C | 30 | 110 |
| Complete; arginase activated at 0°C | 30 | 93 |
| Assayed at pH7.3 | 30 | 12 |
| Assayed at pH7.3; $MnCl_2$ replaced by $CoCl_2$ | 30 | 18 |
| Complete; enzyme boiled (100°C, 5min) | 30 | <1 |

are in agreement with our suggestion that in mammalian tissues the decarboxylation of *S*-adenosylmethionine is closely coupled to spermidine formation and that free decarboxylated *S*-adenosylmethionine does not accumulate (Pegg & Williams-Ashman, 1968*c*, 1969; Williams-Ashman *et al.* 1969). The findings also provide evidence that measurement of the rate of decarboxylation of *S*-adenosylmethionine in the presence of putrescine does indeed provide a reliable measure of the ability of the prostatic preparations to synthesize spermidine.

Some properties and the hormonal control of some other enzymes involved in the metabolism of ornithine in rat prostate were examined. We have suggested that provision of L-ornithine for the biosynthesis of putrescine and polyamines may represent a major function of arginase in extrahepatic tissues (Pegg & Williams-Ashman, 1968*b*; Williams-Ashman *et al.* 1969). The properties of the arginase present in soluble extracts of rat ventral prostate homogenates are summarized in Table 5. Determination of the formation of ornithine from arginine was used to assay arginase activity. For this method to be effective it was necessary to remove free amino acids from the ultracentrifuged ventral-prostate extracts by passage through a Bio-Gel P-2 column. Virtually all of the arginase activity of crude ventral-prostate homogenates remained in solution after ultracentrifugation at 105000*g* for 1h. The arginase activity was proportional to the amount of extract protein added to the test system, and there was stoicheiometry between the amounts of both ornithine and urea formed. Like liver arginase (Greenberg, 1960) the prostate enzyme required preincubation with $Mn^{2+}$ or $Co^{2+}$ for maximal rates of reaction. As with the liver enzyme, $Co^{2+}$ was a more effective activator of prostatic arginase than was $Mn^{2+}$ in assays conducted at

pH 7.3, whereas much greater activity was observed in the presence of $Mn^{2+}$ at pH 9.4 (Table 5). Change of the temperature of the activation process between 0°C and 55°C had little effect on the subsequently determined arginase activity. An approximate $K_m$ of 10mM for L-arginine at pH 9.4 was found for prostatic arginase. Spermidine at concentrations up to 9mM was ineffective as an activator of prostatic arginase; spermidine, spermine and putrescine did not inhibit the enzyme when added in molar concentrations up to six times those of the L-arginine substrate.

Certain characteristics of the L-ornithine–2-oxo acid aminotransferase (ornithine transaminase) in rat ventral prostate were determined. When measured by the methods described above, the activity was proportional to the amount of protein added and to the duration of incubation for periods of up to 30min. Omission of L-ornithine or 2-oxoglutarate decreased the formation of glutamic $\gamma$-semialdehyde to negligible values.

Subcellular fractionation of prostate homogenates prepared in 0.25M-sucrose revealed that more than 80% of ornithine transaminase activity was associated with the mitochondrial fraction sedimenting between 120*g* for 10min and 15000*g* for 10min. The reaction exhibited an optimum between pH 7.6 and 8.0. In general, the properties of the prostatic enzyme were similar to those described for liver ornithine transaminase (Strecker, 1965; Peraino, Bunville & Tahmisian, 1969). However, the rat ventral-prostate enzyme catalyses the formation of only about 4$\mu$mol of 1-pyrolline-5-carboxylate/h per g-equiv. of tissue at 37°C whereas rat liver ornithine transaminase forms about 85$\mu$mol of 1-pyrolline-5-carboxylate/h per g (Strecker, 1965). The difference between the two organs cannot be accounted for solely by variations in the quantity of mitochondrial protein present per unit wet weight of tissue. Addition of spermine or spermidine up to 50mM had no effect on the ornithine transaminase activity of ventral prostate mitochondria, whereas the same concentration of putrescine decreased the activity about 10%.

An experiment dealing with effects of castration and subsequent androgen treatment on the amount of ornithine in the prostate and the activities of prostatic arginase and ornithine transaminase is shown in Table 6. The activity of prostatic arginase was doubled 7 days after castration. Subsequent administration of androgen caused a further but transient increase in arginase activity, which reached a peak 24–48h after the initiation of hormonal treatment. At this point the arginase activity was three to four times that of control animals. Ornithine transaminase activity was very sensitive to the androgen status. At 7 days after castration ornithine transaminase activity, expressed in terms of $\mu$mol of product formed/mg of mitochondrial protein, declined to about 30% of the normal value. Castration leads to a diminution in the mitochondrial population density in the prostatic cells (Edelman, Brendler, Zorgniotti & Edelman, 1963; Williams-Ashman, 1962; Pegg &

Table 6. *Effect of castration and androgens on content of ornithine and activities of arginase and ornithine transaminase in rat ventral prostate*

Details of testosterone treatment are described in Table 2. Ornithine concentrations were determined by amino acid analysis on a Technicon AutoAnalyzer. The samples were prepared in the following manner. A 2.0ml portion of the aqueous phase from the butan-1-ol extraction step used for isolating polyamines was acidified with HCl, and 0.5$\mu$mol of DL-[1-$^{14}$C]ornithine (2.3mCi/mmol) was added to determine recovery during the extraction procedure. The solution was applied to a column (5cm×1cm) of Dowex 50 ($H^+$ form) and the column was washed with 50ml of 0.2M-HCl to remove $Na_2SO_4$. Ornithine was then eluted in 10ml of 5.0M-HCl. The samples were evaporated to dryness at 50°C in a rotary evaporator and the residue was redissolved in a small amount of 0.02M-HCl before amino acid analysis. The percentage recovery was determined (86–94%) and the values were corrected accordingly. The enzymic activities were determined as described in the Experimental section.

| Animals | Time after testosterone treatment (h) | Ornithine content of ventral prostate ($\mu$mol/g wet wt.) | Arginase activity ($\mu$mol of ornithine/30min per mg of protein) | Ornithine transaminase activity ($\mu$mol of product/30min per mg of mitochondrial protein) |
|---|---|---|---|---|
| Normal controls | — | 1.07 | 1.71 | 0.21 |
| Castrated 7 days | — | 0.70 | 3.22 | 0.06 |
| Castrated 7 days | 3 | — | 3.65 | 0.13 |
| Castrated 7 days | 6 | — | 3.46 | 0.29 |
| Castrated 7 days | 12 | — | 4.57 | 0.26 |
| Castrated 8 days | 24 | 0.82 | 6.47 | 0.36 |
| Castrated 9 days | 48 | 0.56 | 5.92 | 0.62 |
| Castrated 10 days | 72 | 0.37 | 4.59 | 0.59 |
| Castrated 14 days | 168 | — | 2.96 | 0.53 |

Williams-Ashman, 1968*a*). The total ornithine transaminase activity/g of prostatic tissue is therefore even less than 30% of normal after castration, since there is a combined effect of a decreased number of mitochondria per unit amount of tissue. Within 3h of the injection of testosterone propionate into animals castrated 7 days previously there was a significant increase in the activity of ornithine transaminase, and by 6h there was a four- to five-fold increase in the activity of this enzyme. Ornithine transaminase activities in excess of normal were quickly achieved and maintained throughout 7 days of testosterone treatment (Table 6). However, administration of similar doses of testosterone propionate to normal non-castrated animals had no effect on either ornithine transaminase or arginase activity after 3 or 7 days of treatment. There was only a small decline in the prostatic ornithine concentration by 7 days after castration, and when prostatic growth was reinitiated by androgen the ornithine content decreased even more (Table 6). This further decrease could conceivably reflect the large androgen-induced increases in the activities of both ornithine decarboxylase and ornithine transaminase (which both serve to remove ornithine), coupled with only a small increase in arginase activity (which produces ornithine). However, the lower ornithine concentration in castrated rats shows that there was no close correlation between ornithine concentration and the activities of these enzymes, because at this time both ornithine decarboxylase and ornithine–2-oxo acid transaminase activities were decreased whereas arginase activity was higher than normal activity.

## DISCUSSION

The above experiments show that after castration there was a large decrease in the content of putrescine, spermidine and spermine in rat ventral prostate, and that on subsequent androgen treatment the concentrations of these substances were greatly increased. The activities of prostatic L-ornithine decarboxylase and also putrescine-dependent *S*-adenosyl-L-methionine decarboxylase similarly decreased after castration, and were rapidly enhanced after injection of testosterone. Although other pathways for putrescine and polyamine formation could be operative in the prostate gland, this close relationship between putrescine and spermidine concentrations on the one hand and activities of their biosynthetic enzymes on the other hints that the enzymes we have studied indeed play a major role in polyamine production *in vivo*. The feeble activities of ornithine decarboxylase and the putrescine-dependent *S*-adenosyl-L-methionine decarboxylase in comparison with the high concentrations of polyamines in rat ventral prostate has prompted suggestions that the polyamine concentrations in this tissue may partly reflect the unusually high activities of these enzymes (Pegg & Williams-Ashman, 1968*b*, 1969). Ornithine concentrations in the prostate were found invariably to be higher than the $K_m$ (0.2mM) for ventral-prostate L-ornithine decarboxylase (Pegg & Williams-Ashman, 1968*b*). Moreover, the activities of arginase and of ornithine–2-oxo acid transaminase in prostate, although much lower than in rat liver, are still greatly in excess of those of ventral-prostate ornithine decarboxylase.

Increased activity of ornithine decarboxylase was also noted in rat liver after partial hepatectomy (Jänne & Raina, 1968; Russell & Snyder, 1968; Fausto, 1969; Schrock, Oakman & Bucher, 1969), in the liver of hypophysectomized rats after treatment with growth hormone (Jänne, Raina & Siimes, 1968; Russell & Snyder, 1969; Jänne & Raina, 1969), in developing chick embryos (Russell & Snyder, 1968) and in cultures of chick-embryo epidermis after stimulation with epidermal growth factor (Stastny & Cohen, 1969). In all of these situations increased accumulation of putrescine and spermidine also occurs, and the suggestion that ornithine decarboxylase (in conjunction with the stimulation of *S*-adenosylmethionine decarboxylase by the putrescine formed) regulates polyamine formation in liver (Jänne, 1967; Jänne & Raina, 1968; Jänne *et al.* 1968; Pegg & Williams-Ashman, 1968*b*,*c*, 1969) seems plausible. But it is clearly shown here that the putrescine-dependent *S*-adenosyl-L-methionine decarboxylase activity is also markedly increased in the prostate after androgen treatment, and this enzyme system has not yet been measured in parallel with ornithine decarboxylase in other tissues.

Another possible site for regulation of polyamine synthesis is the supply of *S*-adenosylmethionine, which participates in a large number of biological transmethylations. The role of *S*-adenosylmethionine as a precursor of polyamines is noteworthy in view of the possible correlation between polyamines and nucleic acids, since this compound can also be regarded as a precursor of rRNA and tRNA by virtue of its functions as a donor of the methyl groups of the minor methylated bases present in these macromolecules (Borek & Srinivasan, 1966). This additional link between polyamines and nucleic acids does not appear to have been previously acknowledged. In situations in which the tissue concentrations of polyamines and RNA are increased, there must be increased utilization of *S*-adenosylmethionine. It is possible that formation of polyamines and methylation of nucleic acids represent only a minor portion of the total utilization of *S*-adenosylmethionine by most tissues, but studies

of the synthesis and methyl-donor functions of this compound under such conditions would seem to be desirable.

We have previously discussed the likelihood that ornithine transaminase could play a regulatory role in polyamine synthesis by removing ornithine available for ornithine decarboxylase (Williams-Ashman *et al.* 1969). Ornithine transaminase activity is decreased in foetal tissue, some tumours and liver after partial hepatectomy or treatment with growth hormone (Herzfeld & Knox, 1968; Räihä & Kekomäki, 1968), which are situations where polyamine formation is increased (Jänne *et al.* 1964; Dykstra & Herbst, 1965; Raina *et al.* 1966; Jänne & Raina, 1968; Kostyo, 1966). However, the experiments described in this paper do not support this hypothesis, since after androgen treatment of castrated animals both polyamine formation and ornithine transaminase activity are markedly increased in the prostate. A parallel between activity of ornithine transaminase and cellular proliferation was observed in synchronized cell cycles of HeLa cells (Volpe, 1969) and in Chang's liver cells cultivated *in vitro* (Strecker & Eliasson, 1966). The very rapid increase in prostatic ornithine -2-oxo acid transaminase activity caused by the administration of testosterone to castrated rats is noteworthy, since this enzyme is located in the mitochondrial fraction and the increase in activity occurs considerably before any detectable changes in the activities of other mitochondrial enzymes or mitochondrial protein synthesis (Pegg & Williams-Ashman, 1968*a*). Swick, Rexroth & Strange (1968) have shown that the turnover of ornithine transaminase and also of alanine transaminase in rat liver is much faster than for most other mitochondrial proteins.

A remarkable feature of the changes in polyamine concentrations in the prostate after castration and androgen treatment is that the fluctuations in spermidine content are much more rapid than the changes in spermine concentration. Thus the spermidine content decreases faster than the spermine content during regression of the prostate after androgen withdrawal, and during androgen-induced growth the spermidine concentration increases continuously over the first 4 days whereas that of spermine remains constant (Table 1). There is therefore a marked decline in the spermidine/spermine molar ratio from 0.9 to 0.5 within 7 days after castration and after 4 days of testosterone treatment the ratio rises from 0.5 to 1.5 (Table 1). A decline in spermidine content with little change or even an increase in the spermine concentration was also reported to occur in rat liver during aging of the animal (Jänne *et al.* 1964). Converse changes in polyamine concentrations occur after partial hepatectomy (Dykstra & Herbst, 1965; Raina *et al.* 1966) or treatment with growth hormone (Kostyo, 1966; Jänne *et al.* 1968), in rat liver during pregnancy or lactation (Neish & Key, 1968*a*,*b*) in lactating mammary gland (Neish & Key, 1968*b*) and in the livers of rats bearing Rd/3 sarcoma (Neish & Key, 1967). These results raise the possibility that there is a specific requirement for spermidine rather than total polyamine or spermine during certain metabolic conditions. Our results for the decrease in polyamines in the rat ventral prostate after castration are not in very good agreement with those reported by Calderera *et al.* (1968). These workers found a more rapid decline in both spermine and spermidine concentrations, reaching values of 0.8 and 0.6 $\mu$mol/g wet wt. of prostate within 6 days of removal of the testes, and did not observe a greater rate of decrease in the spermidine concentration as compared with that of spermine. The reason for this difference in results is not clear, but could be due to changes in the experimental technique and the strain of rats employed.

The secretion of citrate, fructose and other characteristic constituents of the seminal plasma of many mammals ceases within a few days after castration but administration of testosterone promptly restores normal concentrations (Mann & Parsons, 1947; Humphrey & Mann, 1949; Price & Williams-Ashman, 1961; Mann, 1964). The increase in citrate and fructose concentrations in various male accessory glands provides the basis of tests for androgenic activity that are more sensitive than measurements of the changes in the weight of the organs (Mann, 1964). The dependence on the continued presence of androgens for polyamine formation by the rat ventral prostate is therefore not unexpected, since polyamines are also major constituents of the seminal plasma. However, the rapid increase in the activities of the polyamine-synthesizing enzymes is among the earliest known metabolic changes to occur in the prostate after androgen administration, occurring within a few hours of testosterone administration, and at about the same time as increase in nuclear RNA polymerase activity and decline in tissue ATP concentration (Liao *et al.* 1965; Ritter, 1966; Coffey *et al.* 1968*a*). Several groups suggested that polyamine concentrations may be connected with RNA synthesis *in vivo* (Cohen & Raina, 1967; Dykstra & Herbst, 1965; Raina *et al.* 1966; Caldarera *et al.* 1965, 1968; Moruzzi *et al.* 1968; Kremzner & Cote, 1968; Barros & Guidice, 1968; Jänne & Raina, 1969). Although many of these workers suggested that high polyamine concentrations or ornithine decarboxylase activity are associated with rapid tissue growth, this does not necessarily mean that hyperplasia is involved, since similar increases in polyamine synthesis occur in cases where the growth is largely hypertrophic, and does not involve much

increase in DNA synthesis or cell division (Schrock *et al.* 1969; Kostyo, 1966; Jänne *et al.* 1968; Jänne & Raina, 1969).

Since polyamine synthesis appears in general to be increased in metabolic situations where there is an increase in the formation of RNA, and because DNA-dependent RNA polymerase activity is stimulated *in vitro* by addition of polyamines to the assay medium (Krakow, 1963; Fox & Weiss, 1964; Ballard & Williams-Ashman, 1966; Abraham, 1968) the possibility arises that increased polyamine concentrations are causally related to the increase in RNA synthesis induced in the prostate of castrated rats after testosterone administration (see Caldarera *et al.* 1968; Moulton & Leonard, 1969). Moruzzi *et al.* (1968) made a similar suggestion about RNA synthesis in the developing chick embryo. Caldarera *et al.* (1968) support this argument with results showing that the RNA polymerase activity of nuclei isolated from the prostate of castrated rats is increased, by the addition of spermine, to a value similar to that obtained from prostatic nuclei of normal animals. However, they assayed the RNA polymerase activity of normal animals without added polyamine, which does not provide a satisfactory control. Also, spermine was much more effective in stimulating RNA polymerase activity of nuclei from castrated animals in their experiments, whereas we found that the rapid increase in polyamine concentration in the prostate after androgen administration is due to an increase in spermidine with no change in spermine concentrations for several days. Another point against the hypothesis that polyamine concentrations control the activity of nuclear RNA polymerase in the prostate is that there is a very large decline (at least 50%) in nuclear RNA polymerase activity within 24h after castration (Liao & Fang, 1969) and at this time there is little decrease in the concentrations of polyamines in the prostate (A. E. Pegg, unpublished work; Caldarera *et al.* 1968). (However, the actual intracellular concentrations of polyamines under these conditions have not been determined.) In view of the great complexity of the nuclear RNA polymerase system and the lack of knowledge about the role of factors such as nucleases in determining the measurable RNA polymerase activity, a definite conclusion on the physiological role of polyamines in this system cannot yet be made. Further experimental evidence, particularly with regard to the distribution of polyamines within the cell, is also required. However, at present there appears to be no compelling evidence that polyamines mediate the effects of androgens or other hormones on nucleic acid synthesis. An alternative hypothesis on correlations between polyamines and RNA would be that increased production of basic polyamines by cells would aid in the neutralization of the increased negative charges due to newly formed RNA. Another possibility, which stems from the fact that the RNA produced on hormonal stimulation is largely of the ribosomal type, is that spermidine production is somehow necessary (in addition to ribosomal RNA and proteins) for complete assembly of new functional ribosomes. Certainly, in these connexions, the relationship between polyamines and the balance of metal cation (especially $Mg^{2+}$) within the cell needs further experimental study (cf. Cohen & Raina, 1967; Stevens, 1969).

Finally, it should be emphasized that in the rat and certain other mammals high concentrations of polyamines are not characteristic of all lobes of the prostate gland, despite their common embryological origin from the foetal urogenital sinus. Thus in adult rats of the Sprague–Dawley strain the content of spermidine and spermine in the ventral and dorsolateral lobes of the prostate is exceptionally high, whereas the anterior lobe (coagulating gland) contains only low concentrations of these polyamines (Rhodes & Williams-Ashman, 1964). Studies by Brasel, Coffey & Williams-Ashman (1968) have shown that changes in RNA content and also DNA polymerase activity during the growth of the anterior prostate of castrated rats after daily injections of testosterone propionate are quite similar to alterations in these parameters previously determined in rat ventral prostate, despite the remarkable differences in the polyamine content of the two lobes of the gland in sexually mature animals.

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## REFERENCES

Abraham, A. J. (1968). *Eur. J. Biochem.* **5**, 143.
Ballard, P. & Williams-Ashman, H. G. (1966). *J. biol. Chem.* **241**, 1602.
Barros, C. & Guidice, G. (1968). *Expl Cell Res.* **50**, 671.
Borek, E. & Srinivasan, P. R. (1966). *A. Rev. Biochem.* **35**, 275.
Brasel, J., Coffey, D. S. & Williams-Ashman, H. G. (1968). *Med. exp.* **18**, 321.
Caldarera, C. M., Barbiroli, B. & Moruzzi, G. (1965). *Biochem. J.* **97**, 84.
Caldarera, C. M., Moruzzi, M. S., Barbiroli, B. & Moruzzi, G. (1968). *Biochem. biophys. Res. Commun.* **33**, 266.
Chinard, F. R. (1952). *J. biol. Chem.* **199**, 91.
Coffey, D. S., Ichinose, R. R., Shimazaki, J. & Williams-Ashman, H. G. (1968*a*). *Molec. Pharmac.* **4**, 580.
Coffey, D. S., Shimazaki, J. & Williams-Ashman, H. G. (1968*b*). *Archs Biochem. Biophys.* **124**, 184.

Cohen, S. S., Hoffner, N., Jansen, M., Moore, M. & Raina, A. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 721.
Cohen, S. S. & Lichtenstein, J. (1960). *J. biol. Chem.* **235**, 2112.
Cohen, S. S. & Raina, A. (1967). In *Organizational Biosynthesis*, p. 157. Ed. by Vogel, H. J., Lampen, J. O. & Bryson, V. New York: Academic Press Inc.
Colbourn, J. L., Witherspoon, B. H. & Herbst, E. J. (1961). *Biochim. biophys. Acta*, **49**, 422.
Conway, E. J. (1947). *Micro-diffusion Analysis and Volumetric Error*, 2nd ed. London: C. Lockwood and Co.
Dykstra, W. G. & Herbst, E. J. (1965). *Science, N.Y.*, **149**, 428.
Edelman, J. C., Brendler, H., Zorgniotti, A. W. & Edelman, P. M. (1963). *Endocrinology*, **72**, 853.
Fausto, N. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 366.
Fox, C. F. & Weiss, S. B. (1964). *J. biol. Chem.* **239**, 175.
Greenberg, D. M. (1960). In *The Enzymes*, vol. 4, p. 257. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Hershko, A., Amoz, S. & Mager, J. (1961). *Biochem. biophys. Res. Commun.*. **5**, 46.
Herzfeld, A. & Knox, W. E. (1968). *J. biol. Chem.* **243**, 3327.
Huggins, C. (1947). *Harvey Lect.* **42**, 148.
Humphrey, G. F. & Mann, T. (1949). *Biochem. J.* **44**, 97.
Jänne, J. (1967). *Acta physiol. scand.* Suppl. no. 300, p. 1.
Jänne, J. & Raina, A. (1968). *Acta chem. scand.* **22**, 1349.
Jänne, J. & Raina, A. (1969). *Biochim. biophys. Acta*, **174**, 769.
Jänne, J., Raina, A. & Siimes, M. (1964). *Acta physiol. scand.* **62**, 352.
Jänne, J., Raina, A. & Siimes, M. (1968). *Biochim. biophys. Acta*, **166**, 419.
Kosto, B., Calvin, H. I. & Williams-Ashman, H. G. (1967). In *Advances in Enzyme Regulation*, vol. 5, p. 25. Ed. by Weber, G. New York: Pergamon Press Inc.
Kostyo, J. L. (1966). *Biochem. biophys. Res. Commun.* **23**, 150.
Krakow, J. S. (1963). *Biochim. biophys. Acta*, **72**, 566.
Kremzner, L. T. & Cote, L. J. (1968). *Fedn Proc. Fedn Am. Socs exp. Biol.* **27**, 839.
Liao, S. & Fang, S. (1969). *Vitams Horm.* **27**, (in the Press).
Liao, S., Leininger, K. R., Sagher, D. & Barton, R. W. (1965). *Endocrinology*, **77**, 763.
Liao, S. & Williams-Ashman, H. G. (1962). *Proc. natn. Sci. U.S.A.* **48**, 1956.
Liquori, A. M., Constantino, L., Crescenzi, V., Elia, V., Giglio, E., Puliti, R., de Santis Savino, M. & Vitagliano, V. (1967). *J. molec. Biol.* **24**, 113.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mann, T. (1964). *The Biochemistry of Semen and of the Male Reproductive Tract.* pp. 193–200. New York: John Wiley and Sons Inc.
Mann, T. & Parsons, U. (1947). *Nature, Lond.*, **160**, 294.
Martin, R. G. & Ames, B. N. (1962). *Proc. natn. Acad. Sci. U.S.A.*, **48**, 2171.
Moruzzi, G., Barbiroli, B. & Caldarera, C. M. (1968). *Biochem. J.* **107**, 609.
Moulton, B. C. & Leonard, S. L. (1969). *Endocrinology*, **84**, 1461.
Neish, W. J. P. & Key, L. (1967). *Biochem. Pharmac.* **16**, 163.
Neish, W. J. P. & Key, L. (1968*a*). *Biochem. Pharmac.* **17**, 497.
Neish, W. J. P. & Key, L. (1968*b*). *Comp. Biochem. Physiol.* **27**, 709.
Norton, J. W., Erdmann, V. A. & Herbst, E. J. (1968). *Biochim. biophys. Acta*, **155**, 293.
Obrink, K. J. (1955). *Biochem. J.* **59**, 134.
Pegg, A. E. (1969). *Biochim. biophys. Acta*, **177**, 361.
Pegg, A. E. & Williams-Ashman, H. G. (1968*a*). *Endocrinology.* **82**, 603.
Pegg, A. E. & Williams-Ashman, H. G. (1968*b*). *Biochem. J.* **108**, 533.
Pegg, A. E. & Williams-Ashman, H. G. (1968*c*). *Biochem. biophys. Res. Commun.* **30**, 76.
Pegg, A. E. & Williams-Ashman, H. G. (1968*d*). *Biochem. J.* **109**, 32P.
Pegg, A. E. & Williams-Ashman, H. G. (1968*e*). *FEBS 5th Meet.*, Prague, p. 31.
Pegg, A. E. & Williams-Ashman, H. G. (1969). *J. biol. Chem.* **244**, 682.
Peraino, C., Bunville, L. G. & Tahmisian, T. N. (1969). *J. biol. Chem.* **244**, 2241.
Peraino, C. & Pitot, H. C. (1963). *Biochim. biophys. Acta*, **73**, 222.
Price, D. & Williams-Ashman, H. G. (1961). In *Sex and Internal Secretions*, p. 366. Ed. by Young, W. C. Baltimore: Williams and Wilkins Inc.
Räihä, N. C. R. & Kekomäki, M. P. (1968). *Biochem. J.* **108**, 521.
Raina, A. (1963). *Acta physiol. scand.* Suppl. no. 218, p. 1.
Raina, A., Jänne, J. & Siimes, M. (1964). *Acta chem. scand.* **18**, 1804.
Raina, A., Jänne, J. & Siimes, M. (1966). *Biochim. biophys. Acta*, **123**, 197.
Raina, A., Jansen, M. & Cohen, S. S. (1967). *J. Bact.* **94**, 1684.
Ratner, S. (1962). In *Methods in Enzymology*, vol. 5, p. 843. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Rhodes, J. B. & Williams-Ashman, H. G. (1964). *Med. Exp.* **10**, 281.
Ritter, C. (1966). *Molec. Pharmac.* **2**, 125.
Russell, D. & Snyder, S. H. (1968). *Proc. natn. Acad. Sci. U.S.A.* **60**, 1420.
Russell, D. & Snyder, S. H. (1969). *Endocrinology*, **84**, 223.
Schrock, T. R., Oakman, M. J. & Bucher, N. L. R. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 599.
Silman, N., Artman, M. & Engelberg, H. (1965). *Biochim. biophys. Acta*, **103**, 231.
Stastny, M. & Cohen, S. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 599.
Stevens, L., (1969). *Biochem. J.* **113**, 117.
Strecker, H. J. (1965). *J. biol. Chem.* **240**, 1225.
Strecker, H. J. & Eliasson, E., (1966). *J. biol. Chem.* **241**, 5750.
Swick, R. W., Rexroth, A. K. & Strange, J. L. (1968). *J. biol. Chem.* **243**, 3581.

Tabor, C. W. (1962*a*). In *Methods in Enzymology*, vol. 5, p. 756. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Tabor, C. W. (1962*b*). In *Methods in Enzymology*, vol. 5, p. 756. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Tabor, H. & Tabor, C. W. (1964). *Pharmac. Rev.* **16**, 245.

Tabor, H., Tabor, C. W. & Rosenthal, S. M. (1961). *A. Rev. Biochem.* **30**, 579.

Tanner, M. J. A. (1967). *Biochemistry, Easton*, **6**, 2686.

Volpe, P. (1969). *Biochem. biophys. Res. Commun.* **34**, 190.

Walter, G., Zillig, W., Palm, P. & Fuchs, E. (1967). *Eur. J. Biochem.* **3**, 14.

Wickner, R. R., Tabor, C. W. & Tabor, H. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 352.

Williams-Ashman, H. G. (1962). In *Essays in Experimental Biology: On Cancer and Hormones*, p. 325. Chicago: University of Chicago Press.

Williams-Ashman, H. G. (1965*a*). *Cancer Res.* **25**, 1096.

Williams-Ashman, H. G. (1965*b*). *J. cell. comp. Physiol.* **66**, Suppl. 1, p. 111.

Williams-Ashman, H. G., Liao, S., Hancock, R. L., Jurkowitz, L. & Silverman, D. A. (1964). *Recent Prog. Horm. Res.* **20**, 247.

Williams-Ashman, H. G., Pegg, A. E. & Lockwood, D. H. (1969). In *Advances in Enzyme Regulation*, vol. 7, p. 291. Ed. by Weber, G. New York: Pergamon Press Inc.

Williams-Ashman, H. G. & Shimazaki, J. (1967). In *Perspectives in Reproduction and Sexual Behaviour*, p. 241. Ed. by Diamond, M. Bloomington: Indiana University Press.

Williams-Ashman, H. G. & Shimazaki, J. (1968). *Endogenous Factors Influencing Tumor-Host Balance*, p. 31. Ed. by Wissler, R. W., Dao, T. & Wood, S., jun. Chicago: University of Chicago Press.