Metabolic Control Mechanisms in Mammalian Systems

HORMONAL REGULATION OF PHOSPHOFRUCTOKINASE IN THE RAT PROSTATE AND SEMINAL VESICLES

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1. The hormonal regulation of phosphofructokinase was investigated in the accessory reproductive organs of the orchidectomized rat. 2. Phosphofructokinase activities declined to 51% and 47% in the prostate and 9% and 6% of the normal values in seminal vesicles 4 and 8 weeks after castration respectively. Administration of testosterone (100 µg./100g. body wt.) for 3 days reversed substantially the effects of orchidectomy, and phosphofructokinase activity increased to 173% in the prostate and 536% in seminal vesicles as compared with the values of castrated controls. 3. Time-course studies demonstrated that after a single injection of testosterone (5mg./100g. body wt.) phosphofructokinase activity was maximally elevated to 236% in the prostate and 342% in seminal vesicles at 24hr. 4. Dose-response studies revealed that 2.5mg. of testosterone propionate/100g. body wt. was the minimal amount necessary to induce significant increases in enzyme activity in both accessory sex organs; maximal increases were obtained with a dose of 5mg./100g. body wt. 5. The observed enzyme increases induced by testosterone were inhibited by the simultaneous administration of oestradiol-17β, and phosphofructokinase activity in this group of rats remained at 97% in the prostate and 137% of the control values in seminal vesicles. Oestradiol-17β by itself failed to produce any significant effect on enzyme activity in either of these secondary sexual tissues. 6. The nature of the testosterone-induced increases in phosphofructokinase activity was studied by using a variety of inhibitors of RNA and protein synthesis. Cycloheximide, 5-fluorouracil and ethionine largely blocked the androgen-stimulated rise in enzyme activity observed 24hr. after steroid injection. The inhibitory effect of ethionine was completely reversed by the simultaneous administration of methionine. 7. Actinomycin, which is known to inhibit the synthesis of messenger RNA as well as the synthesis of other cellular RNA fractions, when given simultaneously with the hormone, also inhibited the testosterone-induced increases in prostatic and seminal-vesicular phosphofructokinase. However, when the antibiotic was given 6 or 12hr. after injection of the steroid, practically no inhibition of phosphofructokinase induction was obtained. This indicates that, once the enzyme-forming machinery is turned on and allowed to operate for a few hours, actinomycin is incapable of reversing the hormone-induced enzyme responses. 8. The results presented suggest that new RNA and protein synthesis may be involved in the observed androgen-induced increases in phosphofructokinase activity in the prostate and seminal vesicles of the orchidectomized rat.

Studies on the hormonal regulation of enzyme biosynthesis have been advanced largely by an understanding of the mechanisms involved in protein synthesis and its genetic control. Evidence indicates that the process of hormone-stimulated quantitative changes in the amounts of specific proteins (or enzymes) is dependent on new RNA synthesis. Since little information is available on the

action of sex hormones in terms of their effects on enzyme biosynthesis, our attention was directed towards a study of the sex-hormonal control of certain enzymes that are rate-limiting in the process of glycolysis.

In a series of studies on the regulation of enzyme biosynthesis in accessory sex organs of the female, it was shown that oestrogenic hormones are capable

of inducing significant increases in the activities of uterine enzymes involved in glycolysis (Singhal & Valadares, 1967, 1968; Singhal, Valadares & Ling, 1967a,b,c; Valadares, Singhal & Parulekar, 1968). Phosphofructokinase, the enzyme that catalyses a key functional step in the pathway of glycolysis, was shown to be markedly increased by oestradiol-17 β in the uterus of the ovariectomized rat (Singhal *et al.* 1967a). It also was demonstrated that the activities of uterine glucose phosphate isomerase (Singhal *et al.* 1967b; Singhal & Valadares, 1968) and hexokinase (Valadares *et al.* 1968) were increased after administration of oestrogens to ovariectomized rats. Puromycin, ethionine, cycloheximide and 5-fluorouracil, as well as actinomycin, inhibitors of RNA and protein synthesis, largely prevented these hormonal responses, and the suggestion was raised that oestrogenic hormones are capable of inducing synthesis of these enzymes (Singhal *et al.* 1967a,b; Singhal & Valadares, 1968).

Since androgenic hormones are known to play an important homeostatic role in the male, studies were initiated on the hormonal regulation of carbohydrate-metabolizing enzymes in the rat prostate and seminal vesicles. In a preliminary investigation (Singhal, 1967a) it was shown that testosterone administration increased seminal-vesicular phosphofructokinase activity, which reached peak values at 24 hr. The results described in the present paper indicate that the testosterone-induced increases in the activities of prostatic and seminal-vesicular phosphofructokinase represent new enzyme formation, and support the view that stimulation of RNA synthesis may be a primary action of androgenic steroids on secondary sexual tissues of the male (Williams-Ashman, 1965a,b). Preliminary accounts of portions of this work have appeared (Singhal, 1967a; Singhal & Ling, 1967).

MATERIALS AND METHODS

Young male Wistar rats weighing 150–170 g., maintained on Master Laboratory chow and water *ad libitum*, were used throughout this study. The animals were orchidectomized bilaterally via the scrotal route under light pentobarbital anaesthesia. Four weeks were allowed for recovery from surgery before they were subjected to the experimental procedures described below.

Consequence of orchidectomy. To follow the sequential changes in phosphofructokinase activity of the prostate and seminal vesicles after castration, groups of gonadectomized rats were killed after 0.5, 1, 2, 4 and 8 weeks.

Effect of multiple injections of testosterone propionate. Since orchidectomy resulted in marked decreases in phosphofructokinase activity in the prostate and seminal vesicles, experiments were designed to investigate whether the effects of castration could be reversed by administration of testosterone propionate. The hormone was injected in a dose of 100 μ g./100 g. body wt. once a day for 3 days and all

animals were killed 24 hr. after the final injection of the hormone.

Sequence of events after a single injection of testosterone propionate. The time-course of the steroid-induced increases in phosphofructokinase activity was studied for 72 hr. after a single injection of the hormone. Testosterone propionate was administered intramuscularly in a dose of 5.0 mg./100 g. body wt. Groups of rats were killed at 8, 16, 24, 48 and 72 hr.

Dose-response studies with testosterone propionate. Since the above dose of testosterone induced marked increases in phosphofructokinase activity in both prostate and seminal vesicles, a dose-response study was carried out to establish whether smaller doses of the hormone were also capable of inducing significant increases in the activity of this enzyme. Groups of orchidectomized rats were given injections of single intramuscular doses of testosterone propionate (1.0, 2.5, 5.0 and 10.0 mg./100 g. body wt.) and killed after 24 hr.

Influence of actinomycin, cycloheximide, 5-fluorouracil and ethionine on testosterone-stimulated enzyme increases. The nature of the testosterone-induced increases in phosphofructokinase activity was studied with the use of compounds known to inhibit RNA and protein synthesis. The following seven groups of gonadectomized animals were used: (a) control rats; (b) animals injected with testosterone propionate; (c), (d), (e) and (f) testosterone-treated rats given injections of actinomycin, cycloheximide, 5-fluorouracil and ethionine respectively; (g) animals administered testosterone by injection and treated simultaneously with ethionine and methionine. All animals were killed 24 hr. after administration of the hormone.

Effect of delayed administration of actinomycin. The testosterone-induced increases in prostatic and seminal-vesicular phosphofructokinase activity were effectively prevented by actinomycin when administered simultaneously with the hormone. The influence of actinomycin on the androgen-stimulated enzyme increases after this had been initiated was investigated by injecting actinomycin at various times after testosterone. Five groups of castrated rats were employed: (a) control animals; (b) rats treated by injection with testosterone; (c), (d) and (e) testosterone-treated animals given actinomycin at 0, 6 and 12 hr. respectively after hormone injection. Animals from each group were killed 24 hr. after administration of testosterone.

Effect of oestradiol-17 β on the testosterone-induced enzyme response. The known physiological antagonism between androgens and oestrogens (Huggins, 1947; Bever, 1959) prompted us to investigate the effect of oestradiol administration on the testosterone-induced increases in phosphofructokinase activity in these accessory sex glands. The following four groups of castrated animals were used: (a) control rats; (b) animals injected with oestradiol-17 β ; (c) rats injected with testosterone; (d) testosterone-treated rats injected simultaneously with oestradiol-17 β . All animals were killed 24 hr. after steroid administration.

Chemicals and doses. Testosterone propionate, obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), was dissolved in ethanolic 0.9% (w/v) NaCl and administered intramuscularly, in a single dose of 5 mg./100 g. body wt. Control rats received injections of an equal volume of the ethanolic 0.9% NaCl alone. Actinomycin D (Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A.) was dissolved in aq. 0.9% NaCl and injected intraperitoneally (25 μ g./100 g. body wt.) simultaneously with the hormone. 5-Fluorouracil (15 mg./100 g. body wt.) (Hoffmann-La Roche, Basle,

Switzerland) was solubilized in aq. 0.9% NaCl and injected intraperitoneally at the same time testosterone was administered. Cycloheximide (60 μ g./100g. body wt.) (Upjohn Co., Kalamazoo, Mich., U.S.A.), DL-ethionine (50mg./100g. body wt.) and DL-methionine (100mg./100g. body wt.) (both obtained from Sigma Chemical Co.) were dissolved in aq. 0.9% NaCl and injected intraperitoneally twice at 12hr. intervals. Oestradiol-17 β (Sigma Chemical Co.) was dissolved in ethanolic 0.9% NaCl and administered intramuscularly in a single dose of 10 μ g./100g. body wt. These doses and schedules of injections were used and carried out in all experiments unless stated otherwise.

Preparation of tissues and supernatant fluids and assay of enzyme activity. Animals were stunned, decapitated and exsanguinated. The prostate glands and seminal vesicles were excised quickly and cleaned of all adhering tissue, and their fluid contents were extruded under light pressure. The organs were then rinsed in cold homogenizing medium, blotted on filter paper, weighed rapidly on a Roller-Smith torsion balance and placed in beakers immersed in crushed ice. Prostate glands and seminal vesicles were pooled separately, finely minced with scissors and 5% (w/v) homogenates prepared in iso-osmotic KCl solution, pH 7.4. The supernatant fluid was obtained and phosphofructokinase activity assayed immediately according to the procedure of Lea & Walker (1965) as described previously (Weber & Singhal, 1965; Singhal *et al.* 1967a). The method was adapted to prostate and seminal vesicles after linear kinetics had been established individually for these tissues. Enzyme activity was calculated as μ moles of alkali-labile phosphate formed/hr./g. of tissue at 37° times the weight of the organ, as described previously (Singhal, 1967a; Singhal *et al.* 1967a; Valadares *et al.* 1968). The results were analysed statistically and significant differences between the means (calculated as *P* values) are shown. No statistical significance is indicated when the *P* value was > 0.05.

RESULTS AND DISCUSSION

Consequences of orchidectomy on prostatic and seminal-vesicular phosphofructokinase activity. The effects of castration on enzyme activity in accessory sex organs are shown in Fig. 1. Whereas prostatic phosphofructokinase activities remained unaffected up to 1 week after gonadectomy, seminal-vesicular enzyme activity declined to 36% in 4 days and to 30% in 1 week as compared with the values of normal unoperated rats. The earliest significant decrease (67%) in phosphofructokinase activity in the prostate gland was observed 2 weeks after orchidectomy; thereafter enzyme activity declined to 51% and 47% of the control group at 4 and 8 weeks respectively. All subsequent experiments were carried out in rats that had been castrated for 4 weeks, since near-minimal enzyme activities were attained at this time. It is noteworthy that ovariectomy also results in marked decreases in the activity of uterine phosphofructokinase (Singhal & Valadares, 1966; Singhal *et al.* 1967a). These observations suggest that the maintenance of normal phosphofructokinase activities in accessory reproductive organs

Fig. 1. Effect of castration on phosphofructokinase activity in the rat prostate ($\circ$) and seminal vesicles (∇). Each point represents the mean and s.e.m. of three values, each obtained by pooling the prostate glands and seminal vesicles from four or five rats. The results are given in percentages, taking the values of normal unoperated rats as 100%.

of both the male and female may be contingent on an adequate supply of androgens and oestrogens respectively.

Effects of chronic testosterone treatment. Growth and proliferation of sexual target structures stimulated chronically by hormones would be expected to be associated with an enhancement in glycolysis and protein synthesis. To assess the results of chronic testosterone treatment on phosphofructokinase activity, gonadectomized rats were treated by injections of 100 μ g. of the hormone/100g. body wt. once daily for 3 days. The results obtained are illustrated in Fig. 2. The enzyme response in seminal vesicles elicited by testosterone treatment of castrated rats was much greater than that exhibited by the prostate gland. The increases in phosphofructokinase activity in both the prostate and seminal vesicles after administration of testosterone were greater than those in organ weights. These results confirm our previous findings on phosphofructokinase increases in secondary sexual tissues induced by administration of testosterone (1mg./100g. body wt. daily for 3 days) to young rats weighing approx. 100g. (Singhal, 1967b). The results indicate that the depletion in phosphofructokinase activity of the prostate and seminal vesicles produced by orchidectomy can be reversed substantially by testosterone treatment, and support the observations that stimulation of protein synthesis may be an important effect of androgens on sensitive tissues that have been rendered atrophic by castration (Williams-Ashman, 1965a,b).

Fig. 2. Effects of 3 days of testosterone treatment on phosphofructokinase activity in the prostate (a) and seminal vesicles (b). Bars represent the means and S.E.M. of three values. Each enzyme assay was carried out in the prostate glands and seminal vesicles pooled from three rats. Testosterone propionate was injected intramuscularly into castrated rats in a dose of 100 μ g./100g. body wt. once a day for 3 days and the animals were killed on the fourth day. The results are given in percentages, taking the values of control rats as 100%. Key: C, controls; T, testosterone-treated. * Statistically significant difference as compared with the values of control rats ($P < 0.05$).

Time-course studies after a single injection of testosterone propionate. Since significant increases in phosphofructokinase activity were observed after chronic administration of testosterone propionate, the sequential changes in enzyme activity during a 72 hr. period were studied after a single injection of 5mg. of the steroid/100g. body wt. The results summarized in Table 1 demonstrate that the earliest detectable significant increases in enzyme activity were obtained 16 hr. after hormone treatment. Enzyme activity was maximal at 24 hr. and was 236% in the prostate and 342% of the controls in seminal-vesicular tissue.

The earliest detectable biochemical responses have been considered to be crucial to an understanding of hormone action at the molecular level

Table 1. *Time-course of testosterone-induced increase in phosphofructokinase activity in the rat prostate and seminal vesicles*

The results represent means $\pm$ S.E.M. of three determinations of enzyme activity. Each determination was carried out in the prostate glands and seminal vesicles pooled from two or three rats. Castrated rats were injected with testosterone propionate (5mg./100g. body wt.) intramuscularly and killed 8, 16, 24, 48 and 72 hr. later. The results are also given in percentages, taking the values of control rats as 100%.

Time (hr.)	Phosphofructokinase activity in prostate		Phosphofructokinase activity in seminal vesicles	
	(activity)	(% of control)	(activity)	(% of control)
Control	23.1 $\pm$ 1.0	100	2.6 $\pm$ 0.2	100
8	24.5 $\pm$ 0.2	106	2.8 $\pm$ 0.1	108
16	32.6 $\pm$ 0.1	141*	3.7 $\pm$ 0.1	143*
24	54.6 $\pm$ 0.3	236*	8.9 $\pm$ 0.2	342*
48	47.5 $\pm$ 2.2	206*	7.2 $\pm$ 0.4	277*
72	42.7 $\pm$ 2.7	185*	7.2 $\pm$ 0.1	277*

* Statistically significant difference as compared with the values of control rats ($P < 0.05$).

(Weber & Singhal, 1964). Earlier studies in this Laboratory demonstrated that after a single injection of oestradiol-17 β a significant increase in uterine phosphofructokinase activity was detectable at 4 hr. and reached peak values at 16 hr. (Singhal *et al.* 1967a). The rapid increase in uterine phosphofructokinase observed after oestrogen treatment contrasts with the comparatively slower androgen-induced stimulation of prostatic and seminal-vesicular phosphofructokinase. Oestrogens are known to produce physiological effects such as uterine hyperaemia and water imbibition, responses that become manifest more rapidly than those produced by the known actions of androgens on male accessory sexual tissues (Williams-Ashman, 1965a).

Dose-response studies with testosterone propionate. Since a dose of 5mg. of testosterone/100g. body wt. induced significant increases in phosphofructokinase activity at 24 hr. in both accessory reproductive organs, it was considered important to establish the minimal dose of the steroid necessary to produce statistically significant increases in enzyme activity. A dose of 2.5mg./100g. body wt. appears to be the minimal amount necessary to induce significant increases (165% in prostate and 223% in seminal vesicles) in the activity of phosphofructokinase (Table 2). Maximal increases in enzyme activity were observed with a dose of 5.0mg. of the hormone/100g. body wt. It is noteworthy that the dose of testosterone (5mg./100g. body wt.) that produced the maximal enzymic response in male

Table 2. *Effect of various doses of testosterone propionate on phosphofructokinase activity in the rat prostate and seminal vesicles*

The results represent means $\pm$ s.e.m. of three determinations of enzyme activity. Each enzyme assay was carried out in the prostate glands and seminal vesicles pooled from two or three rats. Groups of ten to 12 castrated rats were given testosterone propionate in doses varying from 1.0 to 10.0 mg./100 g. body wt. and killed after 24 hr. The results are also given in percentages, taking the values of control rats as 100%.

Testosterone (mg./ 100 g.)	Phosphofructokinase activity in prostate		Phosphofructokinase activity in seminal vesicles	
	(activity)	(% of control)	(activity)	(% of control)
Control	23.1 $\pm$ 1.0	100	2.6 $\pm$ 0.2	100
1.0	26.6 $\pm$ 0.7	114	3.2 $\pm$ 0.1	123
2.5	38.1 $\pm$ 1.3	165*	5.8 $\pm$ 0.2	223*
5.0	54.6 $\pm$ 0.3	236*	8.9 $\pm$ 0.2	342*
10.0	50.0 $\pm$ 1.9	222*	8.7 $\pm$ 0.1	335*

* Statistically significant difference as compared with the values of control rats ($P < 0.05$).

accessory reproductive organs is about 500 times the dose of oestradiol-17 β (10 μ g./100 g. body wt.) necessary for achieving a similar sequela in uterine tissue (Singhal *et al.* 1967a).

Influence of actinomycin, cycloheximide, 5-fluorouracil and ethionine on testosterone-induced increases in phosphofructokinase. Experiments were designed to study the nature of the androgen-induced enzyme response by using compounds that inhibit RNA and protein synthesis, although recently questions have been raised with regard to their non-specific toxic effects when administered *in vivo* (Lippe & Szego, 1965; Williams-Ashman, 1965b; Cohen, 1966; Nicolette & Mueller, 1966). Notwithstanding these caveats, such inhibition studies have provided considerable insight into the actions of steroid hormones at the molecular level (Williams-Ashman, 1965b). The results presented in Table 3 show that phosphofructokinase activity was elevated to 236% of the control values in the prostate gland 24 hr. after administration of testosterone propionate. Actinomycin, which inhibits DNA-directed RNA synthesis by binding to the deoxyguanosine bases of DNA (Reich, 1963), produced an almost complete inhibition (116% of the control values) of the steroid-induced enzymic response. Cycloheximide, an antibiotic that suppresses protein synthesis by inhibiting either the transfer of aminoacyl-transfer RNA to ribosomes or the formation of peptide bonds (Ennis & Lubin, 1964; Siegel & Sisler, 1964), also prevented the testosterone-stimu-

Table 3. *Effect of inhibitors of RNA and protein synthesis on testosterone-induced increases in phosphofructokinase activity in the rat prostate*

The results represent means $\pm$ s.e.m. of four determinations of enzyme activity. Each determination was carried out in the prostate glands pooled from two or three rats. Rats were injected with testosterone propionate (5 mg./100 g. body wt.) intramuscularly and were killed after 24 hr. Actinomycin D (25 μ g./100 g. body wt.) and 5-fluorouracil (15 mg./100 g. body wt.) were given intraperitoneally concurrently with testosterone. Cycloheximide (60 μ g./100 g. body wt.), ethionine (50 mg./100 g. body wt.) and methionine (100 mg./100 g. body wt.) also were injected intraperitoneally, twice at 12 hr. intervals. The results are also given in percentages, taking the values of control rats as 100%.

Group no.	Treatment	Phosphofructokinase activity	
		(activity)	(% of control)
1	Control	23.1 $\pm$ 1.0	100
2	Testosterone	54.6 $\pm$ 0.3	236*
3	Testosterone + actinomycin	26.8 $\pm$ 1.5	116†
4	Testosterone + cycloheximide	27.0 $\pm$ 3.2	117†
5	Testosterone + 5-fluorouracil	29.2 $\pm$ 0.5	126†
6	Testosterone + ethionine	29.4 $\pm$ 2.1	127†
7	Testosterone + ethionine + methionine	51.9 $\pm$ 0.4	218*

* Statistically significant difference as compared with the values of control rats ($P < 0.05$).

† Statistically significant difference as compared with the values of testosterone-injected rats ($P < 0.05$).

lated rise in prostatic phosphofructokinase activity, which remained at 117% of the control values. Experiments were also carried out with 5-fluorouracil, which by being incorporated into the RNA molecule leads to the formation of biologically inactive RNA (Jacob & Monod, 1961). This anti-metabolite, when administered concurrently with testosterone, was capable of preventing the hormone-induced enzyme response. Phosphofructokinase activity in this group of animals remained at 126% as compared with the values of control rats. Additional evidence for the involvement of both RNA and protein synthesis in the observed increases in prostatic phosphofructokinase activity induced by testosterone was obtained by the use of ethionine. This compound is thought to inhibit the synthesis of protein through an inhibition of RNA synthesis as well as by lowering ATP concentrations (Farber, Shull & Villa-Trevino, 1963; Villa-Trevino, Shull & Farber, 1966). Ethionine, when administered by injection to testosterone-treated animals, effectively prevented the steroid-induced increases

Fig. 3. Influence of inhibitors of RNA and protein synthesis on testosterone-induced increases in phosphofructokinase activity in rat seminal vesicles. Bars represent the means and S.E.M. of four enzyme values. Each enzyme assay was carried out in seminal vesicles pooled from three or four rats. Castrated rats were injected with testosterone propionate (5mg./100g. body wt.) intramuscularly and killed after 24hr. Actinomycin D (25 μ g./100g. body wt.) was injected intraperitoneally with testosterone. 5-Fluorouracil (15mg./100g. body wt.) was also injected intraperitoneally at the same time testosterone was administered. Cycloheximide (60 μ g./100g. body wt.), ethionine (50mg./100g. body wt.) and methionine (100mg./100g. body wt.) were given intraperitoneally, twice at 12hr. intervals. The results are given in percentages, taking the values of control rats as 100%. Key: C, controls; T, testosterone-treated; A, actinomycin-treated; CH, cycloheximide-treated; F, 5-fluorouracil-treated; E, ethionine-treated; M, methionine-treated. * Statistically significant difference as compared with the values of control rats ($P < 0.05$). † Statistically significant difference as compared with the values of rats receiving testosterone alone ($P < 0.05$).

(127%) in phosphofructokinase activity in the prostate gland. Since the administration of methionine counteracts most of the effects of ethionine, including those related to protein synthesis (Villa-Trevino *et al.* 1966), it was decided to investigate whether methionine was able to reverse the inhibitory effects of ethionine on the androgen-induced increases in phosphofructokinase. In testosterone-treated rats injected concurrently with ethionine and methionine, prostatic phosphofructokinase activity exhibited an almost complete return (to 218% of the control values) to the value observed in rats receiving testosterone alone (Table 3).

The effects of these four inhibitors of RNA and protein synthesis on the testosterone-induced increase in seminal-vesicular phosphofructokinase are

illustrated in Fig. 3. The steroid elevated phosphofructokinase activity to 342% of the control value 24hr. after its administration. Fig. 3 shows that the increased seminal-vesicular enzyme activity observed in testosterone-injected castrated animals is almost completely blocked when inhibitors are administered along with the hormone. As in the prostate gland, the inhibitory effect of ethionine on the androgen-induced increase in seminal-vesicular phosphofructokinase can be reversed by concurrent treatment with methionine.

The above studies *in vivo* involving the use of a variety of inhibitors of RNA and protein synthesis raise the possibility that both new RNA and new protein synthesis may be involved in the observed testosterone-induced increases in phosphofructokinase activity in the prostate glands and seminal vesicles. Since actinomycin inhibits DNA-dependent synthesis of RNA, the observed inhibition of testosterone-induced increases in phosphofructokinase activity by actinomycin in accessory reproductive organs indicates the participation of messenger-RNA synthesis at the gene locus.

Effects of delayed administration of actinomycin. It is generally believed that the stimulation of RNA synthesis is restricted to an early phase of hormone-type inductions of specific proteins (Greengard, 1967). Further evidence for the involvement of an early stimulation of RNA synthesis in the hormonal induction of phosphofructokinase was obtained from experiments wherein actinomycin was administered at intervals (6 and 12hr.) after the administration of testosterone. The results obtained from such an experiment are illustrated in Fig. 4. When actinomycin was injected concurrently with testosterone (zero time), the steroid-induced increases in phosphofructokinase activity in the prostate and seminal vesicles were completely blocked and the values remained at 111% and 112% respectively, as compared with the controls. However, if the administration of actinomycin was delayed for 6hr. or longer, little or no inhibition of the testosterone-induced enzyme response was obtained. Thus, when actinomycin was given either at 6 or 12hr. after the hormone was injected, phosphofructokinase activities in the prostate remained close to those observed with testosterone alone (236%) and were 212% and 222% of the control values respectively. Likewise, seminal-vesicular enzyme activity was 331% and 319% of the control values in animals receiving actinomycin 6 and 12hr. after hormone injection; these values were in the same range as those observed in rats receiving testosterone alone (342%). Since actinomycin inhibits the synthesis of all RNA fractions in the cell (Acs, Reich & Valanju, 1963; Revel & Hiatt, 1964; Drysdale & Munro, 1966), the above experiments suggest that an early stimulation of RNA synthesis may be in-

Fig. 4. Effect of delayed administration of actinomycin on testosterone-induced increases in phosphofructokinase activity in the prostate (a) and seminal vesicles (b). Bars represent the means and s.e.m. of three values each obtained by pooling the prostate glands and seminal vesicles from three or four rats. Orchidectomized rats were treated by injection with testosterone propionate (5 mg./100 g. body wt.) intramuscularly and killed after 24 hr. Actinomycin D (25 μ g./100 g. body wt.) was injected intraperitoneally, either concurrently with the steroid (0 hr.) or 6 and 12 hr. after hormone injection. The results are given in percentages, taking the values of control animals as 100%. Key: C, controls; T, testosterone-treated; A, actinomycin-treated (at times indicated). * Statistically significant difference as compared with the values of control rats ($P < 0.05$). † Statistically significant difference as compared with the values of rats receiving testosterone alone ($P < 0.05$).

involved in the testosterone-induced enzyme response, and that once the enzyme-forming machinery has been set into motion actinomycin exerts little or no blocking effect.

The importance of early RNA synthesis was stressed by Greengard, Gordon, Smith & Acs (1964) and Greengard (1967) in their investigations on the induction of egg phosphatase with oestrogen in the male chicken. Actinomycin was shown to prevent this induction phenomenon completely if injected together with oestrogen, but, if given a few hours later, actinomycin failed to inhibit the biosynthesis of phosphatase. These experiments were interpreted to indicate that the hormone acts by increasing the concentration of an RNA species and that this species must have been stable for several hours (Greengard, 1967). Ui & Mueller (1963) demonstrated that, when actinomycin was administered at the same time as or 1 hr. after oestrogen, it inhibited the hormone-induced increases in uterine wet weight and incorporation of labelled glycine into protein. However, if actinomycin injection was delayed for 2 hr., the antibiotic failed to restrict the oestrogenic response. In addition, Tarentino, Richert & Westerfeld (1966) have shown that the twofold increases in hepatic malate dehydrogenase

and α -glycerophosphate dehydrogenase that occurred after the injection of 3,3',5-tri-iodo-L-thyronine could be blocked by the simultaneous treatment with actinomycin. However, when administration of the antibiotic was delayed for 6 hr. or longer, little or no inhibition of either of the enzyme responses was obtained, suggesting that the synthesis of messenger RNA for both enzymes may have occurred within 6 hr. after injection of the hormone. The inability of actinomycin to interfere with the process of enzyme induction when administered subsequent to the hormone also has been demonstrated in experiments in which hepatic xanthine oxidase was induced by feeding animals on a high-protein diet (Rowe & Wyngaarden, 1966). The picture that emerges from such studies emphasizes the importance of an intact RNA-synthesizing system for specific protein induction; once this machinery has been switched on and permitted to operate for a few hours, actinomycin is incapable of reversing the hormone-initiated enzyme responses.

Influence of oestradiol-17 β on testosterone-induced phosphofructokinase activity. Since oestrogens and androgens are known to produce physiological responses that are mutually antagonistic (Huggins, 1947; Bever, 1959), we investigated the effects of

Fig. 5. Effect of oestradiol-17 β on testosterone-induced increases in phosphofructokinase activity in the prostate (a) and seminal vesicles (b). Bars represent the means and S.E.M. of six values. Each enzyme assay was carried out in the prostate glands and seminal vesicles pooled from three or four animals. Castrated rats were treated by injection with testosterone propionate (5mg./100g. body wt.) intramuscularly and killed after 24hr. Oestradiol-17 β (10 μ g./100g. body wt.) was also given intramuscularly, either alone or concurrently with testosterone. The results are given in percentages, taking the values of control rats as 100%. Key: C, controls; T, testosterone-treated; O, oestradiol-17 β -treated. * Statistically significant difference as compared with the values of control rats ($P < 0.05$). † Statistically significant difference as compared with the values of testosterone-treated rats without oestradiol administration ($P < 0.05$).

oestradiol-17 β on the testosterone-induced increases in prostatic and seminal-vesicular phosphofructokinase activity. The results illustrated in Fig. 5 show that testosterone enhanced phosphofructokinase to 176% in the prostate and to 305% of control values in seminal vesicles. The observed increases in enzyme activity in the accessory sex organs were inhibited by the administration of oestradiol-17 β to testosterone-treated rats, although the oestrogen by itself failed to produce any significant effect. Thus when testosterone-treated rats were given oestradiol-17 β , the androgen-induced biochemical response in the prostate was completely suppressed (97%). Likewise, seminal-vesicular enzyme activity, which was elevated by testosterone to 305% of the control values, was almost completely blocked by simultaneous treatment with oestradiol (137%). Recent studies indicate that the increases in hexokinase and glucose 6-phosphate dehydrogenase activities induced by

testosterone in these male accessory reproductive organs also were inhibited by oestradiol (R. L. Singhal, unpublished work). A similar antagonism by testosterone of the oestrogen-stimulated rise in the activities of uterine phosphofructokinase and glucose phosphate isomerase has been reported (Valadares, Singhal & Ling, 1967; Singhal & Valadares, 1968). It was suggested that oestradiol-17 β and testosterone may act as inducer and suppressor respectively of uterine enzyme biosynthesis (Singhal & Valadares, 1968). The present experiments emphasize the desirability of investigating the role of RNA synthesis in androgen-oestrogen interactions in both male and female sex organs, since such studies may shed additional light on the sex-hormonal control of specific protein formation.

The experimental evidence available to date is by and large consistent with the view that androgenic steroids initiate and maintain growth and functional differentiation of the prostate gland and seminal vesicles by regulating RNA and protein biosynthesis. Wicks & Vilee (1964) demonstrated that RNA synthesis in rat seminal vesicles was evoked by testosterone propionate at a time when there was no detectable effect on protein and lipid metabolism. Further, Wicks & Kenny (1964) have shown that injection of testosterone to orchidectomized rats results in rapid increases in RNA turnover rate in seminal vesicles. The specific radioactivity of seminal-vesicular RNA was found to be double that of untreated controls 1hr. after an intraperitoneal injection of testosterone. Their analysis of the distribution of 32 P in the nucleotides of the newly synthesized RNA suggested that testosterone increases the incorporation of 32 P into a mixture of ribosomal and DNA-like RNA species. The ability of testosterone to enhance RNA synthesis in the rat prostate was demonstrated by Liao, Barton & Lin (1966), who also found that this process could be selectively suppressed by actinomycin. In addition, Liao & Williams-Ashman (1962) have suggested that testosterone governs the synthesis or utilization (or both) of messenger RNA in the ventral prostate. Liao, Leininger, Sagher & Barton (1965) found that the RNA polymerase activity (when measured at relatively low ionic strength) of prostatic nuclear extracts was significantly elevated within 1hr. after injection of testosterone and that this increase also was blocked by actinomycin. The stimulating effect of testosterone on nuclear RNA polymerase activity lends additional support to the concept that steroid hormones act primarily by regulating the transcription of the genetic message from DNA to RNA molecules (Liao *et al.* 1965). However, conclusive evidence for the direct participation of androgenic hormones in

the nuclear RNA-synthesizing systems has yet to be obtained.

Weber, Singhal & Srivastava (1965a,b) and Weber, Singhal, Stamm, Lea & Fisher (1966) have suggested that certain hormones exert their effects on the target organ by attacking receptor sites at the source of enzyme production and turn on or off the biosynthetic action of 'whole genetic units' governing the synthesis of functionally related key rate-limiting enzymes. These investigators have provided evidence suggesting that the three key enzymes of hepatic glycolysis, namely glucokinase, phosphofructokinase and pyruvate kinase, may be produced on the same genic unit because of their similar behaviour under a variety of hormonal and nutritional influences. Recent work in this Laboratory (Valadares *et al.* 1968) as well as an earlier report by Smith & Gorski (1967) indicate that uterine tissue contains little or no glucokinase (high- K_m enzyme) and that glucose is phosphorylated in this tissue by the low- K_m hexokinase. Analogous to these findings are the observations that hexokinase rather than glucokinase is chiefly responsible for phosphorylating glucose in both the prostate and seminal vesicles. Testosterone also is capable of increasing prostatic and seminal-vesicular hexokinase activity. The observed increases in hexokinase activity may also represent new enzyme synthesis, since they are effectively blocked by inhibitors of RNA and protein synthesis (R. L. Singhal unpublished work). These findings, along with the results described in previous papers (Singhal *et al.* 1967a; Singhal & Valadares, 1968; Valadares *et al.* 1968), suggest that both male and female sex hormones play vital roles in the regulation of key glycolytic enzymes in their respective target structures.

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