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Effects of α-Difluoromethylornithine, an Enzyme-Activated Irreversible Inhibitor of Ornithine Decarboxylase, on Testosterone-Induced Regeneration of Prostate and Seminal Vesicle in Castrated Rats

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1. Castration of adult rats markedly decreases the amounts of polyamines (putrescine, spermidine and spermine) and of RNA and DNA in the ventral prostate and the seminal vesicle. 2. Daily injections of testosterone propionate to rats castrated 7 days previously increase polyamine and nucleic acid contents more rapidly in the seminal vesicle than in the ventral prostate. 3. After 7 days of androgen treatment, polyamine and nucleic acid contents of the seminal vesicle are significantly higher than those of intact animals. Nucleic acid, but not polyamine, contents return to normal values during the next 4 days of continued treatment. In the prostate, androgen treatment increases polyamine and nucleic acid contents to, but not above, normal values. 4. Repeated doses of α-difluoromethylornithine, a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase, totally blocked the testosterone-induced increase of putrescine and spermidine in the ventral prostate and of putrescine in the seminal vesicle. They slowed significantly the accumulation of spermine in the ventral prostate and of spermidine in the seminal vesicle. α-Difluoromethylornithine also retarded the testosterone-induced accumulation of RNA in the ventral prostate. However, no clear correlation was apparent between accumulation of polyamines and of nucleic acids in the two organs. 5. α-Difluoromethylornithine markedly slows the testosterone-induced weight gain of the prostate, but not of the seminal vesicle. Cytological studies suggest that this effect on the prostate is due to inhibition of the androgen-induced restoration of the secretion content of prostatic acini.

Castration of adult rats results in a rapid and pronounced loss of weight of accessory reproductive organs such as seminal vesicles or ventral prostate. Cytological and metabolic changes, particularly a marked decrease of nucleic acid synthesis, occur during the involution of these organs (Butler & Schade, 1958; Kochakian & Harrison, 1962; Brandes & Groth, 1963; Coffey *et al.*, 1968; Higgins *et al.*, 1976; Takyi *et al.*, 1977). Attention has been focused on the concomitant decrease in activity of the two rate-limiting enzymes in the biosynthesis of polyamines, i.e. ornithine decarboxylase (L-ornithine carboxy-lyase) (EC 4.1.1.17) and *S*-adenosylmethionine decarboxylase (*S*-adenosyl-L-methionine carboxy-lyase) (EC 4.1.1.50) (Pegg *et al.*, 1970; Takyi *et al.*, 1977; Piik *et al.*, 1977). These effects of castration can be reversed by injections of testosterone. Changes in the concentrations of putrescine, spermidine and spermine in the ventral prostate caused by castration of rats and by subsequent testosterone treatment have been studied (Pegg *et al.*, 1970; Takyi *et al.*, 1977). In spite of a good correlation between alterations in RNA accumulation and total spermidine concentrations, there appeared to be no evidence that polyamines mediate the effects of androgens on nucleic acid synthesis in this model (Williams-Ashman *et al.*, 1975*a*). New approaches were required to clarify the roles of polyamine in prostate and in seminal vesicle.

The present paper reports the effects of α-difluoromethylornithine (RMI 71.782), a potent enzyme-activated irreversible inhibitor of ornithine decarboxylase *in vitro* and *in vivo* (Metcalf *et al.*, 1978; Seiler *et al.*, 1978), on biochemical and cytological changes during testosterone-induced regrowth of ventral prostate and seminal vesicles in castrated rats.

Experimental

Chemicals

The following compounds were purchased: tetrasodium EDTA (Calbiochem, San Diego, CA, U.S.A.); L-ornithine, pyridoxal phosphate, $(NH_4)_2SO_4$, sucrose, buffer reagents, diphenylamine, 3,5-dihydroxytoluene, Haematoxylin, and Eosin (Merck, Darmstadt, Germany); dithiothreitol, testo-

sterone propionate, yeast RNA (type XI) and calf thymus DNA (type I) (Sigma, St. Louis, MO, U.S.A.); acetaldehyde (Aldrich Chemical Co., Milwaukee, WI, U.S.A.); Light Green (Prolabo, Paris, France); DL-[1-^{14}C]ornithine (sp. radioactivity 58Ci/mol) (The Radiochemical Centre, Amersham, Bucks., U.K.). α-Difluoromethylornithine (RMI 71.782) was synthesized in our laboratories (Metcalf *et al.*, 1978).

Animals

Castrated or intact male rats of the Sprague–Dawley strain (300–310g body wt.) were purchased from Charles River, Saint-Aubin-les-Elbeuf, France. Castration was performed by the supplier, 1 day before delivery. Animals had access to standard diet and water *ad libitum* and were kept under a constant 12h light/12h dark lighting schedule.

Testosterone propionate (1mg) was given subcutaneously in 0.2ml of sesame oil. α-Difluoromethylornithine (200mg/kg body wt.) dissolved in 0.9% NaCl was injected intraperitoneally.

Rats were killed by decapitation between 11:00h and 12:00h on the given days to minimize effects due to diurnal fluctuations.

Preparation of tissue extracts

The two lobes of the seminal vesicle were clamped off to prevent the loss of secretions and removed after the coagulating glands and connective tissue had been dissected away. One lobe, containing secretions, frozen in liquid N_2, and the other lobe, emptied of secretions, were weighed separately on a microbalance. Ventral prostate was likewise removed, freed of fat and connective tissue, and weighed. Immediately thereafter the organs were homogenized at 4°C in 5ml of 5% (w/v) trichloroacetic acid and the homogenates were centrifuged at 3000*g*. Supernatants and precipitates were used respectively for polyamine and nucleic acid determination.

Determination of polyamines and α-difluoromethylornithine

The supernatants were filtered through a Millipore membrane (pore size 0.22μm), before measurement of their polyamine content with a Durrum D-500 (Durrum Instruments Corp., Palo Alto, CA, U.S.A.) amino acid analyser by the method of Marton *et al.* (1974) as modified by Bartos *et al.* (1977). α-Difluoromethylornithine was determined with the same amino acid analyser by the method of Lee (1973) modified in such a way that a single lithium citrate buffer (pH4.6, 0.668M) eluted this compound in 62min. The detection of the amines was made fluorimetrically by using *o*-phthaldialdehyde as the reactant.

Nucleic acid determination

RNA and DNA were measured by the procedure described by Schneider (1957) modified as follows. The trichloroacetic acid precipitate was resuspended in 2ml of 0.3M-KOH for 20h at 37°C. Thereafter, 5ml of 1.6M-$HClO_4$ was added, and the suspension was cooled to 0°C for 1h and then centrifuged to 3000*g* for 10min. The supernatant was used to determine the RNA by the orcinol reaction (Mejbaum, 1939) with purified yeast RNA as standard. The precipitate was resuspended in 2ml of 0.5M-$HClO_4$ and digested for 20min at 90°C. The suspension was centrifuged at 2000*g* for 10min. The supernatant was used to determine DNA by the diphenylamine method (Burton, 1968) with calf thymus DNA as reference material.

Assay of time-dependent inhibition of ornithine decarboxylase (in vitro)

Ventral prostates were removed from ten untreated intact rats. To obtain sufficient activity of ornithine decarboxylase, seminal vesicles were excised from 16 rats castrated 8 days earlier, 20h after subcutaneous injection of 2.5mg of testosterone propionate. Ornithine decarboxylase activities from these two organs were purified by using steps 1 and 2 [105000*g* centrifugation and $(NH_4)_2SO_4$ precipitation] of the method described by Jänne & Williams-Ashman (1971).

Assays of time-dependent inhibition of ornithine decarboxylase and measurements of the kinetic constants of the inhibition were performed essentially as described previously (Metcalf *et al.*, 1978).

Staining of sections of the prostate gland

Ventral prostates were fixed for 20h in Carnoy's medium and sections were stained in the modified Gomori's trichrome (Haematoxylin–Light Green–Eosin) similarly to that described by Mangan *et al.* (1973).

Results

Changes in weight of seminal vesicle and of ventral prostate

Prostates and seminal vesicles rapidly atrophied after bilateral orchidectomy, and this atrophy was readily reversed by testosterone injections (Fig. 1). Indeed, repeated injections of testosterone into castrated rats caused an overgrowth of the seminal vesicle. Testosterone-induced growth of this organ was not impaired by the concomitant administration of α-difluoromethylornithine (Figs. 1*a* and 1*b*).

In contrast, the testosterone-induced weight gain of ventral prostate was markedly slowed by α-difluoromethylornithine treatment (Fig. 1*c*). After 11 days of testosterone injections the prostates of

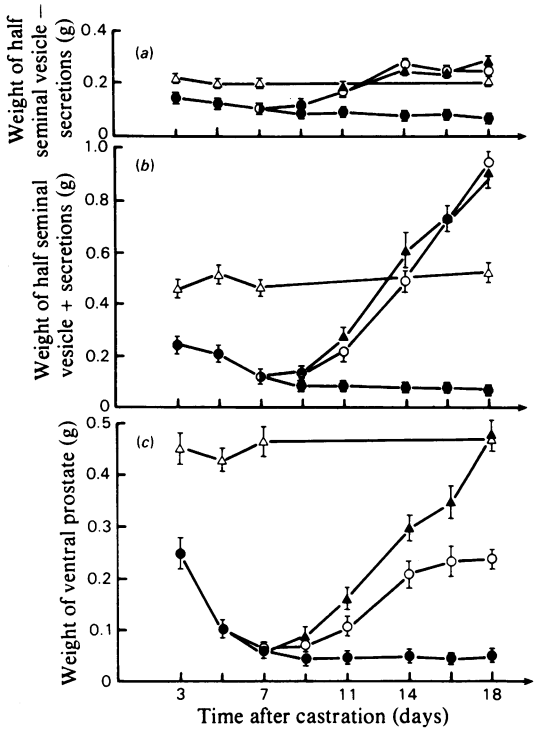

Fig. 1. *Influence of administration of androgen and of combined administration of androgen plus α-difluoromethylornithine on the weight of seminal vesicle and of ventral prostate of castrated rats*

The animals used in this experiment weighed 300–310g. Castration was performed at day zero. Testosterone treatment consisted of subcutaneous injections of 1mg of testosterone propionate in 0.2ml of sesame oil, given each day beginning on day 7. α-Difluoromethylornithine (200mg/kg body wt.) was injected intraperitoneally every 6h beginning on day 6. Control animals received vehicles only. At given days, the animals were killed. One seminal vesicle lobe emptied of secretions (*a*), the other seminal lobe containing secretions (*b*) and the ventral prostate (*c*) were weighed as described in the Experimental section. Each value is the mean ± S.E.M. for five animals. Symbols: △, intact animals injected with sesame oil and saline solution; ●, castrated animals injected with sesame oil and saline solution; ▲, castrated animals injected with testosterone propionate and saline solution; ○, castrated animals injected with testosterone propionate and α-difluoromethylornithine.

castrated rats had regained the same weight as those of normal rats, whereas the prostates of animals concomitantly receiving α-difluoromethylornithine weighed only half as much.

Influence of α-difluoromethylornithine on the morphological changes of the ventral prostate induced by testosterone treatment

The morphological structure of two prostates from each of the four groups was studied. α-Difluoromethylornithine had no effect on restoration of epithelium height by testosterone, but impaired restoration of secretions and of the size of the acini (Table 1). α-Difluoromethylornithine did not cause any apparent change in the morphology of epithelial cells.

Polyamine content of ventral prostate and seminal vesicle

Polyamine content of the prostate decreased concomitantly with prostatic weight after castration (Fig. 2*a*). Subsequent treatment with testosterone increased the content of putrescine and spermidine to control values within 7 and 11 days respectively. Spermine content increased more slowly and was only 40% of the control values at the end of the experiment. α-Difluoromethylornithine blocked the increase of putrescine and spermidine and markedly slowed that of spermine in testosterone-treated rats (Fig. 2*a*).

The spermidine and spermine contents of the seminal vesicle were markedly decreased as early as 3 days after castration (Fig. 2*b*). Testosterone injections caused a very fast increase of spermidine and spermine contents. At 4 days after the beginning of the testosterone treatment, spermidine and spermine contents were 200 and 100% respectively of those found in intact rats. Thereafter, the spermidine content stabilized, whereas spermine content continued to increase slowly and finally stabilized at 150% of that found in intact animals. Initial putrescine content was low both in intact and in castrated rats. After testosterone injections, the putrescine content increased dramatically and after 4 days of treatment was 7 times higher than in intact rats. Thereafter, it returned to normal during 7 days of continued treatment. α-Difluromethylornithine blocked the testosterone-induced increase in putrescine content, slowed that of spermidine and had no significant effect on the increase in spermine concentration (Fig. 2*b*).

α-Difluoromethylornithine concentrations measured during treatment were in the same range in ventral prostate and in seminal vesicle (Table 2).

Nucleic acid content of ventral prostate and of seminal vesicle

Nucleic acid content of ventral prostate decreased after castration. RNA content decreased more rapidly than DNA content and increased immediately with testosterone injections (Fig. 3). The DNA content of ventral prostate, which 7 days after

Table 1. *Effect of α-difluoromethylornithine on prostatic morphology*

Details of the experiment are described in the legend to Fig. 1. On day 18, two ventral prostates of each group of rats were kept for histological study. The tissues were sectioned at 4μm and stained as described in the Experimental section. Diameters of acini and of their secretion content (measured from the length and width of the theoretical rectangles that would enclose these areas), and height of epithelial cells lining acini were measured with an ocular micrometer in 50 to 70 acini/prostate, located in three different regions. Values in the Table are means ± S.E.M. for pooled measurements. The significance of the differences (compared with the group of castrated rats receiving only testosterone) was calculated by Student's *t* test: $*P<0.005$.

Treatment	Epithelium height (μm)	Acini diameter (μm)		Secretion diameter (μm)	
		Maximum	Minimum	Maximum	Minimum
Intact control	17.0±0.2*	252±12*	134±5	177±11*	86±4*
Castration	8.0±0.2*	83±7*	34±3*	23±4*	9±2*
Castration+testosterone	18.9±0.2	204±11	120±5	123±9	67±5
Castration+testosterone+α-difluoromethylornithine	18.6±0.2	160±9*	90±4*	95±8*	45±4*

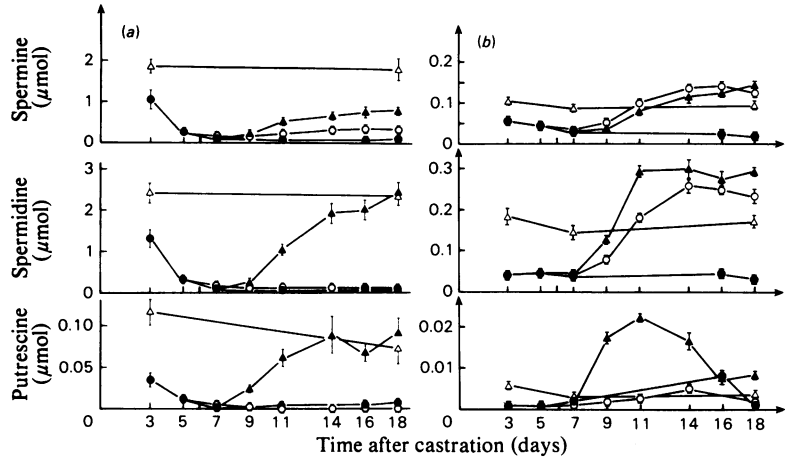

Fig. 2. *Influence of administration of androgen and of combined administration of androgen plus α-difluoromethylornithine on polyamine content of ventral prostate and seminal vesicle of castrated rats*

Details of the experiment are described in the legend to Fig. 1. Polyamines were measured as described in the Experimental section in the ventral prostate (*a*) and in one lobe (containing secretions) of the seminal vesicle (*b*). Each value is the mean ± S.E.M. for five animals. Symbols: △, intact rats injected with sesame oil and saline solution; ●, castrated rats injected with sesame oil and saline solution; ▲, castrated rats injected with testosterone propionate and saline solution; ○, castrated rats injected with testosterone propionate and α-difluoromethylornithine.

castration was 50% of that found in intact animals, continued decreasing significantly for 2 days after testosterone injections. After dosing with testosterone for 11 days, both RNA and DNA contents were not significantly different from those found in intact animals.

α-Difluoromethylornithine injected into testosterone-treated rats significantly slowed accumulation of RNA in the ventral prostate. At 12 days after the beginning of the combined treatment, the RNA content was 60% of that of ventral prostate of castrated rats treated with testosterone alone. The testosterone-induced accumulation of prostatic DNA, however, was not significantly affected by α-difluoromethylornithine.

Castration caused a rapid and marked decrease in RNA content of seminal vesicles, whereas the decrease in DNA content was much slower and less marked (Fig. 3). As in the prostate, testosterone injections led to an immediate and fast accumulation of RNA, whereas a significant increase of DNA content was observed only after 48h. The RNA and DNA content reached a maximum, significantly higher than in intact animals, after 4 days of testosterone injections, and thereafter returned to values found in intact animals. α-Difluoromethylornithine

Table 2. *Concentrations of α-difluoromethylornithine in ventral prostate and seminal vesicle*

Details of the experiment are described in the legend to Fig. 1. α-Difluoromethylornithine concentration was measured in the ventral prostate and in one lobe of the seminal vesicle (containing secretions), as described in the Experimental section, on 3 treatment days, 5h after dosing. Values are means ± S.E.M. for five animals.

Days of α-difluoromethylornithine treatment	α-Difluoromethylornithine (μmol/g wet wt. of tissue)	
	Ventral prostate	Seminal vesicle
1	0.10 ± 0.06	0.28 ± 0.13
5	0.21 ± 0.02	0.17 ± 0.02
12	0.19 ± 0.01	0.13 ± 0.02

was without effect on these changes in nucleic acid contents.

Kinetic constants of inhibition of ornithine decarboxylase from ventral prostate and seminal vesicle by α-difluoromethylornithine in vitro

Since ornithine decarboxylase activity in ventral prostate is much higher than in seminal vesicle (Piik *et al.*, 1977), properties of this enzyme could conceivably be different in the two organs, leading to different susceptibilities to the inhibition by α-difluoromethylornithine. The kinetic constants for time-dependent inhibition of partially purified ornithine decarboxylases from ventral prostate and seminal vesicle were measured as described in the Experimental section. Apparent dissociation constants were 9.2 and 9.6 μM, and half-lives of ornithine decarboxylase activities extrapolated for an infinite concentration of α-difluoromethylornithine were 3.5 and 3.2 min. respectively for ventral prostate and seminal vesicle.

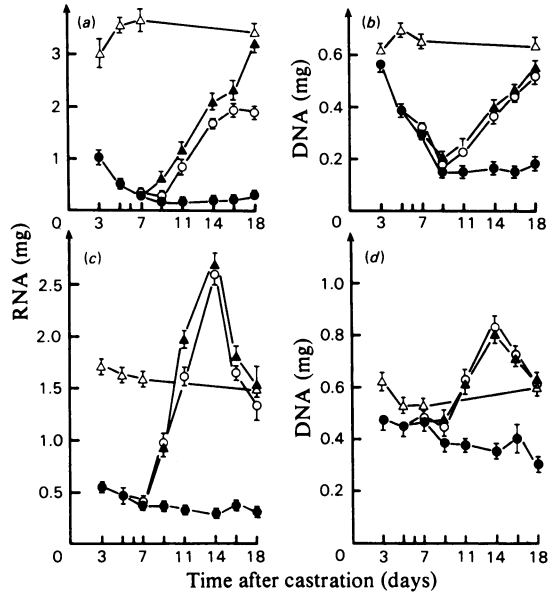

Fig. 3. *Influence of administration of androgen and combined administration of androgen plus α-difluoromethylornithine on nucleic acid contents of ventral prostate and seminal vesicle of castrated rats*

Details of the experiment are described in the legend to Fig. 1. RNA contents of ventral prostate (*a*) and of one lobe (containing secretions) of the seminal vesicle (*c*) and DNA contents of the ventral prostate (*b*) and of one lobe (containing secretions) of the seminal vesicle (*d*) were measured as described in the Experimental section. Each value is the mean ± S.E.M. for five animals. Symbols: △, intact rats injected with sesame oil and saline solution; ●, castrated rats injected with sesame oil and saline solution; ▲, castrated rats injected with testosterone propionate and saline solution; ○, castrated rats injected with testosterone propionate and α-difluoromethylornithine.

Discussion

The early effects of androgen treatment differ considerably in ventral prostate and in seminal vesicles of sexually mature castrated rats. Testosterone treatment leads to a more rapid regrowth of seminal vesicle than of prostate. Accumulation of RNA and DNA is also more rapid in seminal vesicle than in prostate. In addition, repeated injections of testosterone causes in seminal vesicle, but not in ventral prostate, a marked 'overshoot' of both RNA and DNA contents, followed by a return to normal values. Such an 'overshoot' of RNA content of seminal vesicle has been observed previously in castrated mice after similar androgen treatment (Kochakian & Harrison, 1962). These results show that early steps in the restoration of seminal vesicle are more sensitive to testosterone action than those of the ventral prostate, in agreement with a similar finding in the immature rat (Sheppard *et al.*, 1965) and suggest differences in the homeostatic constraint mechanisms (Coffey *et al.*, 1968; Bruchovski *et al.*, 1975) of these two organs.

These differences in the effects of testosterone in the two organs are not readily explained. Differences in the reduction of testosterone by NADPH-dependent 3-oxo-5α-steroid–$NADP^+$ Δ^4-oxidoreductase (EC 1.3.1.22) to 5α-dihydrotestosterone, accepted as the most active metabolite of testosterone (Mainwaring, 1977), are unlikely, since the same amount of this compound has been found in ventral prostate and seminal vesicle after injection of [^{3}H]testosterone to rats (Bruchovski & Wilson, 1968). It is more likely that differences in the binding of testosterone and dihydrotestosterone on their specific receptors or in the number and in the functional role

of these receptors (Mainwaring, 1977) may be involved. In addition, the role of other active metabolites of testosterone may be underestimated.

Relationships between testosterone-induced accumulations of nucleic acid and of polyamines have been studied previously in the ventral prostate of castrated rats (Williams-Ashman *et al.*, 1975*a*), but not in the seminal vesicle. Unlike the prostate, the seminal vesicle does not secrete any polyamine (Williams-Ashman & Lockwood, 1970), so that polyamines measured in this organ are most likely intracellular. Therefore, accumulation of polyamines induced during the testosterone treatment can be more directly related to tissue growth.

Figs. 1(*a*) and 2(*b*) show a close parallelism between the increase of weight of the seminal vesicle emptied of secretions, and the accumulation of spermine, whereas the spermidine content changes in a slightly different fashion. The time courses of testosterone-induced increase of polyamines and of nucleic acids are similar only during the initial stages. During the first 7 days of testosterone treatment, polyamine and nucleic acid contents increase above those of intact animals, but thereafter polyamine contents remain constant, whereas both RNA and DNA contents return to values for intact rats. This may suggest a regulatory role of polyamines in the early steps of nucleic acid accumulation in the seminal vesicle, but shows that, later on, any such role is not predominant.

Previous attempts to investigate the role of polyamines in the testosterone-induced regrowth of ventral prostate with an inhibitor of *S*-adenosylmethionine decarboxylase, methylglyoxal bis(guanylhydrazone) (Williams-Ashman & Schenone, 1972), have not been successful (Williams-Ashman *et al.*, 1975*b*). In the present study, repeated doses of α-difluoromethylornithine totally blocked accumulation of putrescine and spermidine in ventral prostate and of putrescine in seminal vesicle. Accumulation of spermine in ventral prostate and of spermidine in seminal vesicle were only slowed down, and spermine accumulation in seminal vesicle was not impaired by the drug. The difference in the effects of α-difluoromethylornithine on polyamine accumulation in the two organs cannot be attributed to differences in the nature of ornithine decarboxylase in these organs, since kinetic constants of the inhibition *in vitro* of these enzymes were nearly identical. It cannot be due to differences in α-difluoromethylornithine concentrations, since these were similar in prostate and seminal vesicle. It could, however, be related to the marked difference of ornithine decarboxylase turnover in the two organs, as has been discussed previously (Danzin *et al.*, 1979).

The effects of the ornithine decarboxylase inhibitor on polyamine contents were not accompanied by parallel changes in DNA accumulation in either organ studied. Takyi *et al.* (1977) have previously shown that, in cultured prostate, androgen-induced synthesis of DNA and cell division can occur despite depletion of the polyamine content. In the seminal vesicle repeated administrations of α-difluoromethylornithine also failed to affect RNA accumulation. In the ventral prostate, RNA accumulation was slightly, but significantly, slowed by α-difluoromethylornithine treatment. This may have been due to the inhibition of polyamine accumulation, since physiological concentrations of spermidine and spermine have been previously shown to enhance the RNA polymerase activities of rat ventral prostate cell nuclei (Barbiroli *et al.*, 1971; Caldarera *et al.*, 1978).

Due to the presence of polyamines in prostatic secretions (Williams-Ashman & Lockwood, 1970), it is difficult to estimate the intracellular content of polyamines in the ventral prostate. The patterns of polyamine accumulation inside the cell could be different from those observed in the whole ventral prostate. It is possible that α-difluoromethylornithine could selectively inhibit polyamine accumulation in prostatic secretions. The spermidine and spermine measured might then be almost entirely intracellular and the intracellular concentrations would be in the same range as in normal liver (Chadwick *et al.*, 1978) or in normal seminal vesicle, i.e. 200–350 pmol/μg of DNA. Such concentrations might allow a nearly normal growth of prostatic cells, as observed in the present study.

The possibility that the ornithine decarboxylase inhibitor exerts its main effect on polyamines in prostatic secretions is suggested by the cytological findings. α-Difluoromethylornithine markedly inhibits the testosterone-induced restoration of the normal content of prostatic secretions without affecting the normalization of epithelial cells. The high concentrations of polyamines in the ventral prostate may play a role in the regulation of the secretory activity of this organ. Fuller *et al.* (1975) have previously suggested that in the rat ventral prostate ornithine decarboxylase activity reflects secretory activity rather than growth. The present results support this hypothesis.

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