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INDUCTION OF PARTICLE-BOUND RENIN AND ARGININE ESTERASE BY TESTOSTERONE IN THE MOUSE*

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Abstract We studied the induction of renin and a protease (arginine esterase) in the washed 105,000 *g* sediment of the homogenized submaxillary gland of 758 young female mice. Administration of testosterone propionate, α-methyltestosterone or an anabolic steroid such as nandrolone phenpropionate increased the particle-bound renin and arginine esterase activity in the animals sacrificed 7 days after the first injection. The enzyme activity rose significantly within 4 days. Actinomycin D blocked the induction of renin and arginine esterase. In addition, testosterone increased the weight of the animals, of the submaxillary glands and of the kidneys, the microsomal protein concentration of the submaxillary glands and the RNA content of the microsomal fraction of the glands and kidneys. Actinomycin D blocked these effects of testosterone as well. The particle-bound kallikrein activity of the submaxillary and the particle-bound renin activity of the kidney were not changed by testosterone administration. The 105,000 *g* sediment of kidney homogenate has arginine esterase activity which increased an average of 63 per cent after the injection of testosterone.

Submaxillary glands of the white Swiss male mouse contain both renin and kallikrein, but the glands of the female mouse have very little renin [1, 2]. Testosterone administration stimulates the development of secretory granules and increases renin concentration in the gland of female mice [3, 4]. Renin and kallikrein activity are present in a granular layer isolated from the submaxillary gland of male mice [5], but the particles containing renin can be separated from kallikrein granules by repeated centrifugations [6]. Continuing these investigations, we have also found enzymic activity in a high-speed sediment, the microsomal-ribosomal fraction of homogenized kidney and submaxillary gland. This fraction from glands of male or female mice contains active renin and kallikrein. The renin activity increased in the microsomal fraction of the submaxillary gland after treating young female animals with testosterone or with an anabolic steroid. We also studied the induction of a benzoyl-arginine ethyl ester (BAEe) esterase by testosterone in the gland and in the kidney of the mouse.

MATERIALS AND METHODS

Materials. Testosterone propionate was purchased from Schwarz-Mann, 17α-methyltestosterone from Sigma Co., nandrolone phenpropionate (Durabolin) from Organon, (Asp^1-Ile^5)-angiotensin I, bradykinin and the synthetic tetradecapeptide renin substrate from Schwarz-Mann, and hog renin from Miles Labs. Norbolethone (WY3475) was a gift from Dr. M. I. Gluckman of Wyeth Labs. Kits for radioimmunoassay of renin were purchased from Squibb & Sons. Actinomycin D was obtained from Schwarz-Mann and DFP from Aldrich Chemical Co.

Animals and tissue preparations. A total of 758 4-week-old white female Swiss–Webster mice were used. They were obtained from A. Sutter, Co., Springfield, Mo. (series I), or from Sutter, Co., or Simonsen Labs., Gilroy, Calif., or Microbiol. Assoc., Walkersville, Md. (series II) at the age of 3 weeks. On day 0, groups of eight to nine animals were injected s.c. with 0·1 ml sesame oil (S) or with 0·1 ml sesame oil containing either 1 mg testosterone propionate (Tp), 1 mg 17α-methyltestosterone (Ta), 0·25 mg norbolethone (N) or 0·25 mg nandrolone phenylpropionate (Durabolin) (D). The D injection was not repeated.

On days 2, 4 and 6, injections of 0·1 ml S or 0·1 ml sesame oil containing 0·25 mg Tp, N, or Ta were repeated. The animals were designated as C, S, Ta, Tp, D and N mice. Control animals (C) were not handled. Actinomycin D (ACT)-treated animals were injected i.p. with 2·5 μg ACT in 0·1 ml saline 30 min prior to each androgen injection. D-treated animals were similarly injected with ACT on day 0, and also on days 2, 4 and 6. On day 7, eight animals per group were killed by exsanguination under sodium pentobarbital anesthesia. One kidney and both submaxillary glands were removed from each animal. The glands and the kidneys were pooled separately and cooled in an ice-cold solution of 0·25 M sucrose in 0·05 M Tris–HCl buffer, pH 7·4. All subsequent treatments were performed at 0–5°. Tissues were cleaned of fat and membrane, weighed and chopped with scissors in 2 or 3 ml of fresh sucrose buffer. Eighteen strokes in a glass-glass Potter–Elvehjem tissue grinder (Kontes size 23) at 1200 rev/min for 1 min were used to homogenize the tissue. The resulting homogenate was further diluted to a final concentration of 1:10 (w/v) with sucrose buffer. Aliquots were

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retained for DNA determination. The remainder was centrifuged at 120 *g* in a refrigerated Sorvall centrifuge for 10 min at 4°, then for 30 min at 10,900. The precipitate was resuspended in approximately 2 ml of sucrose buffer and centrifuged for 30 min at 10,900 *g*. The combined supernatant plus wash were then centrifuged for 1 hr at 105,000 *g*. The 105,000 *g* supernatant was kept (final supernatant). The sediment from this high-speed centrifugation was resuspended in sucrose buffer and centrifuged again. The drained precipitate obtained after the second centrifugation (microsomal fraction) was resuspended in 2 ml of 0·1 M, pH 6·8, sodium phosphate buffer by 12 strokes with a small (Kontes size 21) glass glass Potter Elvehjem homogenizer. The 10,900 *g* precipitate was resuspended in the same fashion. All fractions were frozen for storage and usually thawed only once at the time of assay. Fourteen-day samples were obtained from animals which had been injected with the same amounts of androgen on alternate days, sacrificed on day 14 and were prepared in the same manner as day 7 samples.

Methods. Microsomal fractions were solubilized with 0·1% Triton X-100 and centrifuged in a Brinkmann desk-top centrifuge for 2·5 min and the protein in the supernatant was determined [7]. Bovine serum albumin was used as protein standard. RNA in the microsomal fraction was determined by the modified Schmidt Thannhauser method [8]. DNA in the homogenate was measured by the diphenylamine method. Samples were extracted twice with 1 M perchloric acid for 20 min at 70° [9].

Hog renin substrate was prepared as described previously [5]. (One mg of substrate preparation contained 0·86 mg protein and produced 224 ng angiotensin I when incubated with excess commercial hog renin.) The K_m equaled 0·8 mg/ml in our system. Samples were preincubated for 30 min at 37° with enzyme inhibitors (10^{-3} M *o*-phenanthroline, 8×10^{-3} M BAL and 10^{-3} M DFP in 0·05 M sodium phosphate buffer, pH 6·8). Hog renin substrate was added to ice-cold preincubation mixtures to a final concentration of 4 mg/ml. The samples were incubated for 5–15 min at 37° in a shaking incubator. During this time not more than 25 per cent of substrate was converted to angiotensin I. The reaction was stopped by freezing samples in a −50° bath. Angiotensin I was assayed with the Squibb Angiotensin I Immunotope kit. A similar protocol was used substituting 10 μg tetradecapeptide ml of incubation mixture for hog renin substrate. In this assay, the reaction was stopped by placing the incubation tube in a boiling water bath for 15 min and subsequently freezing, thawing and centrifuging it. The angiotensin I liberated in the supernatant was assayed on the systemic arterial blood pressure of the anesthetized rat [5]. (Asp^1-Ile^5) angiotensin I was used as the standard. The animals received 50 mg/kg of sodium pentobarbital and 10 mg/kg of pentolinium tartarate. Female Wistar Furth (WF fmai), so-called dextran resistant [10], rats were used. These animals are particularly suitable for experiments lasting up to 6–8 hr, since they usually do not develop edema.

Human plasma kininogen was prepared by diluting fresh plasma 1:1 with saline and heating it to 56° for 30 min. Microsomal kallikrein preparation was incubated at 37° for 20 min in 0·03 M Tris HCl buffer, pH 7·4, containing 10^{-3} M *o*-phenanthroline. After kininogen was added, aliquots were withdrawn every 5 min and the kinin released was assayed on the isolated rat uterus [11], using synthetic bradykinin as standard. BAEe esterase assay was done as mentioned previously [12]. Microsomal samples were solubilized with 0·1% Triton X-100 and assayed with 10^{-3} M BAEe in 0·05 M Tris HCl buffer, pH 8·0, in a Cary 15 or 118C recording u.v. spectrophotometer.

RESULTS

Effect of testosterone. The weight of all treated and untreated animals increased in 7 days including C, S, Ta and Tp mice, but only Ta mice weighed significantly more ($P < 0·01$) than the control (Table 1). Mice which were treated with ACT did not gain but lost weight.

The weights of submaxillary glands and kidneys of C and S animals were similar. Treatment with testos-

Table 1. Effect of testosterone and actinomycin in young female mice

Series	Treatment	Weight of animals (g)	Submaxillary gland: Weight (mg/pair of glands)	Submaxillary gland: Microsomal protein (mg/pair of glands)	Submaxillary gland: Microsomal RNA-P (μg/pair of glands)	Kidney: Weight (mg/kidney)	Kidney: Microsomal protein (mg/kidney)	Kidney: Microsomal RNA-P (μg/kidney)
I	C	29·9 ± 0·5 (N = 40)*	93·7 ± 5·7	0·462 ± 0·092	2·68 ± 0·15	153·2 ± 11·8	1·36 ± 0·13	7·97 ± 1·07
	S	31·2 ± 0·5 (N = 56)	102·7 ± 5·6	0·445 ± 0·065	4·45 ± 0·28†	165·4 ± 7·6	1·39 ± 0·13	10·73 ± 1·17
	Ta	33·5 ± 0·4† (N = 64)	122·2 ± 5·9†‡	0·570 ± 0·085	8·35 ± 0·80†‡	197·6 ± 10·5‡	1·63 ± 0·11	12·92 ± 1·48
II	S	27·8 ± 0·8 (N = 79)	90·1 ± 6·1	0·261 ± 0·040	1·41 ± 0·25	149·0 ± 13·8	0·796 ± 0·117	9·84 ± 0·90
	Tp	28·0 ± 0·8 (N = 86)	125·9 ± 9·4§	0·445 ± 0·021§	5·24 ± 0·74§	196·1 ± 12·0	1·111 ± 0·143	19·72 ± 1·25§
	Tp + ACT	19·6 ± 0·5¶ (N = 42)	74·8 ± 8·6¶	0·288 ± 0·080	2·71 ± 1·05	133·8 ± 5·7¶	0·703 ± 0·083	12·55 ± 1·02¶

* N = number of animals.
† $P < 0·01$.
‡ S.D. from C, $P < 0·05$.
§ S.D. from S, $P < 0·01$.
Significantly different from S, $P < 0·05$.
¶ S.D. from Tp, $P < 0·01$.

Table 2. Induction of BAEe esterase activity in a microsomal fraction of the submaxillary gland of female mice*

No. of group	Control C	Control S	Treated Tp	Treated Ta	Treated D	Treated N
1		0·2	12·2 (6·1)		6·6 (3·6)	
2		1·1	8·9 (7·4)			12·5 (7·4)
3	2	1·4	10·8 (6·1)	4·3 (1·8)		
4		0·6	8·2 (7·2)		7·8 (4·3)	8·8 (4·3)
5		1·0	14·1	7·3		
6	2·8				7·1	
Mean ± S. E.	2·4	0·9 ± 0·2	10·8 ± 1·1†	5·8	7·2	10·65
ACT-treated (mean ± S. E.)			6·7 ± 0·3‡	1·8	3·95	5·85

* Each figure was obtained from eight pairs of submaxillary glands. Figure in parentheses: mice treated with ACT. Activity: μmoles BAEe hydrolyzed/min/mg of protein.

† Significantly different from S, $P < 0·01$.

‡ S.D. from Tp, $P < 0·05$.

terone increased significantly the weight of the submaxillary gland and the kidney and the solubilized microsomal protein content of the gland (TP). However, all of these changes were less than 71 per cent. The increase in organ weights in the second series of animals was greater; these animals also weighed less initially than the mice in the first series.

The increase in the microsomal RNA was more remarkable. The injection of both Ta and Tp increased the RNA content of the washed 105,000 *g* sediment. RNA rose in the glands of Ta and Tp mice 88 and 272 per cent and in the kidneys 68 and 100 per cent, respectively, compared to S animals. In three exploratory studies, we found no significant changes in the DNA content of the gland of the treated animals. Treatment with ACT blocked the effect of testosterone on the weight of organs, and on the protein and RNA concentrations.

Submaxillary gland

Esterase. BAEe esterase increased 11-fold ($P < 0·01$) in the microsomal fraction of the animals treated with Tp. Table 2 shows the results obtained in the second series of experiments. Ta, Tp, N or a single injection of D raised the microsomal arginine esterase activity. ACT blocked the increase partially. This indicated that the increase in the esterase activity of the microsomal fraction after testosterone or anabolic steroid treatment can be attributed to induction of the enzyme.

The microsomal preparation also had kallikrein activity as shown by assaying the amount of kinin released from human plasma kininogen on the rat uterus. In four experiments done with three different preparations, no consistent differences were found between the S and Tp animals.

The rate of hydrolysis of BAEe increased within 48 hr after the first injection of Ta and kept rising with time after treatment. Table 3 shows the microsomal enzyme activity in a group of Ta mice sacrificed at the times indicated. The activity rose significantly after 4 days of treatment.

The relative concentration of the esterase in the 105,000 *g* fraction also increased during testosterone administration. In the C group, only 2 per cent of the esterase activity sedimented from the final supernatant. This increased to 3 per cent after 48 hr, to 5 per cent after 96 hr and to 8 per cent after 7 days. There was a corresponding decrease in the esterase in the supernatant from 95 to 68 per cent after 7 days. The activity in the 10,900 *g* sediment rose parallel with the microsomal fraction from 4 to 25 per cent on the average.

Renin. Microsomal fractions of the homogenized gland of young female mice contained renin, although much less than the adult male (Table 4). Ta, Tp and

Table 3. Time course of induction of BAEe esterase in a microsomal fraction of the submaxillary gland*

	Control					Ta-treated				
	C			S						
Group	Day 0	Day 4	Day 7	Day 4	Day 7	Day 1	Day 2	Day 3	Day 4	Day 7
0					1·98	1·0	1·85	2·28	4·99	11·82
1	0·98					1·22	2·24	3·88	6·10	14·40
2	0·99	1·42		1·98	3·78				9·9	15·4
3	0·8		2·32	1·36	2·16				4·38	9·6
4	1·17		1·71		2·24				2·50	15·38
5	0·75		1·29		1·52				2·42	8·88
6	1·09		1·37		1·63					6·81
7	1·74		1·63		2·22					12·15
Mean ± S. E.	1·07	1·42	1·66	1·67	2·22	1·11	2·04	3·08	5·05	11·80
	± 12		± 0·18		± 0·28				± 1·13	±1·13†

* Each figure was obtained from eight pairs of submaxillary glands. Activity: μmoles BAEe hydrolyzed/min/mg of protein.

† Significantly different from S7, $P < 0·01$.

Table 4. Induction of renin in a microsomal fraction of the submaxillary gland*

No. of group	Control C	Control S Day 7	Control S Day 14	Treated (1 week) Ta	Treated (1 week) Tp	Treated (1 week) N	Treated (1 week) D	Treated (2 weeks) Tp
1	6	3·8		65·6	108·8			
2		23·2			181·2 (110·4)			
3		7·4			344·4			
4			58·8					395
5	32·5	9·2		56·4 (22·8)	259·6 (58·8)			
6		19·8		122·2	209·4			
7		11·6			394·8 (122·4)			
8			25·2					271 (181·4)
9		23·0			123		184·8 (51·6)	
10		6·2			465 (207·6)	401·2 (98·6)		
11		4·4			134·4 (117·4)	187 (46·2)	197·4 (42·6)	
Mean ± S.E.	19·4	12·1 ± 2·6	42	81·4 ± 20·6	246·7 ± 42·8†	294·1	191	333
ACT-treated (mean ± S.E.)				22·8	123·3 ± 23·9	72·4	47·1	181·4

* Figure in parentheses: mice treated with ACT. Each figure was obtained from eight pairs of submaxillary glands. Activity: ng angiotensin I/min/mg of protein. Group 12: adult male mice; activity: 722. Source of mice: groups 1–6 and 12, Sutter; 7–9, Simonsen; 10 and 11, MBA.

† Significantly different from S, $P < 0{\cdot}01$.

‡ S.D. from Tp, $P < 0{\cdot}05$.

the anabolic steroids enhanced the renin content of the submaxillary gland (Table 4). Ta was the weakest inducer. Renin in the microsomal fraction of Ta mice rose 7-fold, in the Tp mice it went up 20-fold, 24-fold in D mice and 16-fold in the other anabolic steroid-treated N mice. The amount of angiotensin I generated by the fraction from purified hog angiotensinogen in 1 min was 247 ± 43 ng angiotensin/mg of protein in Tp mice treated for 1 week as established by radioimmunoassay. Treating animals for 2 weeks enhanced the renin activity even more, to 333 ng. Administration of ACT lowered induced renin action by 50 per cent.

The specific activity of renin also increased in the 10,900 *g* sediment and in the final supernatant of the homogenized gland after 1 week of administration of Tp. The activity increased in the supernatant from 145 to 2886 and in the 10,900 *g* sediment from 335 to 2752 ng/mg of protein. Figure 1 shows the time course of induction in one group of mice receiving Ta. The renin content of the microsomal fraction rose considerably after 4 days.

The enzyme in the microsomal fraction was hypertensive as shown by injecting it, i.v., into anesthetized rats. The mean systemic arterial blood pressure of the rats pretreated with a ganglion blocker rose 60 per cent after injection of 23 μg of microsomal protein. There was tachyphylaxis to this effect on the blood pressure.

The microsomal renin preparation released more angiotensin I from the synthetic tetradecapeptide substrate than from angiotensinogen. Tp mice liberated 25 μg/mg of protein in 1 min at pH 6·8. This was established by bioassay of the peptide on the rat blood pressure. Much less (3·4 μg) angiotensin I was released when the incubation of renin and substrate was carried out at pH 4·5.

Kidney

Renin. The microsomal fraction of the female mouse kidney contained renin as established by radioimmunoassay and by bioassay. We detected no significant differences among C, S and Tp groups. Angiotensin I [8·8 (S.E. ± 1·7) ng] was released by 1 mg protein in 1 min by the Tp group as measured by radioimmunoassay. The renal fraction of female mice in S group and in Tp group treated with ACT liberated 10·2 (±1·8) and 9·9 (±2·8) ng angiotensin I. The microsomal renin released more angiotensin I from nephrectomized rat plasma than from purified hog angiotensinogen; 0·1 μg/mg of angiotensin I was liberated in 1 min in radio immunoassay. Even more angiotensin I (1·7 μg) was released from the synthetic tetradecapeptide substrate estimated in bioassay done under the conditions described in Methods.

Injection of 300 μg of the microsomal protein into rat raised the mean systemic arterial blood pressure by 90 per cent. During the length of the experiment, the blood pressure did not return to preinjection level. Repeated injection of the material was much less effective because of tachyphylaxis.

Esterase. Because storage at −20° may affect the BAEe esterase in the microsomes, in the same group of C, S, Ta and Tp mice the enzyme was tested the

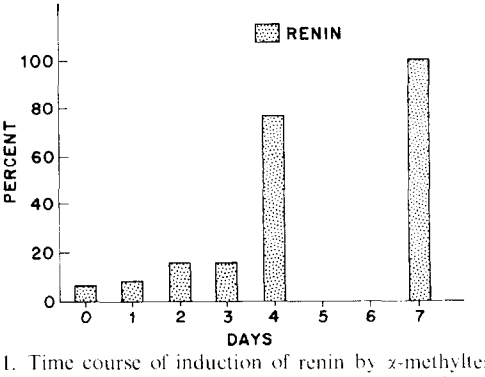

Fig. 1. Time course of induction of renin by α-methyltestosterone in the submaxillary gland of the young female mouse.

Table 5. BAEe esterase activity in a microsomal fraction of female mouse kidney*

Series	No. of group	Control C	Control S	Treated Ta	Treated Tp	Increase in treated groups (%)
I	1	7	8	12		50
	2	20	21	28		33
	3	20	28	46		64
	4	11	10	19		90
II	5		18		29 (16)	61
	6		9		20 (14)	122
	7	34	37		46 (30)	24
Mean ± S.E.		18·4 ± 4·7	18·7 ± 4·1	26·2 ± 7·4	31·7 ± 7·6	63·4 ± 12·7†
ACT-treated (mean ± S.E.)					20 ± 5·03	

Figure in parentheses: mice treated with ACT. Each figure was obtained by pooling six to eight kidneys from a group of eight mice. Activity: μmoles BAEe hydrolyzed/min/mg of protein.

† By the sign test probability of S. D. is <0·01.

same day and the results were expressed as per cent increase in Ta or Tp mice (Table 5). The BAEe esterase activity of the microsomal fraction of the homogenized kidney cortex was similar in C and S groups. Treating the animals with Ta or Tp, however, enhanced the action of the enzyme in the microsomal fraction. The mean increase in the Tp mice was 63 per cent. However, treatment with ACT abolished this increase.

DISCUSSION

Increasing evidence indicates that renin exists in several forms in tissue and in blood. For example, blood samples taken from patients suffering from Wilm's tumor of the kidney contain a higher molecular weight variety of renin [13]. In addition, renin may be coupled to a protein in the kidney [14]. A precursor of renin exists in the amniotic fluid [15] and many tissues contain pseudorenin [16].

Several groups of investigators observed that there is renin in the salivary glands of the white Swiss male and that the enzyme is also present in glands of the young female mice which are treated with testosterone [1,2,5,17–20]. Some reports indicating the complete lack of renin in untreated females were based on using bioassay, which was not sensitive enough to detect a low level of the enzyme [17].

Our experiments have shown that a portion of the renin content of the submaxillary gland and kidney of mice is particle bound and sediments with the washed microsomal fraction. This preparation released angiotensin I as established by radioimmunoassay and bioassay. It cleaved the purified hog angiotensinogen, and the synthetic tetradecapeptide and liberated angiotensin I also from nephrectomized rat plasma. The enzyme also raised the blood pressure of the rat when injected i.v. Treating 28-day-old female mice with testosterone elevated the renin content in the submaxillary gland. The specific activity of microsomal renin increased more than the weight of the organ or the protein or RNA content of the fraction. That the rise in renin activity was due to induction of the enzyme was shown in experiments where administration of ACT blocked most of the effect of testosterone.

The activity of a BAEe esterase in the particle fraction of the submaxillary gland rose parallel to renin. ACT also blocked the rise in the esterase action. This BAEe esterase in the submaxillary gland, however, was not identical with kallikrein. The presence of BAEe esterase in the gland and the effect of testosterone on the enzyme were shown by a number of investigators but its functions are not known [21,22]. Because of its substrate specificity it may cleave peptide bonds involving the carboxyl group of arginine. Two peptides, the nerve growth factor and epidermal growth factor present in the gland, have C-terminal arginine [23,24]. If they exist in tissues in the form of a longer peptide chain, such as proinsulin in the pancreas [25], then the arginine esterase, or maybe even kallikrein, might liberate them at the arginine residue.

In addition to the two testosterone preparations used, a single injection of an anabolic steroid also induced both renin and BAEe esterase in the gland.

The microsomal renin content of the kidney was not stimulated by testosterone. However, this hormone acts on the epithelial cells of the proximal tubules of the kidney [26] and these cells probably do not contain renin. The microsomal arginine esterase content of the kidney increased moderately in the Ta and Tp mice. We did not study the kininogenase (kallikrein) activity of the preparations. The arginine esterase activity in human urine is due to kallikrein, and in rat kidney it is at least partially attributed to kallikrein [27,28]. This renal enzyme may originate from the proximal tubules [29], although recently it was located in the distal tubules [30].

Granules of submaxillary gland contain kallikrein and renin [5], and juxtaglomerular granulation is the site of renin storage in the kidney [31]. Previously we used a Dounce homogenizer for isolating granules from the submaxillary gland at 37° because the granules lyse at 4° [5,6,12]. In the present experiments, we used a different technique and no precautions were taken to extract the granules intact; neither was the first 120 *g* sediment containing unbroken cells and debris re-extracted to improve the yield. We wanted to establish the existence of a particle-bound renin in the tissues of the mouse.

The particle-bound renin described here is different from the pseudorenin [16]. The microsomal renin is active at neutral pH and releases angiotensin I from plasma *in vitro* and *in vivo*. Pseudorenin is inactive under these conditions.

Renin is released from the kidney after β-adrenergic receptor stimulation [32] and its half-life in the circulation can be measured in minutes [33]. As we observed in the submaxillary gland, its concentration in the tissue increased relatively slowly, since we detected a significant rise in microsomal renin only after 4 days of testosterone treatment. It remains to be established whether this form of microsomal renin is released into the circulation or whether it is retained in the organ for an intrarenal function.

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