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Localization of 25-hydroxyvitamin D_3 1α-hydroxylase and 24-hydroxylase along the rat nephron

(1α,25-dihydroxyvitamin D_3/24,25-dihydroxyvitamin D_3/parathyroid hormone/proximal convoluted tubules/proximal straight tubules)

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ABSTRACT **Defined nephron segments were microdissected from the kidney of vitamin D-deficient rats, normal rats, and normal rats treated with 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$]. Tubule segments were incubated with ^{3}H-labeled 25-hydroxyvitamin D_3 and the rates of production of ^{3}H-labeled 1α,25-$(OH)_2D_3$ and 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] were determined. Nephron segments tested include the glomerulus, proximal convoluted tubules (PCT), proximal straight tubules (PST), medullary and cortical thick ascending limbs of Henle's loop, distal tubules, and collecting tubules. Production of 1α,25-$(OH)_2D_3$ was detected only in PCT of vitamin D-deficient rats (mean ± SEM, 0.70 ± 0.05 fmol/mm per hr); the value decreased to 0.11 ± 0.05 after parathyroidectomy. By contrast, significant 24,25-$(OH)_2D_3$ production occurred in PCT of normal rats (0.23 ± 0.05 fmol/mm per hr). After administration of 1α,25-$(OH)_2D_3$ to normal rats, the rate of production of 24,25-$(OH)_2D_3$ in PCT increased to 0.64 ± 0.06 fmol/mm per hr and also became apparent in PST (1.07 ± 0.21). The rates of production of 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ in these nephron segments were linear with tubule length over a wide range of lengths per incubation and with the incubation time. The results define the localization of 25-hydroxyvitamin D_3 1α-hydroxylase and 24-hydroxylase along the rat nephron: PCT is capable of producing both 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ and PST can produce 24,25-$(OH)_2D_3$. The use of defined nephron segments may be useful for study of the distribution and regulation of 25-hydroxyvitamin D_3 hydroxylases in the kidney.**

It is well established that vitamin D_3 must undergo sequential hydroxylations, first in the liver to 25-hydroxyvitamin D_3 (25-OH-D_3) and then in the kidney to either 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2D_3$] or 24,25-dihydroxyvitamin D_3 [24,25-$(OH)_2D_3$] before it exerts its biological actions (1). Under normal physiological conditions, the kidney is believed to be the principal site of synthesis of 1α,25-$(OH)_2D_3$ which is thought to be the primary hormonal form of vitamin D_3 (1). The kidney seems also to be the major site of 24,25-$(OH)_2D_3$ production because plasma levels of 24,25-$(OH)_2D_3$ are markedly decreased in anephric patients and rats (2, 3). The activities of 25-$(OH)D_3$ 1α- and 24-hydroxylase, the enzymes responsible for the production of 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$, respectively, are finely regulated by various ions and hormones, including calcium, phosphate, parathyroid hormone, and vitamin D; in general, there are reciprocal changes in the activities of these two enzymes (1).

Recent studies have demonstrated the exclusive localization of 1α-hydroxylase in the proximal tubules in chicken and fetal rabbit kidneys (4, 5). However, the precise location of 1α-hydroxylase in the mature mammalian kidney is not known. Furthermore, the location of 24-hydroxylase in the kidney has not been studied. Determination of the precise location of both 1α-hydroxylase and 24-hydroxylase in the kidney may provide further insight into the regulatory mechanisms of these two hydroxylase systems.

Using single nephron segments microdissected from rats, we found that both 1α-hydroxylase and 24-hydroxylase are located only in the proximal convoluted tubules (PCT) of vitamin D-deficient and normal rats, respectively, and that 24-hydroxylase is present in both the PCT and proximal straight tubules (PST) in normal rats treated with 1α,25-$(OH)_2D_3$.

MATERIALS AND METHODS

Animals. To create vitamin D deficiency, male Holtzman rats were fed a vitamin D-deficient diet, containing 0.45% calcium and 0.3% phosphorus, for 7–10 weeks after weaning (6). The mean (±SEM) plasma concentrations of calcium and inorganic phosphate in vitamin D-deficient rats at the time of study were 4.4 ± 0.5 and 4.8 ± 0.5 mg/100 ml, respectively. Normal male rats (Holtzman or Wistar) weighing 200–300 g were fed a laboratory rat chow; some were given daily subcutaneous injections of 1α,25-$(OH)_2D_3$ (25 ng/kg) for 3–10 days. Mean (±SEM) plasma concentrations of calcium and inorganic phosphate in normal rats were 9.9 ± 0.1 and 6.7 ± 0.3 mg/100 ml, respectively; in rats treated with 1α,25-$(OH)_2D_3$ they were 11.8 ± 0.3 and 7.1 ± 0.4 mg/100 ml, respectively.

Preparation of the Nephron Segments. The animals were anesthetized with sodium pentobarbital, 40 mg/kg of body weight intraperitoneally. The right renal and superior mesenteric arteries were ligated and, immediately before perfusion of the left kidney, the aorta was ligated above the renal arteries. The left kidney was then perfused for a few seconds through a PE 100 polyethylene catheter inserted in the abdominal aorta below the renal arteries; the initial perfusate was chilled Krebs–Ringer pH 7.4 bicarbonate buffer containing 8.3 mM glucose (KRBG) and equilibrated with 95% O_2/5% CO_2; this was followed by a rapid perfusion with chilled KRBG containing 0.1% bovine serum albumin (fraction V, Sigma) and 0.1% collagenase (type I, Sigma).

The left kidney was removed and slices (thickness, ≈0.5–1.0 mm) were cut in the corticomedullary plane. Slices were incubated for 30 min at 30°C in KRBG containing 0.1% bovine serum albumin and 0.1% collagenase, with constant bubbling with 95% O_2/5% CO_2. The kidney slices were rinsed three times with 30–50 ml of ice-cold modified Hanks' solution

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Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; PCT, proximal convoluted tubules; PST, proximal straight tubules; MTAL, medullary thick ascending limb of Henle's loop; CTAL, cortical thick ascending limb of Henle's loop; DT, distal tubules; CT, collecting tubules.

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(137 mM NaCl/5.2 mM KCl/0.34 mM Na_2HPO_4/0.44 mM KH_2PO_4/1.0 mM $CaCl_2$/0.49 mM $MgCl_2$/0.42 mM $MgSO_4$/4.2 mM $NaHCO_3$ pH 7.4), and defined single nephron segments were dissected manually in the ice-cold Hank's solution under a stereomicroscope. PCT were dissected with the glomerulus attached, and then the glomerulus was separated. PST were dissected from the juxtamedullary portion of the cortex and retrogradely dissected from the early portion of its descending limb of Henle's loop in the outer medulla. The medullary thick ascending limb of Henle's loop (MTAL) was dissected from the outer medulla. The cortical thick ascending limbs of Henle's loop (CTAL) and distal tubules (DT) were dissected together from the medullary ray of the cortex, and then the CTAL and DT were separated at the macula densa. Collecting tubules (CT) were dissected from either the cortex or outer medulla.

Incubation. After the tubule length was measured with the aid of a drawing tube attached to the microscope (Wild M5 dissecting microscope), the dissected tubule segments were transferred to Hanks' solution containing 5 mM pyruvate, 8.3 mM glucose, and 10 mM Hepes at pH 7.4. Each group of defined tubule segments was incubated in a Microflex tube (Kontes, Vineland, NJ) in 20 μl of incubation medium (Hanks' solution/5mM pyruvate/8.3 mM glucose/10 mM Hepes, pH 7.4) with room air as the gas phase, and with 0.5 μM 25-OH-[26,27-*methyl*-^{3}H]D_3 (specific activity, 9.8 or 22.3 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) or 25-OH-[23,24(*n*)-^{3}H]D_3 (specific activity, 51 Ci/mmol; both isotopes from Amersham. The latter labeled 25-OH-D_3 was used only to measure 1α-hydroxylase activity. Each incubation mixture contained the specific segments from one animal, but no more than one incubation mixture was prepared for a given segment from each animal. The incubation was carried out at 37°C for 60 min with 120 oscillations per min and was terminated by the addition of 50 μl of chloroform/methanol, 1:2 (vol/vol). Lipid extraction was performed according to the method of Bligh and Dyer (7).

Measurement of 25-OH-D_3 1α- and 24-Hydroxylase Activities. Initially, lipid extracts were subjected to sequential chromatographic separations to verify the authenticity of labeled metabolites of 25-(OH)-[^{3}H]D_3. First, the extract was applied to a thin-layer chromatography plate (silica gel 60 on aluminum foil, E. Merck, Darmstadt, Germany) and developed continuously with benzene/ethyl acetate, 1:1(vol/vol) (8). After drying under N_2, the plate was cut transversely at 0.5-cm intervals and the radioactivity of each slice was determined by using Beckman model LS8100 liquid scintillation counter.

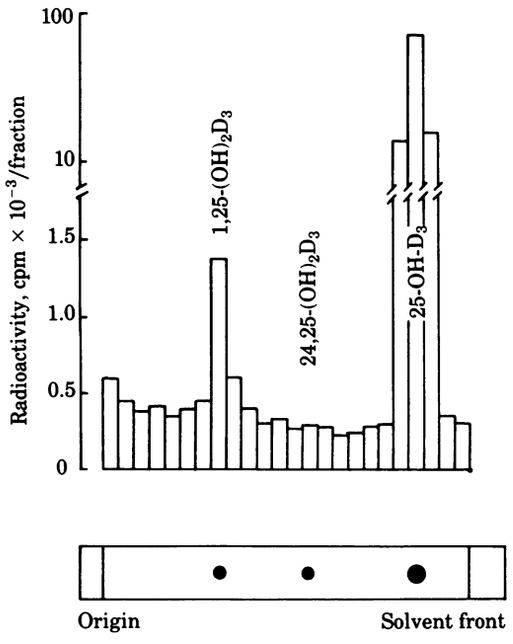

FIG. 1. Thin-layer chromatography profile of the extract of the PCT of a vitamin D-deficient rat. Radioactive peaks (0.5-cm fractions) correspond to authentic 25-OH-D_3 and 1α,25-$(OH)_2D_3$ as shown at the bottom. No radioactivity was seen at the 24,25-$(OH)_2D_3$ position.

The combined fractions of either 1α,25-$(OH)_2$[^{3}H]D_3 or 24,25-$(OH)_2$[^{3}H]D_3 were extracted again with chloroform and subjected to high-performance liquid chromatography (model 6000, Waters, Milford, MA) on a μPorasil column (0.39 × 30 cm). The solvent system was 10% isopropanol/*n*-hexane; the elution rate was 1.5 ml/min at 500 psi. Fractions (1 or 0.5 min) were collected over 20 min and the radioactivity in each fraction was determined by scintillation spectrometry. The metabolites of 25-(OH)-[^{3}H]D_3 were identified by cochromatography with authentic 1α,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ (kindly supplied by A. W. Norman, University of California, Riverside, CA), or 25-OH-D_3 (a gift from Upjohn). The peak corresponding to 24,25-$(OH)_2D_3$ was also subjected to periodate oxidation for further chemical characterization (9). The amounts of 1α,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ produced were calculated from data obtained by thin-layer chromatography and were expressed in fmol per mm of tubule length or per glomerulus based on the specific activity of 25-OH-[^{3}H]D_3 after correction for recovery (79–89%).

Measurement of Adenylate Cyclase Activity. Effects of synthetic bovine parathyroid hormone (NH_2- terminal amino acids 1–34, Beckman) on adenylate cyclase activity along the rat nephron were examined by a method described by Chabardes *et al.* (10) with a modification (11).

The results are expressed as mean ± SEM; statistical comparisons were made with the Student *t* test.

RESULTS

Distribution of 1α-Hydroxylase. The PCT prepared from a vitamin D-deficient rat metabolized 25-OH-[^{3}H]D_3 mainly to a polar peak that comigrated with authentic 1α,25-$(OH)_2D_3$ (Fig. 1). When the radioactive peaks corresponding to 1α,25-$(OH)_2D_3$ on thin-layer chromatography were subjected to high-performance liquid chromatography, the radioactivity again appeared in the same position as authentic 1α,25-$(OH)_2D_3$ (Fig. 2). Significant production (0.70 ± 0.05 fmol/mm per hr) of

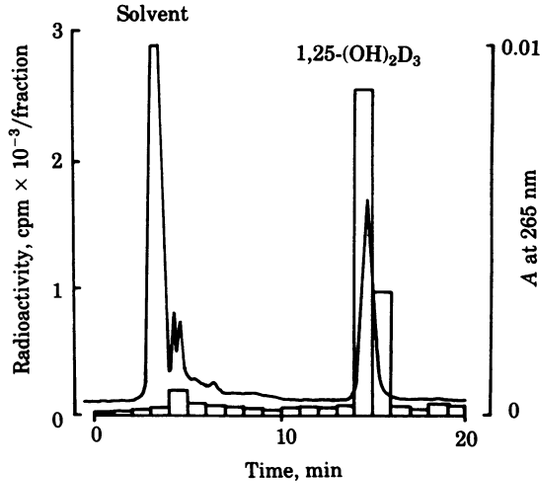

FIG. 2. High-performance liquid chromatography profile of peaks corresponding to 1α,25-$(OH)_2D_3$ in Fig. 1. Solid line, absorbance at 265 nm; bars, radioactivity in each 1.5-ml fraction.

Table 1. Distribution of 25-OH-D_3 1α-hydroxylase and 24-hydroxylase along rat nephron

Nephron segment	n	Tubule length per incubation, mm	Hydroxylase activity,* fmol/mm/hr
1α-Hydroxylase in vitamin D-deficient rat			
Glomerulus	4	80–120†	ND
PCT	10	39.6–111.3	0.70 ± 0.05
PST	5	23.6– 76.1	ND
MTAL	4	35.6– 85.0	ND
CTAL	4	19.9– 89.6	ND
DT	4	15.9– 43.1	ND
CT	4	18.9– 65.2	ND
24-Hydroxylase in normal rat			
Glomerulus	4	30–100†	ND
PCT	9	40.5– 87.0	0.23 ± 0.05
PST	4	37.0– 89.0	ND
MTAL	3	43.0– 85.0	ND
CTAL	5	38.0– 89.0	ND
DT	5	31.0– 47.0	ND
CT	4	41.1–109.0	ND
24-Hydroxylase in normal rat given 1α,25-$(OH)_2D_3$			
Glomerulus	4	30–100†	ND
PCT	8	36.9–106.0	0.64 ± 0.06
PST	13	34.2–196.3	1.07 ± 0.21
MTAL	4	22.4– 46.0	ND
CTAL	4	17.0– 81.2	ND
DT	4	25.1– 36.1	ND
CT	4	20.0– 51.5	ND

* Mean ± SEM. ND, not detected.
† Number of glomeruli per incubation.

1α,25-$(OH)_2D_3$ occurred only in the PCT of vitamin D-deficient rats (Table 1). No 1α,25-$(OH)_2[^3H]D_3$ was produced in the heat-denatured PCT of vitamin D-deficient rats. No radioactivity could be found in the position corresponding to 24,25-$(OH)_2D_3$ in any of the nephron segments from the vitamin D-deficient rats. The rate of generation of 1α,25-$(OH)_2[^3H]D_3$ was linear with respect to tubule length per incubation and to the duration of incubation (Fig. 3).

Because the secondary hyperparathyroidism of vitamin D deficiency may play an important role in stimulating the activity of 1α-hydroxylase, the effect of parathyroidectomy on the activity of this enzyme was examined in the PCT from vitamin D-deficient rats. The production of 1α,25-$(OH)_2D_3$ decreased

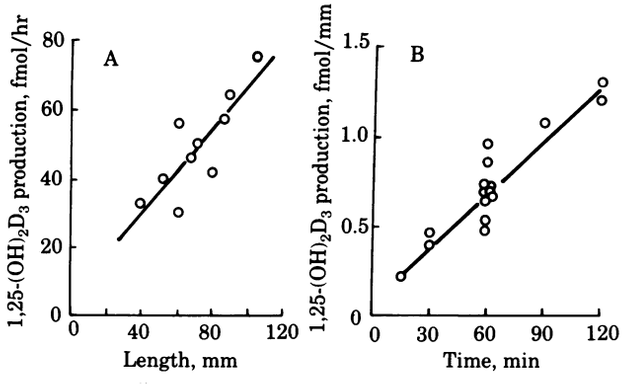

FIG. 3. (*A*) Relationship between length of PCT (vitamin D-deficient rats) and 1α,25-$(OH)_2D_3$ produced (r = 0.82). (*B*) Relationship between duration of incubation and 1α,25-$(OH)_2D_3$ produced by PCT (r = 0.92). Each point represents one incubation.

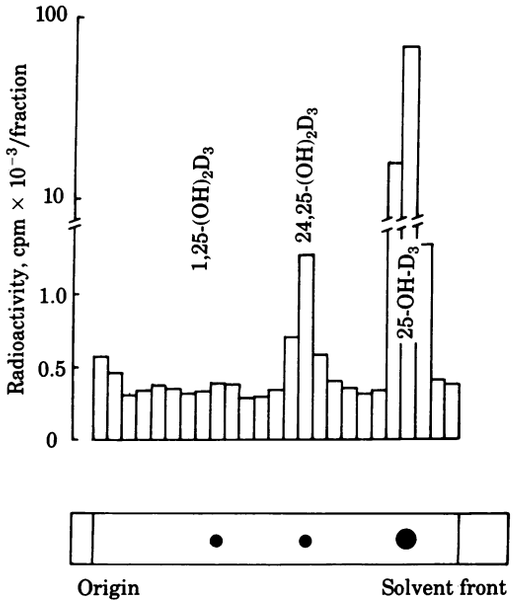

FIG. 4. Thin-layer chromatography profile of the extract of PCT of a normal rat. Radioactive peaks (0.5-cm fractions) correspond to authentic 25-OH-D_3 and 24,25-$(OH)_2D_3$ as shown at the bottom. No radioactivity was seen at the 1α,25-$(OH)_2D_3$ position.

markedly to 0.11 ± 0.05 fmol/mm/hr at 24 hr after parathyroidectomy.

Distribution of 24-Hydroxylase. PCT from a normal rat metabolized 25-OH-$[^3H]D_3$ primarily to a polar peak, that comigrated with authentic 24,25-$(OH)_2D_3$ (Fig. 4). The radioactive peaks corresponding to 24,25-$(OH)_2D_3$ on thin-layer chromatography were collected and subjected to high-performance liquid chromatography. The radioactivity again appeared in the same position as authentic 24,25-$(OH)_2D_3$ (Fig. 5); it completely disappeared after periodate treatment. Significant production of 24,25-$(OH)_2D_3$ occurred in the PCT of normal rats (Table 1). The results obtained in Holtzman and Wistar rats were not different, and the data have been combined. No significant 24,25-$(OH)_2[^3H]D_3$ production could be detected in either the remaining nephron segments or in the incubations of the heat-denatured PCT of normal rats.

It has been shown that 1α,25-$(OH)_2D_3$ can induce 24-hy-

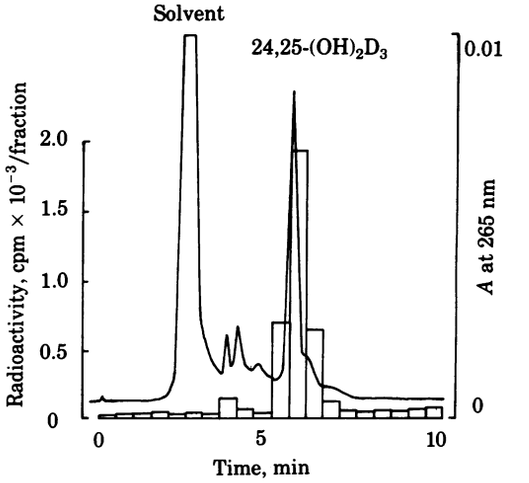

FIG. 5. High-performance liquid chromatography profile of peaks corresponding to 24,25-$(OH)_2D_3$ in Fig. 4. Solid line, absorbance at 265 nm; bars, radioactivity in each 0.75-ml fraction.

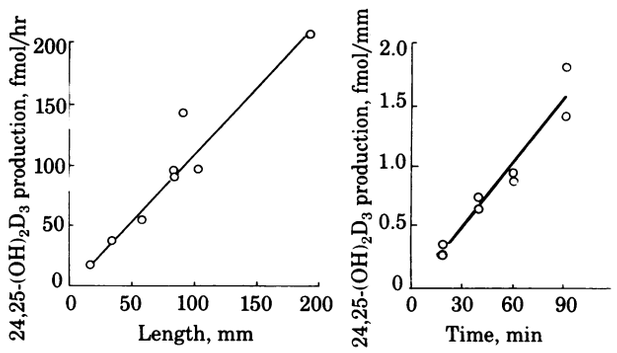

FIG. 6. (*A*) Relationship between production of 24,25-$(OH)_2[^3H]D_3$ and length of PST in normal rats given 1α,25-$(OH)_2D_3$ ($r = 0.84$). (*B*) Relationship between incubation period and rate of 24,25-$(OH)_2D_3$ production by PST ($r = 0.90$). Each point represents one incubation.

droxylase in the kidney (1). Therefore, the effect of *in vivo* administration of 1α,25-$(OH)_2D_3$ on 24-hydroxylase activity was examined in the PCT and other nephron segments from normal rats. The rates of 24,25-$(OH)_2D_3$ production in the PCT increased significantly, from 0.23 ± 0.05 to 0.64 ± 0.06 fmol/mm/hr ($P < 0.01$) (Table 1). Of interest was the finding that there was a marked increase in the rate of production of 24,25-$(OH)_2[^3H]D_3$ in the PST, from undetectable in normal rats to 1.07 ± 0.21 fmol/mm/hr in rats treated with 1α,25-$(OH)_2D_3$; this value is higher than that in the PCT ($P < 0.01$). No 24,25-$(OH)_2[^3H]D_3$ production was detected in the other nephron segments tested. The rate of 24,25-$(OH)_2D_3$ production was linear with both the length of tubules per incubation and the incubation period (Fig. 6).

Distribution of Parathyroid Hormone-Sensitive Adenylate Cyclase. Parathyroid hormone (0.5–1.0 unit/ml) markedly stimulated the adenylate cyclase activity of the PCT, CTAL, and DT in normal rats; hormone-sensitive enzyme activity was less in the PST (Table 2). The near-maximal to maximal stimulation of adenylate cyclase activity was obtained at the concentrations of parathyroid hormone used in the present studies.

DISCUSSION

Incubation of defined tubule segments microdissected from vitamin D-deficient and normal rats has made possible the demonstration of the precise locations of 25-OH-D_3 1α- and 24-hydroxylase activities along the mature mammalian nephron. As shown in Table 1, significant 1α-hydroxylase activity was localized exclusively in the PCT of vitamin D-deficient rats; none was found in other nephron segments, including the PST. The results are similar to those of Brunette *et al.* (4) who demonstrated that 1α-hydroxylase is located exclusively in the PCT and cortical thick loops in rachitic chicks. A recent study by Akiba *et al.* (5) showed, however, that both PCT and PST are the sites of 1α-hydroxylation in the fetal rabbit kidney, with the activity higher in PST than in PCT. The lack of enzyme activity in the PST of the young rats in the present study may be due to several factors, including species difference and the ontogeny of the enzyme activity. It is possible that the occurrence of much lower activities of parathyroid hormone-sensitive adenylate cyclase in the PST of the rat kidney compared to those in the rabbit PST (10) may account for the absence of significant 1α-hydroxylase activity in PST of the rat because parathyroid hormone plays a major role in stimulating 1α-hydroxylase activity (see below). It is also possible that the distribution of 1α-hydroxylase may change during the development of animals and a greater demand for 1α,25-$(OH)_2D_3$ may be met under certain circumstances, such as *in utero*, by having a larger portion of the tubule (i.e., PCT plus PST) capable of producing 1α,25-$(OH)_2D_3$.

Table 2. Parathyroid hormone-sensitive adenylate cyclase activity along the rat nephron

Nephron segment	Adenylate cyclase activity, fmol cyclic AMP produced/mm/30 min	
	Control	With hormone
PCT	6.5 ± 1.1	309.2 ± 30.5
PST	12.2 ± 2.0	43.7 ± 4.3
MTAL	46.9 ± 1.5	33.0 ± 2.3
CTAL	40.9 ± 19.3	485.9 ± 49.4
DT	11.4 ± 2.2	370.8 ± 51.6

Values are the mean ± SEM of three to nine incubations. Parathyroid hormone concentration was 1 unit/ml except for DT it was 0.5 unit/ml. The presence of hormone sensitive adenylate cyclase has been shown in the glomerulus (12). The effect of hormone on enzyme activity was not tested in CT, the segment devoid of parathyroid hormone-sensitive adenylate cyclase (10, 13).

It has been shown (14, 15) that parathyroid hormone is a potent stimulator of 1α-hydroxylase and that cyclic AMP functions as the cellular mediator of the hormone's action on this enzyme. The present observation that parathyroidectomy caused an 84% reduction in 1α-hydroxylase activity in PCT of vitamin D-deficient rats is in accord with the earlier *in vivo* observations and with the thesis that secondary hyperparathyroidism causes a maximal stimulation of 1α-hydroxylase activity in vitamin D deficiency (15). Further, the presence of parathyroid hormone-sensitive adenylate cyclase together with 1α-hydroxylase in PCT is consistent with the role of cyclic AMP as a mediator of this hormone's action. The role of parathyroid hormone sensitive adenylate cyclase in distal portions of the nephron is not clear; it may be involved in the regulation of the tubular transport of calcium and other ions (11).

Significant production of 24,25-$(OH)_2D_3$ occurred only in the PCT of normal rats. In rats treated with 1α, 25-$(OH)_2D_3$, the 24-hydroxylase activity increased markedly in the PCT. Furthermore, with 1α,25-$(OH)_2D_3$ treatment, significant 24-hydroxylase activity appeared in the PST, and the enzyme activity in the PST exceeded that in the PCT. The functional properties of both 1α-hydroxylase and 24-hydroxylase are quite similar, and one might suspect that they represent a single enzyme system with different catalytic capabilities. The present results clearly demonstrate that the localization of the two enzyme activities along the nephron can be different, an observation which may suggest that they represent two different systems. The levels of major regulators of 1α-hydroxylase activity—e.g., parathyroid hormone, calcium, phosphorus, and 1α,25-$(OH)_2D_3$—favor the maximal stimulation of 1α-hydroxylation in vitamin D deficiency. Despite this, there was no activity of the 1α-hydroxylase in the PST where 24-hydroxylase could be detected. It is possible, however, that some other factor may be capable of inducing 1α-hydroxylase in the PST of the mature mammalian kidney.

In contrast to the case in avian kidney, *in vitro* demonstration of 1α-hydroxylase activity in the mammalian kidney has been difficult because of the presence of potent inhibitors of the enzyme (16). Recently, several investigators were able to determine the 1α-hydroxylase activity of the mammalian kidney *in vitro* by using the mitochondrial fraction, homogenates, isolated kidney cells, and isolated perfused kidney (17–20). In contrast to these previous methods, a use of microdissected, well-defined nephron segments to measure 1α-hydroxylase and 24-

hydroxylase activities, as shown in this communication, has a definite advantage for defining the exact localization and the mechanisms of the physiological regulation of the enzyme system(s) in the mammalian kidney.

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