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In vitro metabolism of 25-hydroxyvitamin D_3 by isolated rat kidney cells

(1,25-dihydroxyvitamin D_3/24,25-dihydroxyvitamin D_3/vitamin D deficiency/calcium deficiency)

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ABSTRACT **Cells were dispersed from rat kidney after enzymatic digestion of the extracellular matrix. When the cells were suspended in a serum-free medium and incubated with ^{3}H-labeled 25-hydroxyvitamin D_3 (25-OH-D_3) several polar metabolites, including 1,25-$(OH)_2$[^{3}H]D_3 and 24,25-$(OH)_2$[^{3}H]D_3 were produced. The specific activities of the 25-OH-D_3:1- and 24-hydroxylases in isolated rat kidney cells were 10–100 times greater than in avian kidney homogenates. The rates of production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were linear over a wide range in cell densities (0.65–5.0 × 10^6 cells per ml) and substrate concentrations (3.5–70 nM). The rate of production of 24,25-$(OH)_2$[^{3}H]D_3 from 25-OH-[^{3}H]D_3 by cells isolated from rats fed control diet was linear with time for up to 30 min, while the synthesis of 1,25-$(OH)_2$[^{3}H]D_3 was linear for over 90 min. The specific activity of the 25-OH-D_3:1-hydroxylase was increased in kidney cells from vitamin D-deficient rats (11.5 fmol/min per 10^6 cells) as well as calcium-deficient rats (8.1 fmol/min per 10^6 cells) when compared to cells from rats fed the control diet (2.0 fmol/min per 10^6 cells). Also, the specific activity of the 25-OH-D_3:24-hydroxylase was reduced in cells from the vitamin D-deficient rats (<0.2 fmol/min per 10^6 cells) and calcium-deficient rats (5.1 fmol/min per 10^6 cells) compared to the controls (15.2 fmol/min per 10^6 cells). On the basis of these results, as well as previous *in vivo* studies, we conclude that the metabolism of 25-OH-D_3 by freshly isolated rat kidney cells reflects the *in vivo* activities of the renal vitamin D-metabolizing enzymes and may prove useful as an assay.**

Vitamin D functions as a prohormone. Subsequent to its synthesis in dermal cells by photolysis of 7-dehydrocholesterol, vitamin D undergoes a series of hydroxylations, the first occurring at carbon 25 to produce 25-OH-D_3. 25-OH-D_3 is further hydroxylated in the kidney to more polar metabolites, including 1,25-$(OH)_2D_3$, the most potent known vitamin D metabolite in enhancing intestinal mineral transport and in mobilizing bone mineral, and 24,25-$(OH)_2D_3$, a major vitamin D metabolite whose physiological significance is poorly understood (1–3).

Some progress has been made toward understanding the control of 25-OH-D_3 metabolism by *in vivo* studies (reviewed in ref. 2). However, because there are many variables involved in whole animal studies, an *in vitro* model would prove useful in delineating the mechanisms of regulation of 1,25-$(OH)_2D_3$ synthesis in mammals. Previous attempts to develop an assay for rat kidney 25-OH-D_3-metabolizing enzymes have been unsuccessful (4–6). In this report we describe a method for isolation and short-term incubation of intact rat kidney cells that allows *in vitro* expression of the 25-OH-D_3:1- and 24-hydroxylases. We also provide evidence that the metabolism of 25-OH-D_3 by freshly isolated rat kidney cells reflects the *in vivo* activities of the vitamin D-metabolizing enzymes.

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MATERIALS AND METHODS

Weanling male Sprague–Dawley rats were individually housed in suspended wire cages and fed a semisynthetic diet (7). Untreated rats were fed our control diet containing 0.6% calcium, 0.6% phosphorus, and 2 units of vitamin D_3 per g of diet. The calcium-deficient (−Ca) rats received the control diet, except that calcium carbonate was omitted. The −Ca diet contained by calcium analysis 0.01% Ca. The vitamin D-deficient (−D) animals were housed in the dark and received the control diet, except that vitamin D was omitted.

The animals were maintained on their respective diets for 4 wk and then sacrificed. In one experiment the rats were fed the −Ca diet for 4 wk followed by the control diet for 1 wk. The rats were anesthetized with ether and the kidneys were removed and placed in sterile buffer (137 mM NaCl/4 mM KCl/0.4 mM NaH_2PO_4/9.4 mM $NaHCO_3$/11 mM glucose; pH 7.4) (8). The kidneys were rinsed several times and minced to about 1-mm pieces. Fragments were placed in 25-ml erlenmeyer flasks with the above buffer (5 ml) containing 50 mg of collagenase (EC 3.4.4.19, Worthington) and 30 mg of hyaluronidase (EC 3.2.1.35, Sigma type 1), and incubated at 37°C for 15 min in a water bath shaker. Five milliliters of a 5% trypsin/0.2% EDTA (GIBCO) solution was added, and the suspension was shaken for an additional 5 min. Vigorous pipetting was also used to aid in the cell dispersion. Finally, the cell suspension was washed with Hanks' balanced salt solution at 4°C, centrifuged (120 × *g*, 12 min, 4°C), and then resuspended. This washing procedure was done three times. Between each washing large tissue fragments were allowed to settle and were discarded. The cells were counted with a hemacytometer, using trypan blue exclusion as a test for viability. Pairs of kidneys yielded an average of 1 × 10^8 cells; erythrocytes were not included in the count.

The isolated kidney cells were incubated in silicone-treated 25-ml erlenmeyer flasks in 5.0 ml of McCoy's 5a medium (9) (GIBCO); usually at a density of 3–4 × 10^6 cells per ml, media contained 25-OH-[^{3}H]D_3 (25-hydroxy-[26(27)-*methyl*-^{3}H]-cholecalciferol; 11.3 Ci/mmol; Amersham; 1 Ci = 3.7 × 10^{10} becquerels) at a concentration of 26 nM. The cell suspension was gassed with 5% CO_2 in air. The flasks were stoppered tightly and incubated for 15 min in a shaker bath at 37°C. After incubation the medium and the cells were removed for subsequent extraction of the radioactive vitamin D metabolites with dichloromethane (10).

Dichloromethane extracts of the cultured cells and medium were evaporated to dryness under a gentle stream of N_2, resuspended in a small volume of hexane/chloroform/methanol

Abbreviations: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; −Ca, calcium-deficient; −D, vitamin D-deficient.

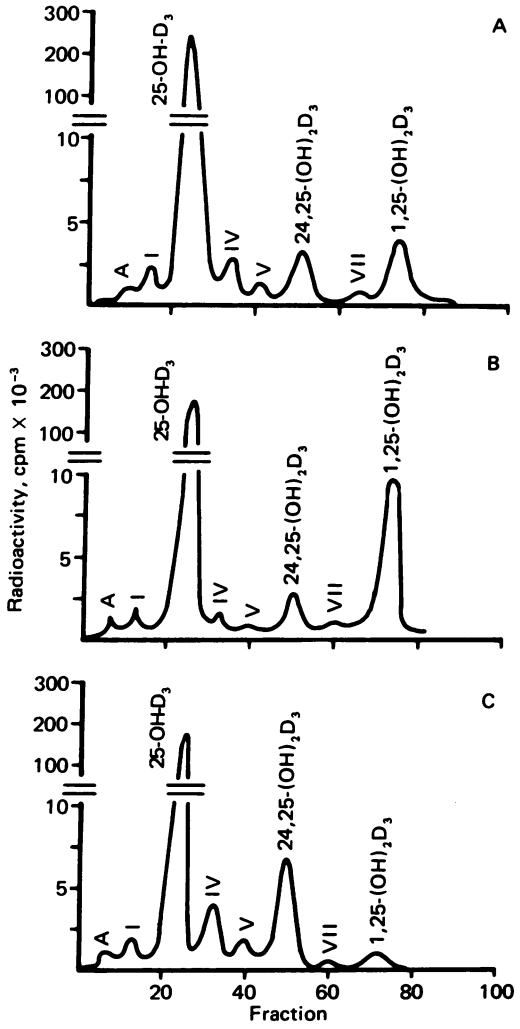

FIG. 1. Separation of metabolites of 25-OH-[^{3}H]D_3 by Sephadex LH-20 column chromatography. Freshly isolated rat kidney cells (3–4 × 10^6 cells per ml of incubation medium) were incubated for 15 min with 25-OH-[^{3}H]D_3 (26 nM) and extracted with dichloromethane. The extracted metabolites (4.5 × 10^5 cpm) were separated on Sephadex LH-20 by elution with hexane/chloroform/methanol (9:1:1). (*A*) Kidney cells pooled from two Ca-deficient rats. (*B*) Kidney cells pooled from two vitamin D-deficient rats. (*C*) Kidney cells pooled from two rats fed a control diet. Roman numerals designate uncharacterized radioactive peaks. A, void volume for the column.

(9:1:1, vol/vol), and chromatographed on Sephadex LH-20 columns (Pharmacia) (1 × 56 cm, 15 g of LH-20) previously equilibrated with the same solvent system. Three-milliliter fractions were collected for determination of the radioactivity (10). About 80–95% of the radioactivity added to kidney cells was recovered after separation of vitamin D metabolites by LH-20 column chromatography.

Polar metabolites of 25-OH-[^{3}H]D_3 were identified by comparing their elution volumes with those of authentic standards on Sephadex columns and by periodate (aqueous) cleavage of the products (10). The peak identified as 1,25-$(OH)_2D_3$ lost 5% of the radioactivity after treatment with periodate, whereas the peak identified as 24,25-$(OH)_2D_3$ lost more than 95% of the radioactivity.

Fractions that migrated with synthetic 1,25-$(OH)_2D_3$ during Sephadex LH-20 column chromatography were pooled, dried under N_2, and redissolved in hexane/isopropanol (90:10, vol/vol). These pooled fractions were chromatographed with synthetic 1,25-$(OH)_2D_3$ (donated by M. Uskokovic, Hoffmann–La Roche, Nutley, NJ) on a high-pressure liquid chromatography system (Laboratory Data Control) using a Zorbax-Sil (4.6 mm × 25 cm) column and a solvent system of hexane/isopropanol (90:10) at a flow rate of 1 ml/min. The radioactivity in the 1,25-$(OH)_2D_3$ region after Sephadex chromatography was found to migrate with the authentic 1,25-$(OH)_2D_3$ by high-pressure liquid chromatography.

RESULTS

Freshly isolated rat kidney cells metabolized 25-OH-D_3 to more polar compounds, including 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ (Fig. 1). The *in vitro* incubation conditions were optimized for cell concentration (Fig. 2), incubation time (Fig. 3) and substrate concentration (Fig. 4). The net production of the dihydroxyvitamin D metabolites was linear over wide ranges in cell density (from 0.65 × 10^6 to 5.0 × 10^6 cells per ml of incubation medium) and substrate concentration (from 3.5 to 70.0 nM). The apparent K_m values for the 25-OH-D_3:1- and 24-hydroxylases were similar to those previously reported for those enzymes in isolated avian mitochondria (approximately 0.1 μM) (11). These results suggested that the intact plasma membrane in isolated rat kidney cells did not prevent diffusion of the substrate to the site(s) of 1- and 24-hydroxylations.

The net production of 24,25-$(OH)_2D_3$ by kidney cells from rats fed the control diet was linear for approximately 30 min while the production rate for 1,25-$(OH)_2D_3$ was linear for at least 90 min, at which time 26% of the initial substrate had been metabolized to more polar compounds. To minimize substrate depletion and possible regulation of enzyme activity by product inhibition our standard incubation conditions were chosen to be as follows: 3–4 × 10^6 cells per ml incubated with 26 nM 25-OH-[^{3}H]D_3 for 15 min. Although it was expected that the activities of the 25-OH-D_3:1- and 24-hydroxylases in rat kidney cells would eventually have become altered due to culture conditions, previous results with quail kidney cells indicated that such modulations required incubation for much longer intervals (2–6 hr) than in the assay used in this study (12).

As expected, rats that had been fed diets deficient in either calcium or vitamin D became hypocalcemic (Table 1). Fur-

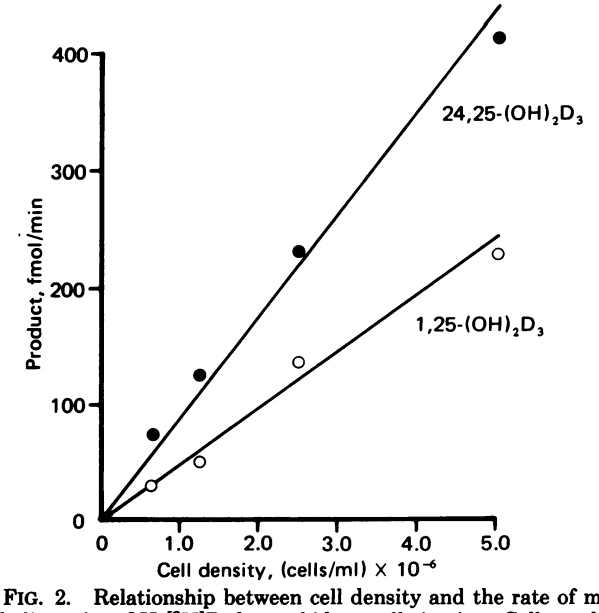

FIG. 2. Relationship between cell density and the rate of metabolism of 25-OH-[^{3}H]D_3 by rat kidney cells *in vitro*. Cells pooled from the kidneys of two rats fed a −Ca diet for 4 wk followed by the control diet for 1 wk were incubated for 15 min with 25-OH-[^{3}H]D_3 (26 nM) and the products were separated by Sephadex LH-20 column chromatography as in Fig. 1.

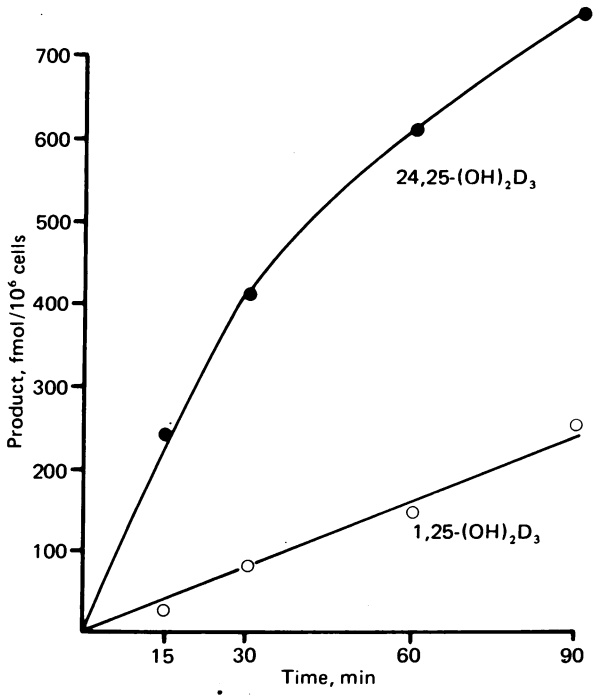

FIG. 3. Effect of incubation time on the metabolism of 25-OH-[^{3}H]D_3 by rat kidney cells. 25-OH-[^{3}H]D_3 (26 nM) was incubated with cells isolated from kidneys pooled from two rats fed control diets (3.7 × 10^6 cells per ml of incubation medium) and the products were separated by Sephadex LH-20 column chromatography as in Fig. 1.

thermore, the specific activities of the 25-OH-D_3:1- and 24-hydroxylases measured in freshly isolated kidney cells depended upon the calcium and vitamin D status of the rats former diet (Fig. 1 and Table 1). The specific activity of the 1-hydroxylase in kidney cells isolated from rats fed a −D diet was 575% of that in cells from animals fed the control diet. Similarly, enzyme activity in cells from animals fed a −Ca diet was elevated to 400% of the control value. In contrast, the specific activity of the 24-hydroxylase was reduced to less than 1% of the control value in cells isolated from rats fed the −D diet and to 34% in cells from animals fed the −Ca diet. When calcium-depleted rats were fed the control diet for 1 wk, both serum calcium and the specific activity of the renal 24-hydroxylase increased to

Table 1. Effect of diet on serum calcium and hydroxyvitamin D_3 hydroxylases in rat kidney cells

Diet	*n*	Hydroxylase specific activity,* fmol/min per 10^6 cells: 1-Hydroxylase	24-Hydroxylase	Serum calcium,† mg/dl
Control‡	3	2.0 ± 0.2	15.2 ± 0.9	10.9 ± 0.3
−D‡	3	11.5 ± 1.1	<0.2	6.9 ± 0.8
−Ca‡	6	8.1 ± 0.5	5.1 ± 0.8	6.0 ± 0.7
−Ca/control§	5	9.2 ± 0.8	18.9 ± 1.0	10.7 ± 0.5

* Mean ± SD.
† Serum calcium was determined at sacrifice by atomic absorption spectrometry (13).
‡ Rats were fed the indicated diet for 4 wk.
§ Rats were fed the −Ca diet for 4 wk followed by the control diet for 1 wk.

a near control level but the activity of the 1-hydroxylase remained elevated (460% of the control value) (Table 1). Previous studies had shown that, after 1 wk of calcium repletion, osteoclast number in the tibia diaphysis and serum parathyroid hormone were decreased to below the control level, whereas gut fractional calcium absorption remained elevated (14). If this preliminary result [continued elevated 1,25-$(OH)_2D_3$ production] were verified it would suggest that a factor other than parathyroid hormone regulates the renal 25-OH-D_3:1-hydroxylase during calcium repletion.

Freshly isolated rat kidney cells metabolized 25-OH-D_3 to several products whose structures have not yet been determined (Fig. 1). Radioactive peaks (I, IV, V, and VII) eluted from Sephadex LH-20 columns after incubation of rat kidney cells with 25-OH-[^{3}H]D_3 comigrated with the metabolites produced by Japanese quail kidney homogenates (10) and by cultured Japanese quail kidney cells (12). Peak IV has also been identified in rat plasma (15). Peak VII has migration properties on Sephadex LH-20 similar to those of 25,26-$(OH)_2D_3$ and may represent that metabolite (16).

25-OH-[^{3}H]D_3 was stable in McCoy's 5a medium at 37°C for periods of at least 2 hr. In the absence of rat kidney cells 25-OH-[^{3}H]D_3 was not altered. Kinetic studies have shown that peaks I, IV, V, and VII were produced from 25-OH-D_3 but not from 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ (unpublished results). Furthermore, cells with elevated 24-hydroxylase activity (isolated from rats fed the control diet) did not metabolize added

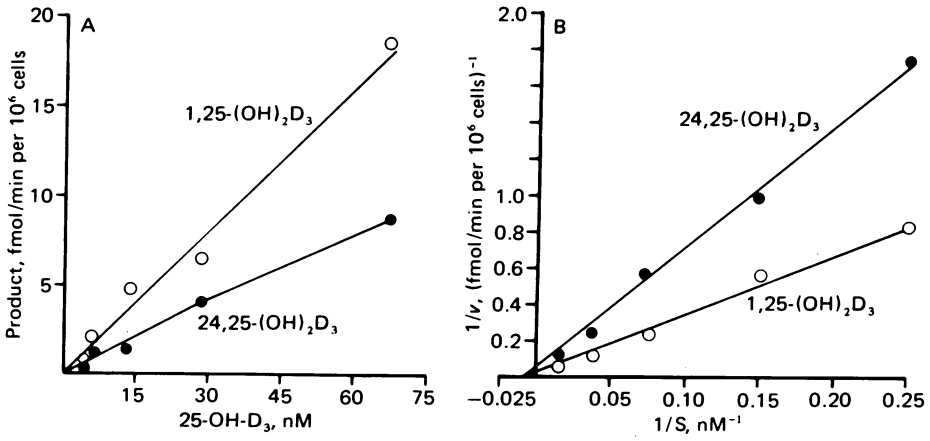

FIG. 4. (*A*) Effect of substrate concentration on the *in vitro* metabolism of 25-OH-[^{3}H]D_3 by rat kidney cells. Cells (4 × 10^6 cells per ml of incubation medium) isolated from kidneys pooled from two rats fed a −Ca diet for 4 wk were incubated with 25-OH-[^{3}H]D_3 for 15 min and the metabolites were separated as in Fig. 1. (*B*) Lineweaver–Burk plot of the data in *A*. The apparent K_m values for the 25-OH-D_3:1- and 24-hydroxylases were both approximately 0.1 μM. The apparent V_{max} for the 1-hydroxylase was 33 fmol/min per 10^6 cells and that for the 24-hydroxylase was 17 fmol/min per 10^6 cells.

24,25-$(OH)_2[^3H]D_3$ to further products. Similarly, cells isolated from rats fed the −D diet and having elevated 1-hydroxylase activity did not metabolize added 1,25-$(OH)_2[^3H]D_3$. These results suggested that the net production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ measured *in vitro* was a true measure of the rates of production.

DISCUSSION

The kidney is the principal (if not exclusive) location for the biosynthesis of 1,25-$(OH)_2D_3$. The evidence establishing a renal site for the 1-hydroxylation reaction is as follows: (*i*) nephrectomy prevents the synthesis of 1,25-$(OH)_2D_3$, and (*ii*) anephric patients have no detectable 1,25-$(OH)_2D_3$ in their plasma (3, 17). Furthermore, the renal 25-OH-D_3:1-hydroxylase is easily assayed *in vitro* in homogenates of either chicken or Japanese quail kidney (10, 18, 19). In chickens the enzyme has been further localized to the mitochondria of the proximal renal tubules (19, 20).

In contrast to avian kidney, demonstration of rat renal 1-hydroxylase activity *in vitro* has proven elusive. Rat serum has been found to contain a potent inhibitor that prevents expression of the 25-OH-D_3:1-hydroxylase in chicken kidney mitochondria (4–6). Contamination by this inhibitor is thought to be responsible for the absence of 1-hydroxylase activity when the enzyme is assayed in rat kidney homogenates (4–6). Precipitation of vitamin D-binding protein from serum with a specific antibody to the protein was only partially successful in removing inhibitor activity (6).

Inability to assay 25-OH-D_3:1-hydroxylase in rat kidney has proven to be a major handicap in elucidating the mechanism regulating the renal production of 1,25-$(OH)_2D_3$ in mammals. In this study we have investigated the possibility that intact cells isolated from rat kidney maintain their capacity to metabolize vitamin D when assayed *in vitro*. Indeed, we have found that isolated kidney cells hydroxylated 25-OH-$[^3H]D_3$ more efficiently than quail kidney homogenates (12).

Although monkey kidney cells have been reported to 24-hydroxylate 25-OH-D_3 in culture, no 25-OH-D_3:1-hydroxylase activity was detected (21). We have been unable to detect 1-hydroxylase activity in rat kidney cells incubated in media containing 10% fetal calf serum (unpublished results). This result suggests that the use of serum as a growth promoter in previous studies may have been at least partially responsible for the apparent absence of 25-OH-D_3:1-hydroxylase in cultured mammalian kidney cells (21). In the absence of serum, however, isolated kidney cells 1-hydroxylated up to 26% of the substrate—25-OH-$[^3H]D_3$—in 90 min.

Diet has a major influence on the subsequent metabolism of 25-OH-D_3 assayed *in vitro*: compared to kidney cells isolated from rats fed the control diet, the specific activity of the 1-hydroxylase was elevated and the 24-hydroxylase was reduced in cells from animals fed −Ca and −D diets. The *in vitro* production of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ was altered with diet in the same direction as the circulating levels of the metabolites in intact rats (22, 23). The *in vitro* results were also in accord with the *in vivo* rates of renal production of the dihydroxyvitamin D metabolites estimated from the appearance of radioactive vitamin D metabolites in plasma after intraperitoneal administration of 25-OH-$[^3H]D_3$ (14, 24). Together, these findings provide convincing evidence that the metabolism of 25-OH-$[^3H]D_3$ by freshly isolated rat kidney cells reflects the *in vivo* activities of the renal vitamin D-metabolizing enzymes.

We optimized our *in vitro* incubation conditions for cell density, substrate concentration, and incubation time to ensure that these parameters did not spuriously alter the interpretation of the results obtained on the metabolism of 25-OH-$[^3H]D_3$ by isolated rat kidney cells. This was particularly pertinent in regards to incubation time; previous studies have shown that vitamin D metabolism can become altered *in vitro* due to the presence of 25-OH-D_3 in the incubation medium (12). We did not test the effect of various medium calcium and phosphorus concentrations on vitamin D metabolism *in vitro*. Plasma calcium and phosphorus are potent regulators of vitamin D metabolism *in vivo*. However, in a previous study, medium calcium was varied from 1.8 to 3.1 mM and medium phosphorus from 0.8 to 1.9 mM without altering 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ production by Japanese quail kidney cells after 4 days in culture (unpublished results).

In summary, freshly isolated cells from rat kidney are capable of expressing 25-OH-D_3:1- and 24-hydroxylase activities when assayed in a serum-free medium. This *in vitro* mammalian model appears to reflect *in vivo* vitamin D metabolism and should be useful in studies of the regulation of 1,25-$(OH)_2D_3$ synthesis.

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