# Regulation of the Metabolism of 25-Hydroxyvitamin $D_3$ in Primary Cultures of Chick Kidney Cells

U. Trechsel, J-P. Bonjour, and H. Fleisch with the technical assistance of
A. Monod and J. Portenier, *Department of Pathophysiology, University of Berne,*
*Murtenstrasse 35, 3010 Berne, Switzerland*

ABSTRACT  A primary chick kidney cell culture is described, capable of forming 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], 24,25-dihydroxyvitamin $D_3$ [24,25-$(OH)_2D_3$], and 1,24,25-trihydroxyvitamin $D_3$ [1,24,25-$(OH)_3D_3$] over several days. The apparent $K_m$ values were 0.125 $\mu M$ for the 1-hydroxylase and 2.1 $\mu M$ for the 24-hydroxylase. Exogenous 1,25$(OH)_2D_3$ decreased 1-hydroxylase and increased 24-hydroxylase within 4 h. 24,25$(OH)_2D_3$ produced similar effects, but only in the absence of fetal calf serum. R and S isomers of 1,24,25$(OH)_3D_3$ were about five times less active than 1,25$(OH)_2D_3$. Bovine parathyroid hormone stimulated the 1- and reduced the 24-hydoxylase in 6 h, but this only occurred in cultures either previously treated with 1,25$(OH)_2D_3$ and EGTA to lower Ca to 0.8 mM or in cultures grown in the presence of 25-hydroxyvitamin $D_3$ (25$(OH)D_3$). Under the latter condition, the sensitivity to bovine parathyroid hormone was enhanced, 0.04 U/ml producing a maximum response. Synthetic aminoterminal tetratriacontapeptide (1-34) human parathyroid hormone was equally effective. In the absence of D metabolites, estradiol for 6 h produced a dose-dependent inhibition of the 1-hydroxylase, but no change in the 24-hydroxylase. Progesterone, testosterone, and corticosterone had no significant effect. In cultures grown in the presence of 25$(OH)D_3$ no reproducible effects were obtained with either 1 $\mu M$ estradiol or 1 $\mu M$ testosterone, alone or in combination, but 5 $\mu M$ corticosterone decreased the 1- and increased the 24-hydroxylase. Changes in Ca and P concentrations of the medium as well as addition of ethane-1-hydroxy-1,1-diphosphate for 48 h did not affect any of the hydroxylase activities. The modulation of the hydroxylase activities by vitamin $D_3$ metabolites and parathyroid hormone suggests that these factors regulate the renal hydroxylase by direct actions, whereas it would appear that ethane-1-hydroxy-1,1-diphosphate, Ca, P, and steroids may exert their influence indirectly.

## INTRODUCTION

It is now well established that the physiological functions of vitamin $D_3$ are exerted by hydroxylated metabolites rather than by the parent vitamin itself (1–4). Vitamin $D_3$ is hydroxylated to 25-hydroxyvitamin $D_3$ [25$(OH)D_3$][1] in the liver. The kidney is the site of further hydroxylation, to 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], to 24,25-dihydroxyvitamin $D_3$ [24,25-$(OH)_2D_3$], or to 1,24,25-trihydroxyvitamin $D_3$ [1,24,25$(OH)_3D_3$]. 1,25$(OH)_2D_3$ is the most potent known metabolite in stimulating intestinal calcium absorption (5, 6) and bone resorption (7, 8) and must be regarded as a hormone that plays a central role in calcium and phosphate homeostasis. The physiological importance of the other metabolites is unknown. The production of 1,25$(OH)_2D_3$ is regulated in reference to the mineral requirements of the organism. Several factors such as parathyroid hormone (PTH), plasma calcium and phosphate, sex steroids, prolactin, and 1,25$(OH)_2D_3$ itself have been reported to influence the renal 25$(OH)D_3$-1- and 25$(OH)D_3$-24-hydroxylases. The experimental evidence concerning the regulation of the metabolism of vitamin $D_3$ has been obtained through studies in vivo as well as through in vitro techniques with kidney homogenates, isolated mito-

chondria, or isolated kidney tubules. However, the experiments performed in vivo have the disadvantage that direct or indirect effects cannot be distinguished, and the available in vitro techniques are too short-lived to permit the study of the long-term regulation mechanisms likely to be involved in the metabolism of vitamin D.

We have therefore developed a primary chick cell culture system that can metabolize $25(OH)D_3$ to $1,25(OH)_2D_3$, $24,25(OH)_2D_3$, or $1,24,25(OH)_3D_3$ for several days. In these cultures, we have studied the effects of agents shown to alter vitamin D metabolism in vivo, namely $1,25(OH)_2D_3$ (9–11), PTH (11–14), steroid hormones (15–18), and ethane-1-hydroxy-1,1-diphosphonate (EHDP) (19–21). $24,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ were investigated in view of their potential therapeutic value in the management of metabolic bone disease (22). Finally, the effects of Ca and P concentrations in the culture medium were studied.

## METHODS

10- to 20-d-old Hubbard cockerels raised from hatching on a vitamin D-deficient diet (kindly provided by B. Giboulot, Fabrique de Mélanges Alimentaires Expérimentaux, La Minière, F-78 Versailles, France) were used. Three to four animals were killed by decapitation, skinned, rinsed in isopropanol, and their kidneys flushed with 10 ml of 0.15 M NaCl through a catheter introduced into the aorta. The kidneys were quickly removed and rinsed in ice-cold minimum essential medium with Earle's salt solution (23) containing 227 mg of $NaHCO_3$/liter, 60 $\mu$g of penicillin/ml, 100 $\mu$g of streptomycin/ml, and 0.25 $\mu$g of Fungizone/ml (E. R. Squibb & Sons, Princeton, N. J.). This medium was used for the entire cell preparation procedure. A method similar to that used by Shain (24) was used to produce isolated kidney tubules. The kidneys were minced with scissors and digested for 15 min in medium added with 160 mg/100 ml collagenase and 100 mg/100 ml hyaluronidase, 5 ml/kidney, in a shaking water bath, at 37°C. Tissue dispersion was then aided by gentle pipetting, and the suspension obtained was sieved through a 85-$\mu$m nylon mesh. The volume of the filtrate was adjusted to 25 ml/kidney with ice-cold medium. 10-ml portions of the suspensions were centrifuged in a refrigerated centrifuge at 50 $g$ for 60 s. The pellet containing mainly tubular fragments was washed once with 10 ml of ice-cold medium. The pellet containing the tubular preparation was resuspended in 20 ml of medium without enzymes. Individual cells were obtained by passing the suspension 5–10 times through 20-ml graduated pipette at room temperature. The remaining tubular fragments were recovered by centrifugation as before, the pellet was resuspended and again passed through the pipette. After repeating the procedure two or three times, the combined supernates containing individual cells and small aggregates were centrifuged in 10-ml portions at 350 $g$ for 15 min. All centrifugations were performed at 4°C. The cells were suspended in minimum essential medium with Earle's salt solution, antibiotics, and 10% fetal calf serum, counted under the microscope, and plated in plastic Petri dishes, 3.5 cm in diameter, with 1.5 ml medium. The number of cells plated was chosen in reference to the time at which confluency was

to be reached. With some 1.2 million cells/dish, confluency was obtained after 3–4 d of culture. Cultures were kept in a humidified incubator in an atmosphere of 5% $CO_2$ in air at 37°C. 24 h after plating, the medium containing nonadherent cells was discarded, the plates were washed once with medium, and 1.5 ml of fresh medium with antibiotics and 10% fetal calf serum was added. The calcium concentration in the medium was either 1.8 or 1.2 mM as indicated for every experiment individually.

The predominant aspect of the cells in confluent cultures was epitheloid. About 2 d after reaching confluency, the cultures deteriorated, the ability to metabolize $25(OH)D_3$ disappeared, and the cells no longer adhered to the dish. We have not yet been able to keep the confluent cultures longer than 2 d.

DNA was determined by the method of Burton (25) after treatment of the cells according to Leyva and Kelley (26).

*Assessment of $25(OH)D_3$ metabolism.* The medium was discarded and replaced by 1 ml of minimal essential medium with antibiotics, but without fetal calf serum to avoid binding of [$^3$H]$25(OH)D_3$ to serum protein (27, 28). Except where stated, 100 pmol of purified tritiated $25(OH)D_3$ was then added in 10 $\mu$l of ethanol, and the preparation incubated for 30 min in the cell culture incubator. Gentle shaking considerably enhanced the metabolism of $25(OH)D_3$. The reaction was stopped by adding 1 ml of methanol. The cells were scraped with a rubber policeman, the suspension was transferred to a glass test tube, and the dish was washed with 1 ml of methanol which was added to the test tube, followed by 1 ml of chloroform. The preparations were stored at −20°C at least overnight. The vitamin D metabolites were then extracted in chloroform by a modification of the method of Bligh and Dyer (29). 1 ml of chloroform and 1 ml of water were added to the tubes, resulting in the separation into two phases, which were accelerated by low speed centrifugation. The chloroform phase was transferred to a small test tube and the water-methanol phase washed twice with 2 ml of chloroform. The chloroform extract was evaporated to dryness under a stream of nitrogen and redissolved in 250 $\mu$l of a solvent mixture containing 20% isopropanol in n-hexane. Twice 20 $\mu$l were transferred to scintillation counting vials to determine recovery of tritium label. In a typical experiment with 56 incubations under standard conditions, 93±4% (mean±SD) of the radioactivity added to the cell cultures was recovered.

*High-pressure liquid chromatography (HPLC).* The equipment consisted of a Waters HPLC pump (model 6000 A, Waters Associates, Inc., Milford, Mass.), a Rheodyne sample injector (model 7120, Rheodyne Inc., Berkeley, Calif.), a stainless steel column 0.3 × 50 cm (Portmann Instruments, Therwil, Switzerland) packed in our laboratory with small-particle silica (LiChrosorb S160, 5 $\mu$M mean particle size, Merck, AG, Darmstadt, West Germany), a flow-through spectrophotometer for HPLC (model LC55, Perkin-Elmer Corp., Norwalk, Conn.), and a fraction collector (model 3403B, LKB) which was adapted for collection into scintillation counting vials.

The solvent system used was similar to that described by Jones and DeLuca (30). For all extracts of cultures incubated with 100 pmol of [$^3$H]$25(OH)D_3$ for 30 min, 12.5% isopropanol in n-hexane and a flow rate of 1.4 ml/min were used. The retention times for synthetic $25(OH)D_3$, $24R,25(OH)_2D_3$, and $1,25(OH)_2D_3$ were 3.5, 6, and 13 min, respectively. 33 fractions of 30 s were collected into plastic vials for scintillation counting, and the radioactivity was determined. 91±3% (mean±SD) of the radioactivity applied to the HPLC column was recovered in the chromatograms of 56 extracts of confluent cultures incubated with 100 pmol of [$^3$H]$25(OH)D_3$

for 30 min. The elution of metabolites more polar than 1,25(OH)$_2$D$_3$ was achieved with 20% isopropanol in n-hexane. This system was used for extracts of cultures incubated with [$^3$H]24,25(OH)$_2$D$_3$ or [$^3$H]1,25(OH)$_2$D$_3$.

*Presentation of results.* The production of tritiated metabolites is expressed as the percentage of the radioactivity recovered in HPLC, with the exception of the kinetic data where amounts formed were calculated. The results were not related to cell number, cell protein, or DNA, because these measurements could not be performed in the dishes assayed for hydroxylase activity. However, in certain experiments, the DNA content of separate culture dishes was determined to detect alterations of cell number in different groups, and the values are reported. Values are means±SEM. The significance of the differences between groups was evaluated by a two-sided *t* test.

## Materials

*Tritiated vitamin D metabolites.* [26,27-$^3$H]25(OH)D$_3$ (The Radiochemical Centre, Amersham, U. K., specific activity 7.3 Ci/mmol) was purified with HPLC before use. [$^3$H]24,25-(OH)$_2$D$_3$ and [$^3$H]1,25(OH)$_2$D$_3$ were produced biologically with chick kidney homogenate as described by Boyle et al. (31). For the production of [$^3$H]1,25(OH)$_2$D$_3$, chicks were raised on a vitamin D-deficient diet containing 0.15% Ca and 0.25% P for 4 wk from hatching. Conversion to [$^3$H]24,25-(OH)$_2$D$_3$ was obtained with kidney homogenate from vitamin D-deficient chicks receiving, from hatching, a diet with 1% Ca and 1% P and for the last 7 d a daily supplement of 1 $\mu$g of 1$\alpha$-hydroxyvitamin D$_3$ and 10 mg P/kg of EHDP. The specific activity of [$^3$H]1,25(OH)$_2$D$_3$ and [$^3$H]24,25(OH)$_2$D$_3$ could not be determined. However, as these metabolites were produced with kidney homogenates from vitamin D-deficient chicks, we may assume that it was similar to that of the substrate [$^3$H]25(OH)D$_3$ (7.3 Ci/mmol).

*Nonradioactive vitamin D$_3$ metabolites, steroids, PTH, and EHDP.* Synthetic 25(OH)D$_3$ and the R as well as the S isomer of 24,25(OH)$_2$D$_3$ and 1,24,25(OH)$_3$D$_3$ were generously supplied by F. Hoffmann-La Roche & Co., Basel. The metabolites were added to cultures in 10–20 $\mu$l of ethanol. $\beta$-Estradiol, progesterone, testosterone, and corticosterone (Sigma Chemical Co., St. Louis, Mo.) were added in 10 $\mu$l of ethanol. Bovine PTH (bPTH) (TCA extract, 248 U/mg; Inolex Corp, Glenwood, Ill.) was added to cultures in 10 $\mu$l of 0.15 M NaCl containing 0.01 M HCl, 1.4 mM mercaptoethanol, and 1 mg/ml of bovine serum albumin. Synthetic amino terminal tetratriacontapeptide(1-34) of human PTH [hPTH(1-34)] was a generous gift from Dr. G. Tregear and Dr. M. Rosenblatt of the Massachusetts General Hospital, Boston, Mass. It was added in the same solvent as bPTH. EHDP was kindly provided by the Procter & Gamble Co., Cincinnati, Ohio. It was added to cultures in 15 $\mu$l of distilled water, pH 7.4. Each of the control dishes received its respective vehicle alone.

Eagle's minimum essential medium, antibiotics, and fetal calf serum were from Paisley PA 3 4 EP Renfrewshire, Scotland, U. K. Collagenase (type I) and hyaluronidase (type I) were from Sigma Chemical Co. Organic solvents and all other chemicals were analytic grade.

## RESULTS

*Identification of tritiated material.* The labeled material present in extracts of cultures incubated for 30 min with [$^3$H]25(OH)D$_3$ or [$^3$H]24,25(OH)$_2$D$_3$ comigrated with synthetic 25(OH)D$_3$, 24R,25(OH)$_2$D$_3$, 1,25(OH)$_2$D$_3$,

and 1,24R,25(OH)$_3$D$_3$. No other significant peaks of radioactivity were observed under these conditions. No tritiated material other than [$^3$H]25(OH)D$_3$ was observed if the incubation with [$^3$H]25(OH)D$_3$ were performed with cells that had been killed, either with methanol or by drying.

*Kinetic studies.* The formation of [$^3$H]1,25(OH)$_2$D$_3$ in confluent cultures was linear for at least 1 h. All routine incubations were performed with an incubation time of 30 min.

The relationship between 25(OH)D$_3$ substrate concentration and the velocity of 24,25(OH)$_2$D$_3$ or 1,25-(OH)$_2$D$_3$ formation showed saturation kinetics. Lineweaver-Burk plots presented in Fig. 1A and B yielded apparent $K_m$ values of 2.1 $\mu$M for the 24-hydroxylase and 0.125 $\mu$M for the 1-hydroxylase.
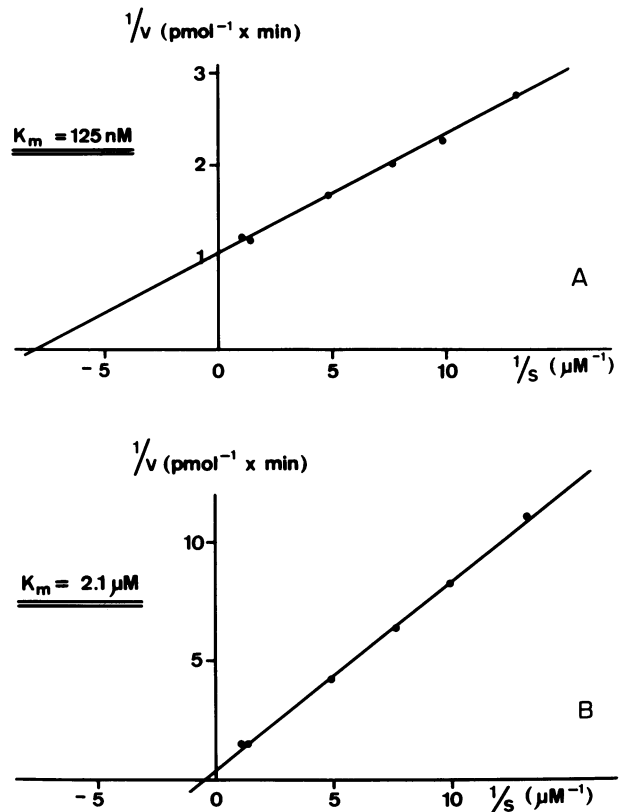


FIGURE 1  Lineweave-Burk plots for the 25(OH)D$_3$-1-hydroxylase (A) and the 25(OH)D$_3$-24-hydroxylase (B). (A) Confluent cell cultures (1.2 mM Ca) were incubated for 30 min with one of six concentrations of [$^3$H]25(OH)D$_3$ ranging from 75 nM to 0.9 $\mu$M, extracted and chromatographed as described in Methods. Percent of radioactivity recovered as [$^3$H]1,25-(OH)$_2$D$_3$ in HPLC was used to calculate the amount of metabolite produced per minute per dish. Every point represents the mean of three incubations. (B) Procedure as in A except that the 24-hydroxylase had previously been induced with 1,25(OH)$_2$D$_3$ (32 pmol/ml). Every point is the mean of three incubations.

TABLE I

**Metabolism of [³H]24,25(OH)₂D₃ or [³H]1,25(OH)₂D₃ to [³H]1,24,25(OH)₃D₃ in Kidney Cell Culture. Comparison with Hydroxylation of [³H]25(OH)D₃**

| Substrate | Induction of 24-hydroxylase with 1,25(OH)₂D₃ | Percent radioactivity in HPLC | | | |
|---|---|---|---|---|---|
| | | 25(OH)D₃ | 24,25(OH)₂D₃ | 1,25(OH)₂D₃ | 1,24,25(OH)₃D₃ |
| [³H]25(OH)D₃ | − (3) | 79.2±0.8 | 0.8±0.2 | 16.8±0.6 | UD |
| [³H]24,25(OH)₂D₃ | − (3) | UD* | 71.1±0.8 | UD | 20.3±0.7 |
| [³H]25(OH)D₃ | + (4) | 84.8±0.6 | 3.3±0.1 | 6.9±0.2 | UD |
| [³H]1,25(OH)₂D₃ | + (4) | UD | UD | 85.7±0.3 | 5.1±0.1 |

32 pmol/ml of 1,25(OH)₂D₃ was added to confluent cultures. 15 h later the medium was replaced by 1 ml of medium without fetal calf serum and 100 pmol/ml of tritiated substrate added. Incubation time was 30 min. Extraction and chromatography were performed as described in Methods. Results are means±SEM. Number of incubations in parentheses.
* Undetectable.

*Formation of 1,24,25(OH)₃D₃.* Table I shows the ability of confluent cultures to metabolize [³H]24,25-(OH)₂D₃ or [³H]1,25(OH)₂D₃ to a labeled material comigrating with synthetic 1,24R,25(OH)₃D₃ on HPLC. Similar conversion rates were obtained with [³H]25-(OH)D₃ as with the dihydroxylated compounds, indicating similar affinitites of the hydroxylases for either substrate. [³H]1,25(OH)₂D₃ was converted to [³H]1,24,25(OH)₃D₃ only in cultures in which the 24-hydroxylase had previously been induced with 1,25(OH)₂D₃. [³H]1,24,25(OH)₃D₃ was also formed when [³H]25(OH)D₃ was used as a substrate. However, during a 6-h incubation of confluent cultures with 500 pmol/ml of [³H]25(OH)D₃ only about 2% of the labeled substrate was recovered as 1,24,25(OH)₃D₃. In our standard incubation conditions, the production of the trihydroxylated material was below the limit of detection.

*Effect of vitamin D₃ metabolites on hydroxylating enzymes.* All vitamin D metabolites tested were able to induce the 24-hydroxylase. The activity of this enzyme was very low in cultures that were not exposed to any D metabolites other than those added with the fetal calf serum. Furthermore, all the D metabolites were capable of reducing the 1-hydroxylase activity. Addition of 25(OH)D₃ for the last 2 d of culture produced the hydroxylase activities of the control dishes of the experiments presented in Tables IV–VI, and in Figs. 5–8. 50 pmol/ml of synthetic 25(OH)D₃ added for 6 h to confluent cultures produced a twofold increase in the 24-hydroxylase and a three-fold decrease of the 1-hydroxylase activity which were both statistically significant ($P < 0.02$), but this only in the absence of fetal calf serum. 1,25(OH)₂D₃ added to confluent cultures produced a time-dependent inhibition of the conversion of [³H]25(OH)D₃ to [³H]1,25(OH)₂D₃ and an increased conversion to [³H]24,25(OH)₂D₃ (Fig. 2). Both effects occurred simultaneously, being statistically significant after 4 h of treatment with

1,25(OH)₂D₃. Similar effects were produced also by 24,25(OH)₂D₃ (epimeric mixture) and by both the R and the S isomers of 1,24,25(OH)₃D₃ (Fig. 3). 1,24,25-(OH)₃D₃ was about five times less potent than 1,25(OH)₂D₃ in stimulating the 24-hydroxylase activity but equally effective in inhibiting the 1-hydroxylase. 24,25(OH)₂D₃, however, influenced the hydroxylases only at a much higher concentration level. Interestingly,
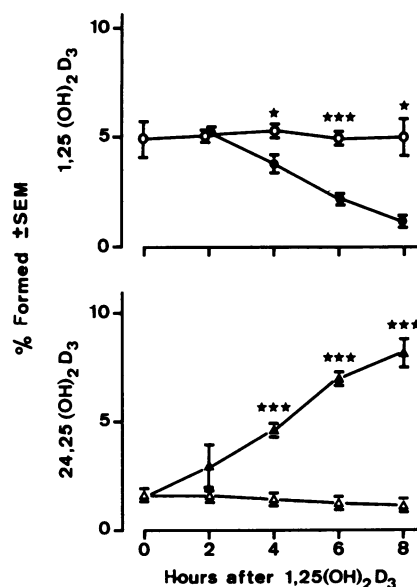


FIGURE 2 Time-course of the effect of 1,25(OH)₂D₃ on metabolism of [³H]25(OH)D₃ in kidney cell culture. Confluent cell cultures (1.8 mM Ca) were preincubated with 24 pmol of synthetic 1,25(OH)₂D₃ (16 pmol/ml) for the times indicated. The medium was then replaced by 1 ml of medium without fetal calf serum and without 1,25(OH)₂D₃, and the cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, n = 3. *, $P < 0.02$; ***, $P < 0.001$ as compared with the value of controls of the same time point; ○△, control; ●▲, 1,25(OH)₂D₃.
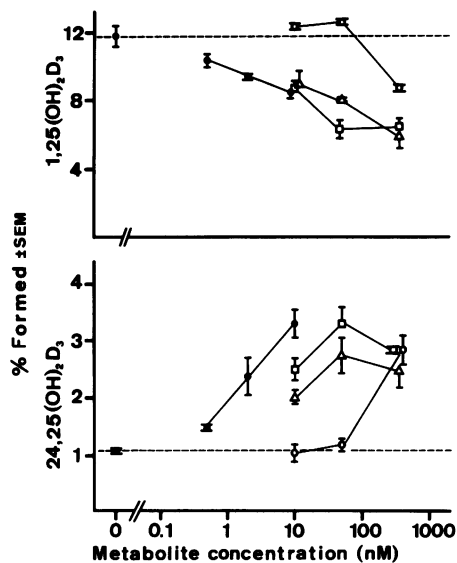
FIGURE 3 Effect of 1,25(OH)$_2$D$_3$ (●), 24,25(OH)$_2$D$_3$ (○) (epimeric mixture), 1,24R,25(OH)$_3$D$_3$ (△), and 1,24S,25(OH)$_3$D$_3$ (□) on metabolism of [$^3$H]25(OH)D$_3$ in kidney cell culture. Upper panel: conversion to [$^3$H]1,25(OH)$_2$D$_3$. Lower panel: conversion to [$^3$H]24,25(OH)$_2$D$_3$. The D metabolites were added to confluent cultures without changing the medium (1.8 mM Ca). 6 h later the medium was replaced by 1 ml medium without fetal calf serum and without unlabeled metabolites, and the cultures were incubated with [$^3$H]25-(OH)D$_3$, extracted, and chromatographed as described in Methods. Results are means±SEM, $n$ = 3. All values were significantly different from control values ($P < 0.05$) for both hydroxylases, with the exception of those with 0.5 pmol/ml of 1,25(OH)$_2$D$_3$, 10 and 50 pmol/ml of 24,25(OH)$_2$D$_3$.



FIGURE 4 Effect of 24R,25(OH)$_2$D$_3$ on metabolism of [$^3$H]25(OH)D$_3$ in kidney cell cultures without fetal calf serum in the medium. The medium of confluent cultures grown in medium (1.2 mM Ca) with 10% fetal calf serum was replaced by 1.5 ml of medium without fetal calf serum but with 24R,25(OH)$_2$D$_3$ added. 6 h later the medium was discarded and 1 ml of fresh medium without fetal calf serum and without 24R,25(OH)$_2$D$_3$ added. The cultures were incubated with [$^3$H]25(OH)D$_3$, extracted, and chromatographed as described in Methods. Results are expressed as percent of radioactivity recovered in HPLC. Values are means±SEM, $n$ = 3.

hydroxylase in cultures in which the 24-hydroxylase had not previously been induced and the 1-hydroxylase decreased with 1,25(OH)$_2$D$_3$. In cultures treated with 1,25(OH)$_2$D$_3$, bPTH produced a small but statistically significant stimulation of the 1-hydroxylase and a nonsignificant decrease of the 24-hydroxylase, but only when the Ca concentration in the medium was lowered to 0.8 mM with EGTA. However, when cells were cultured in the presence of 25(OH)D$_3$ which led to an induction of the 24-hydroxylase and a decrease of the

much lower concentrations of 24R,25(OH)$_2$D$_3$ were effective if fetal calf serum were omitted from the medium (Fig. 4). In these conditions there was an optimum concentration of 24R,25(OH)$_2$D$_3$ (100 pmol/ml) for the stimulation of the 24-hydroxylase but not for the reduction of the 1-hydroxylase. The effects of 1,25(OH)$_2$D$_3$ and of 1,24R and S,25(OH)$_3$D$_3$ in the absence of fetal calf serum are shown in Table II. Both isomers of 1,24,25(OH)$_3$D$_3$ were about five times less potent than 1,25(OH)$_2$D$_3$ in inducing the 24-hydroxylase and inhibiting the 1-hydroxylase. Although all these compounds appeared to be somewhat more active in the absence than in the presence of fetal calf serum, the effect of fetal calf serum was not nearly as dramatic as it was for 24,25(OH)$_2$D$_3$. It is possible that the small differences in activity were the result of alterations in cell metabolism in the presence or absence of serum.

*Effect of PTH on hydroxylases.* Under certain conditions PTH produced a small but statistically significant increase of the 1-hydroxylase activity and a decrease of the 24-hydroxylase activity. As shown in Table III, 5 U/ml of bPTH had no effect on either
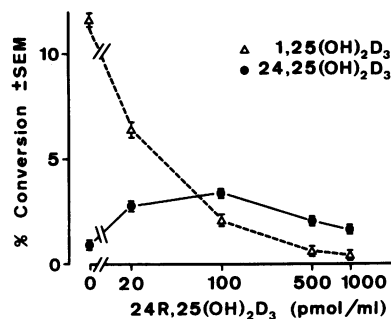
TABLE II
*Effect of 1,25(OH)$_2$D$_3$, 1,24R,25(OH)$_3$D$_3$, and 1,24S,25(OH)$_3$D$_3$*
*in the Absence of Fetal Calf Serum on Metabolism*
*of [$^3$H]25(OH)D$_3$ in Kidney Cell Culture*

| Metabolite | Concen- tration | $n$ | Percent conversion to | |
|---|---|---|---|---|
| | | | [$^3$H]24,25(OH)$_2$D$_3$ | [$^3$H]1,25(OH)$_2$D$_3$ |
| 0 | | 6 | 0.8±0.1 | 11.9±1.1 |
| 1,25(OH)$_2$D$_3$ | 2 nM | 3 | 5.0±1.1* | 5.5±0.6* |
| | 10 nM | 3 | 6.2±1.4* | 3.9±0.4* |
| 1,24R,25(OH)$_3$D$_3$ | 2 nM | 3 | 2.6±0.6‡ | 9.7±1.5 |
| | 10 nM | 3 | 4.7±1.1* | 7.0±1.2‡ |
| 1,24S,25(OH)$_3$D$_3$ | 2 nM | 3 | 2.1±0.4‡ | 10.9±1.3 |
| | 10 nM | 3 | 4.2±1.0‡ | 7.0±1.1‡ |

The metabolites were added to confluent cultures (1.2 mM Ca) after replacing the medium by 1 ml of medium without fetal calf serum. 6 h later [$^3$H]25(OH)D$_3$ was added, and the cultures incubated, extracted, and chromatographed as described in Methods. Results are means±SEM.
* $P < 0.001$.
‡ $P < 0.01$.

1-hydroxylase similar to the effects obtained with 1,25(OH)₂D₃, much smaller concentrations of bPTH were required to produce significant effects on both hydroxylases. Fig. 5 shows the effect of 1 U/ml of bPTH on the hydroxylases. The ratio of the 1- and the 24-hydroxylase activities is also represented. This expression is useful because it eliminates the influence of variations in cell number in different dishes. Table IV summarizes the results of two dose-response experiments performed with bPTH and hPTH(1-34). In both experiments maximum effects were obtained with 0.04 U/ml of either bPTH or hPTH(1-34). Dose-response experiments with lower doses of bPTH (Fig. 6) and of hPTH(1-34) (Fig. 7) showed a dose-dependent increase of the 1-hydroxylase up to 0.05 U/ml for bPTH and up to 0.01 U/ml for hPTH(1-34). With both hormones, the dose-response curve for the 24-hydroxylase did not exhibit a simple correlation, and we cannot exclude a biphasic process.

*Effect of steroid hormones.* As shown in Table V, no consistent effects on either hydroxylase were obtained with estradiol and testosterone, alone or in combination, added for 22 h to cultures grown in the presence or absence of 25(OH)D₃. In the presence of 25(OH)D₃, both steroids tended to increase the 24- and reduce the 1-hydroxylase activity. Corticosterone produced a dramatic increase of the 24- and a decrease
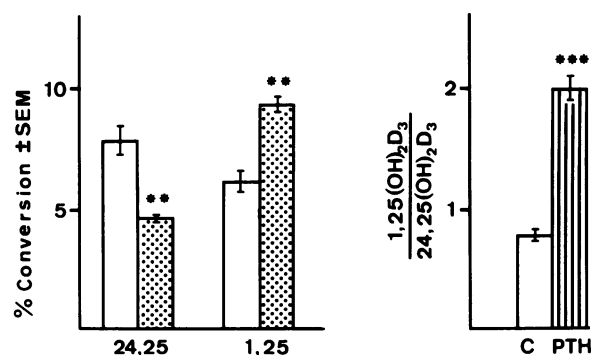


FIGURE 5 Effect of bPTH on the metabolism of [³H]25-(OH)D₃ in kidney cell culture. Cells were grown in medium (1.2 mM Ca) with 10% fetal calf serum, in the presence of 25(OH)D₃. 300 pmol of synthetic 25(OH)D₃ was added 24 h and 48 h after plating. 1 U/ml of bPTH was added to confluent cultures in 15 μl of 0.15 M NaCl containing 0.01 M HCl, 1.4 mM mercaptoethanol, and 1 mg/ml bovine serum albumin. Controls received solvent alone. 6 h later the medium was replaced by 1 ml of medium without fetal calf serum and without hormone, and cultures were incubated with [³H]25-(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 3$. Controls (C), open bars; PTH treated, dotted and hatched bars. **, $P < 0.01$; ***, $P < 0.001$.

of the 1-hydroxylase activity in cultures grown with 25(OH)D₃. When cultures without 25(OH)D₃ were exposed to 1 μM estradiol for 6 h (not shown), the 1-hydroxylase activity was decreased by 39% ($P < 0.001$), but the 24-hydroxylation was unchanged. Testosterone and progesterone in concentrations between 10 nM and 1 μM and corticosterone between 50 nM and 5 μM did not alter either hydroxylase activity under these conditions.

*Effect of Ca and P in the medium.* As shown in Table VI, changing Ca or P in the medium did not significantly affect the hydroxylase activities, either in the presence or in the absence of 25(OH)D₃. The variations in the DNA content of cultures with different media were small, indicating that cell numbers were similar, and the enzyme activities per microgram of DNA were not significantly changed by variations of Ca and P in the medium.

*Effect of EHDP.* No effect was observed with the diphosphonate on either hydroxylase, in the presence or absence of 25(OH)D₃ (Fig. 8). In a separate experiment, the DNA content of cultures treated in the same way was determined. 0.25 mM EHDP did not influence the DNA content of the cultures, indicating that the cell number was unaffected.

## DISCUSSION

The development of kidney cell cultures by our laboratory and by others (32–36) has for the first time offered a system for the investigation in vitro of long-

TABLE III

*Effect of PTH on Metabolism of [³H]25(OH)D₃ in Kidney Cell Culture*

| PTH | [Ca] | 1,25(OH)₂D₃ | Percent conversion to | |
|---|---|---|---|---|
| | | | [³H]24,25(OH)₂D₃ | [³H]1,25(OH)₂D₃ |
| | *mM* | | | |
| − | 1.8 | − | 1.1±0.1 | 12.6±1.2 |
| + | 1.8 | − | 1.1±0.1 | 14.6±0.7 |
| − | 0.8 | − | 1.2±0.1 | 13.6±0.5 |
| + | 0.8 | − | 1.1±0.1 | 13.7±1.6 |
| − | 1.8 | + | 6.6±0.5 | 4.9±0.6 |
| + | 1.8 | + | 6.4±0.3 | 5.6±0.6 |
| − | 0.8 | + | 7.4±0.7 | 4.9±0.4 |
| + | 0.8 | + | 5.2±0.7 | 6.7±0.3* |

16 pmol/ml of synthetic 1,25(OH)₂D₃ was added to confluent cultures (1.8 mM Ca) 15 h before PTH. PTH (5 U/ml) was added in 10 μl of 0.15 M NaCl containing 0.01 M HCl, 1.4 mM mercaptoethanol, and 1 mg/ml bovine serum albumin. Controls received solvent alone. The addition of 15 μl of 100 mM EGTA decreased the biologically effective Ca concentration to 0.8 mM. Controls received 15 μl of 0.15 M NaCl. 6 h later the medium was replaced by 1 ml of medium without fetal calf serum and without PTH, and cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are expressed as percent±SEM, $n = 3$.
* $P < 0.05$ as compared with control.

TABLE IV
Effect of bPTH and hPTH(1–34) on the Metabolism of [³H]25(OH)D₃
in Kidney Cell Culture

| Hormone preparation | Dose | Percent conversion to | | | |
| --- | --- | --- | --- | --- | --- |
| | | [³H]24,25(OH)₂D₃ | | [³H]1,25(OH)₂D₃ | |
| | | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| | U/ml | | | | |
| bPTH | 0 | 6.3±0.5 | 4.3±0.1 | 9.0±0.2 | 5.2±0.2 |
| bPTH | 0.04 | 5.0±0.5 | 3.9±0.1* | 11.5±0.7‡ | 6.2±0.1* |
| bPTH | 0.2 | 4.5±0.3‡ | 3.8±0.2‡ | 12.2±0.5§ | 6.4±0.1* |
| bPTH | 1.0 | 4.9±0.5 | 3.9±0.2‡ | 11.2±0.8‡ | 6.8±0.2* |
| bPTH | 5.0 | 4.3±0.2‡ | 3.5±0.1§ | 12.0±0.7* | 7.2±0.2§ |
| hPTH (1–34) | 0 | 6.2±0.8 | 5.8±0.2 | 8.8±0.6 | 4.4±0.1 |
| hPTH (1–34) | 0.04 | 3.8±0.1‡ | 5.1±0.2‡ | 11.2±0.7‡ | 5.1±0.2* |
| hPTH (1–34) | 0.2 | 3.9±0.2‡ | 5.0±0.4 | 12.0±0.5* | 5.3±0.2‡ |
| hPTH (1–34) | 1.0 | 3.8±0.1‡ | 5.1±0.3 | 11.5±0.6‡ | 5.3±0.2‡ |

Cells were grown in medium (1.2 mM Ca) with 10% fetal calf serum, in the presence of 25(OH)D₃. 300 pmol of synthetic 25(OH)D₃ was added 24 and 48 h after plating. Hormones were added to confluent cultures in 15 µl of 0.15 M NaCl containing 0.01 M HCl, 1.4 mM mercaptoethanol, and 1 mg/ml bovine serum albumin. Controls received solvent alone. 6 h later the medium was replaced by 1 ml of medium without fetal calf serum and without hormone, and cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 4$.
* $P < 0.01$.
‡ $P < 0.05$.
§ $P < 0.001$.

term effects on both renal hydroxylases. Several studies on the effects of 1,25(OH)₂D₃ (32–36), PTH (34, 35), and steroid hormones (36) have been reported.

In our system we have studied the properties of the hydroxylases in cultured cells and we have found that they were similar to those of the enzymes in fresh chick kidney preparations. Cultures grown in the absence of any vitamin D metabolites except for those added with the fetal calf serum behaved like kidneys from vitamin D-deficient chicks with a high 1-hydroxylase activity and a low 24-hydroxylase activity. However, the presence during culture of 25(OH)D₃, the circulating form of vitamin D in animals, produced an induction of the 24-hydroxylase and a decrease of the 1-hydroxylase. This preparation resembled the kidney of a vitamin D-replete bird. The apparent $K_m$ values for both hydroxylases were in agreement with those obtained with chick kidney homogenates (13, 37) or isolated mitochondria (38, 39) with the exception that in one study with mitochondria (40), the $K_m$ of the 1-hydroxylase was found to be higher by about one order of magnitude. The enzymes were also capable of hydroxylating 24,25(OH)₂D₃ and 1,25(OH)₂D₃ to 1,24,25(OH)₃D₃. This metabolite has been isolated from animal tissues (31) and produced in vitro with kidney preparations (41).

The response of the hydroxylases to exogenous 1,25(OH)₂D₃, namely an induction of the 24-hydroxylase and a reduction of the 1-hydroxylase activity, is in agreement with data obtained in vivo (9–11) and in vitro in a variety of systems (34, 36, 42, 43). The time-course of the response to 1,25(OH)₂D₃ (Fig. 2) might have suggested that the change of the 24-hydroxylase preceded the inhibition of the 1-hydroxylase. However, in subsequent experiments 1,25(OH)₂D₃ added for 2 h had either no effect or influenced both hydroxylases. Of interest, in the study of Spanos et al. (36) the inhibition of the 1-hydroxylase by 1,25(OH)₂D₃ was found to precede the induction of the 24-hydroxylase.

Our results with 25(OH)D₃, 24,25(OH)₂D₃, and 1,24,25(OH)₃D₃ show that D metabolites other than 1,25(OH)₂D₃ also are able to produce similar effects. Exogenous 24,25(OH)₂D₃, in the presence of fetal calf serum, was active only in large concentrations. This might be attributed to the binding of 24,25(OH)₂D₃ to protein in serum as demonstrated by other authors (44, 45), because in the absence of fetal calf serum lower concentrations were required. There was an optimum concentration for the stimulation of the 24-hydroxylase. Product inhibition of the 24-hydroxylase with high concentrations of 24,25(OH)₂D₃ might explain this dose-response relationship (39). The results with 1,24R,25(OH)₃D₃, the naturally occurring isomer (46),
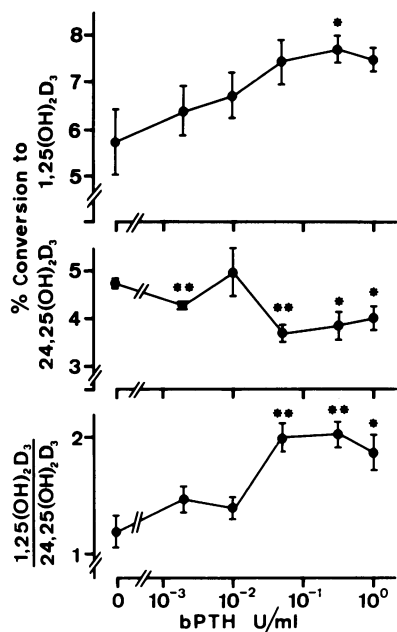
FIGURE 6 Effect of bPTH on the metabolism of [³H]25(OH)D₃ in kidney cell culture. Cells were grown in the presence of 25(OH)D₃ as in the experiments shown in Fig. 5 and Table IV. bPTH was added in 15 μl of solvent. 6 h later the medium was replaced by 1 ml of medium without fetal calf serum and without hormone, and cultures were incubated with [³H]25-(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 4$. *, $P < 0.05$; **, $P < 0.01$.

are in agreement with the inhibition of the 1-hydroxylase obtained in vivo in chicks (47). In our system 1,24S,25(OH)₃D₃ was also tested and found to be equally effective, indicating that the stereochemical configuration of the 24-hydroxyl group does not affect the response of the hydroxylases to 1,24,25(OH)₃D₃. The dose-response relationship for the induction of the 24-hydroxylase suggests that, as for 24,25(OH)₂D₃, there was an optimum concentration of 1,24,25(OH)₃D₃ for the effect on this enzme. Contrary to the response seen with 24,25(OH)₂D₃, the dramatic effects of fetal calf serum were not observed with 1,25(OH)₂D₃ or 1,24,25(OH)₃D₃, suggesting that these metabolites are not as tightly bound to serum protein as 24,25(OH)₂D₃. Because all the D metabolites appeared to elicit comparable responses of the hydroxylases, and because 25(OH)D₃ as well as 24,25(OH)₂D₃ may be further hydroxylated very rapidly, we cannot conclude whether these compounds themselves or metabolites thereof were responsible for the effects on the hydroxylases.

The modulation of the hydroxylases by vitamin D metabolites has certain implications for studies in vivo in experimental animals because the administration of one of these compounds is likely to alter the pattern of metabolites produced endogenously. Preliminary

results suggest that 25,26-dihydroxyvitamin D₃, another naturally occurring derivative of vitamin D, produces similar effects on the hydroxylases in cell culture as the metabolites tested in this study (unpublished results). A possible alteration of vitamin D metabolism should also be considered when vitamin D metabolites are used clinically in the management of metabolic bone disease.

With respect to the influence of PTH on the renal enzymes affecting vitamin D metabolism, in vivo experiments have shown that PTH can stimulate the renal 1-hydroxylase and reduce the 24-hydroxylase (11–14). However, the question of whether PTH acts directly at the kidney level can only be answered by experiments with kidney preparations in vitro. Although some evidence has been presented indicating that PTH can stimulate the 1-hydroxylase and decrease the 24-hydroxylase activities in vitro (35, 42, 48), the limited data available are inconclusive. In our culture system, a small but reproducible and statistically significant stimulation of the 1-hydroxylase and a decrease of the 24-hydroxylase activity were observed, which is in contrast to the data obtained by Henry (34) who found no effect.

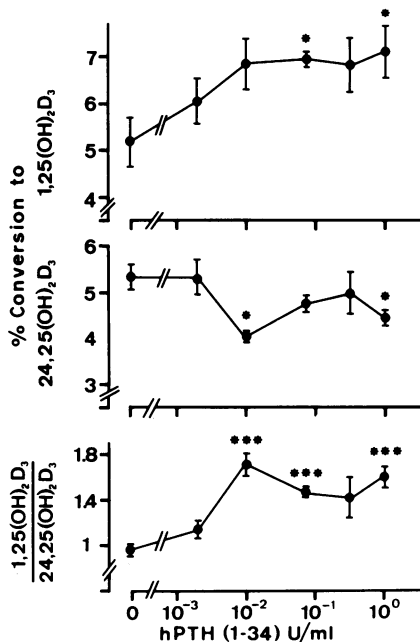The concentrations of either bPTH or hPTH(1-34)



FIGURE 7 Effect of hPTH(1-34) on the metabolism of [³H]25(OH)D₃ in kidney cell culture. Cells were grown in the presence of 25(OH)D₃ as in the experiments shown in Fig. 5 and Table IV. hPTH(1-34) was added in 15 μl of solvent. 6 h later the medium was replaced by 1 ml of medium without fetal calf serum and without hormone, and cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 4$. *, $P < 0.05$; ***, $P < 0.001$.

to elicit a response were in the range of those shown to stimulate bone resorption in vitro (49). The minimum incubation time with 1 U/ml of bPTH for these effects to be detectable was 4 h. This is in contrast to the results obtained with kidney tubules where exposure to PTH for <1 h resulted in a stimulation of the 1- and a reduction of the 24-hydroxylase activities (42, 48). However, delayed response of cultures to

## TABLE V
### Effect of Steroids on the Metabolism of [³H]25(OH)D₃ in Kidney Cell Culture

| Steroid | Concen-tration | 25(OH)D₃ | [³H]24,25(OH)₂D₃ | | [³H]1,25(OH)₂D₃ | |
|---|---|---|---|---|---|---|
| | | | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| | $\mu M$ | | | | | |
| 0 | | − | 0.8±0.1 | 0.8±0.1 | 20.1±0.9 | 15.7±0.5 |
| Estradiol | 1 | − | 1.6±0.5 | 1.6±0.4 | 20.5±0.5 | 15.3±0.5 |
| Testosterone | 1 | − | 1.0±0.1 | 1.0±0.1 | 20.4±0.6 | 14.4±0.3 |
| Estradiol + testosterone | 1 + 1 | − | 0.9±0.1 | 1.0±0.1 | 19.9±0.8 | 14.9±0.4 |
| Corticosterone | 5 | − | 1.0±0.1 | 1.2±0.2 | 19.0±1.0 | 11.0±0.7* |
| 0 | | + | 6.5±0.3 | 5.3±0.1 | 7.8±0.3 | 4.9±0.1 |
| Estradiol | 1 | + | 6.9±0.3 | 6.4±0.3‡ | 7.5±0.7 | 4.8±0.1 |
| Testosterone | 1 | + | 7.8±0.5 | 6.1±0.1* | 6.4±0.3‡ | 4.2±0.4 |
| Estradiol + testosterone | 1 + 1 | + | 7.8±0.7 | 6.5±0.1§ | 5.8±0.5‡ | 4.3±0.1 |
| Corticosterone | 5 | + | 10.4±0.3§ | 9.7±0.3§ | 3.5±0.3§ | 2.6±0.1§ |

Cells were grown in medium (1.2 mM Ca) with 10% fetal calf serum, in the presence of 25(OH)D₃. 300 pmol of synthetic 25(OH)D₃ was added 4 and 48 h after plating. The steroids were added to nearly confluent cultures (1.2 mM Ca) without changing the medium. 22 h later the medium was replaced by 1 ml of medium without fetal calf serum and without steroids, and the cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 4$ in experiment 1, $n = 3$ in experiment 2.
* $P < 0.01$.
‡ $P < 0.05$.
§ $P < 0.001$.

## TABLE VI
### Metabolism of [³H]25(OH)D₃ in Cell Cultures Grown with Different Concentrations of Calcium and Phosphate

| Ca | P | 25(OH)D₃ | DNA* | [³H]24,25(OH)₂D₃ | [³H]1,25(OH)₂D₃* |
|---|---|---|---|---|---|
| $mM$ | $mM$ | | $\mu g/dish$ | | |
| 1.2 | 1.0 | − | 3.2±0.03 (5) | 1.3±0.1 | 12.3±0.5 (5) |
| 1.2 | 1.0 | + | 3.3±0.02 (3) | 6.0±0.5 | 4.3±0.2 (4) |
| 0.5 | 1.0 | − | 3.0±0.13 (5) | 1.4±0.2 | 12.0±0.3 (5) |
| 0.5 | 1.0 | + | 3.5±0.16 (3) | 6.0±0.4 | 4.3±0.3 (4) |
| 2.5 | 1.0 | − | 3.4±0.05 (4) | 1.3±0.1 | 11.8±0.2 (5) |
| 2.5 | 1.0 | + | 3.4±0.03 (4) | 7.2±0.4 | 3.8±0.1 (5) |
| 1.2 | 0.3 | − | 3.3±0.05 (5) | 1.3±0.1 | 12.6±0.4 (5) |
| 1.2 | 0.3 | + | 3.2±0.01 (3) | 6.4±0.4 | 4.6±0.2 (4) |
| 1.2 | 3.0 | − | 3.4±0.05 (5) | 1.4±0.2 | 13.7±0.3 (5) |
| 1.2 | 3.0 | + | 3.5±0.04 (5) | 7.1±0.6 | 4.7±0.1 (5) |

Cells were grown to confluence in media containing indicated Ca and P concentrations, with 10% fetal calf serum. 300 pmol of synthetic 25(OH)D₃ was added 24 and 48 h after plating. All cultures were confluent 72 h after plating. Hydroxylase activities were assayed as described in Methods, and DNA was determined in separate dishes. Results are means±SEM.
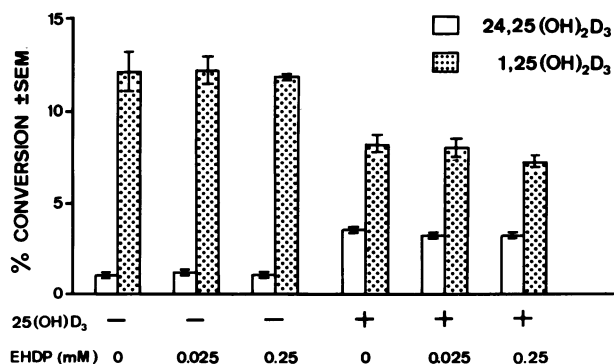* Number of replicates in parentheses.

FIGURE 8 Absence of effect of EHDP on metabolism of [³H]25(OH)D₃ in kidney cell culture. Cells were grown in medium (1.2 mM Ca) with 10% fetal calf serum. EHDP was added 24 h after plating, after changing the medium. Cultures grown in the presence of 25(OH)D₃ received 300 pmol of synthetic 25(OH)D₃ 24 and 48 h after plating. Confluence was reached after 72 h of culture. At that time the medium was replaced by 1 ml of medium without fetal calf serum and without EHDP, and the cultures were incubated with [³H]25(OH)D₃, extracted, and chromatographed as described in Methods. Results are means±SEM, $n = 3$ for cultures without 25(OH)D₃, $n = 4$ for cultures with 25(OH)D₃.

PTH agrees with the results obtained in vivo where several hours were required to produce an effect (11–13). In our culture system, the vitamin D and calcium status of the system influenced the response to PTH which would support similar findings by Larkins et al. (42) with kidney tubules. In relation to the vitamin D status, it is of interest that the sensitivity of the system to PTH was much greater in the presence of 25(OH)D₃ than in the presence of 1,25-(OH)₂D₃. This finding suggests that D metabolites other than 1,25(OH)₂D₃, such as 25(OH)D₃ or metabolites of 25(OH)D₃ formed during culture, might play a role in modulating the response of the hydroxylases to PTH.

Steroid hormones have been reported to influence the renal 25(OH)D-hydroxylases in birds. Estradiol given to quails (15–17) or chicks (15) stimulated the renal 1-hydroxylase and inhibited the renal 24-hydroxylase. However, supplementation with estradiol of quails treated with an antiestrogen produced the opposite effect, namely an inhibition of the 1-hydroxylase and a stimulation of the 24-hydroxylase (50). Testosterone and a stimulation of the 24-hydroxylase (50). Testosterone has been shown to potentiate the effect of estradiol in immature male chickens (15) and to decrease the 24-hydroxylase activity when given to mature male quails (17). Corticosterone has also recently been shown to stimulate the 1-hydroxylase when given to chicks (18). The mode of action of steroids on vitamin D metabolism is unknown, but in view of the conflicting evidence obtained with estradiol in vivo, it would seem that the effect of this steroid in vivo might be mediated indirectly. Our results would support the concept of

an indirect mechanism. We were unable to show a stimulatory effect on the 1-hydroxylase in cell culture by any of the steroids tested, whereas under certain conditions a decrease of the 1-hydroxylase activity was seen. The decrease of the 1-hydroxylase and the induction of the 24-hydroxylase by corticosterone presented in Table V were not observed when cultures treated with 1,25(OH)₂D₃ were incubated for 6 h with 5–5,000 nM corticosterone. In a similar culture system, Spanos et al. (36) obtained no change of the 1-hydroxylase with estradiol.

Other physiological factors that have been suggested to play a role in the regulation of the renal hydroxylases are plasma Ca and P (51, 52). We could not demonstrate any effect of rather large variations in Ca and P concentrations of the medium. Therefore it appears that if extracellular Ca and P play a role in the regulation of the renal enzymes, an additional factor might be required for the regulation to occur.

When EHDP is given to animals in large doses, it has been shown to block bone mineralization (53–55) and to impair calcium absorption in the intestine, by decreasing the production of 1,25(OH)₂D₃ in the kidney (19–21). This effect has been suggested to be an indirect homeostatic response to the inhibition of bone mineralization, protecting the organism from being overloaded with calcium (56). In short-term experiments in vitro with isolated kidney mitochondria, an inhibition of the 1-hydroxylase was observed with the diphosphate (20, 57) but only with high levels of EHDP, which are unlikely to occur in vivo. In our long-term treatment of kidney cells with EHDP, no effect on either hydroxylase was observed. Because the concentration of EHDP used was high compared with the plasma concentration of diphosphate in animals treated with high doses of EHDP (unpublished results), and because this concentration has been shown to affect metabolic reactions in cultures of other cells (58), it seems very unlikely that a direct effect of EHDP on the renal enzymes is responsible for the impairment of 1,25-(OH)₂D₃ production in EHDP-treated animals. The factor that mediates the response is unknown.

On the basis of this study, it is clear that the kidney cell culture as described presents a useful alternative for studying the regulation of vitamin D metabolism by the kidney. Moreover, the system has the distinct advantage that long-term effects of hormones and agents can now be monitored which should enable a greater understanding of the mechanisms underlying Ca homeostasis.

## ACKNOWLEDGMENTS

## REFERENCES

1. Omdahl, J. L., and H. F. DeLuca. 1973. Regulation of vitamin D metabolism and function. *Physiol. Rev.* **53**: 327–372.

2. Norman, A. W., and H. Henry. 1974. 1,25-Dihydroxycholecalciferol—a hormonally active form of vitamin D. *Recent Prog. Horm. Res.* **30**: 431–480.

3. Fraser, D. R. 1975. Advances in the knowledge of the metabolism of vitamin D. *Proc. Nutr. Soc.* **34**: 139–143.

4. DeLuca, H. F., and H. K. Schnoes. 1976. Metabolism and mechanism of action of vitamin D. *Annu. Rev. Biochem.* **45**: 631–666.

5. Boyle, I. T., L. Miravet, R. W. Gray, M. F. Holick, and H. F. DeLuca. 1972. The response of intestinal calcium transport to 25-hydroxy and 1,25-dihydroxy vitamin D in nephrectomized rats. *Endocrinology.* **90**: 605–608.

6. Wong, R. G., A. W. Norman, C. R. Reddy, and J. W. Coburn. 1972. Biologic effects of 1,25-dihydroxycholecalciferol (a highly active vitamin D metabolite) in acutely uremic rats. *J. Clin. Invest.* **51**: 1287–1291.

7. Raisz, L. G., C. L. Trummel, M. F. Holick, and H. F. DeLuca. 1972. 1,25-Dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science (Wash. D. C.).* **175**: 768–769.

8. Reynolds, J. J., M. F. Holick, and H. F. DeLuca. 1973. The role of vitamin D metabolites in bone resorption. *Calcif. Tissue Res.* **12**: 295–301.

9. Henry, H. L., R. J. Midgett, and A. W. Norman. 1974. Regulation of 25-hydroxyvitamin $D_3$-1-hydroxylase in vivo. *J. Biol. Chem.* **249**: 7584–7592.

10. Tanaka, Y., and H. F. DeLuca. 1974. Stimulation of 24,25-dihydroxyvitamin $D_3$. *Science (Wash. D. C.).* **183**: 1198–1200.

11. Tanaka, Y., R. S. Lorenc, and H. F. DeLuca. 1975. The role of 1,25-dihydroxyvitamin $D_3$ and parathyroid hormone in the regulation of chick renal 25-hydroxyvitamin $D_3$-24-hydroxylase. *Arch. Biochem. Biophys.* **171**: 521–526.

12. Garabedian, M., M. F. Holick, H. F. DeLuca, and I. T. Boyle. 1972. Control of 25-hydroxycholecalciferol metabolism by the parathyroid glands. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 1673–1676.

13. Fraser, D. R., and E. Kodicek. 1973. Regulation of 25-hydroxycholecalciferol-1-hydroxylase activity in kidney by parathyroid hormone. *Nat. New Biol.* **241**: 163–166.

14. Mawer, B. E., J. Backhouse, L. F. Hill, G. A. Lumb, P. de Silva, C. M. Taylor, and S. W. Stanbury. 1975. Vitamin D metabolism and parathyroid function in man. *Clin. Sci. Mol. Med.* **48**: 349–365.

15. Tanaka, Y., L. Castillo, and H. F. DeLuca. 1976. Control of renal vitamin D hydroxylases in birds by sex hormones. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 2701–2705.

16. Baksi, S. N., and A. D. Kenny. 1977. Vitamin $D_3$ metabolism in immature Japanese quail: effects of ovarian hormones. *Endocrinology.* **101**: 1216–1220.

17. Castillo, L., Y. Tanaka, H. F. DeLuca, and M. L. Sunde. 1977. The stimulation of 25-hydroxyvitamin $D_3$-1α-hydroxylase by estrogen. *Arch. Biochem. Biophys.* **179**: 211–217.

18. Spanos, E., K. W. Colston, and I. MacIntyre. 1977. Effects of glucocorticoids on vitamin D metabolism. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* **75**: 73–76.

19. Hill, L. F., G. A. Lumb, E. B. Mawer, and S. W. Stanbury. 1973. Indirect inhibition of the biosynthesis of 1,25-dihydroxycholecalciferol in rats treated with a diphosphate. *Clin. Sci.* **44**: 335–347.

20. Baxter, L. A., H. F. DeLuca, J-P. Bonjour, and H. A. Fleisch. 1974. Inhibition of vitamin D metabolism by ethane-1-hydroxy-1,1-diphosphate. *Arch. Biochem. Biophys.* **164**: 655–662.

21. Taylor, C. M., E. B. Mawer, and A. Reeve. 1975. The effects of a diphosphonate and dietary calcium on the metabolism of vitamin $D_3$ (cholecalciferol) in the chick. *Clin. Sci. Mol. Med.* **49**: 391–400.

22. Kanis, J. A., G. Heynen, R. G. G. Russell, R. Smith, R. J. Walton, and G. T. Warner. 1977. Biological effects of 24,25-dihydroxycholecalciferol in man. In Vitamin D. Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism. A. W. Norman, K. Schaefer, J. W. Coburn, H. F. DeLuca, D. Fraser, H. G. Grigoleit, and D. v. Herrath, editors. Walter De Gruyter & Co., Berlin. 793–795.

23. Eagle, H. 1959. Amino acid metabolism in mammalian cell cultures. *Science (Wash. D. C.).* **130**: 432–437.

24. Shain, S. A. 1972. In vitro metabolism of 25-hydroxycholecalciferol by chick intestinal and renal cell preparations. *J. Biol. Chem.* **247**: 4393–4403.

25. Burton, K. 1956. A study of the conditions and mechanisms of the diphenylamine reaction for colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* **62**: 315–323.

26. Leyva, A., Jr., and W. N. Kelley. 1974. Measurement of DNA in cultured human cells. *Anal. Biochem.* **62**: 173–179.

27. Haddad, J. G., Jr., and K. J. Chyn. 1971. 25-Hydroxycholecalciferol-binding globulin in human plasma. *Biochim. Biophys. Acta.* **248**: 471–481.

28. Edelstein, S. D., E. M. Lawson, and E. Kodicek. 1973. The transporting proteins of cholecalciferol and 25-hydroxycholecalciferol in serum of chicks and other species. *Biochem. J.* **135**: 417–426.

29. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37**: 911–917.

30. Jones, G., and H. F. DeLuca. 1975. High-pressure liquid chromatography: separation of the metabolites of vitamins $D_2$ and $D_3$ on small-particle silica columns. *J. Lipid Res.* **16**: 448–453.

31. Boyle, I. T., J. L. Omdahl, R. W. Gray, and H. F. DeLuca. 1973. The biological activity and metabolism of 24,25-dihydroxyvitamin $D_3$. *J. Biol. Chem.* **248**: 4174–4180.

32. Henry, H. L. 1977. Regulation of the metabolism of 25(OH)$D_3$: 1,25(OH)$_2D_3$-parathyroid interactions. In Vitamin D. Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism. A. W. Norman, K. Schaefer, J. W. Coburn, H. F. DeLuca, D. Fraser, H. G. Grigoleit, and D. v. Herrath, editors. Walter De Gruyter & Co., Berlin. 125–133.

33. Trechsel, U., J-P. Bonjour, and H. Fleisch. 1977. Metabolism of 25-hydroxyvitamin $D_3$ [25-(OH)$D_3$] in primary chick kidney cell culture. In Vitamin D. Biochemical, Chemical and Clinical Aspects Related to Calcium Metabolism. A. W. Norman, K. Schaefer, J. W. Coburn, H. F. DeLuca, D. Fraser, H. G. Grigoleit, and D. v. Herrath, editors. Walter De Gruyter & Co., Berlin. 879–881.

34. Henry, H. L. 1977. Metabolism of 25-hydroxyvitamin $D_3$ by primary cultures of chick kidney cells. *Biochem. Biophys. Res. Commun.* **74**: 768–774.

35. Juan, D., and H. F. DeLuca. 1977. The regulation of

24,25-dihydroxyvitamin $D_3$ production in cultures of monkey kidney cells. *Endocrinology.* **101:** 1184–1193.

36. Spanos, E., D. I. Barrett, K. T. Chong, and I. MacIntyre. 1978. Effect of oestrogen and 1,25-dihydroxycholecalciferol on 25-hydroxycholecalciferol metabolism in primary chick kidney-cell cultures. *Biochem. J.* **174:** 231–236.

37. Colston, K. W., I. M. A. Evans, L. Galante, I. MacIntyre, and D. W. Moss. 1973. Regulation of vitamin D metabolism: factors influencing the rate of formation of 1,25-dihydroxycholecalciferol by kidney homogenates. *Biochem. J.* **134:** 817–820.

38. Bikle, D. D., and H. Rasmussen. 1974. A comparison of the metabolism of 25-hydroxyvitamin $D_3$ by chick renal tubules, homogenates and mitochondria. *Biochim. Biophys. Acta.* **362:** 439–447.

39. Knutson, J. C., and H. F. DeLuca. 1974. 25-Hydroxyvitamin $D_3$-24-hydroxylase. Subcellular location and properties *Biochemistry.* **13:** 1543–1548.

40. Gray, R. W., J. L. Omdahl, J. G. Ghazarian, and H. F. DeLuca. 1972. 25-Hydroxycholecalciferol-1-hydroxylase. Subcellular location and properties. *J. Biol. Chem.* **247:** 7528–7532.

41. Holick, M. F., A. Kleiner-Bossaller, H. K. Schnoes, P. M. Kasten, I. T. Boyle, and H. F. DeLuca. 1973. 1,24,25-Trihydroxyvitamin $D_3$. A metabolite of vitamin $D_3$ effective on intestine. *J. Biol. Chem.* **248:** 6691–6696.

42. Larkins, R. G., S. J. MacAuley, A. Rapoport, T. J. Martin, B. R. Tulloch, P. G. H. Byfield, E. W. Matthews, and I. MacIntyre. 1974. Effects of nucleotides, hormones, ions and 1,25-dihydroxycholecalciferol on 1,25-dihydroxycholecalciferol production in isolated chick renal tubules. *Clin. Sci. Mol. Med.* **46:** 569–582.

43. Larkins, R. G., S. J. MacAuley, and I. MacIntyre. 1974. Feedback control of vitamin D metabolism by a nuclear action of 1,25-dihydroxycholecalciferol on the kidney. *Nature (Lond.).* **252:** 412–414.

44. Haddad, J. G., Jr., C. Min, J. Walgate, and T. Hahn. 1976. Competition by 24,25-dihydroxycholecalciferol in the competitive protein binding radioassay of 25-hydroxycholecalciferol. *J. Clin. Endocrinol. Metab.* **43:** 712–715.

45. Taylor, C. M., S. E. Hughes, and P. de Silva. 1976. Competitive protein binding assay for 24,25-dihydroxycholecalciferol. *Biochem. Biophys. Res. Commun.* **70:** 1243–1249.

46. Tanaka, Y., L. Castillo, and H. F. DeLuca. 1977. The 24-hydroxylation of 1,25-dihydroxyvitamin $D_3$. *J. Biol. Chem.* **252:** 1421–1424.

47. Procsal, D. A., H. L. Henry, E. J. Friedlander, and A. W. Norman. 1977. Studies on the mode of action of calciferol. Biological activity of 1α24R,25-trihydroxyvitamin $D_3$ in the chick. *Arch. Biochem. Biophys.* **179:** 229–234.

48. Rasmussen, H., M. Wong, D. Bikle, and D. B. P. Goodman. 1972. Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. *J. Clin. Invest.* **51:** 2502–2504.

49. Raisz, L. G. 1965. Bone resorption in tissue culture: factors influencing the response to parathyroid hormone. *J. Clin. Invest.* **44:** 103–116.

50. Baksi, S. N., and A. D. Kenny. 1977. Effects of administration of antiestrogen (Tamoxifen) in vivo on the meabolism of 25-hydroxyvitamin $D_3$ in vitro in the Japanese quail. *Biochem. Pharmacol.* **26:** 2439–2443.

51. Boyle, I. T., R. W. Gray, and H. F. DeLuca. 1971. Regulation by calcium of in vivo synthesis of 1,25-dihydroxycholecalciferol and 21,25-dihydroxycholecalciferol. *Proc. Natl. Acad. Sci. U. S. A.* **68:** 2131–2134.

52. Tanaka, Y., and H. F. DeLuca. 1963. The control of 25-hydroxyvitamin D metabolism by inorganic phosphorus. *Arch. Biochem. Biophys.* **154:** 566–574.

53. King, W. R., M. D. Francis, and W. R. Michael. 1971. Effect of disodium ethane-1-hydroxyl-1,1-diphosphate on bone formation. *Clin. Orthop. Relat. Res.* **78:** 251–270.

54. Gasser, A. B., D. B. Morgan, H. A. Fleisch, and L. J. Richelle. 1972. The influence of two diphosphates on calcium metabolism in the rat. *Clin. Sci.* **43:** 31–45.

55. Schenk, R., W. A. Merz, R. Mühlbauer, R. G. G. Russell, and H. Fleisch. 1973. Effect of ethane-1-hydroxy-1,1-diphosphate (EHDP) and dichloromethylene diphosphonate ($Cl_2$MDP) on the calcification and resorption of cartilage and bone in the tibial epiphysis and metaphysis of rats. *Calcif. Tissue Res.* **11:** 196–214.

56. Trechsel, U., R. Schenk, J-P. Bonjour, R. G. G. Russell, and H. Fleisch. 1977. Relation between bone mineralization, Ca absorption, and plasma Ca in phosphate-treated rats. *Am. J. Physiol.* **232:** E298–E305.

57. Henry, H. L., and A. W. Norman. 1975. Biochemical and physiological regulation of 25-hydroxycholecalciferol-1-hydroxylase. *In* Vitamin D and Problems Related to Uremic Bone Disease. A. W. Norman, K. Schaefer, H. G. Grigoleit, D. v. Herrath, and E. Ritz, editors. Walter De Gruyter & Co., Berlin. 6–7.

58. Fast, D. K., R. Felix, C. Dowse, W. F. Neuman, and H. Fleisch. 1978. The effects of diphosphonates on the growth and glycolysis of connective-tissue cells in culture. *Biochem. J.* **172:** 97–107.