# The Ionic Control of 1,25-Dihydroxyvitamin D<sub>3</sub> Production in Isolated Chick Renal Tubules

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A B S T R A C T Isolated renal tubules prepared from vitamin D-deficient chicks catalyze the 1*a*-hydroxylation of 25-hydroxyvitamin D<sub>8</sub> (250HD<sub>8</sub>) in vitro. The effect of calcium and phosphate on the rate of synthesis of the product, 1,25-dihydroxyvitamin D<sub>8</sub> (1,25(OH)<sub>2</sub>D<sub>9</sub>), was studied at two levels : the long-term effects of various dietary calcium and phosphate contents on the ability of the tubules to produce 1,25(OH)<sub>2</sub>D<sub>8</sub>, and the acute effects of different calcium and phosphate concentrations in the incubation medium on the rate of synthesis of 1,25(OH)<sub>2</sub>D<sub>8</sub> by the tubules.

Manipulation of dietary calcium and phosphate sufficient to produce marked changes in the concentration of calcium and phosphate in the serum led to altered rates of  $1,25(OH)_2D_8$  synthesis by the isolated renal tubules. The renal tubules isolated from chicks raised on a vitamin D-deficient diet containing 0.43% calcium and 0.3%P as inorganic phosphate showed the highest rate of synthesis of  $1,25(OH)_2D_8$ . Diets containing more or less of either calcium or phosphate produced chicks whose renal tubules had a slower rate of  $1,25(OH)_2D_8$  production.

The calcium, phosphate, and hydrogen ion content of the incubation medium were manipulated to determine the possible factors concerned with the immediate regulation of  $1,25(OH)_{a}D_{a}$  production. A calcium concentration of 0.5–1.0 mM was necessary for optimal enzymatic activity. Concentrations of calcium greater than this optimal concentration inhibited  $1,25(OH)_{a}D_{a}$  production if phosphate was also present, and this inhibition was more pronounced as the phosphate concentration was increased. The stimulation of  $1,25(OH)_{a}D_{a}$  production by calcium was less at pH 6.7 than at 7.4. Raising the phosphate concentration from 0 to 6 mM in the absence of calcium also stimulated the rate of synthesis of 1,25.  $(OH)_{a}D_{b}$ . This stimulatory effect was blocked by 4 mM calcium. However, at 1-2 mM calcium, phosphate had a biphasic influence on 1,25(OH)\_aD\_b production; extracellular concentrations of phosphate from 0.6 to 1.2 mM resulted in less 1,25(OH)\_aD\_b production than higher or lower phosphate concentrations. This biphasic effect was seen both at pH 7.4 and 6.8.

## INTRODUCTION

1,25-Dihydroxyvitamin Ds (1,25(OH)<sub>2</sub>D<sub>8</sub>)<sup>1</sup> is the major biologically active metabolite of vitamin D<sub>8</sub> (1-7). 25-Hydroxyvitamin D<sub>8</sub> (250HD<sub>8</sub>) 1a-hydroxylase, the enzyme that produces 1,25(OH)<sub>2</sub>D<sub>8</sub> from 25OHD<sub>8</sub>, is found exclusively in kidney mitochondria (8). Studies carried out in vivo have indicated that the rate of 1,25(OH)<sub>2</sub>D<sub>8</sub> production is regulated by changes in the plasma concentrations of calcium (9, 10), phosphate (11), parathyroid hormone (12-14), and calcitonin (15). These studies have employed intact animals. Estimations of 1,25(OH)<sub>2</sub>D<sub>8</sub> synthesis have been made by measuring plasma or tissue contents of 1,25(OH)<sub>2</sub>D<sub>8</sub> at a single fixed time after the administration of labeled 25(OH)D<sub>8</sub> to a suitably prepared animal. In nearly all instances the measurements and experimental manipulations have been made over a period of hours or days and presumably represent relatively long-term changes in "synthetic" rate. Because of the design of these experiments, it is also possible that some of the changes in tissue or plasma content of 1,25(OH)2D8 after a particular experimental manipulation resulted from changes in rate of catabolism or further metabolism of 1,25-(OH)<sub>2</sub>D<sub>8</sub> rather than alterations in its rate of synthe-

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<sup>&</sup>lt;sup>1</sup> Abbreviations used in this paper: EGTA, ethylene-glycol-bis ( $\beta$ -aminoethyl ether) N, N, N', N'-tetraacetic acid; 1,25-(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>; 25OHD<sub>3</sub>, 25-hydroxyvitamin D<sub>3</sub>.

sis. A more direct means of assessing  $1,25(OH)_2D_4$ production is to measure its rate of synthesis in vitro by a system that does not further metabolize the product. Isolated renal tubules have been found to fulfill this criterion.

Studies with isolated chick renal tubules have shown that the rate of conversion of  $25OHD_*$  to  $1,25(OH)_*D_*$ can be quite rapid (16), can be altered by the nutritional status of the donor animal (17), and can respond to parathyroid hormone and calcitonin in vitro (18). Studies with isolated renal tubules, therefore, offer a reasonable compromise between the desire for a physiologic system and the advantages of an in vitro one. The purpose of the present study was to examine the roles of calcium and phosphate in the regulation of the rate of synthesis of  $1,25(OH)_*D_*$  in isolated renal tubules obtained from vitamin D-deficient chicks. Calcium and phosphate were adjusted in vivo by manipulating the diet and in vitro by altering the composition of the incubation medium.

#### METHODS

Animals. White Leghorn cockerels (Moyers Chickens, Quakertown, Pa.) were placed on a vitamin D-deficient diet containing, in general, 0.43% calcium and 0.3% P phosphate. For certain experiments diets were prepared containing one of three calcium concentrations, 0.03%, 0.43%, or 3.0%, and one of three phosphate concentrations, 0.023%, 0.3%, or 3.0% P. The animals were sacrificed after 21-25 days. The same batch of chicks was used for any given experiment.

Materials.  $[26,27-^{s}H_{2}]25OHD_{s}$ , sp act 1.2 mCi/µmol was obtained from New England Nuclear (Boston, Mass.). Unradioactive 25OHD<sub>8</sub> was a gift from The Upjohn Company (Kalamazoo, Mich.). Collagenase, type 1, from Clostridium histolyticum and hyaluronidase, type 1, from bovine testes were obtained from Sigma Chemical Co. (St. Louis, Mo.). Whatman SG81 chromatography paper was obtained from H. Reeve Angel & Co., Inc. (Clifton, N. J.). All other chemicals used were reagent grade and were obtained from commercial suppliers.

Incubation conditions. The renal tubules were prepared by the method of Shain (16) employing Krebs-Ringer bicarbonate buffers. The buffers used both for isolation and incubation of the renal tubules in the dietary manipulation studies contained 1 mM calcium and 1.2 mM phosphate at pH 7.4. However, the calcium, phosphate, and hydrogen concentrations of the buffers were varied when the effects of such in vitro manipulations were studied. For example, in the experiment investigating the acute effect of phosphate on  $1,25(OH)_2D_3$  production by the renal tubules at a calcium concentrations of 4 mM, the tubules were prepared in 4 mM calcium and 0 mM phosphate. If the acute effect of calcium was being studied, for example at 1.2 mM phosphate, the buffers used in the preparation of the tubules contained 0.5 mM ethylene-glycol-bis(\beta-aminoethyl ether)-N.N.N',N'-tetraacetic acid (EGTA) and 1.2 mM phosphate without added calcium. The incubation medium contained 0.5 mM EGTA with sufficient calcium added to give the desired free calcium concentration (19). In all cases the pH of the buffers used in the preparation of the tubules was 7.4. Just before the assay, the tubules were divided

into aliquots and washed three times in the final incubation medium containing the appropriate calcium, phosphate, and hydrogen concentration. In all cases 2 g/100 ml bovine serum albumin, 5 mM glucose, and 2 mM sodium pyruvate were included in the incubation medium. The pH of all buffers was determined after equilibration with 95% O<sub>2</sub>: 5% CO<sub>2</sub>. Adjustments in the bicarbonate concentration were used to manipulate pH. 1-ml aliquots of the tubule suspensions, containing 6.0–9.0 mg tubular protein, were preincubated for 12 min at 37°C in an atmosphere of 95% O<sub>2</sub>: 5% CO<sub>2</sub> (except as noted in the text).

In our early studies, we employed 500 pmol of  $25OHD_s$ , sp act 100 µCi/µmol, in 50 µl of 95% ethanol, and carried out our incubations for 20 min. Previous work (20) had shown that in the concentration range of 25-500 pmol/ml of 25OHD<sub>3</sub> the rate of 1,25(OH)<sub>2</sub>D<sub>3</sub> production was essentially a linear function of 25OHD<sub>3</sub> concentration. In later experiments, we found that incubation of tubules with 25 pmol of 25OHD<sub>8</sub> with tubules for 12 min gave equally valid results so that later experiments were carried out under these conditions. The addition of 7.5 ml of CHCl<sub>3</sub>: MeOH, 1:2, terminated the reaction. The CHCl<sub>3</sub> extracts (21) were chromatographed over SG81 paper as previously described (21). The amount of 1,25(OH)<sub>2</sub>D<sub>8</sub> produced was calculated from the percent of total radioactivity in the 1,25(OH)<sub>2</sub>D<sub>3</sub> peak on the radiochromatogram. Under these conditions the production of 1,25(OH)<sub>2</sub>D<sub>3</sub> was linear with time over the entire incubation period and proportional to the tubular protein concentration. Serum calcium was determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 290 (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Serum phosphate was determined by the method of Lowry and Lopez (22). Protein was measured by the method of Lowry, Rosebrough, Farr, and Randall (23) except that the samples were solubilized in 0.4% sodium lauryl sulfate.

### RESULTS

Dietary manipulations of calcium and phosphate. Manipulations of the dietary content of calcium and phosphate produced the changes in the serum calcium and phosphate concentrations shown in Fig. 1. As expected, increasing the dietary content of calcium increased the serum level of calcium. Similarly, increasing the dietary phosphate content led to increased serum phosphate levels. However, in general, increasing the phosphate content of the diet for any given calcium concentration resulted in a lowering of the serum calcium level. Likewise, increasing the calcium content of the diet at any given phosphate level decreased the serum phosphate level.

When the renal tubules were isolated from the animals raised on these eight different diets and assayed for their  $25(OH)D_{\bullet}$  1 $\alpha$ -hydroxylase activity, the results shown in Fig. 2 were obtained. The renal tubules from the different groups of chicks differed in their ability to produce  $1,25(OH)_{\bullet}D_{\bullet}$ , even though the isolation and assay procedures were performed at the same time under identical conditions. The tubules prepared from chicks raised on the diet containing 0.43% calcium and 0.3% P phosphate produced the most  $1,25(OH)_{\bullet}D_{\bullet}$ . Increasing or

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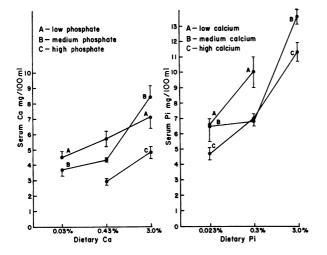


FIGURE 1 The effect of dietary calcium and phosphate content on the serum levels of calcium and phosphate in vitamin D-deficient chicks. Eight groups of chicks from the same original batch were grown on vitamin D-deficient diets differing only in their calcium and phosphate content. Four chicks from each group were sacrificed after 21 d. Renal tubules were prepared from their kidneys, and the calcium and phosphate levels in the serum of each chick were determined. The brackets enclose the mean  $\pm$ SEM of serum calcium (left) and serum phosphate (right) for each group of chicks.

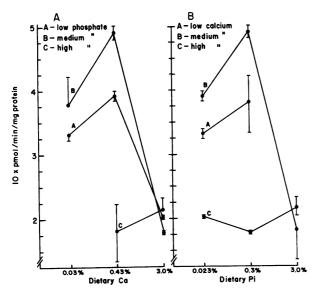


FIGURE 2 The effect of dietary calcium and phosphate content on  $1,25(OH)_{a}D_{a}$  production by the renal tubules isolated from the chicks described in Fig. 1. The brackets enclose the duplicate determinations of  $1,25(OH)_{a}D_{a}$  for each tubule preparation. The data for the eight tubule preparations, shown as a function of dietary calcium in A, are replotted as a function of dietary phosphate in B. 500 pmol of  $25OHD_{a}$  was used as the substrate. The incubation media contained 1 mM calcium, 1.2 mM phosphate, pH 7.4.

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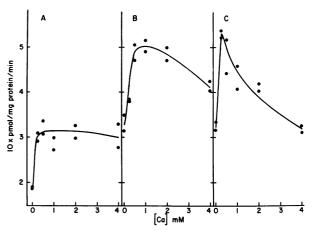


FIGURE 3 The effect of calcium on  $1,25(OH)_{s}D_{s}$  production in renal tubules prepared in 0, 1.2, or 6.0 mM phosphate Krebs-Ringer bicarbonate buffers, pH 7.4, in the absence of calcium. Just before the assay, the tubules were pooled and then divided into six samples, each of which was washed and incubated in a Krebs-Ringer bicarbonate buffer with the indicated phosphate concentration, containing 0.5 mM EGTA and the appropriate amount of calcium to give the free calcium concentrations shown. A = 0 mM phosphate, B = 1.2 mM phosphate, C = 6.0 mM phosphate, 500 pmol of 25OHD<sub>3</sub>.

decreasing either calcium or phosphate in the diet resulted in less 1,25(OH) De production by the isolated renal tubules. The mean serum calcium and phosphate levels of the animals showing optimal  $1\alpha$ -hydroxylase activity were 4.1 and 6.8 mg/100 ml P, respectively. No gross anatomic or microscopic differences in the kidneys from the different groups of chicks were found.

In vitro manipulations of calcium, phosphate, and hydrogen ion. The acute effect of calcium on 1,25-(OH) De production by tubules isolated and incubated in different phosphate concentrations is shown in Fig. 3. Tubules incubated in the presence of calcium produced more 1,25(OH) De than those incubated in its absence. Optimal calcium concentrations were 0.5-1.0 mM. Higher calcium concentrations resulted in less 1,25(OH) De production to an extent dependent on the concentration of phosphate in the medium. In the absence of phosphate, little difference in 1.25(OH).D. production was seen between tubules incubated in 0.5 mM calcium and 4.0 mM calcium (Fig. 3A). However, 4 mM calcium definitely limited 1,25(OH) D. production when the medium contained 1.2 mM phosphate (Fig. 3B), and the inhibitory effect of high calcium was even more pronounced at 6.0 mM phosphate (Fig. 3C).

If the tubules were incubated at pH 6.7 rather than 7.4 (Fig. 4), the 25OHD  $1\alpha$ -hydroxylase activity was less in the presence or absence of calcium.

The effect of hydrogen ion over the pH range of 6.5-8.0 on renal tubular 250HDs 1a-hydroxylase ac-

tivity is shown in Fig. 5. The rate of 1,25(OH)\*D\*production was greater at the more alkaline pH. In this experiment the pH was manipulated by adjusting the bicarbonate concentration of the medium, saturating the buffers with 5% CO\*. Other experiments, using different CO\* concentrations to manipulate the pH at fixed bicarbonate concentrations, indicated that it is the hydrogen concentration, not bicarbonate per se, that influences the rate of 1,25(OH)\*D\* production.

The acute effect of phosphate on 1,25(OH)<sub>2</sub>D<sub>8</sub> production by tubules isolated and incubated in different calcium concentrations is shown in Fig. 6. In the absence of calcium, phosphate stimulated 1,25(OH)2Ds production (Fig. 6A). This stimulation was not found in tubules prepared and incubated in 4 mM calcium (6B). However, in the presence of 1 or 2 mM calcium, conditions which resulted in greater 1,25(OH)2Da production than 0 or 4 mM calcium, the acute effect of phosphate on the 25OHD<sub>8</sub> 1a-hydroxylase activity was biphasic (Fig. 6C and D). Low phosphate concentrations (0.3-1.2 mM) caused less 1,25(OH)<sub>2</sub>D<sub>2</sub> production than the absence of phosphate, and increasing the phosphate concentration above 1.2 mM (2.4 or 6.0 mM) caused an increase in 1*a*-hydroxylase activity. This biphasic effect of phosphate on tubular production of 1,25(OH)<sub>2</sub>D<sub>2</sub> in the presence of 1 mM calcium was found at pH 6.8 as well as 7.4 (Fig. 7).

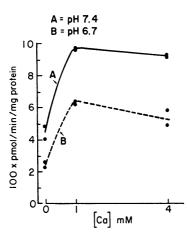


FIGURE 4 The effect of pH on the regulation by calcium of  $1,25(OH)_{2}D_{3}$  production in renal tubules. Renal tubules were prepared in a Krebs-Ringer bicarbonate buffer, pH 7.4, in the absence of calcium. The tubules were then divided into six portions. Three portions were washed and incubated in a Krebs-Ringer bicarbonate buffer, pH 6.7, containing 3.82 mM bicarbonate and 2.0 mM EGTA or sufficient calcium to give a free calcium concentration of 1 and 4 mM (B). The other three aliquots were washed and incubated in a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 25 mM bicarbonate and 2.0 mM EGTA or sufficient calcium to give a free calcium concentration of 1 and 4 mM (A). 25 pmol of  $250HD_{3}$  was used as substrate.

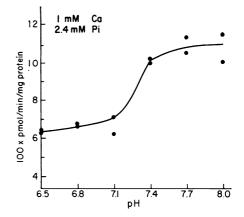


FIGURE 5 The effect of pH on  $1,25(OH)_2D_8$  production by renal tubules prepared in 1 mM calcium. Renal tubules were prepared in Krebs-Ringer bicarbonate buffers, pH 7.4, containing 1 mM calcium and 2.4 mM phosphate. Just before the assay, the tubules were pooled and divided into six aliquots. These were washed and incubated in a Krebs-Ringer bicarbonate buffer with 1 mM calcium and 2.4 mM phosphate containing 3.12 mM, 6.25 mM, 12.5 mM, 25 mM, 50 mM, or 100 mM bicarbonate saturated with 95% O<sub>2</sub>: 5% CO<sub>2</sub>. The pH of each buffer was determined before the assay. 25 pmol of 25OHD<sub>8</sub> was used as substrate.

### DISCUSSION

25OHD:  $1\alpha$ -hydroxylase activity most likely is controlled at two levels: rates of synthesis and degradation of the enzyme, leading to changes in the amount of enzyme present in the cell; and expression of activity of existing enzyme. Factors that control the expression of enzymatic activity should exert their effects immediately, whereas those factors regulating the turnover of the enzyme would be expected to have more prolonged but less immediate effects.

Most investigations of the control of  $1,25(OH)_2D_8$  production have utilized in vivo experiments in which the animal is given the radioactively labeled substrate, 25OHDs, or its precursor vitamin Ds, and the amount of  $1,25(OH)_2D_8$  appearing in the plasma or tissue of interest is determined hours later. The hormonal or nutritional manipulations being investigated preceded the administration of substrate by days or weeks.

Studies of  $1,25(OH)_2D_3$  synthesis in the isolated renal tubule in vitro offer four possible advantages over whole animal experiments: (a) they permit the regulation of calcium, phosphate, and hormone concentrations independent of each other; (b) they permit the measurement of a rate of  $1,25(OH)_2D_3$  production, not just a plasma or tissue content, which could be altered by changes in rates of either synthesis or catabolism; (c) they permit the evaluation of the acute effects of calcium, phosphate, and hormone manipulations as well as the long-term effects induced by the nutritional and hormonal status of the animal; and (d) they facilitate

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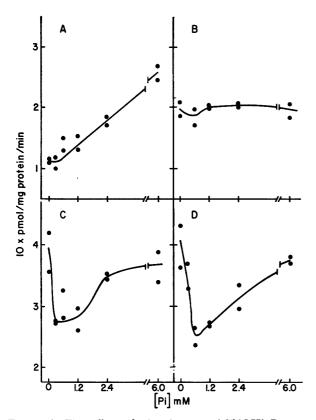


FIGURE 6 The effect of phosphate on  $1,25(OH)_2D_3$  production by renal tubules prepared in different calcium concentrations at pH 7.4. Renal tubules were prepared in 0, 1.0, 2.0, or 4.0 mM calcium Krebs-Ringer bicarbonate buffers, pH 7.4, in the absence of phosphate. Just before the assay the tubules were pooled and then divided into six aliquots, each of which was washed and incubated in a Krebs-Ringer bicarbonate buffer with the indicated calcium (or EGTA) concentration containing the appropriate phosphate concentration: A, 0.5 mM EGTA; B, 4.0 mM calcium; C, 1.0 mM calcium; D, 2.0 mM calcium. 500 pmol cf 25OHD<sub>3</sub> was used as substrate.

the quantitative recovery of the substrate administered and metabolites produced.

The studies in this report demonstrate the acute and long-term control calcium and phosphate exert on the production of  $1,25(OH)_*D_*$  by isolated renal tubules. Dietary manipulations of calcium and phosphate, producing pronounced changes in the serum calcium and phosphate levels, lead to changes in  $1,25(OH)_*D_*$  production by the isolated renal tubules when measured under standard conditions in vitro. The persistence of these differences in vitro suggest that such manipulations exert long-term changes in vivo in the total capacity of the kidney to produce  $1,25(OH)_*D_*$ . On the other hand, changes in the calcium, phosphate, and hydrogen concentrations of the incubation medium lead to differences in  $1,25(OH)_*D_*$  production by renal tubules isolated from the same group of chicks, indicating the

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role of these ions in the acute regulation of  $1\alpha$ -hydroxylase activity.

It is clear from these studies that both calcium and phosphate contribute to the regulation of the 25OHD<sub>a</sub>  $1\alpha$ -hydroxylase at both the long-term and acute level of control. The work from DeLuca's laboratory suggested a dominant role for calcium at first (9, 10), then, more recently, for phosphate (11) in the long-term regulation of 25OHD<sub>a</sub>  $1\alpha$ -hydroxylase activity. It is not clear from their reports that much consideration was given to the interaction of calcium and phosphate in the regulation of 1,25(OH)aDa production. Colston, Evans, Galante, MacIntyre, and Moss (24), however, mentioned their results pointing to such an interaction in the acute regulation of 1,25(OH)aDa production by renal homogenates.

Our data indicated that a vitamin D-deficient diet containing 0.43% calcium and 0.3% phosphate is optimal to achieve high rates of  $1,25(OH)_{a}D_{a}$  production. The mean serum levels of calcium and phosphate of the chicks grown on this optimal diet were 4.1 mg/100 ml calcium and 6.8 mg/100 ml phosphate. The renal tubules from groups of chicks raised on diets other than the above were less effective in  $1,25(OH)_{a}D_{a}$  production, even though some of the diets produced chicks with lower serum calcium or phosphate values. No morphologic differences in the kidney were found to explain this phe-

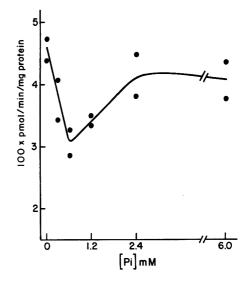


FIGURE 7 The effect of phosphate on  $1,25(OH)_{a}D_{a}$  production by renal tubules incubated at pH 6.8 in the presence of 1 mM calcium. Renal tubules were prepared in Krebs-Ringer bicarbonate buffers, pH 7.4, containing 1 mM calcium but no phosphate. Just before the assay, the tubules were divided into six aliquots, each of which was washed and then incubated in a Krebs-Ringer bicarbonate buffer containing 3.82 mM bicarbonate, 1 mM calcium, the indicated concentration of phosphate, and a pH adjusted to 6.8 after saturation with 95% O<sub>2</sub>:5% CO<sub>2</sub>. 25 pmol of 25OHD<sub>2</sub> was used as substrate.

nomenon. However, such changes in dietary intake of calcium and phosphate no doubt call into play a large number of homeostatic mechanisms, not least of which are parathyroid hormone and calcitonin secretion. Thus, a simple interpretation of these data is not possible. For this reason we chose to study the acute effects of calcium and phosphate on the regulation of the 250 HD<sub>8</sub>  $1\alpha$ -hydroxylase in vitro.

Calcium, phosphate, and hydrogen ion are interrelated complexly in the various cellular and extracellular compartments. Hence, their metabolic effects might be expected to be complex and interrelated. Such an interrelationship has been demonstrated in the control of gluconeogenesis by calcium, hydrogen, and phosphate in isolated renal tubules of the rat (25, 26). Changes in the extracellular concentration of one ion can be expected to change the intracellular concentration not only of the one ion in the various subcellular compartments, but of the other two ions as well.

Our data demonstrate that 0.5–1.0 mM calcium was necessary for optimal production of 1,25(OH)\*D\* by the renal tubules. Higher concentrations of calcium were inhibitory only if phosphate was present. An independent role for phosphate in the control of the  $1\alpha$ -hydroxylase activity was indicated by its ability to stimulate 1,25-(OH)\*D\* production in the absence of calcium. Hydrogen ion appeared to exert its effect primarily by altering basal  $1\alpha$ -hydroxylase activity.

The interrelationship between calcium and phosphate is indicated by the observation that the biphasic effect of calcium or phosphate on  $1,25(OH)_2D_3$  production was seen only when both ions were present. Thus, a high concentration of calcium (4 mM) inhibited 1,25-(OH)\_2D\_3 production acutely only if the tubules were prepared and incubated in the presence of phosphate. Low levels of phosphate (0.3-1.2 mM) inhibited acutely only if the tubules were prepared and incubated in the presence of 1-2 mM calcium.

In previous studies with a similar preparation of isolated renal tubules prepared from D-deficient chicks, Shain reported (27) that calcium had no effect upon the rate of conversion of 25OHDs to 1,25(OH)2Ds. This result is in contrast to the present results (Fig. 3). The differing results can be explained by differences in experimental techniques. As noted in the discussion and in the description of techniques, to obtain a significant and reproducible stimulation of the  $1\alpha$ -hydroxylase by Ca<sup>2+</sup>, the tubules had to be prepared in buffers with low calcium content. Shain did not observe this precaution in his studies. Equally important, as previously reported (20), we found the technique of thin-layer chromatography as employed by Shain (27) an inadequate method for separating 1,25(OH)<sub>2</sub>D<sub>8</sub> from all other metabolites. Because of this fact, the values for 1,25(OH)<sub>2</sub>D<sub>8</sub> re-

covery reported by Shain are open to question. Variable amounts of another as yet unidentified metabolite (20) contaminated his 1,25(OH)<sub>2</sub>D<sub>8</sub> peaks. These differences are probably sufficient to explain our discrepant results.

It is not clear from our studies with the intact cell how much of the effects of calcium, phosphate, and hydrogen ion can be attributed to a direct action on the 25OHD<sub>8</sub> 1 $\alpha$ -hydroxylase within the mitochondrion and how much to an indirect influence via some other aspect of cellular metabolism. To approach this problem, studies with isolated renal mitochondria are necessary. Such studies are reported in the following report (28).

#### ACKNOWLEDGMENTS

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