Regulation of vitamin D metabolism by calcium and phosphate ions in isolated renal tubules

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The acute and long-term effects of Ca^{2+} and P_i on vitamin D metabolism were studied *in vitro* with isolated renal tubules from vitamin D-deficient and vitamin D-supplemented chicks. Ca^{2+} depletion, achieved by isolating renal tubules in Ca^{2+} -free buffers, led to suppression of 1α -hydroxylase activity. Re-introduction of Ca^{2+} during incubation caused an acute stimulation of this enzyme. This stimulatory effect of Ca^{2+} was prevented by prior treatment of Ca^{2+} -depleted renal tubules for 6 h with 1,25-dihydroxycholecalciferol. Ca^{2+} and P_i produced marked acute effects on 1α -hydroxylase activity, which persisted for the whole 8 h experimental period, in Ca^{2+} -depleted renal tubules from vitamin D-deficient chicks. The effects of either ion were influenced by the concentration of the other. However, the effects of these ions could not be reproduced in either Ca^{2+} -depleted renal tubules from vitamin D-supplemented chicks or in renal tubules from vitamin D-deficient chicks, isolated in Ca^{2+} -containing buffers. Isolation of renal tubules from vitamin D-supplemented chicks in Ca^{2+} -containing buffers and subsequent incubation for 8 h in the presence of increased $[\text{Ca}^{2+}]$ led to a modest but statistically significant suppression of 1α -hydroxylase and stimulation of 24-hydroxylase activity. It is concluded that the acute effects of Ca^{2+} and P_i on 1α -hydroxylase activity of Ca^{2+} -depleted renal tubules from vitamin D-deficient chicks are not regulatory but the results of the experimental conditions. In contrast the long-term effects of Ca^{2+} on both hydroxylases of renal tubules from vitamin D-supplemented chicks may be of physiological significance.

Ca^{2+} and P_i were amongst the first factors to be implicated in the regulation of vitamin D metabolism. Studies carried out *in vivo* with vitamin D-supplemented animals have demonstrated that high plasma concentrations of either Ca^{2+} or P_i suppress the production of 1,25-dihydroxycholecalciferol and increase the production of 24,25-dihydroxycholecalciferol in a time-dependent manner (Boyle *et al.*, 1971; Larkins *et al.*, 1973; Tanaka & DeLuca, 1973).

Attempts to show direct effects of these ions *in vitro* produced conflicting results that in many cases were directly opposite to those obtained from studies *in vivo*. It must be emphasized that in all the studies *in vitro* the acute effects of Ca^{2+} and P_i were investigated in renal preparations from vitamin D-deficient animals in contrast with the studies *in vivo*, where the long-term effects of these ions were investigated in vitamin D-supplemented animals.

The aim of the present study was to investigate the nature of the effects of Ca^{2+} and P_i on vitamin D

metabolism *in vitro*. To this end the acute and long-term effects of these ions on vitamin D metabolism were studied in renal tubules from vitamin D-deficient and vitamin D-supplemented chicks.

Materials and methods

Animals

One-day-old cockerels (Rhode Island Red–Light Sussex crossbreed) were raised on a vitamin D-deficient diet containing 0.34% calcium and 0.54% phosphate for 3–5 weeks. Vitamin D_3 supplementation was achieved by injecting vitamin D_3 (20 i.u./day) in arachis oil into the crop.

Chemicals

Hyaluronidase (type I from bovine testes) and collagenase (type I from *Clostridium histoliticum*) were obtained from Sigma Chemical Co., Kingston upon Thames, Surrey, U.K. 25-Hydroxy[26,27-

$Me\text{-}^3H$]cholecalciferol (initial sp. radioactivity 9 Ci/mmol, but diluted to 160 Ci/mol with crystalline 25-hydroxycholecalciferol) and $[U\text{-}^{14}C]$ glucose (sp. radioactivity 288 Ci/mol) were supplied from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were purchased from commercial suppliers.

Preparation of isolated renal tubules

Suspensions of isolated renal tubules were prepared by a modification of the method of Burg & Orloff (1962) as follows. Chicks were killed by decapitation, their kidneys were removed and injected in their substance with 1 ml of an enzyme solution containing 0.1% (w/v) hyaluronidase and 0.15% (w/v) collagenase in Tris/acetate buffer of the following composition: Tris, 15 mmol/litre; NaCl, 125 mmol/litre; KCl, 4 mmol/litre; $MgSO_4$, 1.6 mmol/litre; pH brought to 7.4 with acetic acid. Ca^{2+} and P_i , when present, were added from concentrated stock solutions before the adjustment of the pH. The kidneys were minced and digested with the enzyme solution for 25–30 min with continuous shaking at 37°C. The fine suspension formed was then sieved through a nylon mesh (pore size 80–100 μm). Cold buffer was used to aid the filtration and inactivate the enzyme solution. The renal tubules were collected by centrifugation for 1 min at 50 g, resuspended in cold buffer containing different Ca^{2+} and P_i concentrations, depending on the experiment, and centrifuged again. The renal tubules were washed twice more with buffer containing the appropriate concentrations of Ca^{2+} and P_i and were collected by centrifugation as before. The final pellet was resuspended in the same buffer, but supplemented with 0.1% (w/v) gelatin and 10 mmol of glucose/litre and the renal tubules were evenly dispersed by continuous stirring on a magnetic stirrer. The suspension was then pipetted into exact 1.5 ml or 3 ml portions, depending on the experiment, in glass scintillation vials. The required concentrations of Ca^{2+} and P_i were achieved by adding 20 μl of concentrated stock solutions of $CaCl_2$ and NaH_2PO_4/Na_2HPO_4 in a 1:5 molar ratio, pH 7.4.

Enzyme assays

Vials, containing the isolated renal tubules, were gassed with 100% oxygen for 15 s, placed in a shaking water bath at 37°C (80 cycles/min) and pre-incubated for different time intervals in the presence of the required Ca^{2+} and P_i concentrations. Pre-incubation periods of 10–60 min were used in acute experiments and 8 h in long-term experiments. Enzyme assays were started by the addition of 30 pmol of 25-hydroxy[26,27- $Me\text{-}^3H$]cholecalciferol and the reaction was stopped 5–10 min later by the addition of 3 vol. of methanol/

chloroform (2:1, v/v) per volume of incubation mixture. Extraction, chromatography and identification of cholecalciferol metabolites were as described previously (Spanos *et al.*, 1978). Enzyme activities were calculated from the percentage of radioactivity within each chromatographic peak and expressed as fmol of metabolite produced/min per mg of protein. Estimation of protein was performed by the method of Lowry *et al.* (1951), but sodium citrate was used instead of sodium tartrate to give a more stable colour.

CO_2 production from glucose

The ability of the renal tubules to oxidize glucose was determined by the method of Shain & Barnea (1971).

Results

Tests of biochemical and structural integrity of the renal tubules

A combination of biochemical and morphological studies was used to investigate whether the renal tubules preparation was structurally intact and metabolically functional during the whole experimental period. As it can be seen in Fig. 1 the production of $^{14}CO_2$ from glucose was linear over the 8 h period studied. However, renal tubules incubated in the presence of a low Ca^{2+} con-

Fig. 1. Production of $^{14}CO_2$ from $[U\text{-}^{14}C]$ glucose in isolated renal tubules from vitamin D-deficient chicks incubated in the presence of either 0.2 or 2 mmol of Ca^{2+} /litre

Results are means $\pm$ S.E.M. for three observations.

centration (0.2 mmol/litre) oxidized glucose at a much slower rate than those in the presence of 2 mmol of Ca^{2+} /litre.

When the final cell suspension was examined under the light microscope, it was found to be almost free from damaged or broken cells. The preparation consisted of clumps of 5–20 cells, often aggregated in well-defined segments of tubules. Electron microscopy revealed that the cells were morphologically normal, with intact cell membranes and normal organelles, but the mitochondria were unusual in shape, perhaps as a result of vitamin D deficiency (DeLuca *et al.*, 1960).

Table 1. *Effect of Ca^{2+} depletion on 1 α -hydroxylase activity*

Renal tubules from vitamin D-deficient chicks were prepared in Ca^{2+} -free buffer and incubated for 10 min in the presence of the indicated $[\text{Ca}^{2+}]$ and $[\text{P}_i]$. Data are means $\pm$ S.E.M. for four to six observations. * $P < 0.001$.

$[\text{Ca}^{2+}]$ (mM)	$[\text{P}_i]$ (mM)	1 α -Hydroxylase activity (fmol of 1,25-dihydroxy- cholecalciferol/min per mg of protein)
Expt. 1		
0	2	30.7 $\pm$ 1.8
4	2	73.5 $\pm$ 1.4*
Expt. 2		
0	2	31.8 $\pm$ 1.4
1.2	2	77.1 $\pm$ 2.2*

Optimization of the assay conditions was achieved by studying the influence of substrate, protein concentration and time of incubation on the rate of 1,25-dihydroxycholecalciferol production by isolated renal tubules. As a consequence, all experiments were carried out at 3–5 mg of protein/ml, using incubation periods of 5–10 min.

Experiments in isolated renal tubules from vitamin D-deficient chicks

Renal tubules, isolated and washed three times in Ca^{2+} -free buffers (Ca^{2+} -depleted renal tubules), showed a very-low 1 α -hydroxylase activity when incubated for 10 min in the absence of Ca^{2+} , compared with those incubated in Ca^{2+} -containing media (Table 1). Addition of Ca^{2+} caused an acute stimulation of 1 α -hydroxylase activity in Ca^{2+} -depleted renal tubules that had been incubated for 6 h either in the absence or in the presence of an extremely low $[\text{Ca}^{2+}]$ (0.1 mmol/litre) (Table 2). However, this stimulatory effect of Ca^{2+} was prevented by treating the renal tubules for 6 h with 1,25-dihydroxycholecalciferol (Table 2).

In Ca^{2+} -depleted renal tubules, manipulations in the extracellular Ca^{2+} and P_i concentrations produced marked acute effects on 1 α -hydroxylase activity. Increasing $[\text{Ca}^{2+}]$ in the incubation medium from 0.2 mmol/litre to 5.8 mmol/litre had no effect on 1 α -hydroxylase activity when $[\text{P}_i]$ was 0.4 mmol/litre, but stimulated the enzyme activity in the presence of normal (2.0 mmol/litre) ($P < 0.05$) or high (6.4 mmol/litre) $[\text{P}_i]$ ($P < 0.01$) (Table 3).

Table 2. *Prevention of the stimulatory effect of Ca^{2+} on 1 α -hydroxylase activity of Ca^{2+} -depleted renal tubules, from vitamin D-deficient chicks, by treatment with 1,25-dihydroxycholecalciferol*

In Expt. 1 renal tubules from 16-day-old vitamin D-deficient chicks were prepared in a Tris/acetate buffer containing no Ca^{2+} and 1 mM- P_i . They were pre-incubated in the same buffer for 6 h either with (62.5 nM) or without 1,25-dihydroxycholecalciferol. The tubules were centrifuged for 1 min at 1000 rev./min and resuspended in a fresh buffer, free of 1,25-dihydroxycholecalciferol, but containing the indicated Ca^{2+} concentration, and incubated for 10 min in the presence of the labelled substrate. Data are means $\pm$ S.E.M. for four observations. In Expt. 2 the experimental protocol was exactly the same as in Expt. 1, except that the 6 h pre-incubation period was carried out in the presence of 0.1 mM- Ca^{2+} instead of in the absence of Ca^{2+} . Data are means $\pm$ S.E.M. for six observations.

$[\text{Ca}^{2+}]$ (mM)	1,25-Dihydroxycholecalciferol	1 α -Hydroxylase activity (fmol of 1,25-dihydroxycholecalciferol/min per mg of protein)
Expt. 1		
0	—	29.3 $\pm$ 0.6
1.0	—	50.0 $\pm$ 4.3
0	+	24.5 $\pm$ 0.7
1.0	+	29.7 $\pm$ 1.2
Expt. 2		
0.1	—	24.8 $\pm$ 2.5
1.0	—	45.7 $\pm$ 2.2
3.6	—	46.7 $\pm$ 3.1
0.1	+	22.8 $\pm$ 0.9
1.0	+	22.4 $\pm$ 1.9
3.6	+	20.7 $\pm$ 1.2

Table 3. *Acute effects of Ca^{2+} on 1 α -hydroxylase activity in Ca^{2+} -depleted renal tubules from vitamin D-deficient chicks*

Renal tubules were prepared in Ca^{2+} -free buffer containing 0.4 mM- P_i and incubated for 15 min in the presence of the indicated Ca^{2+} and P_i concentrations. Data are means $\pm$ S.E.M. for three to four determinations.

		1 α -Hydroxylase activity (fmol of 1,25-dihydroxy- cholecalciferol/min per mg of protein)
[Ca^{2+}] (mM)	[P_i] (mM)	
0.2	0.4	45.0 $\pm$ 3.5
1.2	0.4	43.6 $\pm$ 1.2
5.8	0.4	44.8 $\pm$ 1.8
0.2	2.0	33.7 $\pm$ 0.8
1.2	2.0	36.9 $\pm$ 0.6
0.2	6.4	27.5 $\pm$ 1.7
1.2	6.4	34.4 $\pm$ 1.0
0.2	18.4	26.1 $\pm$ 0.5
0.4	18.4	22.8 $\pm$ 1.0

Table 4. *Long-term effects of Ca^{2+} on 1 α -hydroxylase activity in Ca^{2+} -depleted renal tubules from vitamin D-deficient chicks*

Renal tubules were prepared in Ca^{2+} -free buffer containing 0.4 mM- P_i and incubated for 8 h in the presence of the indicated Ca^{2+} and P_i concentrations. Data are means $\pm$ S.E.M. for three to four determinations.

		1 α -Hydroxylase activity (fmol of 1,25-dihydroxy- cholecalciferol/min per mg of protein)
[Ca^{2+}] (mM)	[P_i] (mM)	
0.2	0.4	24.8 $\pm$ 0.6
1.2	0.4	25.2 $\pm$ 1.4
5.8	0.4	27.1 $\pm$ 3.9
0.2	2.0	15.2 $\pm$ 1.6
1.2	2.0	19.1 $\pm$ 0.3
0.2	6.4	14.3 $\pm$ 1.5
1.2	6.4	22.5 $\pm$ 1.0
0.2	18.4	20.1 $\pm$ 0.6
0.4	18.4	17.9 $\pm$ 0.2

Increasing the P_i concentration in the presence of either low (0.2 mmol/litre) or normal [Ca^{2+}] (1.2 mmol/litre) caused a marked suppression of 1 α -hydroxylase activity (Table 3). This inhibitory effect of P_i was partially overcome by increasing the [Ca^{2+}] in the medium (Table 3). Similar results were obtained when the incubation period was extended to 8 h (Table 4).

In contrast, wide variations in the extracellular [Ca^{2+}] and [P_i] had no effect on 1 α -hydroxylase activity in renal tubules prepared in media containing either normal (Table 5) or low [Ca^{2+}] and [P_i] (results not shown).

Table 5. *Effects of Ca^{2+} and P_i on 1 α -hydroxylase activity in Ca^{2+} -repleted renal tubules from vitamin D-deficient chicks*

Renal tubules were prepared in buffers containing either normal [Ca^{2+}] (1.2 mM) and normal [P_i] (2.0 mM) (Expt. 2) or normal [Ca^{2+}] and low [P_i] (0.4 mM) (Expt. 1) and incubated for either 1 (Expt. 1) or 8 h (Expt. 2) in the presence of the indicated ion concentrations. Data are means $\pm$ S.E.M. for five (Expt. 1) and three (Expt. 2) observations respectively.

		1 α -Hydroxylase activity (fmol of 1,25-dihydroxy- cholecalciferol/min per mg of protein)
[Ca^{2+}] (mM)	[P_i] (mM)	
Expt. 1		
0	2.0	50.3 $\pm$ 2.9
0.2	2.0	55.2 $\pm$ 2.6
1.0	2.0	50.4 $\pm$ 2.4
5.0	2.0	53.4 $\pm$ 3.5
Expt. 2		
1.2	2.0	50.5 $\pm$ 0.3
3.6	2.0	54.2 $\pm$ 3.9
1.2	6.4	50.7 $\pm$ 2.9
2.0	6.4	52.1 $\pm$ 3.3

Experiments in isolated renal tubules from vitamin D-supplemented chicks

Renal tubules prepared in Ca^{2+} -free media had a lower 1 α -hydroxylase activity and a higher 24-hydroxylase activity when incubated in the absence of Ca^{2+} compared with those incubated in Ca^{2+} -supplemented media (results not shown). However, provided that as little as 0.2 mmol of Ca^{2+} /litre was present during incubation, wide variations in [Ca^{2+}] and [P_i] in the medium had neither acute nor long-term effects on 24-hydroxylase or 1 α -hydroxylase enzyme activities, even though the renal tubules had been prepared in Ca^{2+} -free buffers (Table 6). That the renal tubules were responsive is indicated by comparing the enzyme activities of the acute and long-term experiments (Table 6, Expts. 1 and 2). Extending the incubation period from 15 min to 8 h in a medium free of 1,25-dihydroxycholecalciferol resulted in a marked suppression of 24-hydroxylase and a profound stimulation of 1 α -hydroxylase activity independently of the [Ca^{2+}] and [P_i] in the medium (Table 6, Expt. 2). When the long term effects of Ca^{2+} were tested in renal tubules prepared in buffers containing normal [Ca^{2+}] and [P_i], increasing the concentration of Ca^{2+} from 0.2 mmol/litre to 3.6 mmol/litre in the presence of normal [P_i] caused a significant inhibition of 1 α -hydroxylase ($P < 0.01$) and a significant stimulation of 24-hydroxylase activity ($P < 0.05$) (Fig. 2). Ca^{2+} and P_i had no acute effects on the renal hydroxylases of similarly prepared renal tubules.

Table 6. Effects of Ca^{2+} and P_i on the renal 25-hydroxycholecalciferol hydroxylases from vitamin D-supplemented chicks

Renal tubules from 14-day-old vitamin D-supplemented chicks (20 i.u./day) were prepared in Ca^{2+} -free buffers containing 0.4 mM- P_i and incubated for either 15 min (Expt. 1) or 8 h (Expt. 2) in the presence of the indicated ion concentrations. Data are means $\pm$ S.E.M. for three observations.

[Ca ²⁺] (mM)	[P _i] (mM)	Enzyme activity (fmol of metabolite produced/min per mg of protein)	
		24-Hydroxylase	1α-Hydroxylase
Expt. 1			
0.2	0.4	55.8 ± 3.5	14.0 ± 1.4
1.2	0.4	48.4 ± 3.4	12.4 ± 0.8
5.8	0.4	49.6 ± 3.1	12.8 ± 0.5
0.2	2.0	47.7 ± 2.8	12.9 ± 0.9
5.8	2.0	52.9 ± 1.2	12.4 ± 1.5
0.2	6.4	50.9 ± 3.8	11.9 ± 0.5
1.2	6.4	45.5 ± 3.7	12.8 ± 0.9
Expt. 2			
0.2	0.4	24.9 ± 1.3	27.2 ± 0.8
1.2	0.4	28.7 ± 1.0	22.3 ± 0.9
5.8	0.4	27.5 ± 1.3	29.7 ± 1.2
0.2	2.0	23.2 ± 3.0	22.9 ± 0.6
5.8	2.0	30.5 ± 2.7	30.2 ± 1.7
0.2	6.4	28.4 ± 1.5	26.7 ± 1.7
1.2	6.4	29.7 ± 1.7	24.2 ± 1.7

Fig. 2. Long-term effects of Ca^{2+} on the renal 25-hydroxycholecalciferol hydroxylases

Renal tubules from vitamin D-supplemented chicks (20 i.u./day) were prepared in buffers containing normal [Ca^{2+}] and [P_i] and incubated for 8 h in the presence of normal [P_i] and the indicated [Ca^{2+}]. Open columns represent 24-hydroxylase and hatched columns represent 1 α -hydroxylase activity. Results are means $\pm$ S.E.M. for four to five observations.

Discussion

Our results demonstrate that Ca^{2+} and P_i exerted variable effects on 1 α -hydroxylase activity, depending on the method of isolation of renal tubules and whether they were from vitamin D-deficient or vitamin D-supplemented chicks. This variability can be explained when one considers the influence of Ca^{2+} depletion on vital biochemical processes and the effect of vitamin D-depletion on mitochondrial function and morphology.

The suppression of 1 α -hydroxylase activity after Ca^{2+} depletion and its stimulation on subsequent addition of Ca^{2+} (Table 1) strongly suggests that some Ca^{2+} is essential for renal tubules to fully express their 1 α -hydroxylase activity. But Ca^{2+} is also essential for a number of vital biochemical processes inside the renal cells. O_2 consumption (Burg & Orloff, 1962) and oxidation of glucose (Fig. 1) by isolated renal tubules is very low when the extracellular [Ca^{2+}] is low. Moreover, Ca^{2+} depletion is associated with changes in cell-membrane permeability (Diamond & Wright, 1969; Rasmussen, 1970), mitochondrial respiration (Carafoli & Lehninger, 1971) and inhibition of the activity of a number of mitochondrial enzymes, such as pyruvate carboxylase and succinate dehydrogenase (Kimmich & Rasmussen, 1969; Ogata *et al.*, 1972). It is therefore conceivable that Ca^{2+} depletion, by affecting both the biochemical integrity and the energy production of the renal cells, may limit the supply of reducing equivalents, leading to a non-specific inhibition of the 1 α -hydroxylase activity. Alternatively, since Ca^{2+} has been shown to enhance the proportion of cytochrome *P*-450 bound to the cholesterol substrate in adrenal mitochondria (Simpson & Williams-Smith, 1975), Ca^{2+} depletion may suppress the 1 α -hydroxylase activity by disrupting the mechanism for directing the 25-hydroxycholecalciferol substrate to the cytochrome *P*-450 component of the 1 α -hydroxylase enzyme. In any event, not only are both mechanisms consistent with an essential rather than a regulatory role for Ca^{2+} , but Ca^{2+} depletion also represents an artificially created unphysiological state not likely to occur to such an extreme extent under any conditions *in vivo*. This last point is further supported by the fact that a decrease in mitochondrial Ca^{2+} *in vivo*, as that seen in vitamin D-deficient animals (Kimmich & Rasmussen, 1969) is associated with very-high 1 α -hydroxylase activity, a situation that is exactly opposite to that observed after Ca^{2+} depletion *in vitro*.

Vitamin D-deficiency is associated with altered mitochondrial shape (the present study; DeLuca *et al.*, 1960) and with a decrease in mitochondrial Ca^{2+} (Kimmich & Rasmussen, 1969; Sampson *et al.*, 1970; Pleasure *et al.*, 1979). It has been suggested

that as a result of the Ca^{2+} loss the mitochondrial buffer system is slowly depleted and ceases to function (Borle, 1971, 1972, 1975). The discovery of a Ca^{2+} -binding protein in mitochondria (Sottocasa *et al.*, 1971) is also consistent with vitamin D playing a role in Ca^{2+} binding by these organelles. These changes may explain why renal tubules from vitamin D-deficient chicks are particularly susceptible to Ca^{2+} depletion, a condition that may account for the acute effects of Ca^{2+} and P_i on 1α -hydroxylase activity.

It is known that Ca^{2+} and P_i are linked in a complex manner in the various cellular compartments and our results indicate a close interrelationship between the effects of these ions on 1α -hydroxylase activity (Tables 3 and 4). Borle (1975) also demonstrated that the concentration of Ca^{2+} in the mitochondria is a function of the solubility product constant of the various calcium phosphate precipitates and inversely proportional to the concentration of P_i . A rise in intramitochondrial $[\text{P}_i]$, such as that produced by increasing the extracellular $[\text{P}_i]$, has been shown to increase the sequestration and precipitation of Ca^{2+} and P_i leading to a reduction in free Ca^{2+} in these organelles (Borle, 1975). The limited amount of Ca^{2+} and the possibly impaired mitochondrial buffering capacity of renal tubules from vitamin D-deficient chicks may cause them to become more susceptible to Ca^{2+} -depletion treatment and more sensitive to changes in extracellular concentrations of Ca^{2+} and P_i in the medium. It is therefore possible that, in renal tubules from vitamin D-deficient chicks, a rise in intramitochondrial P_i by decreasing further the already low free Ca^{2+} concentration, may lead to a non-specific inhibition of 1α -hydroxylase activity. In accordance with this interpretation are our observations showing that the inhibition of 1α -hydroxylase activity by phosphate was more pronounced in the presence of low than in the presence of normal $[\text{Ca}^{2+}]$ in the medium (Tables 3 and 4). Moreover, the inhibitory effect of P_i was partially overcome by increasing the Ca^{2+} concentration in the medium (Tables 3 and 4). An alternative explanation may be that the effects of Ca^{2+} and P_i are due to a direct action of these ions on 1α -hydroxylase activity. However, such an explanation could not account for the failure of Ca^{2+} and P_i to affect the 1α -hydroxylase in renal tubules from vitamin D-deficient chicks, which had been isolated in Ca^{2+} -containing buffers (Table 5).

If the acute effects of Ca^{2+} and P_i on 1α -hydroxylase activity were of physiological relevance, as has been suggested (Bikle & Rasmussen, 1975; Horiuchi *et al.*, 1975), one would expect to reproduce them in renal tubules from vitamin D-supplemented chicks. However, wide variations in extracellular $[\text{Ca}^{2+}]$ and $[\text{P}_i]$ had no effect on 1α -

hydroxylase activity of renal tubules from vitamin D-supplemented chicks, even though they had been subjected to the same Ca^{2+} -depletion treatment as those from vitamin D-deficient chicks (Table 6). These results indicate that preparations from vitamin D-deficient chicks may not be suitable systems *in vitro* to study the effects of Ca^{2+} and P_i on vitamin D metabolism. Unfortunately, the concept of acute regulation of 1α -hydroxylase activity by these ions was based exclusively on observations *in vitro* in preparations from vitamin D-deficient chicks. The fact that Ca^{2+} and P_i were shown to have no effect on (Shain, 1972; Larkins *et al.*, 1974), to inhibit (Colston *et al.*, 1973; Fraser & Kodicek, 1973; Henry & Norman, 1974) or to stimulate (Horiuchi *et al.*, 1975; Bikle & Rasmussen, 1975; Bikle *et al.*, 1975) the 1α -hydroxylase activity under different experimental conditions and that K^+ and acetate, not normally thought to play a role in vitamin D metabolism, were shown to stimulate the 1α -hydroxylase activity (Bikle & Rasmussen, 1978a,b), strongly suggests that caution is needed in interpreting results produced in preparations from vitamin D-deficient animals. All these results taken together with observations showing that Ca^{2+} and P_i have no effects on the activity of the reconstituted 1α -hydroxylase enzyme (DeLuca, 1976) make the concept of acute regulation of 1α -hydroxylase by Ca^{2+} and P_i most unlikely.

The evidence from studies *in vivo* strongly suggests that Ca^{2+} and P_i exert long-term effects on the renal hydroxylases of vitamin D-supplemented animals (DeLuca & Schnoes, 1976). High plasma concentrations of these ions were shown to suppress the 1α -hydroxylase and to stimulate the 24 -hydroxylase activity (Boyle *et al.*, 1971; Tanaka & DeLuca, 1973). Our results show that high $[\text{Ca}^{2+}]$ exerts long-term effects similar to those observed *in vivo* in renal tubules from vitamin D-supplemented chicks, isolated in Ca^{2+} -containing buffers (Fig. 2). That Ca^{2+} acts directly to affect vitamin D metabolism has also been demonstrated with preparations with a life span much longer than that of isolated renal tubules. MacIntyre *et al.* (1979), using primary chick kidney cell cultures, were able to show that incubation for 48 h in the presence of high $[\text{Ca}^{2+}]$ caused a marked suppression of 1α -hydroxylase and profound stimulation of 24 -hydroxylase activity. Moreover, in monkey kidney cell cultures, which lacked 1α -hydroxylase activity, Juan & DeLuca (1977) showed that incubation for 5 days in the presence of high $[\text{Ca}^{2+}]$ produced a 3-fold increase in 24 -hydroxylase activity. These results are in accordance with observations *in vivo* and strongly suggest that Ca^{2+} acts directly on the renal cells to affect vitamin D metabolism. However, whether phosphate also exerts similar long-term direct effects on the renal hydroxylases remains to be established.

References

- Bikle, D. D. & Rasmussen, H. (1975) *J. Clin. Invest.* **55**, 292–298
- Bikle, D. D. & Rasmussen, H. (1978a) *Biochim. Biophys. Acta* **538**, 127–138
- Bikle, D. D. & Rasmussen, H. (1978b) *J. Biol. Chem.* **253**, 3042–3048
- Bikle, D. D., Murphy, E. W. & Rasmussen, H. (1975) *J. Clin. Invest.* **55**, 299–304
- Borle, A. B. (1971) in *Cellular Mechanisms for Calcium Transfer and Homeostasis* (Nickols, G. & Wasserman, R. H., eds.), pp. 151–171, Academic Press, New York
- Borle, A. B. (1972) *J. Membr. Biol.* **10**, 45–66
- Borle, A. B. (1975) in *Calcium Regulating Hormones* (Talmage, R. V., Owen, M. & Parsons, J. A., eds.), pp. 217–228, Excerpta Medica, Amsterdam
- Boyle, I. T., Gray, R. W. & DeLuca, H. F. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2131–2134
- Burg, M. B. & Orloff, J. (1962) *Am. J. Physiol.* **203**, 327–330
- Carafoli, E. & Lehninger, A. L. (1971) *Biochem. J.* **122**, 681–690
- Colston, K. W., Evans, I. M. A., Galante, L. S., MacIntyre, I. & Moss, D. W. (1973) *Biochem. J.* **134**, 817–820
- DeLuca, H. F. (1976) *Clin. Endocrinol.* **5**, Suppl. 97s–108s
- DeLuca, H. F. & Schnoes, H. K. (1976) *Annu. Rev. Biochem.* **45**, 631–666
- DeLuca, H. F., Reiser, S., Steenbock, H. & Kaesberg, P. (1960) *Biochem. Biophys. Acta* **40**, 526–530
- Diamond, J. M. & Wright, E. M. (1969) *Annu. Rev. Physiol.* **31**, 581–646
- Fraser, D. R. & Kodicek, E. (1973) *Nature (London)* **241**, 163–166
- Henry, H. L. & Norman, A. W. (1974) *J. Biol. Chem.* **249**, 7529–7535
- Horiuchi, N., Suda, T., Sasaki, S., Ogata, E., Ezawa, I., Sano, Y. & Shimazawa, E. (1975) *Arch. Biochem. Biophys.* **171**, 540–548
- Juan, D. & DeLuca, H. F. (1977) *Endocrinology* **101**, 1184–1193
- Kimmich, G. A. & Rasmussen, H. (1969) *J. Biol. Chem.* **244**, 190–199
- Larkins, R. G., MacAuley, S. J., Colston, K. W., Evans, I. M. A., Galante, L. S. & MacIntyre, I. (1973) *Lancet* **ii**, 289–291
- Larkins, R. G., MacAuley, S. J., Rapaport, A., Martin, T. J., Tulloch, B. R., Byfield, P. G. H., Matthews, E. W. & MacIntyre, I. (1974) *Clin. Sci. Mol. Med.* **46**, 569–582
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- MacIntyre, I., Brown, D. J. & Spanos, E. (1979) in *Vitamin D Basic Research and its Clinical Application* (Norman, A. W., Schaefer, K., von Herrath, D., Grigoleit, H. G., Coburn, J. W., DeLuca, H. F., Mawer, E. B. & Suda, T., eds), pp. 523–529, Walter de Gruyter, Berlin and New York
- Ogata, E., Kondo, K., Kimura, S. & Yoshitoshi, Y. (1972) *Biochem. Biophys. Res. Commun.* **46**, 640–645
- Pleasure, D., Wyszynski, B., Sumner, A., Schotland, D., Feldman, B., Nugent, N., Hitz, G. & Goodman, D. B. P. (1979) *J. Clin. Invest.* **64**, 1157–1167
- Rasmussen, H. (1970) *Science* **170**, 404–412
- Sampson, H. W., Matthews, J. L., Martin, J. H. & Kunin, A. S. (1970) *Calcif. Tissue Res.* **5**, 305–316
- Shain, S. A. (1972) *J. Biol. Chem.* **247**, 4404–4413
- Shain, S. A. & Barnea, A. (1971) *Endocrinology* **89**, 1270–1279
- Simpson, E. R. & Williams-Smith, D. L. (1975) *Biochim. Biophys. Acta* **404**, 304–320
- Sottocasa, G. L., Sandri, G., Panfili, E. & De Bernard, B. (1971) *FEBS Lett.* **17**, 100–103
- Spanos, E., Barrett, D. I., Chong, K. T. & MacIntyre, I. (1978) *Biochem. J.* **174**, 231–236
- Tanaka, Y. & DeLuca, H. F. (1973) *Arch. Biochem. Biophys.* **154**, 566–574