The effect of dietary calcium on the activity of 25-hydroxycholecalciferol-1-hydroxylase and Ca absorption in vitamin D-replete chicks

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(*Received* 9 *August* 1976 – *Accepted* 14 *September* 1976)

1. As most of the studies on the regulation of renal 25-hydroxycholecalciferol-1-hydroxylase (25-HCC-1-hydroxylase) activity have been done in marginally-vitamin D-deficient animals and as it is known that vitamin D administration suppresses the specific activity of the 25-HCC-1-hydroxylase, it was decided to study the effect of dietary calcium on the activity of 25-HCC-1-hydroxylase and on Ca absorption in vitamin D-replete chicks.

2. Chicks, 10 d old, were given diets differing in their Ca contents (65 nmol cholecalciferol/kg diet) for 10 d and the activity of 25-HCC-1-hydroxylase in kidney homogenates, Ca absorption from the duodenum, Ca-binding protein (CaBP) activity in the duodenal mucosa and plasma Ca and phosphate concentrations were all determined.

3. The CaBP activity and the efficiency of Ca absorption both decreased with increasing dietary intake of Ca. Ca absorption and CaBP activity were significantly correlated (r 0·995, $P < 0·01$).

4. The activity of 25-HCC-1-hydroxylase decreased as the dietary level of Ca increased and was significantly correlated with Ca absorption (r 0·900, $P < 0·05$). The plasma Ca concentration and the activity of 25-HCC-1-hydroxylase were inversely related (r −0·940, $P < 0·01$).

5. It is concluded that in the vitamin D-replete chick the efficiency of duodenal Ca absorption is regulated by the renal 25-HCC-1-hydroxylase activity via production of 1,25-dihydroxycholecalciferol and CaBP synthesis.

The efficiency of intestinal calcium absorption has been shown to vary with the requirements of the body for Ca. During periods of rapid growth, pregnancy, lactation and dietary Ca deprivation, there is an increase in the efficiency of Ca absorption (Nicolaysen, Eeg-Larsen & Malm, 1953; Kenny, 1975). Although the mechanism by which this is brought about is uncertain, it is well known that vitamin D is required for this adaptive response to occur (Nicolaysen *et al.* 1953).

Cholecalciferol (CC) is converted by the liver to 25-hydroxycholecalciferol (25-HCC), the major circulating form of CC (Blunt, DeLuca & Schnoes, 1968). This is further hydroxylated in the mitochondria of kidney tubule cells to form 1,25-dihydroxycholecalciferol (1,25-DHCC) (Holick, Schnoes, DeLuca, Suda & Cousins, 1971; Lawson, Fraser, Kodicek, Morris & Williams, 1971). Since 1,25-DHCC is the form of CC which is active in increasing the rate of Ca absorption, probably by stimulating the synthesis of Ca-binding protein (CaBP) (Tsai, Wong & Norman, 1972; Corradino, 1973), it has been suggested that the rate of intestinal Ca absorption could be controlled by modulation of the metabolism of 25-HCC in response to dietary Ca changes (Boyle, Gray & DeLuca, 1971). In rats fed on a low-Ca diet, the major metabolite of 25-HCC was 1,25-DHCC and in those fed on a high-Ca diet it was 24,25-dihydroxycholecalciferol (24-25-DHCC) (Boyle, *et al.* 1971). In accord with these changes in metabolite patterns found in vivo, dietary Ca deprivation in chicks resulted in increased formation of 1,25-DHCC from 25-HCC by kidney homogenates in vitro. Kidneys from chicks fed on a high-Ca diet produced mainly 24,25-DHCC (Omdahl, Gray, Boyle, Knutson & DeLuca, 1972). These studies were done in animals which were

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marginally vitamin D deficient. Since the administration of CC to vitamin D-deficient chicks has been shown to suppress the activity of 25-HCC-1-hydroxylase (Fraser & Kodicek, 1973), it was possible that no marked changes in enzyme activity in response to different Ca intakes would be shown in vitamin D-replete chicks. This study was undertaken to see if such changes do occur and if so, to study the relationship between Ca absorption and the activity of the renal 25-HCC-1-hydroxylase.

MATERIALS AND METHODS

Male, 1-d-old chicks (Type 909; Thornbers, Hebden Bridge, Yorks.) were maintained on a commercial diet (10 g Ca/kg) for 10 d; then they were weighed and divided into six groups. Each group received one of six artificial diets which differed only in their Ca content (see Table 2). The composition of the diet was similar to that described by Morrissey & Wasserman (1971), and contained (/kg) 6·5 g phosphorus, 65 nmol CC.

After a period of 10 d, the chicks were fasted for 4 h and Ca absorption was determined in vivo by the ligated-loop technique (Swaminathan & Care, 1975). Each chick was anaesthetized with diethyl ether and the duodenal loop was exteriorized. A solution containing 0·15 M-sodium chloride, 1 mg Ca/ml and 0·2 μCi ^{47}Ca/ml (pH 7·0) was injected into the proximal loop of the duodenum and the segment was replaced in the abdominal cavity. After 30 min, a blood sample was taken by cardiac puncture and the chick was decapitated. The duodenum and kidneys were removed. The kidney was placed on ice immediately. The ligated duodenal segment was excised and placed in a tube for measurement of radioactivity.

The distal segment of the duodenum was excised and slit open, rinsed thoroughly in ice-cold Tris buffer (pH 7·4), blotted and the mucosa scraped off with a microscope slide. The mucosa was homogenized in 4 vol. ice-cold Tris buffer ($1·37 \times 10^{-2}$ M-Tris-HCl, 0·12 M-NaCl, $4·7 \times 10^{-3}$ M-potassium chloride, pH 7·4). The homogenate was centrifuged at 17500 *g* for 20 min using a refrigerated centrifuge (Omicron; Griffin & George Ltd, Middlesex) at 4°. The supernatant was stored at −20° for analysis of total protein content and CaBP activity.

A kidney homogenate (100 mg/ml) was prepared in 15 mM-Tris-acetate buffer, pH 7·4, containing 1·9 mM-magnesium chloride, 5 mM-sodium succinate, 200 mM-sucrose, by grinding in a Potter-Elvehjem homogenizer. A 1·5 ml portion of this homogenate was placed in a 25 ml Erlenmeyer flask, flushed with oxygen for 30 s and pre-incubated for 5 min at 39°. At the end of the pre-incubation period, 1 nmol 25-[26,27-^{3}H]-HCC (16 Ci/mol; The Radiochemical Centre, Amersham, Bucks.) dissolved in 20 μl aqueous ethanol (980 ml/l) was added to each incubation mixture and the flask gassed with O_2 for an additional 30 s. The amount of 25 HCC used was similar to that used by Henry, Midgett & Norman (1974) and is approximately three times the value for the Michaelis constant of the 25 HCC-1-hydroxylase. Flasks were incubated for 15 min and at the end of the incubation period the incubation mixture was rapidly cooled to −20° and stored until required for extraction.

Lipid extracts for the incubation mixture were prepared by the method described by Mawer & Backhouse (1969). The chloroform was evaporated under nitrogen and the extracted lipids were redissolved in chloroform–hexane (65:35, v/v) to be chromatographed on Sephadex LH20 (Pharmacia Ltd, Uppsala, Sweden) (0·55 m × 0·14 m glass column, flow-rate 0·7 ml/min) with chloroform–hexane (65:35, v/v) as the eluent. Sixteen 20 ml fractions were collected, and dried under air in liquid scintillation vials. Scintillation fluid (containing (/l toluene) 0·05 g POPOP and 4 g PPO) (10 ml) was added to each vial and the amount of radioactivity measured using a liquid-scintillation counter (ICN Tracer Laboratories, Weybridge, Surrey). The elution profiles of the CC metabolites had been previously determined with standards of radioactively-labelled 25-HCC, 24,25-DHCC and 1,25-DHCC.

Table 1. *Influence of dietary calcium level on the in vitro metabolism of 25-[26-27^{3}H]-HCC by kidney homogenates in vitamin D-replete chicks*

(Mean values with their standard errors for four chicks/treatment)

Dietary Ca (g/kg)	CaBP activity of duodenal mucosa (net s:r/mg protein)	Plasma concentration (mmol/l)		In vitro metabolism of 25-HCC† (% 25-HCC converted)	
		Ca	Phosphate	1,25-DHCC	24,25-DHCC
1	0·761 ± 0·0544	2·15 ± 0·22	2·81 ± 0·42	46·4 ± 5·1	7·0 ± 1·1
20	0·376 ± 0·0355**	2·70 ± 0·05*	2·45 ± 0·03	3·9 ± 0·4***	25·2 ± 3·0***

25-HCC, 25-hydroxycholecalciferol; 1,25-DHCC, 1,25-dihydroxycholecalciferol; 24,25-DHCC, 24,25-dihydroxycholecalciferol; CaBP, Ca-binding activity; s:r, total radioactivity in supernatant fraction: total radioactivity in resin (for details, see p. 48).

Significance of difference between groups given high- and low-Ca diets: * $P < 0·05$, ** $P < 0·005$, *** $P < 0·001$.

† For details of procedures, see p. 48.

The results were expressed as pmol 1,25-DHCC or 24,25-DHCC formed/min per mg protein.

The activity of CaBP was determined by the Chelex-resin method (Wasserman, Corradino & Taylor, 1968). A portion of mucosal supernatant fraction was mixed with a suspension of a cation-exchange resin, Chelex 100 (Biorad. Lab, Richmond, Calif., USA), and a tracer amount of ^{45}Ca. After centrifugation, a portion of the supernatant fraction was assayed for ^{45}Ca. The results were expressed as the ratio, total radioactivity in the supernatant fraction: total radioactivity in the resin, relative to the total protein content of the homogenate (mg).

The plasma Ca concentration was determined by the automated method of Gitelman (1967), and plasma phosphate was also determined by an automated method (Technicon Instruments Co. Ltd, 1966). Total protein concentration was determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Radioactivity (^{47}Ca) remaining in the gut loop was measured using a well-type scintillation counter with a sodium iodide crystal (ICN Tracer Laboratories) and ^{45}Ca was measured in a thin-window gas-flow Geiger counter (ICN Tracer Laboratories).

RESULTS

As there was insufficient information on the metabolism of 25-HCC in vitamin D-replete animals, a preliminary experiment was done in vitamin D-replete chicks fed on either a low (1 g/kg) or high- (20 g/kg) Ca diet, to determine whether a difference in the activity of 25-HCC-1-hydroxylase could be demonstrated. The results are shown in Table 1. As expected, the CaBP activity was higher and plasma Ca concentration was lower in the chicks fed on the low-Ca diet. The predominant metabolite formed by the kidney from chicks fed on the low-Ca diet was 1,25-DHCC and that by the kidneys from chicks fed the high-Ca diet was 24,25-DHCC.

The results of the experiment in which the metabolism of 25-HCC was studied in chicks fed at six different Ca intakes are shown in Table 2. With increasing Ca content of the diet, duodenal Ca absorption (%) decreased. Dietary Ca content and duodenal Ca absorption (%) were significantly correlated (r −0·899, $P < 0·01$). CaBP activity showed a similar decrease with increasing dietary Ca content. As shown in Fig. 1, the absorption of calcium (%) was closely correlated with the CaBP activity (r 0·995, $P < 0·01$). Plasma Ca and phosphate concentrations increased with increasing dietary Ca content.

The activity of 25-HCC-1-hydroxylase decreased as the dietary Ca content increased

Table 2. *The effect of dietary calcium level on duodenal Ca absorption, CaBP activity, plasma Ca and phosphate concentration, and the activities of renal 25-HCC-1-hydroxylase and 25-HCC-24-hydroxylase enzymes*

(Mean values with their standard errors; no. of chicks in parentheses)

Dietary Ca (g/kg)	Duodenal Ca absorption (%)		Duodenal CaBP activity (net s:r/mg protein)		Plasma concentration (mmol/l)				Activity of renal enzyme* (pmol/min per mg protein)			
					Ca		Phosphate		25-HCC-1-hydroxylase		25-HCC-24-hydroxylase	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
1·0 (7)	85·3	2·0	0·538	0·067	2·44	0·07	1·46	0·12	0·60	0·080	0·15	0·002
2·5 (9)	78·5	1·7	0·401	0·042	2·49	0·05	1·93	0·18	0·37	0·040	0·16	0·004
5·0 (9)	53·0	2·7	0·314	0·042	2·79	0·05	1·70	0·06	0·17	0·020	0·32	0·003
10·0 (6)	33·0	2·5	0·173	0·018	2·84	0·18	1·82	0·23	0·11	0·002	0·27	0·009
15·0 (7)	30·9	2·3	0·158	0·006	2·94	0·02	2·02	0·09	0·10	0·006	0·37	0·030
20·0 (9)	28·5	1·8	0·141	0·006	2·81	0·04	2·08	0·11	0·11	0·013	0·52	0·019

25-HCC-1-hydroxylase, 25-hydroxycholecalciferol-1-hydroxylase; 25-HCC-24-hydroxylase, 25-hydroxycholecalciferol-24-hydroxylase; CaBP, Ca-binding protein; s:r, total radioactivity in supernatant fraction: total radioactivity in resin (for details, see p. 48).

* For details of procedures, see p. 48.

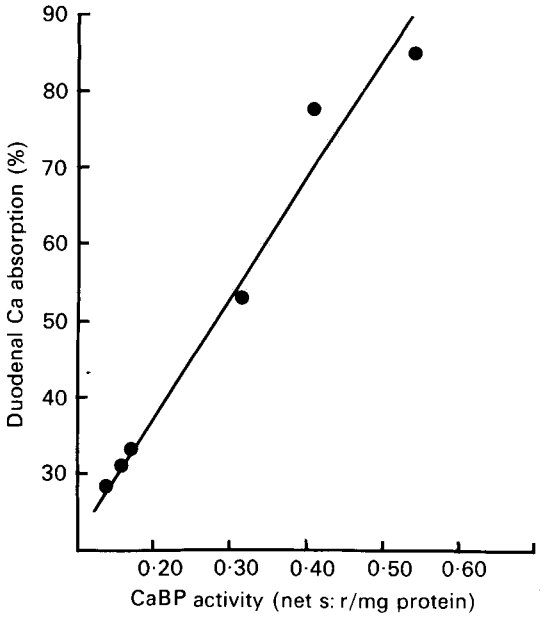

Fig. 1. The relationship between duodenal calcium absorption (%) and Ca binding protein (CaBP) activity (net total radioactivity in supernatant fraction(s): total radioactivity in resin (r)/mg protein) in vitamin D-replete chicks fed on diets differing in Ca content. Chicks (10-d-old) were fed on the diets for 10 d. Duodenal Ca absorption was determined in vivo by the ligated-loop technique and CaBP activity was determined by the Chelex ion-exchange method. For details of diets and procedures, see Table 2 and p. 48.

from 1 g/kg to 10 g/kg. Further increase in dietary Ca content did not alter the activity of this enzyme (Table 2). The activity of the enzyme was significantly correlated with Ca absorption (%) (r 0·932, $P < 0·01$) (Fig. 2), and with CaBP activity (r 0·957, $P < 0·01$). The activity of 25-HCC-24-hydroxylase increased as the dietary Ca content increased from 1 g/kg to 20 g/kg and was directly related to the dietary Ca content (r 0·930, $P < 0·01$) and inversely related to Ca absorption (%) (r −0·843, $P < 0·05$).

To determine whether or not the relationship between the activity of 25-HCC-1-hydroxylase and Ca absorption (%) was reflected in plasma Ca levels, the relationship between plasma Ca concentration and the activity of 25-HCC-1-hydroxylase was studied. The activity of the enzyme decreased as the plasma Ca concentration increased to 2·80 mmol/l but no further change was found at higher concentrations and there was a significant inverse correlation between plasma Ca concentration and the activity of 25-HCC-1-hydroxylase (Fig. 3; r −0·940, $P < 0·01$); the relationship between plasma phosphate concentration and 25-HCC-1-hydroxylase was not significant (r −0·725).

DISCUSSION

Intestinal absorption of Ca as determined by the in vivo technique has been shown to be related to the previous dietary intake of Ca (Morrissey & Wasserman, 1971). This was confirmed in the present studies. It is now recognized that intestinal vitamin D-dependent CaBP plays some part in the process of absorption but the exact role of CaBP is not known. However, CaBP and Ca absorption have been found to be closely related in several circum-

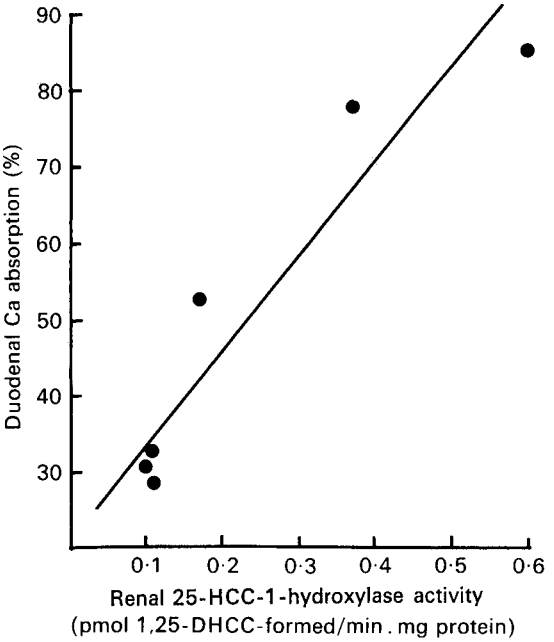

Fig. 2. The relationship between renal 25-hydroxycholecalciferol-1-hydroxylase (25-HCC-1-hydroxylase) activity (pmol 1,25-dihydroxycholecalciferol (1,25-DHCC) formed/min per mg protein) and duodenal calcium absorption (%) in vitamin D-replete chicks fed on diets differing in Ca content. Renal 25-HCC-1-hydroxylase activity was determined in vitro by incubation of tritiated 25-HCC with a kidney homogenate (100 g/l) and duodenal Ca absorption was determined in vivo by the ligated-loop technique. For details of diets and procedures, see Table 2 and p. 48.

stances (Morrissey & Wasserman, 1971; Wasserman & Corradino, 1973; Swaminathan & Care, 1975). This close relationship between CaBP activity and Ca absorption was indicated in our experiments (Fig. 1, Table 2).

The mechanism of the intestinal adaptation to changes in dietary Ca content is far from clear. Nicolaysen *et al.* (1953) suggested the existence of an 'endogenous factor' regulating Ca absorption. They also made the important discovery that adaptation is not possible in the absence of vitamin D. Recent developments in the metabolism of CC outlined earlier suggest that this factor may be 1,25-DHCC. Most of the work was done in marginally-vitamin D-deficient animals; Omdahl *et al.* (1972) studied in vitro the metabolism of 25-HCC in chicks given 260 pmol CC/d, whereas approximately 1·6-3·2 nmol CC/d is necessary for chicks. The marked inhibitory effect of CC on the 25-HCC-1-hydroxylase activity and the dilution of radioactive 25-HCC by unlabelled compounds tending to mask any change were probable reasons for not studying vitamin D-replete chicks. Recently Henry, Midgett & Norman (1974) have studied the enzyme activity in chicks given adequate supplements of CC. They have confirmed the findings of others that an increase in dietary Ca leads to a decrease in the activity of renal 25-HCC-1-hydroxylase. However, they studied only three levels of dietary Ca, and furthermore, the difference between the hydroxylase activity at two of these dietary Ca levels was not marked. In the present study using two different dietary Ca levels, it was first shown that the in vitro technique could differentiate between 25-HCC-1-hydroxylase activities in the kidney of vitamin D-replete chicks given either a high- or low-Ca diet (Table 1). Then, using six different dietary Ca levels, it was demonstrated that the activity of 25-HCC-1-hydroxylase is inversely related to the Ca content of the diet between 1 and 10 gCa/kg, and that the enzyme activity and Ca absorption are

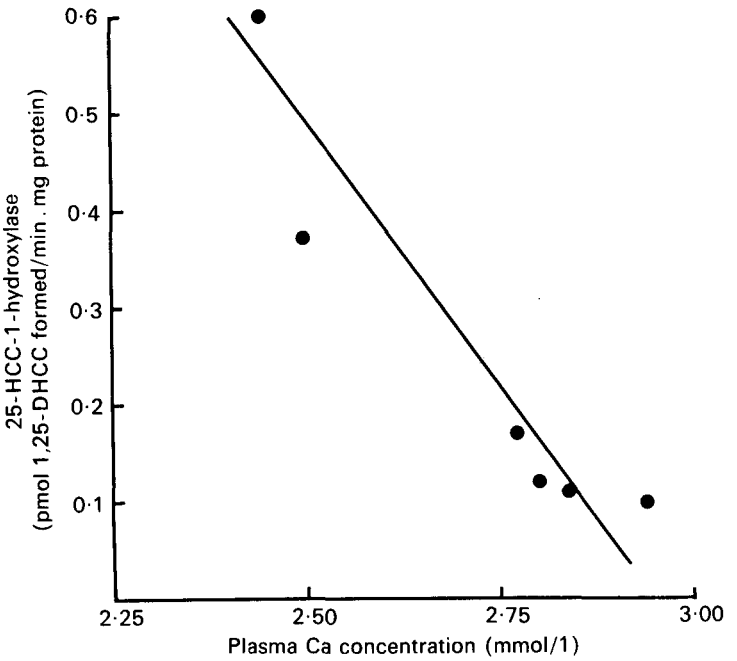

Fig. 3. The relationship between plasma calcium concentration and renal 25-hydroxycholecalciferol-1-hydroxylase (25-HCC-1-hydroxylase) activity (pmol 1,25-dihydroxycholecalciferol (1,25-DHCC)/min per mg protein) in vitamin D-replete chicks fed on diets differing in Ca content. Plasma Ca concentration was determined by the automated method of Gitelman (1967) and renal 25-HCC-1-hydroxylase activity was determined in vitro by incubation of tritiated 25-HCC with kidney homogenates (100 g/l). For details of diets and procedures, see Table 2 and p. 48.

closely correlated (Table 2, Fig. 2). The activity of 25-HCC-24-hydroxylase increased directly with the Ca content of the diet over the range of dietary Ca levels studied. These relationships have not been demonstrated previously. However, by administering radioactively-labelled CC continuously to chicks to produce steady-state conditions, Edelstein, Harell, Bar & Hurwitz (1975) recently demonstrated that in chicks fed on a low-Ca diet the amount of 1,25 DHCC in the intestine was higher than that in chicks fed on a normal-Ca diet.

The present study and that of Edelstein *et al.* (1975) and of Henry *et al.* (1974) show that the response in CC metabolism to changes in dietary Ca content are qualitatively similar in vitamin D-replete chicks to those reported for marginally-vitamin D-deficient animals (Omdahl *et al.* 1972). However, the activity of the enzyme 25 HCC-1-hydroxylase is several times higher in the marginally-deficient chicks (Henry *et al.* 1974).

A further point for discussion is the mechanism of control of 25-HCC metabolism by dietary Ca. Parathyroid hormone (PTH) was shown to increase the 25-HCC-1-hydroxylase activity (Fraser & Kodicek, 1973; Henry *et al.* 1974). Although Galante, Colston, MacAuley & MacIntyre (1972) found that PTH decreased the activity of 25-HCC-1-hydroxylase, it is now generally agreed that PTH stimulates the production of 1,25-DHCC. Other factors thought to influence this enzyme are mitochondrial Ca and phosphate concentration in the renal cortical tubule cells (Colston, Evans, Galante, MacIntyre & Moss, 1973; Tanaka & DeLuca, 1973; Henry & Norman, 1975). In the present study the activity of 25-HCC-1-hydroxylase was inversely related to the plasma Ca concentration, a finding also reported by Henry *et al.* (1974) (Fig. 3). However, how the plasma Ca concentration regulates

25-HCC-1-hydroxylase activity is open to speculation. Plasma Ca concentration also regulates PTH secretion, and the PTH may therefore be the mediator. This implies that in the absence of PTH, adaptation to a low-Ca diet will not take place. Although some workers disagree (Winter, Morava, Horvath, Simon & Sos, 1972), it has been shown by other workers in rats (Kimberg, Schachter & Schenker, 1961), and in pigs (Swaminathan, Fox, Tomlinson & Care, 1974; Fox, 1976), that adaptation to low-Ca diets can occur in the absence of PTH. Favus, Walling & Kimberg (1974) have recently shown that in chronically thyroparathyroidectomized rats fed on a Ca-restricted diet the accumulation of 1,25-DHCC by the intestinal mucosa was not impaired. Thus, at least one mechanism not involving PTH has to be postulated. An important factor in the control of the metabolism of 25-HCC could be the Ca concentration in the renal mitochondria but it is not known to what extent this is influenced by changes in the plasma Ca ion concentration.

It is concluded that in chicks fed on diets differing in their Ca content (1–10 g Ca/kg) and replete in vitamin D, the duodenal absorption (%) of Ca and duodenal CaBP activity are directly related to the activity of renal 25-HCC-1-hydroxylase as studied by an in vitro method.

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Printed in Great Britain