The Relationship between Vitamin D-Stimulated Calcium Transport and Intestinal Calcium-Binding Protein in the Chicken

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1. The rapid stimulation of intestinal Ca^{2+} transport observed in vitamin D-deficient chicks after receiving 1,25-dihydroxycholecalciferol has necessitated a re-evaluation of the correlation hitherto observed between this stimulation and the induction of calcium-binding protein synthesis. By 1 h after a dose of 125 ng of 1,25-dihydroxycholecalciferol, Ca^{2+} transport is increased. This is at least 2 h before calcium-binding protein can be detected immunologically and 1 h before synthesis of the protein begins on polyribosomes, and thus the hormone stimulates Ca^{2+} transport before calcium-binding-protein biosynthesis is induced. 2. The maximum increase in Ca^{2+} transport observed after this dose of 1,25-dihydroxycholecalciferol (attained by 8 h) is similar to that observed after 1.25–25 μg of cholecalciferol, but the stimulation is only short-lived, in contrast with the effect observed after the vitamin. At later times after the hormone, however, when Ca^{2+} transport has declined to its basal rate, the cellular content of calcium-binding protein remains elevated. 3. Calcium-binding protein is synthesized on free rather than membrane-bound polyribosomes, which implies that it is an intracellular protein. 4. Rachitic chicks require the presence of dietary calcium for maximum stimulation of calcium-binding protein production by cholecalciferol. 5. These results suggest that calcium-binding protein is an intracellular protein, and that its synthesis may be a consequence of the raised intracellular calcium content of the intestinal epithelial cells resulting from 1,25-dihydroxycholecalciferol-stimulated Ca^{2+} transport. We propose that calcium-binding-protein synthesis is necessary for maintaining the stimulated rate of Ca^{2+} transport, which is initiated by other factors.

There is now considerable evidence that, in the intestine at least, part of the mechanism of action of the hormonal metabolite of cholecalciferol, 1,25-dihydroxycholecalciferol, involves the stimulation of protein synthesis, and in particular of a protein with calcium-binding activity known as calcium-binding protein. This protein, first identified in the chick intestine (Wasserman *et al.*, 1968) and subsequently found in many other species (see Wasserman *et al.*, 1974b), is the only component of the vitamin D-induced Ca^{2+} -transport system that has so far been identified. Calcium-binding protein is not found in the intestine of vitamin D-deficient animals and is synthesized *de novo* in response to cholecalciferol (Emtage *et al.*, 1974a) and to 1,25-dihydroxycholecalciferol (Spencer *et al.*, 1976a).

The appearance of calcium-binding protein as one of the earliest responses of the deficient chick intestine to cholecalciferol and its metabolites was suggestive of a role for the protein in the intestinal absorption of Ca^{2+} ions. Supporting evidence for such a role includes a number of situations in which the amounts of calcium-binding protein change in a manner corresponding to changes in the ability of

the intestine to absorb Ca^{2+} (for reviews see Taylor & Wasserman, 1969; Wasserman *et al.*, 1974a,b), but a more crucial requirement is that calcium-binding protein should be synthesized before or at the same time as an increase in intestinal Ca^{2+} absorption is observed. Such a correlation between the rate of appearance of calcium-binding protein and the increase in Ca^{2+} absorption induced by cholecalciferol was observed, provided that the protein concentration was measured by immunological procedures (Ebel *et al.*, 1969; Emtage *et al.*, 1974b) rather than by the less sensitive Chelex assay, which assesses total Ca^{2+} -binding activity and not exclusively calcium-binding protein, and with which a correlation was not observed (Harmeyer & DeLuca, 1969). Typically in the rachitic chick the biosynthesis of calcium-binding protein on polyribosomes begins 7 h after a physiological dose of the vitamin, and the appearance of increasing amounts of immunologically detectable calcium-binding protein by 12 h occurs in parallel with a significant increase in Ca^{2+} transport first observed at the same time (Emtage *et al.*, 1974b). Thus it has been accepted that calcium-binding protein plays a major role in Ca^{2+} absorption,

although the mechanism of this role has still to be described.

In comparison with changes observed after administration of the vitamin, those in response to 1,25-dihydroxycholecalciferol occur more rapidly, but nevertheless the available information seemed to confirm a correlation between the appearance of calcium-binding protein and increased intestinal Ca^{2+} absorption (Wasserman *et al.*, 1974b; Norman, 1974). We have been interested in the initial biochemical changes induced in the chick intestine by this hormone, and as a result it has become apparent that conditions can be found when Ca^{2+} absorption occurs before the synthesis of calcium-binding protein. In the present studies, performed *in vivo* and *in vitro*, we show that at short and longer time-intervals after receiving the hormone the calcium-binding protein concentrations in the chick intestine do not correlate with its ability to absorb Ca^{2+} ions. We also demonstrate the probable intracellular location of the protein, and that rachitic birds require the presence of dietary calcium for the maximum stimulation of calcium-binding protein production by the vitamin. These findings have led us to propose that the synthesis of intestinal calcium-binding protein in the chick is a consequence of 1,25-dihydroxycholecalciferol-stimulated Ca^{2+} transport rather than the mediator of this process. A preliminary report of some of these findings has been published (Spencer *et al.*, 1976b).

Experimental

Chemicals

L-[4,5- ^3H]Leucine (57Ci/mmol) and 25-hydroxy-[26- ^3H]cholecalciferol (12.2Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. 1,25-Dihydroxy[26- ^3H]cholecalciferol was prepared by Dr. D. R. Fraser (of this laboratory) by incubating 25-hydroxy[26- ^3H]cholecalciferol with chick kidney homogenates (Fraser & Kodicek, 1970). 1,25-Dihydroxycholecalciferol was a gift from Dr. M. Uskoković, Hoffman-LaRoche, Nutley, NJ, U.S.A. The source of other biochemicals was given by Emtage *et al.* (1974a).

Animals

Chicks (1 day old; Rhode Island Red $\times$ Light Sussex) obtained from the National Institute for Research in Dairying (Shinfield, Reading, Berks., U.K.) were fed on a cholecalciferol-deficient diet containing 1.0% Ca and 0.7% P (Lawson & Wilson, 1974) and used after 3–4 weeks (unless otherwise stated). In some experiments birds were fed on the same diet but containing <0.02% Ca, 0.9% P (calcium-free diet) or 4.0% Ca, 2.8% P (high-calcium

diet), and were given glass-distilled water to drink. Physiological doses of cholecalciferol (5 or 12.5 μg) and 1,25-dihydroxycholecalciferol (125 ng) were administered in 0.1 ml of 10% (v/v) ethanol/90% (v/v) propylene glycol, either orally or intracardially as indicated in the text.

Methods

Preparation of chick intestinal polyribosomes and polypeptide synthesis in vitro. Chick duodenal mucosa was homogenized in rat liver cell sap and polyribosomes were prepared as a routine by the method of Emtage *et al.* (1974a). Occasionally other methods of preparing polyribosomes were used, as indicated in the text, thus.

(1) Treatment of the postmitochondrial supernatant with 0.1% (w/v) sodium deoxycholate before processing in the usual way (Emtage *et al.*, 1974a).

(2) Membrane-bound polyribosomes were prepared from a crude microsomal pellet (prepared in rat liver cell sap) treated with 0.5% (v/v) Nonidet P.40 (Harrison *et al.*, 1974). The microsomal supernatant was used as the source of free polyribosomes.

(3) Total ribosomes, polyribosomes and messenger ribonucleoprotein complexes were precipitated by Mg^{2+} treatment of a mucosal homogenate containing 2% (v/v) Triton X-100 as described by Palmiter (1974).

Polypeptide synthesis *in vitro*, immunoprecipitation of the products with antiserum against calcium-binding protein, and subsequent analysis of the immunoprecipitate by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis were conducted as described previously (Spencer *et al.*, 1976a).

Preparation of intestinal cytosols. Intestinal mucosa was homogenized at 0°C with an equal volume of buffer containing 0.12M-NaCl, 4.74mM-KCl, 1mM- β -mercaptoethanol, and 13.7mM-Tris/HCl, pH 7.4, and centrifuged for 1 h at 100000g_{av}, 4°C.

Assay of calcium-binding protein in intestinal cytosols. Monospecific antiserum was prepared against chick intestinal calcium-binding protein purified to electrophoretic homogeneity by the method of Wasserman *et al.* (1968), as described previously (Spencer *et al.*, 1976a). Calcium-binding protein was measured in intestinal cytosols by immunoelectrophoresis (Laurell, 1966) by using this antiserum and pure chick intestinal calcium-binding protein as the standard.

Measurement of intestinal Ca^{2+} absorption in vivo. The ability of chicks to absorb $^{45}\text{Ca}^{2+}$, given orally, into the blood was measured as described by Emtage *et al.* (1974b).

Measurement of Ca^{2+} transport in vitro. Sacs constructed from 3.0 cm of everted ileum (distal to the yolk-sac remnant) were filled with 0.3 ml of medium containing 198mM-mannitol, 25mM-KCl, 1.2mM-

NaH_2PO_4 , 25 mM- NaHCO_3 , 1.2 mM- MgSO_4 , 20 mM-glucose, 0.5 mM- CaCl_2 and $^{45}\text{CaCl}_2$ (40000 c.p.m./ml) (Hurwitz *et al.*, 1967) and incubated with 3.0 ml of the same medium gassed with O_2/CO_2 (19:1). The incubation was performed in 25 ml Erlenmeyer flasks for 90 min at 37°C, with shaking. Portions (100 μl) were then removed from the serosal and mucosal solutions (inside and outside respectively) for radioactivity measurements.

Other methods. Plasma calcium was measured by atomic absorption spectroscopy. Protein was determined by the method of Warburg & Christian (1941).

Results

Sequence of intestinal events after cholecalciferol and 1,25-dihydroxycholecalciferol

Absorption of $^{45}\text{Ca}^{2+}$ from the intestine by vitamin D-deficient chicks was measured *in vivo* at various times after injecting cholecalciferol or 1,25-dihydroxycholecalciferol intracardially, and the calcium-binding protein concentrations in the mucosa of other birds at the time of killing were determined by immunoelectrophoresis. As shown in Fig 1(a), after 5 μg of cholecalciferol, calcium-binding protein was first detectable at the same time that $^{45}\text{Ca}^{2+}$ absorption was significantly increased above control values (12 h). Thereafter both phenomena increased rapidly until 18 h, beyond which time $^{45}\text{Ca}^{2+}$ transport remained elevated for at least 72 h and calcium-

binding protein continued to increase. However, in birds treated with 125 ng of 1,25-dihydroxycholecalciferol, an increase in $^{45}\text{Ca}^{2+}$ absorption was observed more rapidly than after cholecalciferol and was measurable 4 h before calcium-binding protein could be detected (at 4 h and 8 h respectively) (Fig. 1b). Further, after 18 h, when the absorption of $^{45}\text{Ca}^{2+}$ was declining rapidly, calcium-binding protein continued to increase.

To investigate this discrepancy between the initial appearance of calcium-binding protein in the intestine of vitamin D-deficient chicks and the first time at which a significant increase could be detected in Ca^{2+} absorption, the experiments were repeated to improve the precision of the calcium-absorption measurements. The results are shown in Fig. 2. The ability of chicks to transport Ca^{2+} across the intestine after dosing with 1,25-dihydroxycholecalciferol was measured *in vitro* by using everted intestinal sacs. With this technique, Ca^{2+} transport was increased by 1 h, but significantly only after 2 h. Calcium-binding protein, however, was not reproducibly detectable by immunoelectrophoresis until 7 h after dosing. Further, although intestinal polyribosomes obtained from these birds can synthesize calcium-binding protein by 2 h after dosing, the activity is very low (less than 1% of the maximum activity attained at 13 h) and does not increase markedly until 4 h, by which time Ca^{2+} transport has reached 70% of its maximum rate.

The sequence of events occurring in the chick intestine after a single injection of 125 ng of 1,25-

Fig. 1. Intestinal calcium-binding protein and Ca^{2+} absorption in rachitic chicks after dosing with either cholecalciferol (a) or 1,25-dihydroxycholecalciferol (b)

Groups of four rachitic chicks were dosed intracardially with either cholecalciferol (5 μg) or the hormone (125 ng), and at the times indicated the absorption of $^{45}\text{Ca}^{2+}$ was measured *in vivo* as described under 'Methods'. Other groups of chicks similarly treated were killed at the times indicated and intestinal calcium-binding protein was measured by immunoelectrophoresis. ○, $^{45}\text{Ca}^{2+}$ absorption; the bar indicates S.E.M. for the determinations at each time; ●, calcium-binding-protein concentration in the pooled mucosal tissue.

Fig. 2. Stimulation of Ca^{2+} transport, measured *in vitro*, and calcium-binding-protein synthesis by 1,25-dihydroxycholecalciferol

For each experiment, rachitic chicks were dosed intracardially with 125 ng of 1,25-dihydroxycholecalciferol and killed at the indicated times. Ca^{2+} transport ($\circ$) was measured *in vitro* at each time by using everted intestinal sacs as described under 'Methods'. Each point is the mean of a group of seven to ten chicks; vertical bars indicate $\pm$ s.e.m. Calcium-binding protein ($\square$) was measured in the pooled duodenal cytosols obtained from six chicks by immunoelectrophoresis as described under 'Methods'. Polyribosomes were prepared at each time from the pooled duodenal mucosa of groups of four chicks, and were incubated in the protein-synthesizing system *in vitro* as described under 'Methods'. Duplicate 20 μl portions of the post-ribosomal supernatants were removed for the determination of acid-precipitable radioactivity. The remainder were immunoprecipitated with antiserum to calcium-binding protein. The dissociated immunoprecipitates were electrophoresed and the gels sliced and processed for radioactivity counting. ^3H radioactivity (d.p.m.) in the calcium-binding protein area of the gels ($\blacksquare$) is expressed as a percentage of the total acid-precipitable incorporation into released proteins (corrected up to the maximum value obtained, i.e. 1.4×10^6 d.p.m./ml).

dihydroxycholecalciferol can be summarized as shown in Fig. 3, where at each time all phenomena are expressed as percentages of their maximum response. After a tracer dose (1.8 ng) of 1,25-dihydroxy[26- ^3H]cholecalciferol the intestinal mucosa accumulates the hormone, reaching maximum concentrations by 2 h (data taken from Lawson & Emtage, 1974). This is followed by the increase in Ca^{2+} transport, which reaches its maximum at 8 h after the injection. The curve of calcium-binding protein biosynthesis is parallel to that of increased Ca^{2+} transport, but lies about 2.5 h behind it, and the accumulation of calcium-binding protein in the cytoplasm is evident about 2.5 h after the translation of calcium-binding protein mRNA has begun. Figs. 2 and 3 also show that at later times there is no correlation between the amounts of calcium-binding protein in the cytoplasm and Ca^{2+} transport. After reaching its maximum at 8–12 h Ca^{2+} transport declines quite rapidly, and by 21 h, when calcium-binding protein concentrations are approaching their maximum, Ca^{2+} transport is not significantly higher than that observed in control animals. This last result, which has also been observed by Wasserman, *et al.* (1974a), in itself demonstrates that the 1,25-dihydroxycholecalciferol-induced Ca^{2+} transport in

Fig. 3. Sequence of intestinal events occurring after dosing rachitic chicks with 125 ng of 1,25-dihydroxycholecalciferol

All phenomena are expressed as percentages of the maximum response obtained. $\bullet$, Accumulation of a tracer dose (1.8 ng) of 1,25-dihydroxy[26- ^3H]cholecalciferol in intestinal mucosa; $\circ$, calcium transport *in vitro*; $\blacksquare$, synthesis of calcium-binding protein on polyribosomes; $\square$, calcium-binding protein in intestinal mucosa.

Table 1. *Stimulation of Ca^{2+} transport by chick intestine in vitro in response to cholecalciferol or 1,25-dihydroxycholecalciferol*
 Rachitic birds were dosed with cholecalciferol orally (groups 3, 4) or intracardially (groups 1, 2) or with 1,25-dihydroxycholecalciferol intracardially (group 5), and were killed at the indicated times afterwards. The ability of the intestine to transport calcium was measured *in vitro* by using the everted-sac technique (see under 'Methods'). Stimulation of $^{45}\text{Ca}^{2+}$ transport is expressed as the serosal/mucosal ratio of $^{45}\text{Ca}^{2+}$ concentration in sacs from dosed relative to deficient birds.

Group	No. of expts.	Metabolite given	Time of dosing before killing (h)	^{45}Ca concentration serosal/mucosal ($\pm$ S.E.M.)	Stimulation of $^{45}\text{Ca}^{2+}$ transport relative to control
1	4	Control	—	0.66 ± 0.02	1.38
		Vitamin D_3 (0.25 μg)	20	0.91 ± 0.07	
2	2	Control	—	0.80 ± 0.01	1.59
		Vitamin D_3 (1.25 μg)	20	1.27 ± 0.10	
3	4	Control	—	0.80 ± 0.04	1.47
		Vitamin D_3 (12.5 μg)	41	1.18 ± 0.05	
4	4	Control	—	0.88 ± 0.06	1.59
		Vitamin D_3 ($2 \times 12.5 \mu\text{g}$)	42, 18	1.40 ± 0.11	
5	10	Control	—	0.71 ± 0.33	1.44
		1,25-Dihydroxycholecalciferol (0.125 μg)	8	1.02 ± 0.5	

the chick is dependent on factors other than calcium-binding protein. The ability of intestinal polyribosomes to synthesize calcium-binding protein also declines very rapidly after 12–13 h. This decline in calcium-binding protein biosynthesis is similar to the decline in Ca^{2+} transport observed after 8 h, and by 20 h both phenomena are occurring at only about 20% of their maximum rate. The lag in the initiation of calcium-binding protein biosynthesis after the increase in Ca^{2+} transport, and the subsequent decline of both phenomena, suggest that the biosynthesis of calcium-binding protein is dependent on 1,25-dihydroxycholecalciferol-stimulated Ca^{2+} transport rather than vice versa.

The maximum amount of Ca^{2+} transported by everted intestinal sacs obtained from chicks dosed with 125 ng of 1,25-dihydroxycholecalciferol was similar to the amount transported *in vitro* by sacs from birds dosed with various amounts of cholecalciferol. As shown in Table 1, when measured at a time when the response was maximal for the metabolite and method of administration, the amount of Ca^{2+} transported *in vitro* was independent of the dose of cholecalciferol within the range 1.25–25 μg (50–1000 i.u.). Measurements of Ca^{2+} flux in isolated intestinal preparations obtained from birds dosed with 0.75–2.5 μg of cholecalciferol gave similar results (Norman, 1974). Thus there is apparently a maximum rate of Ca^{2+} transport (approx. 50% greater than in

the controls) attainable that is independent of the dose of cholecalciferol (above 1.25 μg /bird) and of the method of administration. As shown in Fig. 1, the difference in response between birds dosed with cholecalciferol and 1,25-dihydroxycholecalciferol is that the maximum rate of Ca^{2+} transport is maintained for several days after the vitamin, but only for a few hours after the hormone. This reflects the continued production in the animal of the hormone from the vitamin over an extended period of time.

Site of calcium-binding-protein synthesis

The site of synthesis of calcium-binding protein within the intestinal cell was investigated by testing free and membrane-bound polyribosomes for their ability to synthesize calcium-binding protein in a cell-free system. Table 2 lists the recoveries of polyribosomal RNA obtained from chick intestine by various methods. Centrifugation of a post-mitochondrial supernatant through a discontinuous sucrose density gradient as a routine yields 0.6–0.9 mg of polyribosomal RNA/g of mucosa, consisting mainly of free polyribosomes with some monomeric ribosomal contamination (Emtage *et al.*, 1974a). Treatment of the post-mitochondrial supernatant with 1% (w/v) sodium deoxycholate increases this yield to 1.1–1.4 mg of polyribosomal RNA/g of mucosa, consisting of both free and membrane-bound

Table 2. *Yield of polyribosomes obtained from chick intestine by different methods*

Polyribosomes from the ileum and duodenum (after careful removal of the pancreas) were prepared from groups of two 4-week-old vitamin D-deficient chicks (Expts. 1-3) or from vitamin D-deficient chicks 72 h after dosing with 12.5 µg of cholecalciferol (Expts. 4-5) as described under 'Methods'. All chicks were starved overnight before being killed. In Expts. 1-3 yields of two separate experiments are given. The average yields of the different preparations are expressed as percentages of the yield obtained in Expt. (1) (Mg²⁺ precipitation).

No. of expt.	Method of polyribosome preparation	Polyribosome yield	Average yield (% of that in Expt.1)
		(mg of RNA/g of mucosa)	
1	Mg ²⁺ precipitation of detergent-treated homogenate (total ribosomes)	1.95, 2.65	100
2	Centrifugation of postmitochondrial supernatant (free polyribosomes)	0.83, 0.89	37.4
3	Centrifugation of detergent-treated post-mitochondrial supernatant (free+membrane-bound polyribosomes)	1.39, 1.16	55.6
4	As Expt. (2)	0.70	30.4
5	Centrifugation of detergent-treated crude microsomal pellet (membrane-bound polyribosomes)	0.23	10.0

Table 3. *Synthesis of calcium-binding protein by free and membrane-bound polyribosomes in vitro*

Two vitamin D-deficient chicks were dosed orally with 12.5 µg of cholecalciferol 72 h before being killed. The scraped pooled mucosal tissue was used for the separate preparation of free and membrane-bound polyribosomes (Harrison *et al.*, 1974), which were tested for their ability to incorporate [³H]leucine into completed polypeptide chains and into immunoprecipitable calcium-binding protein in a system *in vitro*. The dissociated immunoprecipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the ³H localized in the calcium-binding-protein peak was determined (see under 'Methods'). Calcium-binding-protein synthesis was estimated by relating the radioactivity in the calcium-binding protein peak to the total acid precipitable incorporation into completed polypeptide chains.

Type of polyribosomes	Percentage of total polyribosomes prepared	Acid-precipitable radioactivity (d.p.m./0.1 mg of polyribosomal RNA)	Radioactivity in immunoprecipitable calcium-binding protein (d.p.m./0.1 mg of polyribosomal RNA)	Calcium-binding protein (% of total proteins synthesized)
Free	74.5	160 987	12987	8.1
Membrane-bound	25.5	53 747	0	0

polyribosomes. The preparation of free and membrane-bound polyribosomes separately yielded a total of 0.9 mg of polyribosomal RNA/g of mucosa, of which 25 % were membrane-bound polyribosomes. This yield was lower than that obtained with the mixed preparation, but enabled an assessment of which polyribosome group was synthesizing calcium-binding protein to be made. As shown in Table 3, only the free polyribosomes synthesize calcium-binding protein.

Calcium-binding protein, dietary calcium and vitamin D

Normal chicks adapt to changes in their dietary calcium intake by altering their rate of Ca²⁺ absorption and mucosal content of calcium-binding protein.

Thus after several days on a low-calcium diet (0.2-0.02 %) the rate of active Ca²⁺ absorption across the intestine (measured *in vitro*) is increased relative to the controls maintained on a normal calcium diet (1.0-1.2 %), and the amounts of calcium-binding protein are also higher. On a high-calcium diet (2.3 %), active intestinal Ca²⁺ absorption is decreased and the amounts of calcium-binding protein are lower. This adaptation has been interpreted as evidence for the intimate involvement of calcium-binding protein in vitamin D-mediated Ca²⁺ absorption (Morrissey & Wasserman, 1971).

Chicks raised on a vitamin D-deficient diet (containing 1.0 % calcium) do not, however, exhibit the same types of adaptation to changes in their dietary calcium intake. Norman (1974) has reported

Table 4. *Effects of vitamin D status and dietary calcium content on plasma calcium and intestinal calcium-binding protein in chicks*

Chicks were raised from hatching on a vitamin D-deficient diet containing 1.0% Ca as described in the Experimental section. After 3 weeks on this diet, groups of chicks were transferred to the experimental diets (0% or 4.0% Ca) for the indicated number of days before being killed. In addition chicks in Expts. 4–6 were dosed orally with 12.5 µg of cholecalciferol 72 h before being killed. Chicks in Expt. 7 were dosed orally twice weekly from hatching with 2.5 µg of cholecalciferol and were killed at 3 weeks of age. Plasma calcium and intestinal calcium-binding-protein concentrations were determined as described under 'Methods'. Values given are the average for each group $\pm$ S.E.M. and were tested for significance by Student's *t* test: * $P < 0.001$ compared with all other values; ** $P < 0.01$ compared with control value (Expt. 4); *** $P < 0.001$ compared with control value (Expt. 4).

Expt. no.	Vitamin D status of birds	No. of chicks/group	Added calcium content of experimental diet (%)	Time on experimental diet (days)	Plasma calcium (mg%)	Calcium-binding protein (mg/g of mucosa)
1	Deficient	4	1.0	0	8.3 $\pm$ 0.6	0
2		2	0	3	5.1 $\pm$ 1.5*	0
3		2	4.0	7	9.1 $\pm$ 0.1	0
4	Deficient + 12.5 µg	4	1.0	0	8.8 $\pm$ 0.3	2.10 $\pm$ 0.11
5	of vitamin D ₃	4	0	3	8.5 $\pm$ 0.5	1.03 $\pm$ 0.20**
6	72 h before being killed	3	0	6	7.9 $\pm$ 0.6	0.39 $\pm$ 0.05***
7	2.5 µg of vitamin D ₃ twice weekly from hatching	4	1.0	0	9.1 $\pm$ 0.3	0.73 $\pm$ 0.19***

that in rachitic chicks the rate of active Ca²⁺ absorption remains unchanged as the dietary content of calcium is varied, and, as shown in Table 4 (Expts. 1 and 2), such chicks are unable to maintain their plasma calcium concentration when changed to a calcium-free diet. However, if dosed with cholecalciferol at the same time as they are transferred to the calcium-free diet, vitamin D-deficient chicks can maintain their plasma calcium above 8.5 mg% (Expt. 5), presumably by vitamin D-dependent mobilization of internal calcium stores.

Table 4 also summarizes the effects of changes in vitamin D status and dietary calcium on the amounts of calcium-binding protein in chicks. In the absence of vitamin D, no calcium-binding protein is produced when chicks are transferred from a normal to a calcium-free diet (Expts. 1 and 2). Chicks maintained on the vitamin D-deficient normal-calcium (1.0%) diet but dosed orally with cholecalciferol 72 h before being killed (Expt. 4) produce very large amounts of calcium-binding protein in the intestinal mucosa (2.1 mg of calcium-binding protein/g wet wt. of mucosa). This amount of calcium-binding protein is far higher than that in cholecalciferol-supplemented birds (2.5 µg twice weekly) maintained on the same diet (Expt. 7: 0.73 mg of calcium-binding protein/g of mucosa). When chicks raised on a vitamin D-deficient diet (normal calcium) were starved overnight before dosing with cholecalciferol orally and transfer

to a calcium-free diet for 3 days, the amount of calcium-binding protein produced (1.03 mg/g of mucosa) was about half that which would have been produced if the birds had been kept on the normal calcium diet (Expt. 5). If the birds were pre-adapted to the calcium-free diet for 3 days before being dosed with cholecalciferol, and maintained on the calcium-free diet for a further 3 days, only very low amounts of calcium-binding protein (<0.4 mg/g of mucosa) were produced (Expt. 6).

These results suggest that in rachitic birds the presence of dietary calcium is essential for maximum calcium-binding protein production. The requirement of vitamin D for calcium-binding protein production is apparent, however, since attempts to stimulate calcium-binding protein production in vitamin D-deficient chicks by placing them on a high-calcium diet (4%) for 7 days were unsuccessful, although the plasma calcium concentrations were maintained (Table 4, Expt. 3).

Discussion

In previous descriptions of the mechanism by which 1,25-dihydroxycholecalciferol acts in the intestine of animals, there has been a tendency to invoke intestinal calcium-binding protein as the connexion between the nuclear site of action of the hormone and the physiological response occurring in

the remainder of the cell. The rapid accumulation of the hormone in the intestinal cell nuclei (Lawson & Emtage, 1974), its effect in the intestine on RNA synthesis (Tsai & Norman, 1973), RNA polymerase activity (Zerwekh *et al.*, 1974), DNA template capacity (Zerwekh *et al.*, 1976) and the inhibition of the physiological response *in vitro* by actinomycin D and α -amanitin (Corradino, 1973) indicate that the intestinal nucleus is one, if not the only, site of action of 1,25-dihydroxycholecalciferol. As reviewed in the introduction there are now a number of findings linking calcium-binding protein with 1,25-dihydroxycholecalciferol-stimulated intestinal Ca^{2+} absorption, and so it has appeared most probable that the nuclear events referred to above were those required for the synthesis of this protein. Our own observations on the changes in polyribosomal synthesis of calcium-binding protein in response to the vitamin in particular were consistent with this view (Emtage *et al.*, 1974b). The stimulation of Ca^{2+} absorption after administration of the vitamin was thought to involve the following sequence of events: cholecalciferol $\rightarrow$ 1,25-dihydroxycholecalciferol $\rightarrow$ intestinal gene transcription $\rightarrow$ calcium-binding-protein synthesis $\rightarrow$ Ca^{2+} absorption.

We initiated a series of studies into the nuclear events underlying the action of this hormone, and in the process found that the rate of synthesis and appearance of calcium-binding protein were not appropriate to the rate of increase in Ca^{2+} absorption. Several points have emerged from these studies on intestinal calcium-binding protein that may clarify its role in 1,25-dihydroxycholecalciferol-stimulated Ca^{2+} absorption.

First, Ca^{2+} absorption can be observed at least 2 h before calcium-binding protein can be detected. This observation is confirmed by measurements of polyribosomal calcium-binding protein synthesis, which, although occurring earlier than the protein can be detected immunologically, still does not begin until after Ca^{2+} transport has started. Although these two findings clearly show that other 1,25-dihydroxycholecalciferol-dependent factors are required for intestinal Ca^{2+} transport, there is nevertheless substantial evidence that calcium-binding protein is involved in this process, as referred to in the introduction.

Secondly, the concentration of intestinal calcium-binding protein achieved after a dose of the hormone does not decline until after 48 h. This suggests that the protein is stable, having a turnover time very similar to that of the cells themselves (3–4 days). If a similar phenomenon applies to calcium-binding protein in other cell types that turn over more slowly, such as kidney and brain, it indicates one reason why vitamin D is relatively ineffective in altering the concentrations of this protein in these tissues, as reported by Wasserman *et al.* (1974a).

Thirdly, the data emphasize the importance of renewed investigation into the localization of this protein within the intestine epithelial cells. After the usual homogenization procedure the protein is found in the cytoplasmic fraction, i.e. it is not sedimented by centrifugation at 100000g for 60 min. It is possible, however, that the protein had become detached from a membrane site by the homogenization and that it is not a true cytoplasmic component. The original localization of the protein by immunofluorescent techniques supported the view that it was a membrane-bound protein (Taylor & Wasserman, 1970). Subsequent reports have not confirmed this finding (Morrissey *et al.*, 1976; Arnold *et al.*, 1976), and the results presented here showing that the protein is synthesized on free polyribosomes is not consistent with it being a membrane-bound component, nor with its being destined for export from the cell (Blobel & Dobberstein, 1975). We therefore conclude that the location of calcium-binding protein is intracellular.

Finally, the data here show that in response to 1,25-dihydroxycholecalciferol the rate of decline of polyribosomal calcium-binding protein synthesis is similar to the decline in Ca^{2+} absorption and to the intestinal concentration of this hormone, thereby showing that the other vitamin D-dependent factor(s) required for Ca^{2+} absorption referred to above have a rapid turnover time also.

The large increase in calcium-binding protein in the intestine of rachitic chicks dosed with cholecalciferol has been interpreted (Taylor & Wasserman, 1969) as evidence that factors other than vitamin D control the synthesis of this protein. Whether these factors are also those involved in the initial stage of Ca^{2+} absorption is unknown at present, but our studies on the response of rachitic chicks dosed with the vitamin and transferred to diets of different calcium content have shown how dependent the induction of calcium-binding-protein synthesis by cholecalciferol is on the calcium intake of the animal. The fact that after dosing with cholecalciferol rachitic chicks produce very much less calcium-binding protein on a low-calcium diet than on a normal-calcium diet suggests that a possible controlling factor is the intracellular Ca^{2+} concentration. Again, rachitic chicks transferred from a normal- to a low-calcium diet increase their ability to absorb Ca^{2+} after treatment with vitamin D (Norman, 1974), whereas the amounts of calcium-binding protein decline. This is another situation where the capacity of the intestine to absorb Ca^{2+} does not correlate with the amount of intestinal calcium-binding protein. This observation contrasts with the finding that normal animals adapted for some time to a low-calcium diet have both a high capacity to absorb Ca^{2+} and higher amounts of intestinal calcium-binding protein. Support for the view that different

relationships can be found at different times between Ca^{2+} absorption and calcium-binding protein can be found in the data of other workers (Omdahl & Thornton, 1972). Table 4 also shows that the concentration of calcium-binding protein attained during the different dietary regimes is not related to the plasma calcium concentration of the birds.

The experiments reported in the present paper have led us to conclude that the induction of intestinal calcium-binding-protein biosynthesis occurs after an earlier 1,25-dihydroxycholecalciferol-mediated event that leads to increased Ca^{2+} absorption across the brush-border membrane and a consequent rise in intracellular calcium. We have also found that calcium-binding protein is synthesized by free ribosomes and, as previously reported (Spencer *et al.*, 1976a), as a molecule of the same size as the native protein. Both findings suggest an intracellular location for calcium-binding protein, in which case the protein may act as a 'buffer' for intracellular calcium permitting continued transport of Ca^{2+} across the mucosal cell. It is proposed that the presence of calcium-binding protein is necessary for maintaining the increased rate of Ca^{2+} absorption induced by 1,25-dihydroxycholecalciferol. Therefore, even if the direct mechanism of Ca^{2+} absorption turns out not to involve new protein synthesis, abolition of calcium-binding protein biosynthesis by inhibitors of protein synthesis could result in abolition of the increased Ca^{2+} absorption.

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