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Identification of intestinal cells responsive to calcitriol (1,25-dihydroxycholecalciferol)

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1. The location of intestinal cells taken from the base of the crypt to the tip of the villus responsive to calcitriol (1,25-dihydroxycholecalciferol) and the distribution of [^{3}H]calcitriol within the intestinal epithelium has been determined in vitamin D-deficient rats. 2. The calcitriol responses examined were (*a*) CaBP (Ca^{2+}-binding protein) levels as measured by immunodiffusion and (*b*) alkaline phosphatase levels as determined cytochemically. 3. Calcitriol had no effect on villus structure or on enterocyte kinetics. This made it possible to compare levels of CaBP and alkaline phosphatase activity in enterocytes at different ages in rats at known times after hormone injection. 4. Cells from both the crypt and villus synthesized CaBP in response to calcitriol. 5. Alkaline phosphatase activity was not detectable in crypt cells, although a pool of precursor was produced in these cells in response to calcitriol. Enzyme activity was increased in all villus cells in response to calcitriol, but the quantitative description of this effect was very different from that found for calcitriol effects on CaBP synthesis. 6. Calcitriol injected into vitamin D-deficient rats was detected, within 2 h, in all cells of the crypt and villus. Most of the binding was to sites having a high affinity for the injected hormone.

One of the major roles of the steroid hormone calcitriol is in permitting the transfer of Ca^{2+} across intestinal mucosal cells as a contribution to the maintenance of Ca^{2+} homoeostasis. This calcitriol-dependent Ca^{2+} absorption, although perhaps not quantitatively the most important route, can nevertheless be of critical importance in many physiological conditions, e.g. growth. Consequently the molecular mechanism by which Ca^{2+} absorption occurs and the role of calcitriol in this process have been extensively investigated in recent years. Calcitriol is known to effect a number of biochemical changes including an obligatory role in the synthesis of CaBP (Wasserman *et al.*, 1978). It also stimulates the activity of a number of enzymes, including alkaline phosphatase. This latter response may be due in part to an increase in the synthesis of this enzyme, although there is no obligatory requirement for calcitriol in this synthesis (Wilson & Lawson, 1982).

During the migration of enterocytes from their origin in the crypts to the villus tip, their structure and function undergo continual change. Both the structure of the microvillus and the peptidase and disaccharidase contents of the brush-border membrane become fully expressed earlier than for alkaline phosphatase. The ability of these cells to absorb nutrients occurs even later in development (Smith, 1984). The site of Ca^{2+} absorption along the villus is not known at the present time, although the distribution of CaBP has been studied with conflicting results. Immunocytochemically CaBP has been described as present in villus cells only, but no allowance was made for the migration of enterocytes in the time between dosing with calcitriol and fixing the tissue (Morrissey *et al.*, 1978; Thorens *et al.*, 1982). Consequently it is not possible to decide from this type of study which cell type responds to calcitriol. In other immuno-

Abbreviations used: CaBP, Ca^{2+}-binding protein; calcitriol, 1,25-dihydroxycholecalciferol.

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histochemical studies CaBP was observed in both villus and crypt cells (Arnold *et al.*, 1976; Jande *et al.*, 1981). These conflicting results are probably ascribable, in part at least, to the need for sensitive assay techniques to detect the protein in thin sections. In one previous study in which a sensitive radioimmunoassay system was applied to the detection of CaBP in a relatively large number of cells collected from different regions of the villus and crypt, the protein was found in both cell types. However, the frequency of sampling and method of analysis of the data was insufficient to identify the time of onset of the response of each cell and the capacity of this response (Shinki *et al.*, 1982).

In the present study we have attempted to resolve these differences by analysing CaBP concentrations at several points in cells prepared sequentially from along the villus–crypt axis by the method of Weiser (1973). We also decided to study further the postulated relation between calcitriol effects on CaBP and alkaline phosphatase synthesis by comparing results obtained with CaBP against those obtained for alkaline phosphatase determined using a method of cytochemical analysis similar to that described recently when determining developmental profiles for other enzymes in rat intestine (King *et al.*, 1983). A preliminary account of part of the present work has already been published in abstract form (Lawson *et al.*, 1983).

Methods

Animals

Piebald weanling rats born from females with a low-vitamin D status were raised on a vitamin D-deficient diet having a Ca : P ratio of 4 : 1. After 2–3 weeks plasma calcitriol was undetectable and rickets had developed, as shown by X-ray examination of the tibia.

Experimental

Rats were killed at known times after receiving 100 ng of calcitriol dissolved in propylene glycol by intracardiac injection and the mid-jejunum removed and divided into three parts. One part was opened and fixed in 4% (v/v) glutaraldehyde 2% (w/v) sucrose buffer (0.1 M-phosphate buffer, pH 7.2) for subsequent measurement of villus length and crypt depth. A second part was opened and mounted between two pieces of liver to be frozen in isopentane cooled in liquid N_2 for the subsequent determination of alkaline phosphatase activity. The third part was everted to form a sac, which was shaken in 2 mM-citrate buffer, pH 7.13, and then in 1.5 mM-EDTA to remove enterocytes sequentially for subsequent analysis. About 10–13 samples were recovered from different experiments, each sample varying in protein content. Total protein recovered has been found previously to be associated with near complete removal of enterocytes from the jejunum. It was therefore possible to present percentages of total protein recovered in individual fractions as percentages of the total number of enterocytes originally present in the tissue and normalize the results to represent 10% recovery in each fraction as described previously (Smith & Moor, 1984).

Analytical

The method used to collect cells sequentially from the villus–crypt axis of vitamin D-deficient and calcitriol-injected rats was similar to that described originally by Weiser (1973). The CaBP content of the cytosol of cells prepared in this way was measured by radial immunodiffusion using rabbit antisera produced against pure rat intestinal CaBP (Bruns *et al.*, 1978). The location on the villus–crypt axis from which cells had been collected was monitored, in these experiments, by carrying out parallel assays for γ-glutamyl transferase by the method of Szasz (1969). The specific activity of this enzyme was found, as expected, to show a fourfold fall in moving from cells obtained from the villus tip to those obtained from the crypt. Routine protein determinations were carried out by the method of Markwell *et al.* (1978).

Alkaline phosphatase activity in the brush-border membrane of cells along the villus–crypt axis was determined by microdensitometry of the reaction product formed by incubation of frozen sections with 6-bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide phosphate, with Fast Blue B as a coupling reagent. The time of incubation and the concentration of substrate used were both chosen to ensure that only initial rates of substrate hydrolysis were being measured (Gutschmidt *et al.*, 1980).

Growth curves were fitted to the results obtained to provide quantitative estimates of control and calcitriol-stimulated developmental profiles for alkaline phosphatase appearance during enterocyte development. The minimization procedure used in these analyses was as described previously (King *et al.*, 1983).

Enterocyte lifespan was estimated in some experiments by injecting [^{3}H]thymidine ($1\ \mu Ci \cdot g^{-1}$) intraperitoneally into the rats. In these cases pieces of the mid-jejunum were also examined autoradiographically. The position of the leading edge of thymidine-labelled enterocytes was measured in eosin-stained autoradiographs by using a curtain eyepiece micrometer (King *et al.*, 1983). A similar micrometer was also used to determine villus length and crypt depth in sections stained with haematoxylin and eosin.

Calcitriol high-affinity binding sites

The uptake of [26,27-^{3}H]calcitriol (158 Ci/mmol; Amersham International) was measured by injecting rats with 2.5 μCi of labelled steroid. After 2–3 h the rats were killed and the enterocytes isolated as described above. The radioactivity in the cells was extracted with chloroform/methanol (1 : 2, v/v) and measured with a Packard Tricarb liquid-scintillation counter. In another experiment a rat received 2.5 μCi of [^{3}H]calcitriol together with 200-fold excess of non-labelled steroid and the radioactivity in the isolated cells was measured as described above.

Results and discussion

Enterocyte kinetics and crypt-villus structure

Cholecalciferol has been reported to increase both villus height and enterocyte migration time in both vitamin D-deficient chicks (Spielvogel *et al.*, 1972) and in rats (Sampson & Krawitt, 1976). With our vitamin D-deficient rats, however, a single injection of 100 ng of calcitriol had no effect on villus height, crypt depth or enterocyte migration time measured 3, 6, 12, 18, 24 and 38 h subsequently (mean values $\pm$ S.E.M. of 447 $\pm$ 6 μm, 132 $\pm$ 2 μm and 11.4 $\pm$ 0.7 $\mu m \cdot h^{-1}$ for villus height, crypt depth and enterocyte migration rate respectively).

This ability to dissociate the short-term effects of calcitriol on intestinal CaBP content and alkaline phosphatase activity from any effect on intestinal structure or enterocyte kinetics was exploited in the present study to estimate the time course with which calcitriol-mediated events took place in single enterocytes, challenged by the hormone at different stages of development.

Calcitriol-dependent changes in rat intestinal CaBP and alkaline phosphatase activity

The time course for increase in CaBP and alkaline phosphatase activity after calcitriol injection into vitamin D-deficient rats is shown in Fig. 1. Values for CaBP have been obtained by adding together amounts found in individual cell fractions; those for alkaline phosphatase have been obtained by summing the amount of reaction product formed in brush-border membranes along a section of intestinal villus. Responses first detected 6 h after injection reached maximal values after a further 12–18 h and began to disappear 38 h after injection. The response of CaBP was more noticeable than that seen for alkaline phosphatase. Both the nature and magnitude of CaBP and alkaline phosphatase responses were similar to those reported previously from experiments using more conventional techniques of analysis on homogenates of intestinal mucosa (Armbrecht *et al.*, 1980; Thomasset *et al.*, 1979).

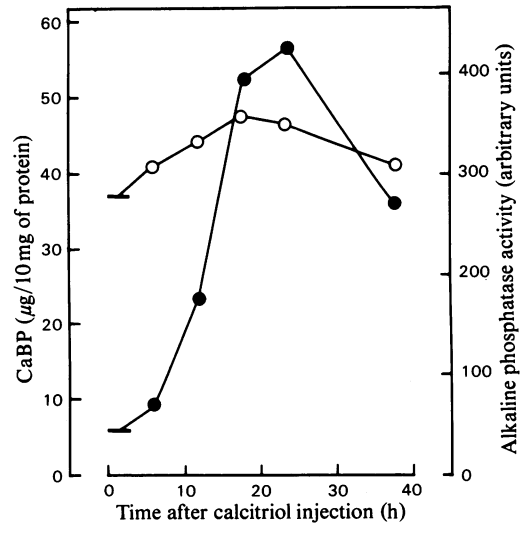


Fig. 1. *Time course for CaBP and alkaline phosphatase response to calcitriol injection into vitamin D-deficient rats*
Values for CaBP (●) were obtained by adding amounts determined from cell-shaking experiments; those for alkaline phosphatase (○) have been obtained by adding activities determined cytochemically from base to tip of villi. Each point gives the mean of determinations carried out on eight rats. The bars give values based on tissues taken from vitamin D-deficient rats before, and 3 h after, injection of calcitriol (16 animals).

The advantage of using cell fractionation and quantitative cytochemistry to measure these effects is that changes can be related to the age of individual enterocytes. The way in which increases in CaBP content are distributed along the crypt–villus axis in response to calcitriol injection is shown in Fig. 2.

There is some residual CaBP in vitamin D-deficient enterocytes and this appears in greatest concentration in villus tip cells (first cells to be collected; 0–3 h; Fig. 2). The amount of CaBP increases with time up to 24 h after injection, and this increase appears to take place along the whole length of the crypt–villus unit. The decay in CaBP levels occurring 38 h after injection also appears to take place in all enterocytes.

Enterocytes born in the crypt at the time of calcitriol injection migrate nearly to the tops of villi during 38 h (shown by the arrows in Fig. 2). By subtracting relevant concentrations of CaBP found in vitamin D-deficient rats from appropriate values found at 6, 12, 18 and 24 h after injection, it is possible to estimate the course of CaBP appearance in enterocytes of different ages. The results of this analysis for enterocytes aged 0.5, 10,

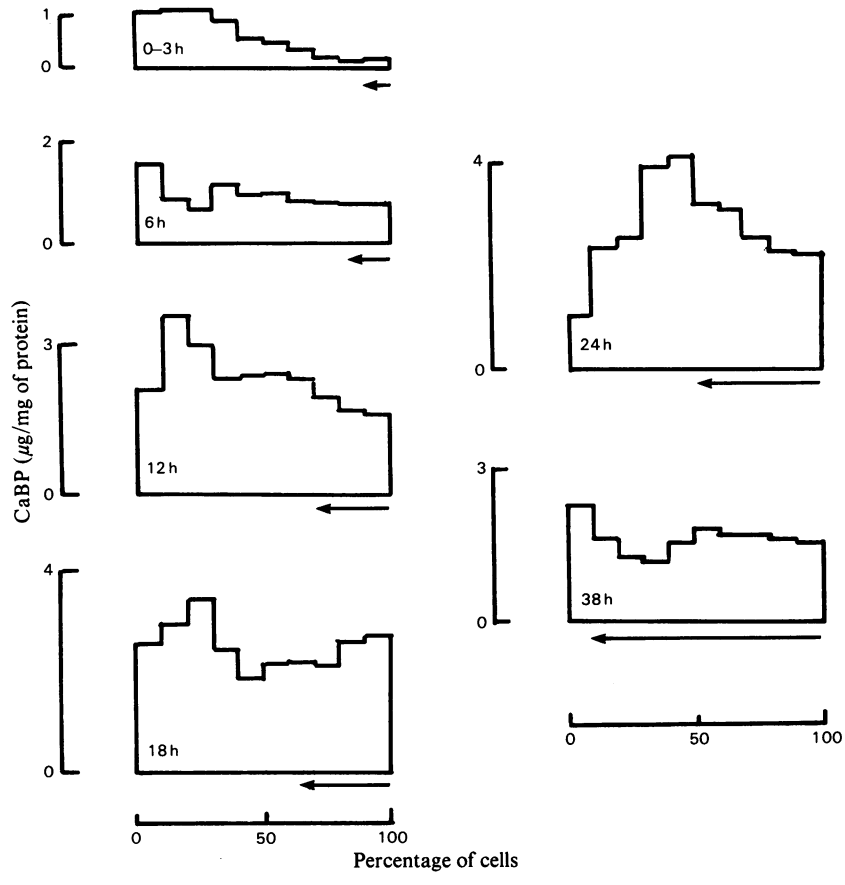
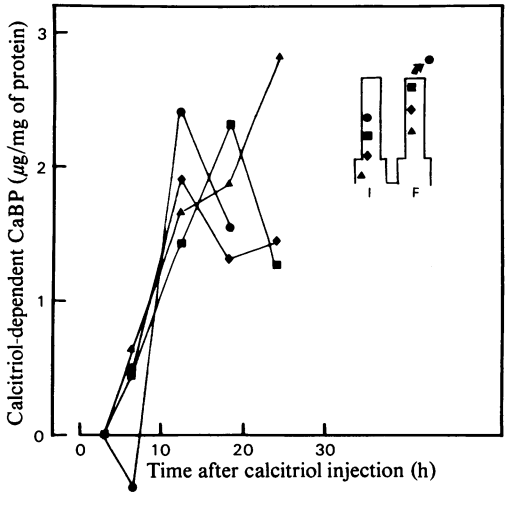


Fig. 2. *Time course for CaBP response to calcitriol injection in rat enterocytes of different ages*
CaBP contents were determined in cell fractions collected sequentially from the villi of vitamin D-deficient rats 0–3, 6, 12, 18, 24 and 38 h after calcitriol injection. The first cell fraction (0–10% cells) came from villus tips; the last cell fraction (90–100% cells) came from the crypt base. Values for control (0–3 h) and test conditions (6, 12, 18, 24 and 38 h) give means based on sixteen and eight rats respectively. The arrows show the progression with time of an enterocyte located at the base of a crypt at the time of calcitriol injection.



20 and 30 h at the time of hormone injection are summarized in Fig. 3. All cells appear to show a similar time course and magnitude of effect in their CaBP response to calcitriol injection.

Fig. 3. *Effect of calcitriol on CaBP content in enterocytes of different ages measured during migration along intestinal villi*
Values for calcitriol-dependent CaBP content have been calculated by subtracting the amounts of CaBP detected at particular levels on villi taken from vitamin D-deficient rats from those found at corresponding points on villi taken from rats killed 6, 12, 18 and 24 h after calcitriol injection. ▲, ◆, ■, ●, Enterocytes aged 0.5, 10, 20 and 30 h respectively at the time of calcitriol injection. I and F, cell positions at the time of injection and 38 h after injection respectively.

Effect of calcitriol on alkaline phosphatase development along rat intestinal villi

Preliminary work showed calcitriol to produce a modest effect on the total levels of alkaline phosphatase in rat small intestine (Fig. 1). Detailed analysis of this effect, however, showed the response to be particularly noticeable in younger enterocytes. The developmental profile for alkaline phosphatase in intestines taken from vitamin D-deficient rats, before and 18h after hormone injection, is shown in Fig. 4. All measurements start from the crypt–villus junction.

Although alkaline phosphatase could not be measured in the crypt cells from either group of rats, the amount of enzyme at the crypt–villus junction of hormone-injected animals (arrows, Fig. 4) was about twice that found in the vitamin D-deficient animal. Alkaline phosphatase in hormone-injected animals then increased more quickly than in control enterocytes. The respective rates of change of alkaline phosphatase activity with time are shown in the inset to Fig. 4. The effect of calcitriol is to reduce the time needed for an enterocyte to express alkaline phosphatase activity at maximal rate and to increase and sharpen the profile of expression. Similar intermediate-type responses were obtained for tissue taken 6, 12, 24 and 38h after hormone injection.

These changes can be expressed quantitatively by fitting growth curves to each set of experimental data according to the equation:

$$y = a + c/\{1 + \exp[-b(x-m)]\},$$

where a is the alkaline phosphatase activity detected when the cell is born (taken as zero), $a+c$ is the maximal alkaline phosphatase activity reached, m is the point of inflexion when expression of alkaline phosphatase takes place at maximal rate and b is a coefficient describing the shape of the developmental curve leading to full expression of the enzyme. Time courses showing the effect of

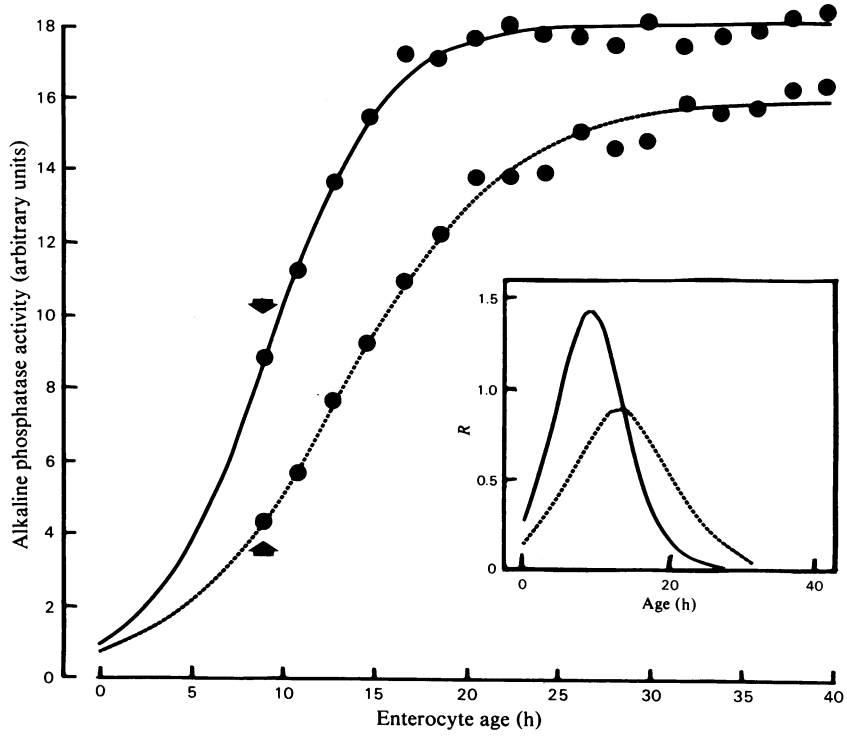


Fig. 4. *Time-dependency of alkaline phosphatase appearance in brush-border membranes of rat enterocytes*
Intestinal tissue taken from vitamin D-deficient rats 0–3h (-----) or 18h (——) after the injection of calcitriol was frozen, and sections were taken for the cytochemical determination of alkaline phosphatase activity as described in the text. Each point gives the mean of 24 or 48 determinations carried out on tissue obtained from 8 or 16 animals (0–3h and 18h curves respectively). The inset shows the rate of change (R) of alkaline phosphatase appearance during the life of an enterocyte. Arrows show the time when enterocytes pass the crypt–villus junction.

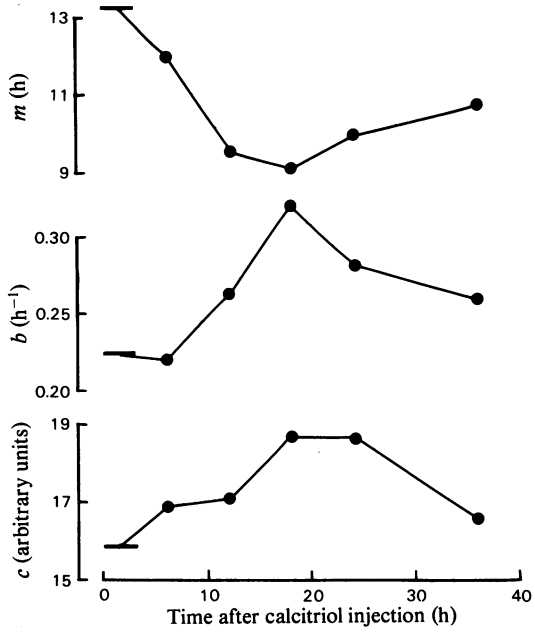


Fig. 5. *Time course for calcitriol effects on logistic curve constants used to describe developmental profiles for alkaline phosphatase appearance in rat intestinal brush-border membranes*

Logistic curves of the form:

$$y = a + c/\{1 + \exp[-b(x-m)]\}$$

were fitted to experimental results obtained 0–3, 6, 12, 18, 24 and 38 h after calcitriol injection into vitamin D-deficient rats. *c*, The maximum activity reached by an enterocyte assuming the starting activity (*a*) to be zero; *m*, the point of curve inflexion; *b*, the exponential coefficient describing the time-dependency of enzyme appearance.

calcitriol on *c*, *m* and *b* have been summarized in Fig. 5. The main effect of hormone is to cause an 18% increase in the final ability of a rat enterocyte to express this enzyme by reducing the parameter *m* and increasing the parameter *b* by 45 and 32% respectively.

Adopting the technique used originally for CaBP it was also possible to estimate the time course for changes in alkaline phosphatase taking place in a single enterocyte as it migrates along the villus. Results for enterocytes aged 3, 10, 20 and 30 h at the time of hormone injection have been summarized in Fig. 6. The magnitude of the response was greatest when given to a 3 h old enterocyte and least when given to 20- and 30 h-old cells. There also appeared to be a quicker response in the 3 h-old enterocyte, but it was not possible to judge whether this was statistically signifciant.

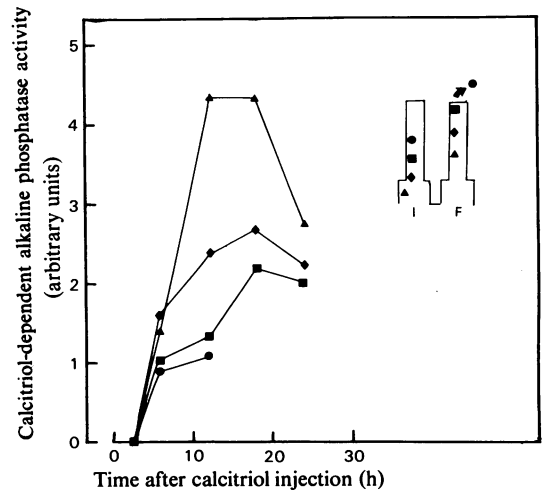


Fig. 6. *Effect of calcitriol on alkaline phosphatase appearance in enterocytes of different ages measured during migration along intestinal villi*

Values for calcitriol-dependent alkaline phosphatase activity were calculated as described for CaBP (Fig. 3). ▲, ◆, ■, ●, Enterocytes aged 3, 10, 20 and 30 h at the time of calcitriol injection. I and F, cell positions at the time of injection and 38 h after injection respectively.

Enterocyte distribution of calcitriol

It was decided to measure calcitriol distribution after intraperitoneal injection into vitamin D-deficient rats to determine, directly, the hormone distribution in enterocytes of different ages. Cell fractions were prepared 2–3 h after injection of labelled calcitriol. The results produced are shown in Fig. 7.

Calcitriol injected in the absence of non-radioactive carrier was found to be present in enterocytes of all ages (Fig. 7*a*). Repeating this experiment in the presence of 200-fold excess of non-radioactive calcitriol reduced considerably the amount of radioactivity found in any one fraction of enterocytes (Fig. 7*b*). It is concluded from this that a large proportion of total radioactivity represented calcitriol binding to high-affinity sites in enterocytes of all ages, a finding consistent with previous reports on the autoradiographic localization of calcitriol (Zile *et al.*, 1978).

In conclusion, calcitriol is found in all cells on the crypt–villus axis and all cells respond to calcitriol irrespective of their age or position. For CaBP, neither the magnitude nor the time course of this effect depends on the age of the enterocyte. In contrast, the youngest enterocytes ultimately contain the highest levels of alkaline phosphatase

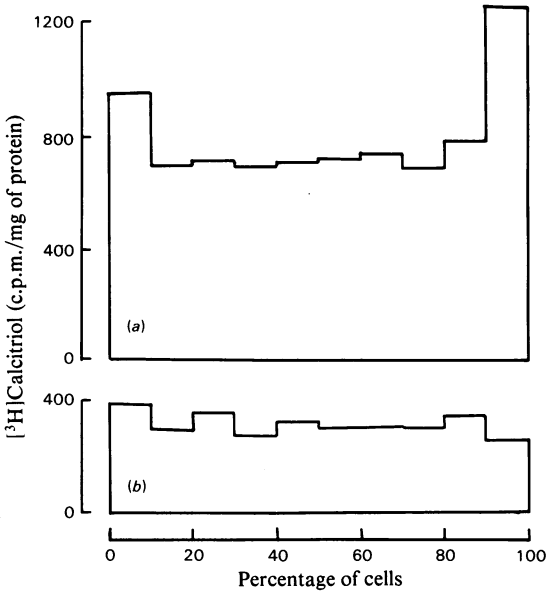


Fig. 7. *Calcitriol distribution in enterocytes of different ages*
[^{3}H]Calcitriol (2.5μCi) was injected intraperitoneally into vitamin D-deficient rats in the absence (*a*) or presence (*b*) of 1.25μg of non-radioactive calcitriol. Rats were killed 2h later and cell fractions prepared and counted for radioactivity as described in the text.

in response to this hormone. It seems that calcitriol increases the pool size of alkaline phosphatase in the crypt cells so that, on migrating on to the villus, their activation results in a much greater response than occurs with vitamin D-deficient cells. Nevertheless, calcitriol increases alkaline phosphatase activity in villus cells by a direct effect. The molecular mechanism of this effect is not shown by the present study, but clearly the extended time for the maximum response to be reached suggests that the effect is to increase the amount of enzyme synthesized.

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