Kinetics of subcellular distribution in rat intestine of 1,25-dihydroxycholecalciferol administered *in vivo*

Evidence for concentration within 5 min into purified nuclei

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To better understand the initial steps in the induction of intestinal Ca^{2+} transport by 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$], we studied the early subcellular localization of $1,25(\text{OH})_2\text{D}_3$ in rat intestine. Vitamin D-deficient rats received 300 pmol of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ intravenously at 5 min to 4 h before being killed. Cells homogenized in buffer of $I = 90$ mmol/litre were fractionated by centrifugation into a crude nuclear pellet, purified nuclei, Golgi and basal-lateral membranes, cytosol and a post-nuclear pellet. Nuclear purification was established by biochemical and morphological criteria and gave a yield of $32 \pm 2\%$ (mean $\pm$ S.E.M.; $n = 21$). Although re-establishment of Ca^{2+} uptake by Golgi is one of the earliest reported intestinal responses to $1,25(\text{OH})_2\text{D}_3$, no direct localization of $1,25(\text{OH})_2\text{D}_3$ to Golgi was detected. Purified nuclei had the highest specific radioactivity at all times studied, with nuclear localization detectable at 5 min and peak nuclear uptake at 1 h. Relative specific radioactivity of nuclei to cytosol increased from 5 min to 30 min, at which time equilibrium between cytosol and nucleus appeared to be attained. Nuclear uptake occurred in all cells from villus to crypt. Of total nuclear binding 10% was resistant to high ionic strength buffer ($I = 365$ mmol/litre); peak nuclear uptake was observed at 30 min in this buffer. This tight binding may represent the active fraction of $1,25(\text{OH})_2\text{D}_3$. These results indicate that localization of $1,25(\text{OH})_2\text{D}_3$ to rat intestinal nuclei precedes the observed Golgi-membrane effects and suggest the existence of high-affinity nuclear $1,25(\text{OH})_2\text{D}_3$ -binding sites.

Very little is known about the mechanism of action of $1,25(\text{OH})_2\text{D}_3$ on intestinal epithelial cells to increase Ca^{2+} transport (Lawson, 1981). Although $1,25(\text{OH})_2\text{D}_3$ first binds to a cytosolic receptor protein and is then concentrated in the nucleus, a definite relationship between subsequent nuclear events and increased Ca^{2+} transport remains to be established. Increased synthesis of CaBP mRNA in chick intestinal nuclei occurs after Ca^{2+} transport has already increased (Spencer *et al.*, 1978); this has not been studied in the rat. It has been reported that increases in nuclear RNA polymerase II activity (Zerwekh *et al.*, 1974), chromatin template activity (Zerwekh *et al.*, 1976) and RNA synthesis (Tsai & Norman, 1973) in response to $1,25(\text{OH})_2\text{D}_3$ precede

stimulation of Ca^{2+} transport; however, more recent measurements indicate that Ca^{2+} transport is increased by 1–2 h after $1,25(\text{OH})_2\text{D}_3$ (Morrissey *et al.*, 1978; Spencer *et al.*, 1978; MacLaughlin *et al.*, 1980), earlier than that observed previously. $1,25(\text{OH})_2\text{D}_3$ could also have a function in the nucleus that is unrelated to transcription, but this has not been studied.

The earliest reported intestinal response to $1,25(\text{OH})_2\text{D}_3$ is enhanced Ca^{2+} uptake by rat intestinal Golgi membrane vesicles, which occurs 15 min after intravenous administration of $1,25(\text{OH})_2\text{D}_3$ and precedes any detectable increase in Ca^{2+} transport by duodenal gut sacs (MacLaughlin *et al.*, 1980). The rapidity of the Golgi response suggests that it might represent a direct effect of $1,25(\text{OH})_2\text{D}_3$ on Golgi membrane or a secondary response to earlier nuclear or extra-nuclear actions of $1,25(\text{OH})_2\text{D}_3$.

In the present paper we describe a method for

Abbreviations used: $1,25(\text{OH})_2\text{D}_3$, $1\alpha,25$ -dihydroxycholecalciferol; $25(\text{OH})\text{D}_3$, 25 -hydroxycholecalciferol; CaBP, vitamin D-dependent intestinal Ca^{2+} -binding protein.

isolating rat intestinal nuclei that appears to overcome problems previously reported (Lawson *et al.*, 1969) and show that by 5 min after an intravenous dose of $1,25(\text{OH})_2\text{D}_3$, nuclear localization has occurred. Concentration of $1,25(\text{OH})_2\text{D}_3$ into Golgi membranes was not observed. A portion of the $1,25(\text{OH})_2\text{D}_3$ in the nucleus is not extracted by a high-ionic-strength buffer ($I = 365 \text{ mmol/litre}$). By analogy with steroid hormones, this tightly bound $1,25(\text{OH})_2\text{D}_3$ may represent the biologically active fraction (Clark & Peck, 1976; Ruh & Baudendistel, 1977). The data are interpreted as evidence that nuclear localization of $1,25(\text{OH})_2\text{D}_3$ occurs early enough for nuclear events to account for the rapid $1,25(\text{OH})_2\text{D}_3$ -dependent recovery of Golgi membrane Ca^{2+} uptake.

Experimental

Materials

Female Holtzman rats (21 days old) were fed on a rachitogenic diet for 12–16 days and then kept on a vitamin D-deficient diet. Normal rats were fed on a control diet throughout. All animals received food *ad lib*.

The three rat diets were purchased from Teklad Test Diets (Madison, WI, U.S.A.) and are based on the work of Guroff *et al.* (1963). The rachitogenic diet (TD79068) contained no added Ca^{2+} or vitamin D; the vitamin D-deficient diet (TD79094) contained no vitamin D but had 0.436% Ca^{2+} ; and the control diet (TD79095) had 0.53% Ca^{2+} plus 2000 units of vitamin D per kg. Vitamin D-deficiency was established by reduced growth, decreased serum Ca^{2+} ($2.51 \pm 0.06 \text{ mmol/litre}$ for controls versus $1.48 \pm 0.06 \text{ mmol/litre}$ for vitamin D-deficient rats), and, in older vitamin D-deficient rats, skeletal deformities.

$1\alpha,25$ -Dihydroxy[$26,27\text{-}^3\text{H}$]cholecalciferol [$1,25(\text{OH})_2[{}^3\text{H}]\text{D}_3$] was purchased from Amersham (Arlington Heights, IL, U.S.A.) at a specific radioactivity of 160 Ci/mmol. Non-radioactive $1,25(\text{OH})_2\text{D}_3$ was a gift from Dr. Michael Holick (Endocrine Unit, Massachusetts General Hospital, Boston, MA, U.S.A.). Concentrations of $1,25(\text{OH})_2\text{D}_3$ were determined in redistilled methanol using $18\,200 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for the molar absorption coefficient at 264 nm (Holick & DeLuca, 1975). For intravenous injection, the $1,25(\text{OH})_2[{}^3\text{H}]\text{D}_3$ was diluted with non-radioactive $1,25(\text{OH})_2\text{D}_3$ to a specific radioactivity of 2.5 or 5.0 Ci/mmol. Solvent was then removed with a stream of N_2 and the $1,25(\text{OH})_2\text{D}_3$ was redissolved in 95% (v/v) ethanol to give 0.75 (or 1.50) $\mu\text{Ci}/300 \text{ pmol}$ of $1,25(\text{OH})_2\text{D}_3$ in a volume of 50 μl .

Other chemicals were purchased as reagent-grade and used without further purification.

Methods

Labelling procedure. Rats (6–14 weeks old) were given 50 μl (300 pmol) of $1,25(\text{OH})_2[{}^3\text{H}]\text{D}_3$ in 95% (v/v) ethanol via the tail vein and were subsequently killed by cervical dislocation.

Purification of nuclei. The procedure developed is a modification of the method described by Lawson *et al.* (1969) for chick intestinal nuclei. The small intestine was removed, rinsed with cold 0.154 M-NaCl + 2.5 mM-dithiothreitol, and then filled with the same solution, the ends clamped, and incubated at 37°C for 3 min in 0.154 M-NaCl. The intestines were then rinsed again with the same solution, blotted to remove excess buffer, slit longitudinally and the mucosa collected by scraping with a glass slide on an ice-cold glass plate. A 10% (w/v) homogenate of the mucosa was prepared in STKMD buffer (approx. 55 mM-Tris was adjusted to pH 7.5 with concentrated HCl and diluted to 50 mM; 0.32 M-sucrose, 25 mM-KCl, 5 mM-MgCl₂, 2.5 mM-dithiothreitol) using 10 strokes with the loose pestle and 10 strokes with the tight pestle of a Dounce homogenizer. The homogenate was centrifuged for 10 min at 1°C and 1250 g ($r_{\text{av}} = 7 \text{ cm}$) in a Beckman JA20 rotor. The pellet was resuspended by vortex-mixing in the same volume of STKMD buffer as used for the initial homogenization and centrifuged again under the same conditions. The supernatant from the first centrifugation was used to prepare the cytosol and 'post-nuclear pellet' fractions (see below).

The crude nuclei (2–3 ml/rat) were purified by suspension in 25 ml of SCD (2.4 M-sucrose/1 mM- CaCl_2 /2.5 mM-dithiothreitol) buffer added slowly with vigorous vortex-mixing, and subsequent centrifugation for 1 h at 1°C at 50 000 g ($r_{\text{av}} = 11.3 \text{ cm}$) in a Beckman SW40 rotor. The purified nuclei formed a white pellet, whereas debris including unbroken cells, brush border and broken and trapped nuclei, collected as a brown layer (ST) on the top of the supernatant. The nuclear pellet was suspended in STKMD buffer.

Electron microscopy. Isolated nuclei were fixed overnight in 4% (v/v) glutaraldehyde (Ladd Research Industries, Burlington, VT, U.S.A.) in Sørensen's buffer (Sørensen, 1909a,b) containing 0.5 mM- CaCl_2 . Nuclei were rinsed in three changes of Sørensen's buffer for 30 min and postfixed for 2 h in 1% (v/v) OsO_4 in Sørensen's buffer. Material was then dehydrated through a graded series of ethanol and acetone, and embedded in Spurr low-viscosity embedding medium (Spurr, 1969). Sectioning was performed using either glass or diamond knives on an LKB Ultratome III (LKB Instruments, Hicksville, NY, U.S.A.). Sections of thickness 1–2 μm were utilized for light microscopy. Thin sections were poststained with uranyl acetate (Watson, 1958) and lead citrate (Venable & Coggeshall, 1965) and observed on a Siemens Elmiskop 1A Electron

Microscope at 80kV (Siemens Corp., Cherry Hill, NJ, U.S.A.).

Experiments with isolated enterocytes. Isolated intestinal epithelial cells were obtained by sequential washing of the intestine as described by Weiser (1973). Nuclei were prepared from these isolated cells by the procedure described above. Seventy strokes with the tight Dounce pestle were required for complete disruption of isolated cells. The yield of nuclei was comparable with that obtained from mucosal scrapings.

Preparation of other subcellular fractions. Golgi and basal-lateral membranes vesicles were prepared as described by Freedman *et al.* (1977) and Weiser *et al.* (1978). Purified microvillus membranes were isolated by the procedure of Forstner *et al.* (1968) as modified by Hopfer *et al.* (1973). Cytosol was obtained from the first supernatant in the nuclear preparation procedure (above) by further centrifugation for 1 h at 1°C and $116\,000\text{ g}$ ($r_{\text{max.}} = 10.8\text{ cm}$) in a Beckman 50.2 Ti rotor. The pellet from this centrifugation ('post-nuclear pellet') contained mitochondria and subcellular membranes.

Assays. Lactate dehydrogenase activity (EC 1.1.1.27) was quantified by following the oxidation of NADH (Wroblewski & LaDue, 1955). Sucrase activity (EC 3.2.1.48) was assayed by the method of Dahlquist (1968). Alkaline phosphatase activity (EC 3.1.3.1) was measured as described by Weiser (1973), except that the final concentration of Zn^{2+} was 3 mM. Succinate: 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride reductase activity was measured by the method of Morré (1971). NADPH-cytochrome *c* reductase activity (EC 1.6.2.4) was quantified by the procedure of Masters *et al.* (1967). Galactosyltransferase activity was measured as described by Podolsky & Weiser (1975). Protein was quantified by the dye-binding method of Bradford (1976) using protein reagent and bovine γ -globulin standards purchased from Bio-Rad Laboratories, Richmond, CA, U.S.A. DNA was measured by the diphenylamine method as modified by Burton (1956).

Radioactivity measurements. Samples ($300\mu\text{l}$ or less, in duplicate) were digested in Protosol (New England Nuclear, Boston, MA, U.S.A.), 15 ml of OSC scintillation cocktail (Amersham) was added, and the radioactivity was measured in a Packard model 3325 Counter (Packard Instruments, Downers Grove, IL, U.S.A.). Counting efficiency of the internal standard ($[^3\text{H}]\text{hexadecane}$; Amersham) was 35–40%.

Results

Purification of nuclei

Failure of previous attempts to purify nuclei from rat intestine has been attributed to the mucus in rat

intestine (Busch *et al.*, 1958; Lawson *et al.*, 1969). The 3 min incubation at 37°C in the presence of 2.5 mM-dithiothreitol and the inclusion of 2.5 mM-dithiothreitol in all buffers served to obviate this problem in our procedure. We recovered more than 98% of the total DNA in the crude nuclear fraction. Yield of purified nuclei was $32 \pm 2\%$ (mean $\pm$ S.E.M.; $n = 21$) based on DNA recovery. Another critical step in the purification appeared to be homogenization. With mucosal scrapings, the yield of nuclei was decreased when more than ten strokes of the tight Dounce pestle were used for the crude homogenate. Rehomogenization of the layer floating on the 2.4 M-sucrose (50 strokes, tight pestle), however, resulted in the release of part of the DNA as additional intact nuclei. In one experiment, yield of nuclei was increased from 25 to 58%. Rehomogenization was not used in the present experiments because it caused the loss of $1,25(\text{OH})_2\text{D}_3$ from the nuclei (30% decrease in pmol/mg of protein), indicative of the loosely bound $1,25(\text{OH})_2\text{D}_3$, described below.

Plate 1 shows a phase-contrast micrograph of nuclei purified by this method. No contamination with brush-border membranes, mitochondria or unbroken cells is apparent and nucleoli are readily visible. Plate 2, an electron micrograph, shows that the nuclear double membrane is intact; broken nuclei are seldom seen. There is little contamination of nuclei with cytoplasm or membranes. Thus, morphologically, the purified nuclei appear undamaged and uncontaminated with other subcellular fractions.

Marker enzyme activities for other subcellular fractions were determined for purified nuclei (Table 1). These results corroborate the morphological evidence of little contamination of the nuclei with other cell fractions. Although galactosyltransferase has been considered a marker for Golgi membranes, the presence of galactosyltransferase activity, together with a slight increase in the specific activity of this enzyme in nuclei, are consistent with previous reports of its partial nuclear localization (Richard *et al.*, 1975; Berthillier *et al.*, 1980). It is unlikely that the nuclear galactosyltransferase activity is due to the presence of Golgi membranes because of the absence of contaminating membranes in Plates 1 and 2 (and in other fields examined but not illustrated) and the low values for enzymes specific for other subcellular membranes (Table 1). The protein/DNA ratio in purified nuclei was 3.1 ± 0.1 (mean $\pm$ S.E.M.). This is similar to values reported for nuclei from chick intestine (Haussler *et al.*, 1968; Lawson *et al.*, 1969) and from non-intestinal tissues of other species (Siebert & Humphrey, 1965; Tsai *et al.*, 1980).

The intestinal epithelium is composed of mitotically active crypt cells, senescent villus absorptive

Table 1. *Biochemical characteristics of purified nuclei*

Nuclear activities were assayed in the resuspended pellet obtained after centrifugation through 2.4 M-sucrose. Assays were performed as described in the Experimental section. Mucosa were homogenized in STKMD buffer. The subcellular location is indicated in parentheses.

Enzyme	Specific activity (nmol of product/min per mg of protein)	
	Homogenate	Nuclei
Lactate dehydrogenase (cytosol)	2420	50
Sucrase (microvilli)	460	100
Alkaline phosphatase (microvilli)	55 000	9000
Succinic:2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride reductase (mitochondria)	600	210
NADP-cytochrome c reductase (microsomes)	4.4	0.6
Galactosyltransferase	0.00042	0.00053
Protein/DNA ratio	20	3.1

cells with pyknotic nuclei and cells in all intermediate stages of differentiation. We attempted to determine whether the purified nuclear pellet was enriched for nuclei from one of these cell types. [^3H]Thymidine (40 μCi) was administered intraperitoneally 2.8 h before being killed as a means of labelling nuclei of those cells that are dividing. Acid-precipitable radioactivity of the homogenate, purified nuclei and ST layer (debris) was measured. There was a slight enrichment for crypt nuclei in the purified nuclear pellet but the increase in specific radioactivity (from 2.0×10^5 d.p.m./mg of DNA in the homogenate to 2.8×10^5 in nuclei) was too small to conclude that the purified nuclei are representative of crypt-cell nuclei. We have also prepared isolated crypt and villus cells by the method of Weiser (1973) and compared the yields of nuclei from these fractions. A slightly higher yield of DNA was obtained from the middle villus (34%) and crypt fractions (25%) compared with the villus tip cells (21%).

Subcellular distribution of injected 1,25(OH) $_2$ [^3H]-D $_3$

Subcellular membrane fractions were prepared from intestines of vitamin D-deficient rats given 1,25(OH) $_2$ [^3H]D $_3$ intravenously 30 min before being killed. The specific radioactivities of the various fractions are shown in Table 2. No membrane fraction had a higher specific radioactivity than the initial homogenate. In particular, there is a lack of concentration of 1,25(OH) $_2$ D $_3$ in Golgi membrane at the time (30 min) when peak Ca $^{2+}$ uptake is observed in these vesicles (MacLaughlin *et al.*, 1980). Within 30 min after injection of 1,25(OH) $_2$ [^3H]D $_3$, most of the homogenate radioactivity that was not soluble after centrifugation at 116 000 g (r_{max} = 10.8 cm) for 60 min was localized to the crude nuclear pellet. The time course of the localization of 1,25(OH) $_2$ D $_3$ to

Table 2. *Distribution of 1,25(OH) $_2$ D $_3$ in subcellular membrane fractions*

Rats (three to four) were labelled intravenously with 0.75 nCi (300 pmol) of 1,25(OH) $_2$ [^3H]D $_3$ 30 min before being killed. Fractionation was done as described in the Experimental section. Results are means $\pm$ S.E.M., where indicated.

Subcellular fraction	1,25(OH) $_2$ D $_3$ (fmol/mg of protein)
Homogenate	26 $\pm$ 6
Microvillus membrane	23
Golgi membrane	20 $\pm$ 3
Basal-lateral membrane	13 $\pm$ 2
50% sorbitol layer + pellet (mitochondria + debris)	24 $\pm$ 7

purified intestinal nuclei and other subcellular fractions was studied further.

Kinetics of nuclear localization of 1,25(OH) $_2$ D $_3$

Vitamin D-deficient rats were injected intravenously with 300 pmol (0.75 or 1.50 μCi) of 1,25(OH) $_2$ [^3H]D $_3$ and killed from 5 min to 4 h later. At all times examined, purified nuclei had the highest specific radioactivity of 1,25(OH) $_2$ [^3H]D $_3$ and the postnuclear pellet had the lowest specific radioactivity of the fractions examined (Fig. 1a). The specific radioactivity of the cytoplasm was intermediate between that of the homogenate and the postnuclear pellet. The maximum specific radioactivity occurred at 1 h in all fractions.

Fig. 1(b) shows the early time points from Fig. 1(a) replotted using an expanded time axis. The ratio of nuclear to cytoplasmic specific radioactivity is shown in parentheses for each time point. Even at 5 min after administration, the nuclear specific radioactivity of 1,25(OH) $_2$ [^3H]D $_3$ is greater than the

EXPLANATION OF PLATE 1

Phase-contrast micrograph of purified rat intestinal nuclei

Purified nuclei (SCD pellet) from a vitamin D-deficient rat were fixed and sectioned as described in the Experimental section (magnification, $\times 3500$).

EXPLANATION OF PLATE 2

Electron micrograph of purified rat intestinal nuclei
Sample was prepared as in Fig. 1. Magnification, $\times 12000$.

cytoplasmic specific radioactivity. The ratio of nuclear to cytoplasmic specific radioactivity increases until 30 min and remains constant thereafter.

Fig. 1. Time course of subcellular localization of $1,25(\text{OH})_2\text{D}_3$ in rat intestinal mucosa homogenized in STKMD buffer

(a) Vitamin D-deficient rats received 300 pmol of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ by tail-vein injection and were killed at the indicated times. Subcellular fractions were prepared as described in the Experimental section. Error bars indicate S.E.M. for three to five rats. (b) Replot of 5 min, 10 min and 30 min points for nuclei and cytosol from (a) using an expanded time scale. Numbers in parentheses indicate the ratio of nuclear to cytosolic specific activity at that time.

Characterization of nuclear binding

The ratio of nuclear to cytoplasmic specific radioactivity is constant at 2.2 from 30 to 240 min. This suggests that nuclear uptake of $1,25(\text{OH})_2\text{D}_3$ which occurs after 30 min, is rapidly reversible. Further evidence for the loose binding of a considerable portion of the $1,25(\text{OH})_2\text{D}_3$ in nuclei purified in STKMD buffer was obtained: (i) washing the purified nuclei five times in STKMD buffer decreased the specific radioactivity by 40–60%; (ii) nuclei obtained by rehomogenizing the ST layer had a lower specific radioactivity than those in the initial nuclear pellet.

Disorganization of the cell structure by homogenization raises the possibility that the nuclear localization of $1,25(\text{OH})_2\text{D}_3$ is an artefact of the homogenization process. We attempted to reproduce the observed nuclear/cytoplasmic distribution by adding $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ directly to the mucosal scrapings after the rats were killed. The results (Table 3) indicate that when $1,25(\text{OH})_2\text{D}_3$ is added *in vitro*, in either of two different media, nuclear concentration does not occur at 0°C . We conclude that the subcellular distribution of $1,25(\text{OH})_2\text{D}_3$ shown in Figs. 1(a) and 1(b) represents the physiological distribution that occurs in the mucosal epithelial cells *in vivo*.

Exposure to buffers of higher ionic strength has been reported to decrease the number of occupied (Haussler & Norman, 1969; Lawson & Wilson, 1974) and unoccupied (Franceschi & DeLuca, 1979) $1,25(\text{OH})_2\text{D}_3$ receptors in chick intestinal nuclei. The calculated ionic strength ($I = 90 \text{ mmol/litre}$) of the STKMD buffer used earlier to purify the nuclei is considered to be low to intermediate (Franceschi & DeLuca, 1979; Walters *et al.*, 1980). We investigated the effect of ionic strength on the subcellular distribution of $1,25(\text{OH})_2\text{D}_3$ in rat intestine by homogenizing in a high-ionic-strength buffer (KTMD) identical with STKMD buffer but containing 300 mM-KCl and no sucrose (calculated $I = 365 \text{ mmol/litre}$). The yield of DNA was comparable in the two buffers provided care was taken to prevent aggregation of the crude nuclear pellet when resuspending in KTMD buffer. Nuclei prepared from KTMD-buffer-based homogenates had a protein/DNA ratio of 2.4 ± 0.1 (mean $\pm$ S.E.M.), lower than for STKMD-buffer-prepared nuclei. This is not surprising since high-ionic-strength buffers are known to extract a number of nuclear proteins (Patel, 1974; Weisbrod & Weintraub, 1979).

The effect of KTMD buffer was studied at 10 min, 30 min and 60 min after the injection of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ (Fig. 2). The specific radioactivities of the postnuclear pellet and nuclei were decreased and the specific radioactivity of cytosol was increased compared with fractions prepared from STKMD-buffer-based homogenates. The maximum concen-

Table 3. *Effect of route of administration of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ on its subcellular distribution in intestinal epithelial cells*
 Animals in group I received $0.75 \mu\text{Ci}$ (300 pmol) of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ intravenously 1 h before being killed. For group II, $0.13 \mu\text{Ci}$ of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ was dissolved in $750 \mu\text{l}$ of serum obtained from a vitamin D-deficient rat. Of this solution, $250 \mu\text{l}$ [$0.04 \mu\text{Ci}$; 15 pmol of $1,25(\text{OH})_2\text{D}_3$] was added to mucosal scrapings from another vitamin D-deficient rat before homogenization. For group III, cytosol was prepared from intestinal mucosa of vitamin D-deficient rats that had been injected intravenously with $0.75 \mu\text{Ci}$ (300 pmol) of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ 1 h before being killed. This cytosol was used as the homogenization medium from mucosal scrapings from uninjected vitamin D-deficient rats. After killing the animals (group I) or addition of label to scrapings (groups II and III), all samples were kept at $0-4^\circ\text{C}$ during subcellular fractionation. All homogenates were prepared in STKMD buffer.

Route of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ administration	Distribution of mucosal $1,25(\text{OH})_2\text{D}_3$ * (%)		Relative specific activity of nuclei to cytosol†
	Cytosol	Nuclei	
I. <i>In vivo</i>	27	30	2.1
II. Serum, <i>in vitro</i>	69	0.3	0.20
III. Cytosol, <i>in vitro</i>	64	7	0.15

* (nCi in cytosol or nuclei/nCi in homogenate) $\times 100$.

† (nCi/mg of protein in nuclei)/(nCi/mg of protein in cytosol).

tration of $1,25(\text{OH})_2\text{D}_3$ in nuclei prepared in the high-ionic-strength buffer occurred at 30 min, at which time the activities in the homogenate and cytosol were still increasing, as was the nuclear concentration of $1,25(\text{OH})_2\text{D}_3$ in nuclei isolated from STKMD-buffer-based homogenates (Fig. 1a). This, together with the attainment of the maximum ratio of nuclear to cytoplasmic labelling in STKMD-buffer-prepared nuclei at 30 min (Fig. 1b), suggest that there is a class of nuclear binding sites for the $1,25(\text{OH})_2\text{D}_3$ -receptor complex that are filled within 30 min after administration of the active metabolite.

Distribution of $1,25(\text{OH})_2\text{D}_3$ along the villus

It has been suggested that $1,25(\text{OH})_2\text{D}_3$ may alter intestinal Ca^{2+} transport, in part, by extranuclear pathways (MacLaughlin *et al.*, 1980; Morrissey *et al.*, 1980) and that such pathways might be active predominantly in the mature villus absorptive cells (MacLaughlin *et al.*, 1980). If so, the subcellular distribution of $1,25(\text{OH})_2\text{D}_3$ in the villus-tip cells might be different from the distribution in the crypt cells. To test this, enterocytes from rats labelled intravenously for 80 min with $1.50 \mu\text{Ci}$ (300 pmol) of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ were isolated as a ten-fraction villus-to-crypt gradient by the method of Weiser (1973). Fractions 1-3, 4-7 and 8-10 were combined to give respectively villus-tip, middle-villus and crypt-cell pools. Each pool was homogenized in STKMD buffer and purified nuclei were prepared. Nuclei from the villus-tip cells had 120 fmol of $1,25(\text{OH})_2\text{D}_3/\text{mg}$ of DNA (450 molecules/nucleus), whereas nuclei from the middle-villus and crypt-cells pools each had 150 fmol of $1,25(\text{OH})_2\text{D}_3/\text{mg}$ of DNA (560 molecules/nucleus).

Partial characterization of the ST layer

The only subcellular fraction other than purified nuclei that had a high specific radioactivity after

Fig. 2. *Time course of subcellular localization of $1,25(\text{OH})_2\text{D}_3$ in rat intestinal mucosa homogenized in KTMD buffer*

Samples were prepared as described in the legend to Fig. 1(a), except that KTMD buffer (calculated ionic strength = 365 mmol/litre) was used as the homogenization medium. Error bars indicate S.E.M. for three to four rats.

labelling *in vivo* with $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ was the ST layer floating on top of the 2.4 M -sucrose. This layer contained two-thirds of the DNA and 56% of the radioactivity in ST could be accounted for by assuming that the DNA in the ST layer consisted of nuclei having the same specific radioactivity as purified nuclei. Attempts at fractionation of the ST layer led us to conclude that most of the remaining radioactivity was present as $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ -

receptor complex non-specifically bound to cellular debris.

Discussion

Ca^{2+} uptake by rat intestinal Golgi membrane vesicles has recovered by 66% within 15 min after giving $1,25(\text{OH})_2\text{D}_3$ to vitamin D-deficient rats (Freedman *et al.*, 1977; MacLaughlin *et al.*, 1980). The rapidity of this response, together with the structural similarities between $1,25(\text{OH})_2\text{D}_3$ and filipin, a polyene antibiotic that increases movement of Ca^{2+} across intestinal microvillus membranes (Norman & Ross, 1979), suggested that $1,25(\text{OH})_2\text{D}_3$ might be interacting directly with Golgi. Table 2 shows that no concentration of radioactivity was seen in Golgi membranes 30 min after the injection of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$, the time of maximal recovery of Ca^{2+} uptake by Golgi. It is possible that $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ was metabolized to a non-radioactive compound capable of causing the observed changes in Ca^{2+} uptake (Esvelt *et al.*, 1979). This is unlikely, however, since most of the $1,25(\text{OH})_2\text{D}_3$ present in the mucosal cells at early times appears unaltered (Frolik & DeLuca, 1972; Jones & Haussler, 1979). Thus, our results do not support a direct effect of $1,25(\text{OH})_2\text{D}_3$ on Golgi Ca^{2+} uptake.

There are a number of reports that $1,25(\text{OH})_2\text{D}_3$ is concentrated in cell nuclei of chick intestine (Lawson & Wilson, 1974; Brumbaugh & Haussler, 1974, 1975; Zile *et al.*, 1978; Jones & Haussler, 1979) and in other tissues from several species (Stumpf *et al.*, 1979; Colston & Feldman, 1980; Gelbard *et al.*, 1980; Eil & Marx, 1981). There are two reports on $1,25(\text{OH})_2\text{D}_3$ localization to the nucleus of rat intestinal mucosal cells (Chen & DeLuca, 1973; Bachelet *et al.*, 1977). The nuclear purification procedure used by Chen & DeLuca (1973) was that of Lawson *et al.* (1969), who stated that the method, when applied to rat intestine, gave a low and variable yield of nuclei that were contaminated with mucus and whole cells. In the other study (Bachelet *et al.*, 1977), no nuclear characterization was reported and the intestinal epithelium was incubated *in vitro* in a buffer containing $1,25(\text{OH})_2\text{D}_3$. Thus, their results may not represent events *in vivo*. Our procedure for purifying rat intestinal nuclei avoids these problems.

We confirmed that $1,25(\text{OH})_2\text{D}_3$ is present in rat intestinal cell nuclei and is concentrated in the nucleus within 5 min after intravenous administration (Fig. 1). This rapidity of localization to nuclei has not been reported previously. If $1,25(\text{OH})_2\text{D}_3$ behaves in a manner analogous to steroid hormones (Lawson, 1978), then the specific activity of hormone binding should initially be higher in the cytoplasm than in the nucleus (O'Malley *et al.*, 1971;

Marver *et al.*, 1972; Brumbaugh & Haussler, 1974; Clark *et al.*, 1976). Our data showing an increasing ratio of nuclear to cytoplasmic $1,25(\text{OH})_2\text{D}_3$ concentration between 5 and 30 min (Fig. 1b) suggest that $1,25(\text{OH})_2\text{D}_3$ must first bind to cytoplasmic receptors and then enter the nucleus as a hormone-receptor complex. Our data represent the first evidence that $1,25(\text{OH})_2\text{D}_3$ administered *in vivo* must be bound first by cytoplasmic receptors before being transferred to the nucleus. Because of the cost of commercially available $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ we were unable to study times before 5 min and document an early phase where the cytoplasmic concentration of $1,25(\text{OH})_2\text{D}_3$ is higher than that of nuclei.

The nuclear concentration of $1,25(\text{OH})_2\text{D}_3$ after administration *in vivo* does not appear to be an artefact of homogenization since addition of $1,25(\text{OH})_2[^3\text{H}]\text{D}_3$ to the homogenate *in vitro* did not result in significant nuclear labelling (Table 3). The nuclear localization may have been higher and more rapid than we detected since a significant proportion of the $1,25(\text{OH})_2\text{D}_3$ receptor, either occupied (Lawson & Wilson, 1974; the present results) or unoccupied (Walters *et al.*, 1980) is easily released from the nucleus.

Our results provide quantification of previous autoradiographic studies in chicks (Zile *et al.*, 1978; Jones & Haussler, 1979) and rats (Stumpf *et al.*, 1979), which show nuclear labelling in all epithelial cells of the villi and crypts. Thus, $1,25(\text{OH})_2\text{D}_3$ could affect epithelial cells in all states of differentiation, although there may be differences among cells in their response to $1,25(\text{OH})_2\text{D}_3$ (MacLaughlin *et al.*, 1980). The villus-to-crypt gradient of decreasing CaBP concentration in rat intestine, recently described by Marche *et al.* (1980), may be an example of such a differential response.

We observed that an increase in ionic strength markedly reduced the amount of $1,25(\text{OH})_2\text{D}_3$ associated with purified rat intestinal nuclei, confirming previous results obtained in chicks. $1,25(\text{OH})_2\text{D}_3$ bound to chick intestinal nuclei was reduced 70–80% when nuclei were prepared in a buffer similar in ionic strength to ours (90 mmol/litre) and subsequently treated with a buffer of higher ionic strength (365 mmol/litre) (Haussler & Norman, 1969; Lawson & Wilson, 1974). In our experiments the ionic strength of the homogenization buffer was altered, rather than exposing purified nuclei to different ionic strengths. This allowed establishment of the nuclear/non-nuclear distribution of $1,25(\text{OH})_2\text{D}_3$ -receptor complex in the presence of the homogenate and reduced the likelihood of trapping receptor aggregates in the nuclei during purification in a lower ionic strength buffer (Yamamoto & Alberts, 1974; Franceschi & DeLuca, 1979). This may account for our finding of

a 90–95% reduction in nuclear $1,25(\text{OH})_2\text{D}_3$ on increasing ionic strength. Even at an ionic strength of 90 mmol/litre (STKMD buffer), however, half of the $1,25(\text{OH})_2\text{D}_3$ can be extracted from nuclei by repeated washing at 4°C. Similarly, Lawson & Wilson (1974) found that up to 30% of the $1,25(\text{OH})_2\text{D}_3$ taken up by chick intestinal nuclei *in vivo* was released during a single incubation of the nuclei at 4°C in a buffer of the same ionic strength as STKMD.

The physiological significance of the tightly bound nuclear $1,25(\text{OH})_2\text{D}_3$, that is resistant to high salt solutions, is unclear. This tightly bound $1,25(\text{OH})_2\text{D}_3$ peaks earlier than does the total uptake seen at lower salt concentrations and, by analogy with nuclear uptake of oestrogens in rat uteri (Clark & Peck, 1976; Ruh & Baudendistel, 1977), may represent the active molecules. Based on 6.2 pg of DNA/rat genome (Lewin, 1980), we calculate that each rat intestinal nucleus contains 570 molecules of $1,25(\text{OH})_2\text{D}_3$, 60 min after injecting 300 pmol of $1,25(\text{OH})_2\text{D}_3$, and that about 30 of these molecules are resistant to high ionic strength. Brumbaugh & Haussler (1974) report that peak nuclear uptake in chick intestine is 1400 molecules per nucleus at 2 h after a dose of 390 pmol of $1,25(\text{OH})_2\text{D}_3$. Thus, compared with the chick, rat intestinal nuclei have a smaller amount of $1,25(\text{OH})_2\text{D}_3$ bound in low-ionic-strength buffers. Whether there is a difference in the amount of tightly bound nuclear $1,25(\text{OH})_2\text{D}_3$ between chicks and rats is not clear. After administration of radioactive vitamin D_3 (Haussler & Norman, 1969) or $25(\text{OH})\text{D}_3$ (Lawson & Wilson, 1974) to vitamin D-deficient chicks, approx. 80% of the nuclear label was removed by treatment with 0.3–0.4 M-KCl. Thus, a major fraction of the nuclear $1,25(\text{OH})_2\text{D}_3$ appears extractable by high ionic strength in both species. The nuclear components responsible for binding $1,25(\text{OH})_2\text{D}_3$ have not been determined. Barrack & Coffey (1980) reported finding specific binding sites for oestrogens and androgens on the nuclear matrix of target tissues. They suggested that the nuclear matrix may play a role in several aspects of nuclear function, including DNA synthesis and RNA synthesis and processing. It is possible that some of the $1,25(\text{OH})_2\text{D}_3$ may be associated with the nuclear matrix in intestinal cell nuclei.

Our results showing nuclear localization of $1,25(\text{OH})_2\text{D}_3$ within 5 min after its administration provide a basis to explain the early events mediated by $1,25(\text{OH})_2\text{D}_3$. In addition to Golgi Ca^{2+} uptake (Freedman *et al.*, 1977; MacLaughlin *et al.*, 1980), these include increased incorporation of radioactive amino acids into a mitochondrial protein within 60 min after $1,25(\text{OH})_2\text{D}_3$ administration (Hobden *et al.*, 1980) and into two other intestinal proteins within 2–3 h after $1,25(\text{OH})_2\text{D}_3$ administration

(Wilson & Lawson, 1977, 1978). Other examples are cited in Weiser *et al.* (1982).

In conclusion, rat intestinal epithelial cell nuclei were purified and shown to concentrate $1,25(\text{OH})_2\text{D}_3$ *in vivo* within 5 min. These results indicate that localization of $1,25(\text{OH})_2\text{D}_3$ to rat intestinal nuclei precedes the $1,25(\text{OH})_2\text{D}_3$ -induced increase in Golgi Ca^{2+} uptake. There is no evidence for accumulation of $1,25(\text{OH})_2\text{D}_3$ in Golgi. The kinetics of subcellular distribution support the concept of a rapid transfer of $1,25(\text{OH})_2\text{D}_3$ bound to a cytosolic receptor into the nucleus. Approx. 10% (30 molecules/nucleus) of the nuclear $1,25(\text{OH})_2\text{D}_3$ is tightly bound and may represent the functionally active component. This tight binding suggests the existence of high-affinity nuclear $1,25(\text{OH})_2\text{D}_3$ -binding sites.

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