*Journal of Steroid Biochemistry*, 1977, Vol. 8, pp. 1047–1049. Pergamon Press. Printed in Great Britain

# NUCLEAR UPTAKE OF CHOLECALCIFEROL METABOLITES IN RAT DUODENAL MUCOSA

M. BACHELET, A. ULMANN,* J. F. CLOIX and
J. L. FUNCK-BRENTANO
I.N.S.E.R.M. U.90, Hôpital Necker, 161, rue de Sèvres, 75730,
Paris Cedex 15, France

(*Received* 16 *February* 1977)

SUMMARY

1α, 25-Dihydroxycholecalciferol (1,25-$(OH)_2D_3$) and 25-hydroxycholecalciferol (25-$(OH)D_3$) nuclear uptake was *in vitro* studied in duodenal mucosa cells from vitamin D-deficient rats. Both 1,25-$(OH)_2D_3$ and 25-$(OH)D_3$ were found in association with purified nuclei. However a saturable maximal uptake (1.4 pmol/mg DNA) was demonstrated only for 1,25-$(OH)_2D_3$. This 1,25-$(OH)_2D_3$ nuclear uptake is specific as it is not altered by the presence of either 25-$(OH)D_3$ or 1α-hydroxycholecalciferol.

## INTRODUCTION

Inside chick intestine 1α, 25-dihydroxycholecalciferol (1,25-$(OH)_2D_3$), an active vitamin $D_3$ metabolite [1] may act in a way similar to steroid hormones. This mechanism involves 1,25-$(OH)_2D_3$ binding to a cytoplasmic protein or receptor. The resulting sterol–receptor complex then migrates to the nucleus and binds to the chromatin. This results in genome modification, preceding hormone specific protein's synthesis [2–7].

In order to investigate whether the same mechanism occurs in the rat duodenum, we have studied the 1,25-$(OH)_2D_3$ and 25-hydroxycholecalciferol (25-$(OH)D_3$) nuclear uptake in duodenal mucosa cells *in vitro*.

## MATERIALS AND METHODS

*Sterols.* 25-hydroxy [26(27)-methyl-[$^3$H]] cholecalciferol ([$^3$H]-25-$(OH)D_3$) (7.1–9.7 Ci/mmol) was purchased from Amersham. Tritiated 1,25-$(OH)_2D_3$ ([$^3$H]-1,25-$(OH)_2D_3$) was obtained by incubation of rachitic chick kidney homogenates with [$^3$H]-25-$(OH)D_3$. A 10% (w/v) kidney homogenate was prepared in 50 mM Tris–acetate buffer (pH 7.4) supplemented with $Mg^{2+}$ (1.9 mM), succinate (10 mM) and sucrose (180 mM), as described by Omdahl *et al.* [20]. Eight ml fractions of the homogenate were incubated in 250 ml Erlenmeyer flasks with 150 ng of [$^3$H]-25-$(OH)D_3$ for 1 h at 37° under oxygen. The incubation mixtures were extracted with methanol–chloroform (2:2, v/v). The chloroform extract was chromatographed on a 2 × 60 cm column containing 30 g of Sephadex-LH 20 equilibrated with chloroform–hexane (65:35, v/v), and the [$^3$H]-1,25-$(OH)_2D_3$ obtained was re-chromatographed on a 1 × 60 cm Sephadex-LH 20 column eluted with the same solvent mixture.

Crystalline 1,25-$(OH)_2D_3$ was kindly supplied by Dr. Uskokovic (Hoffman–La Roche Laboratories, Nutley, NJ, U.S.A.) and 1α-hydroxycholecalciferol (1α-$(OH)D_3$) by Leo Laboratories (Aarhus, Denmark). Crystalline 25-$(OH)D_3$ was generously given by Roussel Laboratories.

*Animals.* Animals used were male weanling Wistar rats (60–100 g) raised on a vitamin D-deficient and low calcium diet as described by Suda *et al.* [19]. Their blood calcium level measured at the time of sacrifice was always below 4 mg/100 ml.

*Tissue incubations with sterols and preparation of nuclei.* Animals were killed by cervical dislocation and their duodenum immediately removed, rinsed and blotted at 4°. The mucosa was then scraped from the submucosa with a microscope slide. The mucosa from 1–2 rats were then immediately incubated for 1 h at 37° with 1 ml of buffer A (Tris–HCl: 10 mM, pH 7.4, sucrose: 250 mM, $MgCl_2$: 1 mM) containing the sterols dissolved in small amounts of ethanol (1.5%, final concentration). After incubation, mucosa fragments were extensively washed at 4° and homogenized in the same buffer with a Potter homogeniser (Teflon pestle, 6 strokes at a low speed). All subsequent operations were carried out at 4°. The homogenates were centrifuged at 800 ***g*** for 10 min. The resulting crude nuclear pellets were rinsed 4 times in buffer A, each rinse being followed by a short centrifugation (800 ***g*** for 10 min). The last pellet was then mixed with 10 ml of a 2 M-sucrose solution and centrifuged at 50,000 ***g*** for 60 min in a Sorvall RC-5 centrifuge. The resulting purified nuclear pellets were taken up in 1 ml of buffer A containing Triton-X-100 (1%, final concentration), mixed and left for 10 min at 4°. The nuclei were then recollected and washed twice with Triton-free buffer A, each wash being followed by a short centrifugation (10 000 ***g*** for 2 min).

* To whom correspondence should be sent.



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This method yielded reasonably pure nuclei, as verified by phase-contrast microscopy.

*Additional procedures.* Purified nuclei were taken up in 0.6 ml of a methanol–chloroform mixture (2:1, v/v) and left for 18 h at 4°. They were then centrifuged at 10 000 *g* for 2 min. Radioactivity was measured on two aliquots (0.1 ml) of the supernatant: each aliquot was mixed with 5 ml of a scintillation mixture (Unisolve, K and L) and radioactivity was measured with an Intertechnique Scintillation Counter (efficiency for tritium: 35%). DNA content of the pellet was assayed according to Burton[8] with calf thymus DNA (Sigma) as standard.

## RESULTS

Duodenal mucosa fragments were incubated for 1 h at 37° with [$^3$H]-25-(OH)$D_3$ (5 nM), and radioactivity measured in purified nuclei as described in "Materials and Methods" (Fig. 1, left). 0.78 ± 0.41 pmol/mg DNA (mean ± S.E.M. of 5 experiments) were found in association with nuclei (total nuclear uptake). [$^3$H]-25-(OH)$D_3$ Nuclear uptake was 0.83 ± 0.19 pmol/mg DNA (mean ± S.E.M. of 5 experiments) when incubations were carried out with [$^3$H]-25-(OH)$D_3$ (5 nM) plus unlabelled 25-(OH)$D_3$ (0.5 μM): thus no [$^3$H]-25-(OH)$D_3$ saturable nuclear uptake was demonstrable. When similar experiments were carried out with [$^3$H]-1,25-(OH)$_2D_3$ (8 nM), a 1.42 ± 0.24 pmol/mg DNA (mean ± S.E.M. of 3 experiments) total nuclear uptake was observed (Fig. 1, right). The presence of unlabelled 1,25-(OH)$_2D_3$ in incubation media decreased [$^3$H]-1,25-(OH)$_2D_3$ nuclear uptake to 0.75 ± 0.13 pmol/mg DNA (mean ± S.E.M. of 3 experiments), whereas the presence of unlabelled 25-(OH)$D_3$ (0.8 μM) had no effect on [$^3$H]-1,25-(OH)$_2D_3$ nuclear uptake. In addition

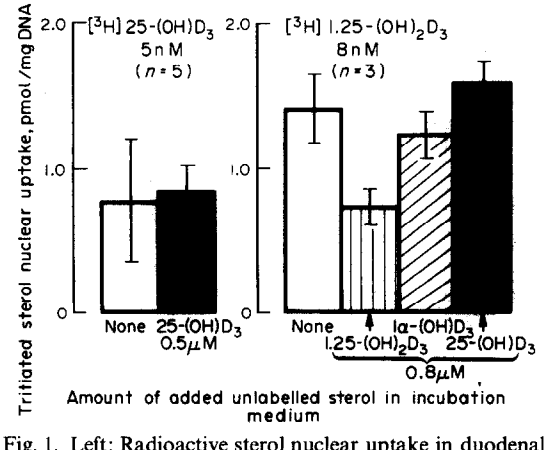


Fig. 1. Left: Radioactive sterol nuclear uptake in duodenal mucosa fragments incubated with [$^3$H]-25-(OH)$D_3$ either alone, or with unlabelled 25-(OH)$D_3$, for 1 h at 37°. Right: Radioactive sterol nuclear uptake in duodenal mucosa fragments incubated with [$^3$H]-1,25-(OH)$_2D_3$ either alone, or in the presence of unlabelled 25-(OH)$D_3$ or 1α-(OH)$D_3$ for 1 h at 37° (vertical bars represent S.E.M.; n: number of experiments).

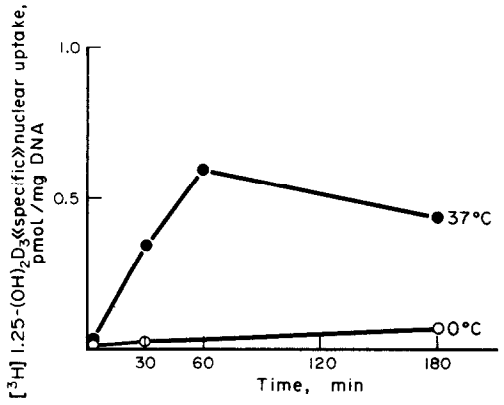


Fig. 2. Duodenal mucosa fragments incubated at 37° or 0° for various times with [$^3$H]-1,25-(OH)$_2D_3$ (5 nM) either alone (total uptake) or in the presence of 5 μM of unlabelled 1,25-(OH)$_2D_3$ (non-specific uptake). Data represent "specific" uptake (total minus non-specific uptake). Each point is the mean of 2 experiments.

Fig. 1 (right) shows that incubations in the presence of unlabelled 1α-(OH)$D_3$ (0.8 μM) resulted in a non-significant decrease of [$^3$H]-1,25-(OH)$_2D_3$ nuclear uptake.

Next, duodenal mucosa fragments were incubated for increasing times (from 0–180 min) at 37° or 0° with [$^3$H]-1,25-(OH)$_2D_3$ (5 nM), either alone (total uptake) or in the presence of unlabelled 1,25-(OH)$_2D_3$ (non specific uptake). Figure 2 shows that at 37° [$^3$H]-1,25-(OH)$_2D_3$ specific nuclear uptake (total minus non-specific uptake) is maximal for a 60-min incubation. At 0°, no significant [$^3$H]-1,25-(OH)$_2D_3$ specific nuclear uptake is demonstrable.

In Fig. 3, duodenal mucosa fragments were incubated with increasing amounts of [$^3$H]-1,25-(OH)$_2D_3$ (from 1–23 nM) either alone (total uptake) or in the presence of a 100-fold relative excess of unlabelled 1,25-(OH)$_2D_3$ (non specific uptake): 1,25-(OH)$_2D_3$ specific nuclear uptake appears to be saturable (maximum specific uptake: 1.4 pmol/mg DNA).

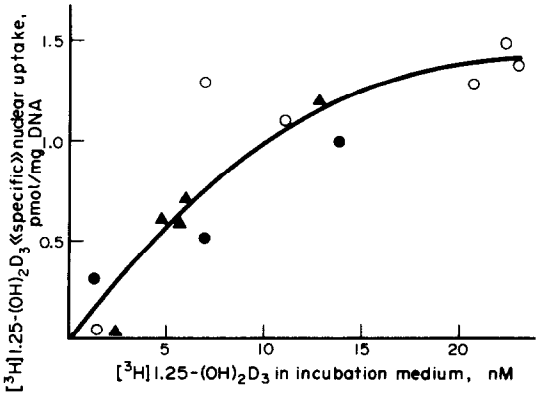


Fig. 3. [$^3$H]-1,25-(OH)$_2D_3$ Specific nuclear uptake when duodenal fragments are incubated with increasing amounts of [$^3$H]-1,25-(OH)$_2D_3$ either alone (total uptake), or in the presence of a 100-fold relative excess unlabelled 1,25-(OH)$_2D_3$ (non specific uptake). Specific uptake: total minus non-specific uptake (different symbols design experiments performed on different days).

## DISCUSSION

Our results show that both [$^3$H]-$1,25\text{-}(OH)_2D_3$ and [$^3$H]-$25\text{-}(OH)D_3$ are found in association with nuclei prepared from rat duodenal mucosa fragments incubated with these sterols. However a saturable nuclear uptake is demonstrated only for $1,25\text{-}(OH)_2D_3$, not for $25\text{-}(OH)D_3$. Maximal $1,25\text{-}(OH)_2D_3$ specific nuclear uptake is approximately 1.4 pmol/mg DNA, which represents about 5000 sterol molecules per nucleus. These experiments can be paralleled with those of Chen and DeLuca [9] who found that $1,25\text{-}(OH)_2D_3$ when injected intravenously to vitamin D-deficient rats binds to a nuclear component inside intestinal mucosa cells whereas $25\text{-}(OH)D_3$ does not. Our results are comparable with those obtained in chick intestine [5–7]: [$^3$H]-$1,25\text{-}(OH)_2D_3$ Specifically binds to chromatin, and only large amounts of unlabelled $25\text{-}(OH)D_3$ decrease this binding. Although we did not ourselves perform any chromatographic analysis of radioactive nuclear content, it seems unlikely from *in vivo* experiments [1, 21] that inside nuclei [$^3$H]-$1,25\text{-}(OH)_2D_3$ is being further metabolized.

These results imply that rat duodenal mucosa cytosol contains specific $1,25\text{-}(OH)_2D_3$ receptors. However, several workers [10–12] including ourselves [13] have found that rat duodenal mucosa cytosol contains macromolecules (sedimentation rate in low or high ionic strength: 5.5–6 *s*) having a higher affinity for $25\text{-}(OH)D_3$ than for $1,25\text{-}(OH)_2D_3$. In addition, such macromolecules have a widespread distribution and have been described in apparently noncholecalciferol target cells [14]. Therefore, these macromolecules are unlikely to represent the $1,25\text{-}(OH)_2D_3$ specific receptors. Such receptors could well be the labile macromolecules recently described by Kream *et al.*[15] in rat duodenal mucosa cytosol (sedimentation rate in high ionic strength: 3.2 *s*).

Such $1,25\text{-}(OH)_2D_3$ nuclear uptake correlates well with the fact that at the intestinal level, $1,25\text{-}(OH)_2D_3$ is the most potent cholecalciferol metabolite [16]. This suggests that in the rat as in the chick $1,25\text{-}(OH)_2D_3$ might work, at least in part, via a nuclear mechanism of action. This action could result in *de novo* protein synthesis, among which could be a calcium-binding protein (CaBP) [17]. Within such a hypothesis, the fact that $1\alpha\text{-}(OH)D_3$ did not decrease [$^3$H]-$1,25\text{-}(OH)_2D_3$ nuclear uptake is in agreement with previous data suggesting that $1\alpha\text{-}(OH)D_3$ acts only after a 25-hydroxylation [18], which cannot happen in rat intestine [18]. Nevertheless, non-specific sterol nuclear association could be of some physiological importance and for example it could perhaps explain why $25\text{-}(OH)D_3$ *per se* has been shown to increase the CaBP content of rat intestinal mucosa cells in culture [17].

*Acknowledgements*—We wish to thank Dr. M. Garabedian (Laboratoire des tissus calcifiés, Hôpital Necker-Enfants Malades, Paris, France) for preparing tritiated $1,25\text{-}(OH)_2D_3$. This work was supported by a grant of the I.N.S.E.R.M.

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