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# Nuclear uptake of 1,25-dihydroxy[$^3$H]cholecalciferol in dispersed fibroblasts cultured from normal human skin

(vitamin $D_3$/receptors/MCF-7 cells/hormone resistance)

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**ABSTRACT** **Because of the relative inaccessibility of known calciferol target tissues (i.e., intestine and bone), we examined fibroblasts derived from normal human skin and grown in tissue culture as a means of evaluating the interaction of 1,25-dihydroxycholecalciferol [1,25(OH)$_2$D$_3$] and its effector system. When dispersed, intact cells were used, nuclear uptake of 1,25-dihydroxy[23,24(*n*)-$^3$H]cholecalciferol [1,25(OH)$_2$[$^3$H]D$_3$] was temperature-dependent, optimal at 45 min at 37°C, and saturable. In competition experiments with other calciferols, the 1,25(OH)$_2$[$^3$H]D$_3$ uptake showed specificity indistinguishable from that reported for 1,25(OH)$_2$D$_3$ receptors from calciferol target tissues. Analysis of 1,25(OH)$_2$[$^3$H]D$_3$ nuclear uptake in fibroblast strains from six normal adults (four male, two female) yielded an average binding capacity ($R_0$) of 10,600 ± 2,000 (SEM) nuclear sites per cell and an apparent dissociation constant ($K_d$) of 0.50 ± 0.07 (SEM) × $10^{-9}$ M. Donor sex, donor age, or anatomic site of origin of the cell line did not affect the characteristics of uptake. Similar nuclear uptake was demonstrable with cultured MCF-7 cells (derived from human breast cancer) when assayed in the same fashion. When hypertonic extracts of nuclei obtained from skin fibroblasts incubated with 1,25(OH)$_2$[$^3$H]D$_3$ were subjected to centrifugation on sucrose gradients, a single peak of radioactivity sedimented at ≈3 S; when excess 1,25(OH)$_2$D$_3$ was coincubated during the cellular uptake phase, this 3S peak was not observed. Molybdate was an essential buffer component for receptor stabilization during cell fractionation and sedimentation analysis. In summary, by using fibroblasts cultured from normal human skin, we have identified a process of nuclear uptake of 1,25(OH)$_2$[$^3$H]D$_3$ with the affinity, saturability, and specificity characteristics of a steroid hormone–receptor interaction. This method should be useful in studying 1,25(OH)$_2$D$_3$ receptor physiology in cells from normal persons as well as in cells from patients who have disorders in the responsiveness of calciferol target tissues.**

Bioactive forms of the calciferols, like those of other steroid hormones, are thought to act via high-affinity receptor proteins (1–3). Once the hormone is bound to the receptor, an "activated" hormone–receptor complex is formed that interacts in the nucleus, where subsequent gene activation occurs. The tissues that calciferol is known to act on (i.e., intestine and bone) contain high-affinity, saturable binding components consistent with this mechanism (4, 5). Inaccessibility of these tissues, however, has hindered the study of vitamin D receptor physiology in man. Other tissues that are similarly inaccessible, such as parathyroid (6, 7), pancreas (8), kidney (7), pituitary (7), and mammary gland (9), have also been found to have high-affinity calciferol receptors. To facilitate the study of 1,25-dihydroxycholecalciferol [1,25(OH)$_2$D$_3$] receptors in humans, we have examined fibroblasts cultured from normal human skin and now report that they show a nuclear uptake process that has the characteristics of 1,25(OH)$_2$D$_3$ receptor mediation.

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## MATERIALS AND METHODS

**Materials.** Collagenase (type 1, from *Clostridium histolyticum*), Tricine [*N*-tris (hydroxymethyl)methyl glycine] and dextran (clinical grade) were purchased from Sigma. Fetal calf serum (mycoplasma and virus screened) was from GIBCO. Tris was from Bethesda Research Laboratories (Rockville, MD). 1,25-Dihydroxy[23,24(*n*)-$^3$H]cholecalciferol [1,25(OH)$_2$[$^3$H]D$_3$; 82 Ci/mmol; 1 Ci = 3.7 × $10^{10}$ becquerels] was from Amersham. Liquid scintillation fluid (Aquasol) was from New England Nuclear. Mixed beef and pork insulin (Iletin, U-100) was from Eli Lilly. Tissue culture flasks (75 and 150 cm$^2$) were from Costar (Cambridge, MA), Falcon, and Corning. Petri dishes, 60 mm in diameter, also were from Falcon. 1,25(OH)$_2$D$_3$ and other calciferol analogs were provided by M. Uskokovic of Hoffman–La Roche. All tissue culture media, trypsin/EDTA solutions, and phosphate-buffered saline (P$_i$/NaCl) were prepared and supplied by the media unit of the National Institutes of Health.

**Cell Culture.** Fibroblast strains were established from skin specimens obtained at surgery or by biopsy using a 4-mm disposable biopsy punch as described (10). The specimens were finely minced in 60-mm-diameter Petri dishes containing 4 or 5 ml of improved Eagle's minimal essential medium supplemented with 10% fetal calf serum/0.1 μM insulin/glutamine (0.584 g/liter)/penicillin (100 units/ml)/streptomycin (100 μg/ml)/collagenase (2 mg/ml). (This medium was freshly prepared for each sample and filtered through a Swinnex-13 Millipore filter immediately before use.) After 24 hr exposure at 37°C in 5% $CO_2$/95% air in a humidified incubator, the medium was changed to a growth medium (medium A) consisting of improved Eagle's minimal essential medium supplemented with penicillin (100 units/ml)/streptomycin (100 μg/ml)/glutamine (0.584 g/liter)/10% (vol/vol) fetal calf serum/0.1 μM insulin. As soon as the cells became confluent (usually in 1–3 weeks), they were detached with 0.05% trypsin/0.02% EDTA in saline at 37°C and passed serially into larger culture flasks, 75 cm$^2$ and then 150 cm$^2$. MCF-7 breast cancer cells were provided by M. E. Lippman.

**Nuclear 1,25(OH)$_2$D$_3$ Uptake.** For analysis, fibroblasts were grown to confluence in four or five 150-cm$^2$ tissue culture flasks. This usually required 4–6 weeks from the time of the initial processing of the skin sample. Two days before the assay, the

Abbreviations: 1,25(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol; 1,25(OH)$_2$[$^3$H]D$_3$, 1,25-dihydroxy[23,24(*n*)-$^3$H]cholecalciferol; 25(OH)D$_3$, 25-hydroxycholecalciferol; P$_i$/NaCl, phosphate-buffered saline.

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medium was changed to medium B (medium A without fetal calf serum). This was repeated 24 hr before the assay. The nuclear uptake of $1,25(OH)_2[^3H]D_3$ was examined by using a modification of the method of Tsai and Samuels (11), as described for the measurement of androgen receptors in dispersed fibroblasts (10). Cells were incubated with 0.01% trypsin/0.02% EDTA in saline for 5–10 min at 37°C, collected by centrifugation at 800 × g, and suspended in cold $P_i$/NaCl; this suspension was centrifuged, and the pellet was suspended at a cell density of ≈1–2 × $10^6$ cells per ml in assay medium (medium C), consisting of medium B without insulin and supplemented with 50 mM Tricine·HCl (pH 7.4). This cell suspension (0.8 ml) was added to 0.2 ml of medium C containing $1,25(OH)_2[^3H]D_3$. The final incubation mixtures consisted of 1.0 ml of medium C in 12 × 75 mm glass test tubes (Kimble, Toledo, OH) containing ≈1 × $10^6$ cells and $1,25(OH)_2[^3H]D_3$ at 0.03–1.0 nM. The radioactive ligand was prepared by evaporating the initial material under a gentle stream of nitrogen and then redissolving it in an equal volume of absolute ethanol. Various amounts of this stock solution were added directly to medium C; 0.2 ml of solution was placed in a scintillation vial containing 1 ml of ethanol and 8.5 ml of Aquasol to determine the total concentration of $1,25(OH)_2[^3H]D_3$ in each incubation. The final concentration of ethanol in the incubation mixtures did not exceed 1.0%. Nonspecific (low-affinity) uptake was determined in a parallel set of tubes by addition of 1 μM unlabeled $1,25(OH)_2D_3$ directly to the empty test tubes from a stock solution in ethanol. The ethanol was evaporated before the assay.

The reaction mixtures were incubated for 45 min at 37°C. After this, 2 ml of $P_i$/NaCl was added, and the cells were collected by centrifugation for 1 min at 1000 × g in a Clay–Adams Sero-FugeII, suspended in 2 ml of $P_i$/NaCl, by mixing in a Vortex, and recentrifuged. The cell pellets were suspended by mixing in a Vortex in 2 ml of buffer D (0.25 M sucrose/20 mM Tris, pH 7.85/1.1 mM $MgCl_2$/0.5% Triton X-100) at 4°C, and the suspension was then centrifuged at 1000 × g for 3 min to collect the nuclei. The nuclear pellet was suspended in 2 ml of buffer D, and the suspension was centrifuged. This nuclear pellet was then suspended in 0.2 ml $P_i$/NaCl. One milliliter of 95% ethanol was added, and the entire mixture was transferred to scintillation vials containing 8.5 ml of Aquasol for spectrophotometric measurement of the radioactivity. For data expression, we quantitated cells with a hemocytometer (precision of ± 3.5%), using a control tube of cells that had been incubated without radioactive ligand and washed but not exposed to buffer D. Scatchard analysis was accomplished by using a computer program (12). Binding capacity was expressed as sites per cell. Competition studies with cholecalciferol analogs were performed with 0.7 nM $1,25(OH)_2[^3H]D_3$; calciferol metabolites were added from stock solutions in ethanol that was evaporated before the assay. Integrity of $1,25(OH)_2[^3H]D_3$ was examined by high-pressure liquid chromatography (13).

**Sucrose Density-Gradient Analysis.** Cells were harvested, incubated as above for estimation of nuclear $1,25(OH)_2[^3H]D_3$ uptake with 0.8 nM $1,25(OH)_2[^3H]D_3$, and washed twice with $P_i$/NaCl. Low-affinity uptake was assessed by the addition of 1 μM $1,25(OH)_2D_3$ during incubation. The cells were suspended in buffer E (10 mM Tris·HCl, pH 7.4/10 mM Na molybdate/1.5 nM EDTA/0.5 mM dithiothreitol) and homogenized with a tight-fitting Dounce homogenizer at 4°C. The crude nuclear pellet, obtained by centrifugation at 1000 × g for 10 min at 4°C, was then suspended in 0.5 ml of buffer E supplemented with 0.3 M KCl and homogenized with a Dounce homogenizer for 30 min at 4°C. To remove unbound $1,25(OH)_2[^3H]D_3$ from the extracts without diluting the samples, pellets were prepared by centrifugation with 0.5 ml of

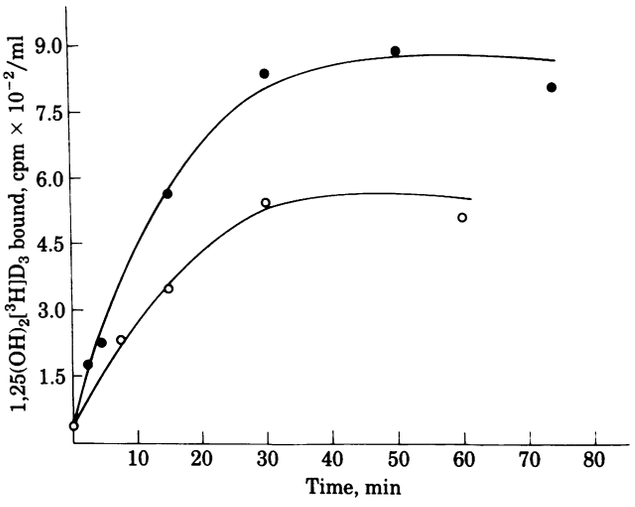


FIG. 1. Time course of high-affinity $1,25(OH)_2[^3H]D_3$ nuclear uptake. Whole cells from dispersed human skin fibroblasts were incubated for various times with 0.4 nM $1,25(OH)_2[^3H]D_3$ at 22°C (○) or at 37°C (●). Low-affinity uptake (not shown) was determined at each time point by the addition of 1 μM unlabeled $1,25(OH)_2D_3$ and subtracted from the total uptake.

dextran-coated charcoal suspension [0.25% (wt/wt) Norit A, 0.0024% (wt/wt) dextran in 0.01 M Tris·HCl, pH 7.4] at 1000 × g for 10 min; then, 0.5 ml of the hypertonic extracts was added and mixed on a Vortex, and the mixtures were allowed to stand 10 min at 4°C. The mixtures were centrifuged for 10 min at 1000 × g at 4°C, and 0.35 ml of the supernatant together with $^{14}C$-labeled bovine serum albumin (New England Nuclear) was layered on a 5–20% sucrose gradient (total vol of 4.3 ml) in buffer E containing 0.3 M KCl. This was centrifuged for 18 hr at 0°C and 250,000 × g (using a Beckman L5-65 centrifuge and an SW 60 rotor), and six drop fractions were collected from the bottom of the gradient, added to 0.5 ml $H_2O$ and 5 ml Aquasol, and assayed for radioactivity. Protein was measured by the method of Lowry *et al.* (14).

## RESULTS

**Uptake of $1,25(OH)_2[^3H]D_3$ by Dispersed Cells.** Dispersed fibroblasts were incubated at 22°C or at 37°C with

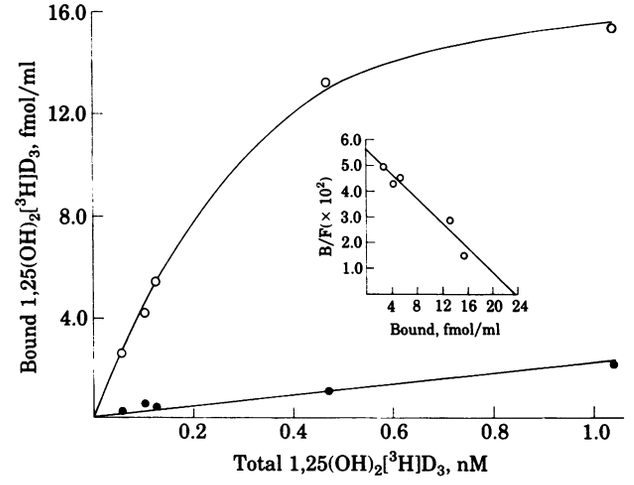


FIG. 2. $1,25(OH)_2[^3H]D_3$ uptake in human skin fibroblast nuclei as a function of concentration. Cells were incubated at various concentrations of $1,25(OH)_2[^3H]D_3$ for 45 min at 37°C in the presence or absence of 1 μM $1,25(OH)_2D_3$. ○, High-affinity uptake; ●, low-affinity uptake. (*Inset*) Scatchard analysis of the data. $R_0$ = 9800 sites per cell; $K_d = 0.42 \times 10^{-9}$ M, $r = -0.97$. B/F, bound/free.

Table 1. $1,25(OH)_2D_3$ nuclear receptor characteristics of cultured cell lines

| Cell line | Donor Sex | Donor Age, years | Site of origin | Nuclear sites per cell* | $K_d$,† $M^{-1} \times 10^9$ |
|---|---|---|---|---|---|
| Fibroblast | M | Neonate | Foreskin | 5,200 | 0.43 |
| Fibroblast | M | 20 | Mons pubis | 9,800 | 0.41 |
| Fibroblast | M | 21 | Mons pubis | 13,200 | 0.50 |
| Fibroblast | M | 22 | Mons pubis | 6,370 | 0.39 |
| Fibroblast | F | 21 | Mons pubis | 8,930 | 0.88 |
| Fibroblast | F | 70 | Abdominal skin | 19,800 | 0.22 |
| MCF-7 human breast cancer | F | 69 | Pleural fluid | 8,660 | 0.39 |

* For fibroblast cell lines, mean ± SEM = 10,600 ± 2000.
† For fibroblast cell lines, mean ± SEM = 0.50 ± 0.07.

$1,25(OH)_2[^3H]D_3$. Because of large amounts of low-affinity uptake, no high-affinity uptake was apparent in whole cells despite repeated washing of the cells with $P_i$/NaCl. This result is in contrast to results from similar experiments with $^3H$-labeled dihydrotestosterone and $^3H$-labeled dexamethasone (10, 15). However, when nuclei were prepared from the incubations with dispersed whole cells, high-affinity uptake was detectable. The time course of $1,25(OH)_2[^3H]D_3$ nuclear uptake showed that equilibrium was established within 45 min for incubations at both temperatures, although the uptake was less at the lower temperature (Fig. 1). The remaining experiments were performed for 45 min at 37°C. A representative saturation plot of the uptake of $1,25(OH)_2[^3H]D_3$ by fibroblast nuclei is shown in Fig. 2. Scatchard analyses of these data (Fig. 2 *Inset*) show that there is one class of sites and that it has a binding capacity of ≈10,000 molecules per cell and a $K_d$ of $0.5 \times 10^{-9}$ M. Similar results were obtained from six fibroblast strains, established from skin specimens of four men and two women [using foreskin (1), mons pubis skin (4), or abdominal skin (1)] (Table 1). Donor age, donor sex, or the anatomic site of origin of the cell line did not affect the characteristics of nuclear uptake. The MCF-7 breast cancer cell line, previously shown to have high-affinity receptors for $1,25(OH)_2D_3$ (16), was also found to have similar nuclear $1,25(OH)_2[^3H]D_3$ uptake parameters. High-pressure liquid chromatography of the $1,25(OH)_2[^3H]D_3$ showed that, after incubation, the radioactive material in the medium was indistinguishable from the starting material. No nuclear uptake was detectable with human lymphocytes freshly prepared from peripheral blood by a modification of the method of Bøyum (17) (results not shown).

**Calciferol Specificity of Nuclear Uptake.** To evaluate the nature of the ligand specificity of the $1,25(OH)_2[^3H]D_3$ uptake in fibroblast nuclei, calciferol metabolites and analogs were incubated with whole cells in the presence of 0.7 nM $1,25(OH)_2[^3H]D_3$ at 37°C. The competition profiles for the nuclear uptake are shown in Fig. 3. As judged by the concentration giving 50% inhibition of the high-affinity binding of $1,25(OH)_2[^3H]D_3$, the metabolites and analogs were weaker competitors than $1,25(OH)_2D_3$ by two or three orders of magnitude. Similar results have been obtained with $1,25(OH)_2D_3$ receptors in hypertonic extracts of cultured mouse bone cells (18), cultured rat osteogenic sarcoma cells (19), and chicken intestine (20).

**Sucrose Density-Gradient Analysis.** Sucrose density-gradient analysis of hypertonic extracts of nuclei obtained from cells incubated for 45 min at 37°C with 0.8 nM $1,25(OH)_2[^3H]D_3$ in the presence or absence of 1 μM unlabeled $1,25(OH)_2D_3$

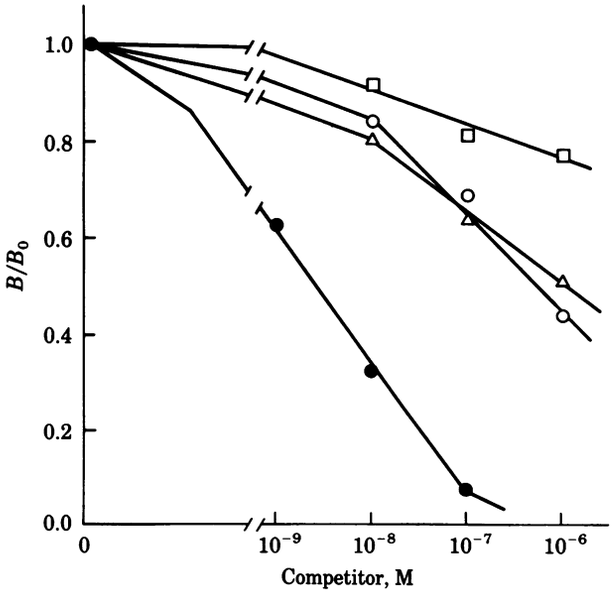


FIG. 3. Ability of other calciferols to compete with $1,25(OH)_2[^3H]D_3$ for uptake in human skin fibroblast nuclei. Cells were incubated for 45 min at 37°C with 0.7 nM $1,25(OH)_2[^3H]D_3$ in the presence of 1 α-hydroxycholecalciferol (□), 24,25-dihydroxycholecalciferol (○), 25-hydroxycholecalciferol (▽), or 1α,25-dihydroxycholecalciferol (●). High-affinity uptake capacity at $B/B_0 = 1.0$ represents uptake of $1,25(OH)_2[^3H]D_3$ in the absence of any inhibitor minus the low-affinity uptake (plotted as $B/B_0 = 0$) of $1,25(OH)_2[^3H]D_3$ in the presence of 1 μM unlabeled $1,25(OH)_2D_3$ (≈15% of the total).

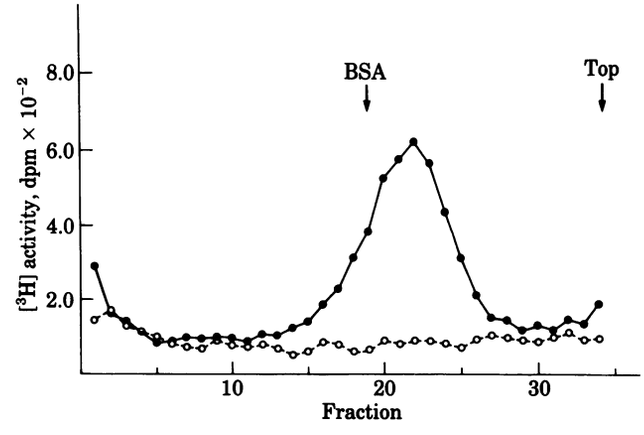


FIG. 4. Sucrose density gradients of $1,25(OH)_2[^3H]D_3$ binding components in extracts from human skin fibroblast nuclei. Extracts (0.9 mg of protein in 0.3 M KCl) were prepared from nuclei of fibroblasts incubated for 45 min at 37°C with 0.8 mM $1,25(OH)_2[^3H]D_3$ alone (●) or in the presence of 1 μM $1,25(OH)_2D_3$ (○) and then subjected to overnight centrifugation on 5–20% sucrose gradients in buffer E/0.3 M KCl. BSA, $^{14}C$-labeled bovine serum albumin marker. (1 dpm = 16.7 mBq.)

showed a peak of binding in the 3S region that was abolished by coincubation of the unlabeled ligand. Omission of 10 mM sodium molybdate from the buffers and the gradients led to marked loss of binding.

## DISCUSSION

Previous work showing that intravenously injected $1,25(OH)_2[^3H]D_3$ is concentrated in the nuclei of dermal and epidermal cells suggests the presence of an effector system for $1,25(OH)_2D_3$ in rat skin (7). Simpson and DeLuca (21) have found a protein indistinguishable from $1,25(OH)_2D_3$ receptors in homogenates of rat epidermis, and Colston *et al.* (9) have shown similar results from murine skin. The nuclear uptake of $1,25(OH)_2[^3H]D_3$, which we have demonstrated with cultured human skin fibroblasts *in vitro* by using the dispersed cell method, has properties characteristic of mediation by $1,25(OH)_2D_3$ receptors (1–3): It is favored at higher temperatures (see Fig. 1), it is a saturable process, and it has sterol specificity and affinity characteristics remarkably similar to those described for $1,25(OH)_2D_3$ receptors obtained from hypertonic homogenates of other cultured cells (18,19). Moreover, by using buffer D, (which contains Triton-X) to prepare the nuclei, we have also confirmed nuclear uptake of $1,25(OH)_2[^3H]D_3$ in MCF-7 breast carcinoma cells, a cell line previously shown to have $1,25(OH)_2D_3$ receptors (16).

Although vitamin $D_3$ itself is synthesized photochemically in the skin (1), the active hormone at the target tissues [$1,25(OH)_2D_3$] is thought to be formed by additional hydroxylation of the vitamin $D_3$ in the liver and kidney (1–3). It is, therefore, not likely that the binding seen here represents interaction of $1,25(OH)_2[^3H]D_3$ with an enzyme involved in cholecalciferol biosynthesis; the $K_d$ is far below the $K_m$ of most known enzymes. Nonetheless, the functional significance of $1,25(OH)_2D_3$ receptors in normal human skin fibroblasts remains to be elucidated. The demonstration that rat skin contains a vitamin D-dependent calcium binding protein (22) suggests that skin might also be a target tissue for $1,25(OH)_2D_3$. A possible role for $1,25(OH)_2D_3$ at the hair follicle in man, a dermal appendage, is suggested by the occurrence of total alopecia in certain patients who have a form of hereditary rickets thought to be due to resistance to the action of $1,25(OH)_2D_3$ (23).

Our method is presumably detecting, in normal cultured cells, the characteristics of $1,25(OH)_2D_3$ receptors and their functional capability for hormone localization within the nucleus. By directly assessing nuclear uptake, this assay system bypasses the need for special precautions to mask low-affinity binding of $1,25(OH)_2[^3H]D_3$ to cytosol components (24). Although not specifically excluded, it is unlikely that the nuclear uptake detected by this assay technique can be accounted for by binding proteins derived from the culture medium. In support of this, we note that 48 and 24 hr before assay the cell growth medium is changed to one lacking serum. When the cells are harvested, they are exposed to trypsin and twice washed with buffer that removes serum. Although a binding protein that has preferential specificity for $25(OH)D_3$ is known to exist in plasma and, possibly, to enter the cell (25, 26), its sedimentation characteristics in association with hypertonic cell extracts (≈6 S) and its binding specificity [greater affinity for $25(OH)D_3$ than for $1,25(OH)_2D_3$] make it an unlikely candidate for the nuclear binding species that we observed.

Measurements of hormone receptors in cultured skin fibroblasts have proven useful in evaluating patients who are resistant to hormones and other circulating factors (27–29) and have provided clues to the mechanisms of action of hormones and nonhormonal regulators. In particular, disorders of androgen action and cholesterol metabolism have been explored extensively by similar techniques. Efforts to find specific receptors for steroid hormones other than androgens and glucocorticoids in skin fibroblasts have thus far been unsuccessful. The demonstration of nuclear uptake of $1,25(OH)_2[^3H]D_3$ in skin fibroblasts with characteristics suggesting mediation by $1,25(OH)_2D_3$ receptors will permit detailed study of the $1,25(OH)_2D_3$ receptor and its functional integrity in normal human cells. Moreover, several forms of hereditary rickets have been described (23, 30–33) involving peripheral resistance to the action of $1,25(OH)_2D_3$, and this technique will allow further investigation of these disorders and of others in which target tissues may show abnormal responsiveness to calciferols.

**Note Added in Proof.** While this manuscript was in preparation, Feldman *et al.* reported high-affinity binding for $1,25(OH)_2D_3$ in hypertonic extracts of fresh and cultured human skin (Feldman, D., Chen, T., Hirst, M., Colston, M., Karasek, M. & Cone, C. (1980) *J. Clin. Endocrinol. Metab.* **51,** 1463–1465).

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