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# Kinetic Differences of the Calcium-Binding Protein in Absorptive Hypercalciuric Renal Stone Formers

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**Summary.** Normal levels of 1.25-dihydroxycholecalciferol and different behaviour of the calcium binding protein kinetics, in a group of absorptive hypercalciuric stone formers indicate that intestinal calcium hyperabsorption in stone formers is due to an altered calcium transport at the intestinal level rather than a phosphate renal leak.

**Key words:** Ca-Binding protein, Hypercalciuria.

## Introduction

Hypercalciuria has been detected in 48.8% of recurrent stone formers [1], and is the result of hyperabsorption, resorption [2] or renal excretion [3]. Intestinal hyperabsorption has been proposed to occur through a renal leak of phosphorous, followed by increasing synthesis of 1.25-dihydroxycholecalciferol ($1.25(OH)_2D_3$) and resultant increased calcium hyperabsorption [4].

Alternatively, it was suggested that intestinal calcium hyperabsorption is due to greater calcium transport without greater synthesis of vitamin $D_3$ metabolites [5].

Intestinal calcium transport appears to be controlled by or mediated through a specific protein existing in the brush border cells [6].

The purpose of study was to compare the roles of the calcium binding protein and of $1.25(OH)_2D_3$ by determining the plasma levels of $1.25(OH)_2D_3$ and the kinetic behaviour of the intestinal calcium binding protein in absorptive hypercalciuric stone-formers.

## Methods and Materials

The subjects were 18 patients (7 men and 11 women, mean age 40 ± 13.8 years) with hypercalciuria who recurrently formed oxalate-phosphate renal stones. The control group comprised 6 men and 6 women (mean age 32 ± 8.8 years) without renal disease. Equipment used was a DBG Beckman spectrophotometer, a refrigerated Sorvall RC-5B centrifuge with an SM-24 rotor, an Isocap-300 liquid scintillation counter, a Shandon-MBI automatic fraction collector cutter, a Millipore Sampling Manifold with filters of 0.025 μm pore size and 2.5-cm diameter, a 5830 Hewlett-Packard gas chromatograph, a 1082B Hewlett-Packard high-pressure liquid chromatograph, and an LKB 80,000 Gamma sample counter. The $^{45}Ca$ (2.1 mCi/ml) and the $1.25(OH)_2D_3$ (85 mCi/mM) was pruchased from Amersham (England). A flexible fiberoptic endoscope was used to obtain duodenal biopsy specimens. The crystalline $1.25(OH)_2D_3$ was a gift from Roche Laboratories, Barcelona (Spain). Wistar male rats of 30–50 g weight were purchased from Panlab, Barcelona (Spain).

### *Detection of Hypercalciuria*

The presence of hypercalciuria was investigated by a two-phase study. First, calcium, magnesium, phosphate, urate and creatinine concentrations were determined in plasma and four separate 24-h urine collections. Oxalate excretion was also determined in the urine [7]. Ammonia, pH, and titratable acidity values were also determined in 2-h urine samples from fasting patients [7]. Hypercalciuria was defined as the urinary excretion of more than 250 mg of calcium per 24 h.

In the investigation of calcium hyperabsorption, the subjects were maintained on a diet of 400 mg of calcium per day. They were then given an oral 250-mg load of calcium chloride containing 25 μCi of $^{45}Ca$ [8]. Intestinal calcium absorption, radio calcium clearance, and the calcium:creatinine ratio were determined [8].

### *1.25-Dihydroxycholecalciferol Determination*

Determination of $1.25(OH)_2D_3$ was based on a radioassay [9, 10] consisting of extracting $1.25(OH)_2D_3$ from a plasma sample and chromatographically separating $1.25(OH)_2D$–$H_3$ from the other vitamin-$D_3$ metabolites. Quantification was performed through a competitive radio-binding assay.

20 ml of plasma were extracted with 200 ml of a methanol: chloroform (120:80) mixture. The extract was evaporated on a Flash evaporator, and the residue was dissolved in a minimal volume of methanol. The dissolved extract was layered in a 1– x 15-cm Sephadex LH 20 column and eluted with chloroform : hexane (64 : 35)

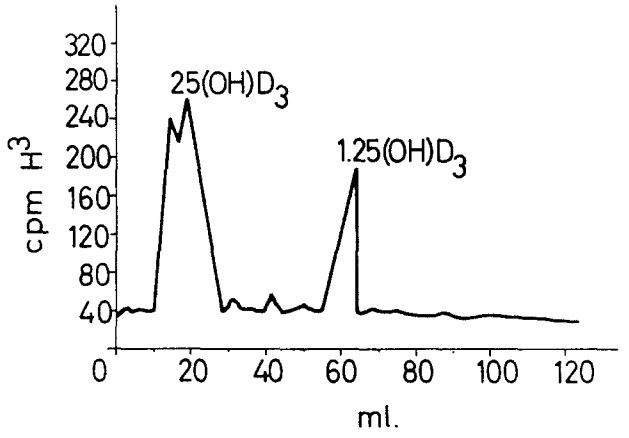


Fig. 1. Typical elution profile of the vitamin $D_3$ metabolites after the LH-20 sephadex column

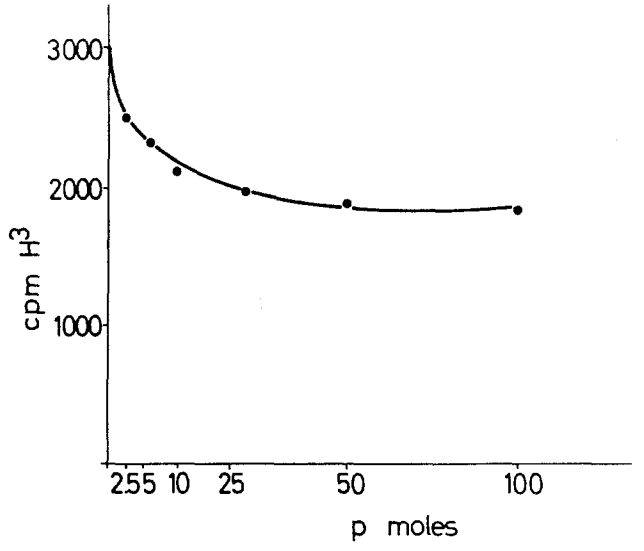


Fig. 2. Competitive displacement curve of the 1.25-dihydroxy-cholecalciferol

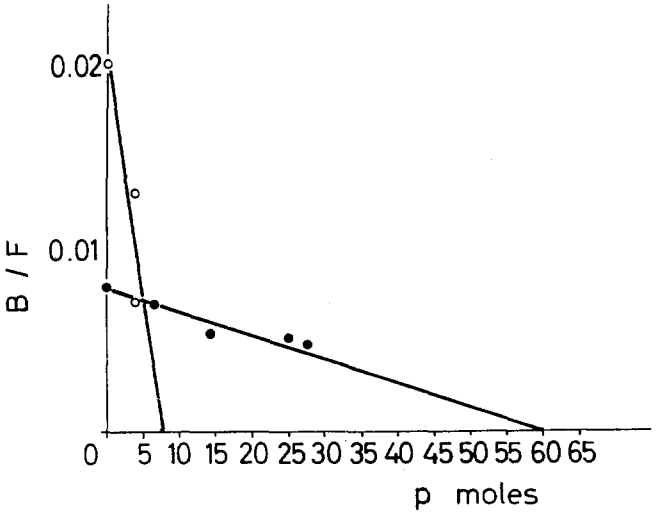


Fig. 3. Scatchard plot of the intestinal calcium binding protein. ○ Control. • Stone former

by collecting 5 ml fractions. Fractions containing $1.25(OH_2D-H_3$ was identified by matching with radioactive $1.25(OH)_2D-H_3$, and its purity (without vitamin-$D_3$ metabolites) was confirmed with a high-pressure liquid chromatograph [11].

Radio-binding assay was performed with $1.25(OH)_2D-H_3$ binding protein obtained from the cytosol fraction of brush border intestinal cells from vitamin $D_3$-deficient Wistar male rats.

Duplicate assays were carried out in 4– x 1-cm propylene tubes containing 1 mg of the aforementioned protein, 20 x $10^3$ cpm of $^3H$-$1.25(OH_3)D-H_3$, and 5 μmol of potassium phosphate buffer (pH 7.4) in 1 ml volume. The standards varied from 0 to 1,000 pmol. Equilibrium was reached after 2 h incubation at 4 °C. Free and bound portions were obtained with 0.5 ml of a charcoal-dextran D (1 : 0.1%) mixture by incubation at 4 °C for 15 min. The sample was centrifuged at 10,000 rpm for 15 min at 4 °C, and the supernatant was decanted into 10 ml PCS solution (solubilizer-phase combining system for liquid scintillation counting of radioactive samples, Amersham/Searle sheet KP/1d, 11/1/7) vials. Radioactivity was then counted for 10 min.

*Calcium-Binding Protein Assay*

The dissociation constant of the calcium-binding protein was calculated by using Scatchard plots [12]. The calcium-binding protein was obtained from the cytosol portion of human intestinal biopsy specimens. Each specimen was homogenized in 0.5 ml of 2.6 mM Tris HCl (pH 7.5) buffer. The cytosol was obtained by centrifugation at 50,000 x g for 17 h.

The assay contained per 0.1 ml final volume: 100 μg of protein from the cytosol fraction, 26 μmol Tris-HCl (pH 7.5) buffer, and increasing concentrations of calcium chloride (from 0 to 250 pmol) with 20 x $10^3$ cpm of $^{45}Ca$ as a tracer. Mixtures were incubated for 2 h at 4 °C. Samples were then diluted with 1 ml of Tris-HCl buffer and filtered through a 25-mm VS Millipore disc (pore size 0.025 μm), by applying 400 mmHg pressure through a Millipore pump. Filters were deposited on 10 ml PPO-POPOP-Toluene (20 mg and 2 mg per 100 ml in Toluene).

Parathyroid hormone [13] and cyclic AMP [14] were determined by radioimmunoassay.

## Results

Each result represents the mean of at least three experiments. The $1.25(OH)_2D_3$ was eluted from the column in the 55–65 ml fraction (Fig. 1). No other peak was detected in this region. The radioactive peak matched that of the purchased $1.25\text{-}D_3(OH)_2\text{-}^3H$. No other peak was detected by high-pressure liquid chromatography. The residue from this peak competitively displaced the radioactive $1.25\text{-}D_3(OH)_2$ from the rat intestinal receptor (Fig. 2). The plasma levels of phosphate were higher in the controls than in the hypercalciuric group, whereas phosphate levels in urine were slightly lower (Table 1). No significant differences in $1.25\text{-}D_3(OH)_2$ plasma levels were found in the control group compared with those in the hypercalciuria patients. In fact, $1.25\text{-}D_3(OH)_2$ levels seem to be lower in the hypercalciuric group (Table 2). The kinetic profile of the calcium-binding protein as shown by the Scatchard plots (Fig. 3) show a steeper line for the controls than for the hypercalciuric patients. The Kd is higher in the control group, whereas there were more binding sites in the hypercalciuric group (Table 3).

**Table 1.** Calcium and phosphate data

| Subjects | Age (year) | Plasma Phosphate (mg/100 ml) | Urine (mg/24 h) | |
|---|---|---|---|---|
| | | | Calcium | Phosphate |
| Controls ($n$ = 12) | 32.5 ± 8.8 | 3.4 ± 0.5 | 132 ± 47 | 676 ± 181 |
| Stone-formers ($n$ = 18) | 40.2 ± 13.8 | 3.0 ± 0.4 | 292 ± 70 | 786 ± 234 |
| | $p > 0.05$ | $p > 0.05$ | $p < 0.05$ | $p > 0.05$ |

**Table 2.** Parathormone, 1.25-dihydroxycholecalciferol and cyclic-AMP values

| Subjects | Plasma | | Urine |
|---|---|---|---|
| | PTH (ng/ml) | $1.25(OH)_2$-$D_3$ (pg/ml) | cAMP (pmol/mg creatinine) |
| Controls ($n$ = 12) | 0.29 ± 0.26 | 40.5 ± 27.7 | 4.24 ± 3.87 |
| Stone-formers ($n$ = 18) | 0.37 ± 0.44 | 19.5 ± 23.5 | 3.41 ± 2.03 |
| | $p > 0.05$ | $p < 0.05$ | $p > 0.05$ |

**Table 3.** Kinetic results of calcium binding in duodenal biopsy specimens

| Subjects | Calcium | |
|---|---|---|
| | Dissociation constant (Kd $10^{-13}$ M/l) | Binding sites (n°/ng protein) |
| Controls ($n$ = 12) | 4.0 ± 4.0 | 8.7 |
| Stone-formers ($n$ = 18) | 26.0 ± 2.6 | 29.2 |
| | $p < 0.05$ | $p < 0.1$ |

## Discussion

The results show no significant differences between plasma levels of parathormone and 1.25-dihydroxycholecalciferol between the control group and the stone-forming patients. In fact the 1.25-dihydroxycholecalciferol were lower in the stone formers.

The cAMP urinary excretion was similar in both groups. The 1.25-dihydroxycholecalciferol levels were slightly lower in the stone-formers. The phosphate concentration in plasma was slightly lower in the hypercalciuric group, but their urinary phosphate was higher.

These results contradict the fingings of Gray et al. [4] who showed that $1.25D_3(OH)_2$ levels were increased in recurrent stone-formers. This discrepancy may be accounted for by two different populations of stone-forming patients; or that patients described by Gray et al. may had a renal type of hypercalciuria.

On the other hand, the Kd of the calcium-binding protein of the control group was higher than that in the group with absorptive hypercalciuria. However, the number of binding sites was more numerous in the stone-formers. These findings might indicate that the calcium-binding protein of the controls has a greater affinity than that in the stone-formers, but that the total capacity for transport of calcium is greater in absorptive hypercalciuric stone-forming patients than in the controls. The patients with absorptive hypercalciuria might be able to transport more calcium than can be controls whose capability for calcium transportation will be diminished more rapidly.

The lack of differences in $1.25D_3(OH)_2$ levels and the presence of kinetic differences in the calcium-binding protein indicate that calcium hyperabsorption may be explained through altered calcium transport at the intestinal level, and not by a renal leak of phosphate.

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