# Effects of Dietary Calcium Restriction and Chronic Thyroparathyroidectomy on the Metabolism of [$^3$H]25-Hydroxyvitamin $D_3$ and the Active Transport of Calcium by Rat Intestine

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ABSTRACT Previous studies have shown that chronically thyroparathyroidectomized (TPTX) rats, fed a diet with restricted calcium but adequate phosphorus and vitamin D content, have higher levels of intestinal calcium absorption than controls. The results of recent acute experiments have suggested that parathyroid hormone (PTH) may be essential for regulating the renal conversion of 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) to 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2$-$D_3$] in response to dietary calcium deprivation. Since 1,25-$(OH)_2$-$D_3$ is the form of the vitamin thought to be active in the intestine, increases in calcium transport mediated by this metabolite would not be expected to occur in the absence of the parathyroid glands if the preceding model is correct. The present study was undertaken to examine the chronic effects of both dietary calcium restriction and the absence of PTH on the metabolism of [$^3$H]25-OH-$D_3$ and duodenal calcium-active transport in rats given thyroid replacement. These relatively long term studies confirm earlier observations which indicated that the adaptation of calcium absorption to a low calcium intake occurs in both sham-operated and TPTX animals.

The present studies also demonstrated that despite reduced levels of 1,25-$(OH)_2$-$D_3$ in the plasma of chronically TPTX animals fed a low calcium diet, the accumulation of this metabolite in at least one target tissue, intestinal mucosa, is identical in both the sham-operated and TPTX groups. A reduced, but continued level of 1,25-$(OH)_2$-$D_3$ production, together with its selective accumulation by intestinal mucosa, probably explains the calcium adaptation which is observed in spite of the chronic absence of the parathyroid glands.

## INTRODUCTION

The efficiency of dietary calcium absorption may vary with body requirements for this element. Periods of growth, pregnancy, lactation, and dietary calcium deprivation may enhance intestinal calcium absorption both in man and experimental animals (1–12). Although the mechanism whereby increased body calcium needs stimulate intestinal calcium absorption remains uncertain, it is known that vitamin D is required for this adaptive response to occur (2, 5).

Vitamin $D_3$ (cholecalciferol) is converted by the liver (13), and probably by kidney and intestinal mucosa (14), to the major circulating form, 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) [1] (15). This metabolite is

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[1] *Abbreviations used in this paper:* CT, calcitonin; HCD, high calcium diet; $J_{net}$, net flux defined as $J_{ms}$-$J_{sm}$; $J_{ms}$, unidirectional flux mucosal to serosal; $J_{sm}$, unidirectional flux serosal to mucosal; LCD, low calcium diet; PD, transmural electric potential difference; PTH, parathyroid hormone; TPTX, thyroparathyroidectomy or thyroparathyroidectomized; 25-OH-$D_3$, 25-hydroxyvitamin $D_3$; 1,25-$(OH)_2$-$D_3$, 1,25-dihydroxyvitamin $D_3$; 24,25-$(OH)_2$-$D_3$, 24,25-dihydroxyvitamin $D_3$.

further hydroxylated, exclusively in mitochondria of renal tubular cells to form 1,25-dihydroxyvitamin $D_3$ [1,25-$(OH)_2$-$D_3$] (16–18). Based on recent studies concerned with the biologic activity of 1,25-$(OH)_2$-$D_3$ and the time course of its formation and localization in target organs, it seems likely that this dihydroxylated metabolite is indeed the major endogenously produced tissue-active form of the parent vitamin in intact animals fed low calcium or low phosphorus diets. Furthermore, it is probable that 1,25-$(OH)_2$-$D_3$ produces the vitamin D-dependent enhancement of intestinal calcium absorption under these conditions (19–25).

Under conditions of dietary calcium restriction the kidney of intact rats predominantly converts 25-OH-$D_3$ to 1,25-$(OH)_2$-$D_3$. On the other hand, after prolonged feeding of a high calcium diet, the major metabolite of 25-OH-$D_3$ produced by the kidney becomes 24,25-dihydroxyvitamin $D_3$ [24,25-$(OH)_2$-$D_3$] (*26, 27*). In accord with these changes in metabolite patterns, dietary calcium deprivation in chicks results in increased activity of 25-OH-$D_3$-1-hydroxylase in kidney homogenates, whereas a period of high calcium intake diminishes the 25-OH-$D_3$-1-hydroxylase activity and 24,25-$(OH)_2$-$D_3$ is preferentially synthesized in vitro (*28*). Although the physiologic role of 24,25-$(OH)_2$-$D_3$ is still unclear, recent reports have suggested that under certain circumstances it may be converted to a more polar trihydroxylated form (e.g., 1,24,25-trihydroxyvitamin $D_3$ [1,24,25-$(OH)_3$-$D_3$]), which is capable of enhancing intestinal calcium transport (*29, 30*).

The formation of 1,25-$(OH)_2$-$D_3$ from its immediate precursor, 25-OH-$D_3$, is closely regulated, but the precise mechanism underlying this regulation is not fully understood. Parathyroid hormone (PTH) secreted in response to serum calcium levels, has been proposed as the tropic factor in the modulation of 1,25-$(OH)_2$-$D_3$ synthesis, and hence the factor responsible for adaptation of intestinal calcium transport to variations in dietary calcium intake (31–33). As will be discussed below, however, this view is not universally held (34–37). Kimberg and his co-workers Schacter and Schenker (5) have demonstrated that thyroparathyroidectomized (TPTX) rats given sufficient vitamin D and inorganic phosphorus are quite capable of adapting to chronic dietary calcium deprivation by increasing intestinal calcium absorption when it is assayed by the everted gut sac technique. If, in the presence of an adequate dietary phosphorus intake, PTH is a required regulator of 1,25-$(OH)_2$-$D_3$ synthesis, an adaptation of calcium transport which involves this metabolite should not occur in the absence of the parathyroid glands. The present studies were undertaken to examine the mechanism underlying the adaptive response to dietary calcium restriction in the chronic absence of PTH.

## METHODS

*Animal preparation.* Male albino rats of the Sherman strain weighing 100 g (Camm Research Institute, Inc., Wayne, N. J.) were raised in the dark and fed a dextrose, corn oil, and casein-based, vitamin D-deficient diet containing 0.8% calcium, 0.4% phosphorus, and 0.24% magnesium for 1 wk to partially deplete vitamin D stores (test diet 69280, General Biochemicals Div., Mogul Corp., Chagrin Falls, Ohio). The animals then underwent either TPTX by blunt dissection or sham surgery under light ether anesthesia. 48 h later blood was obtained by cardiac puncture and calcium was determined by atomic absorption spectrometry. Rats with plasma calcium levels of 7.0 mg/100 ml and below were considered to be TPTX (mean±SEM plasma calcium for sham animals = 9.99±0.09 mg/100 ml, $n = 31$; for TPTX animals = 6.23±0.14 mg/100 ml, $n = 23$).

*Radioactive 25-OH-$D_3$.* [$^3$H-26,27]25-OH-$D_3$ ([$^3$H]25-OH-$D_3$) was obtained from New England Nuclear, Boston, Mass., and the specific activity was adjusted to 600 mCi/mmol by addition of unlabeled 25-OH-$D_3$ (kindly provided by Dr. John C. Babcock, The Upjohn Co., Kalamazoo, Mich.). [$^3$H]25-OH-$D_3$ was dissolved in propylene glycol: 95% ethanol 3:1 (vol/vol) at a concentration of 3.9 nmol/ml.

*Experimental procedures.* Upon verification of thyroparathyroidectomy, TPTX animals were fed either a low calcium diet (LCD) containing 0.02% calcium, 0.4% phosphorus, 0.24% magnesium, and no vitamin D, or a diet of normal calcium content (0.8% calcium) but otherwise identical in composition to the LCD (test diet 69280 with 0.02 or 0.8% Ca, General Biochemicals Div.). Sham-operated animals were fed the LCD. The two groups receiving the LCD (one TPTX group and the sham group) were given 15 IU (937 pmol) of [$^3$H]25-OH-$D_3$ in propylene glycol: 95% ethanol by gastric tube on the 1st day of the dietary regimen and every 3rd day thereafter during the ensuing 20 days (seven doses). The last dose of [$^3$H]-25-OH-$D_3$ was given 48 h before sacrifice. The TPTX group fed the normal calcium diet did not receive [$^3$H]-25-OH-$D_3$, and these animals were raised in the dark to maintain a vitamin D-deficient state. The higher dietary calcium content was used to minimize the mortality in this group. TPTX animals received replacement doses of L-thyroxine, 30 μg subcutaneously every 6 days to maintain euthyroid status (Flint Labs., Div., Travenol Laboratories Inc., Morton Grove, Ill.) (38).

*Intestinal calcium transport studies.* On the 21st day of the test diet and after an overnight fast, the animals in each group were stunned by concussion and exsanguinated. Blood was collected in heparinized tubes for subsequent determinations of plasma calcium and magnesium by atomic absorption spectrometry and of inorganic phosphorus by the method of Fiske and SubbaRow (39). Unidirectional transmural fluxes of calcium across the proximal duodenum were measured in vitro under short-circuited conditions using the apparatus of Ussing and Zerahn (40) with modifications that we have previously described (6, 41). A phosphate-free, bicarbonate-buffered Krebs-Ringer solution (pH 7.4) with a calcium concentration of 0.5 mM was used (42). Steady-state fluxes were calculated as previously described (41) and net fluxes were determined from unidirectional calcium fluxes measured on paired tissues from the same animal with $J_{net} = J_{ms} - J_{sm}$, where $J_{net}$ is net flux and $J_{ms}$ and $J_{sm}$ are the unidirectional

transmural fluxes, mucosal to serosal and serosal to mucosal respectively.

The transmural potential difference (PD) was measured initially and after 50 min of incubation, and was otherwise nulled using the method of compensating for fluid resistance described by Field, Fromm, and McColl (43). The electrical parameters reported are from time intervals when transmural calcium fluxes were at steady state.

*Extraction of radioactivity.* At the time of sacrifice of the sham and TPTX animals fed [$^3$H]25-OH-D$_3$, blood was collected and the proximal 3 cm of duodenum was removed for transport studies as described above. The remaining small intestine was removed, everted over glass rods, and rinsed in ice-cold isotonic saline. The mucosa was separated from underlying coats by scraping on a chilled glass plate with a glass microscope slide. Kidneys were removed, rinsed in ice-cold isotonic saline, and frozen on a Dry Ice-acetone bath. Those tissues not subjected to immediate extraction were stored at −20°C in an atmosphere of nitrogen.

The tissues and plasma were pooled within each group of animals (sham, + 25-OH-D$_3$ vs. TPTX, + 25-OH-D$_3$). Homogenates of intestinal mucosa (10%) and kidney (15%) were prepared in glass-distilled water using a Waring Blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) at medium speed for 30 s. Portions of the homogenates and plasma were analyzed for total tritium content after digestion in a NCS Nuclear-Chicago tissue solubilizer (Amersham/Searle Corp., Arlington Heights, Ill.). Determinations of protein and DNA content were performed by previously described methods (44–46). Homogenates were subjected to lipid extraction by the method of Bligh and Dyer (47) as modified by Lund and DeLuca (48). In addition, the aqueous phase was reextracted with a volume of chloroform. The chloroform phases were then concentrated by evaporation under a stream of nitrogen and dissolved in a small volume of chloroform: hexane, 65: 35 (vol/vol) for Sephadex LH-20 column chromatography (see below).

*Sephadex LH-20 column chromatography.* Chloroform-soluble metabolites extracted from plasma and tissues of sham and TPTX animals fed [$^3$H]25-OH-D$_3$ were eluted from columns containing 20 g of Sephadex LH-20 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) with chloroform: hexane 65: 35 (vol/vol) as previously described (49). More polar material was eluted from the Sephadex LH-20 columns with chloroform: hexane 70: 30 and subsequent batch elution by equilibration of the Sephadex LH-20 in 300 ml of methanol. Radioactivity in portions from each fraction as well as that present in samples of the original chloroform and aqueous phases was measured as previously described with an Intertechnique model SL-40 liquid scintillation spectrometer (Intertechnique Division, Teledyne Isotopes, Westwood, N. J.) (50). Disintegrations per minute in each sample were calculated after internal standardization with [$^3$H]toluene.

*Periodate reaction.* Portions of each of the fractions containing peaks of radioactivity after Sephadex LH-20 column chromatography were pooled, evaporated to dryness under $N_2$, dissolved in 3.0 ml methanol, and subjected to *p*-periodic acid oxidation by previously described methods (51).

*Tissue phosphorus.* Portions of 15% kidney homogenates from sham and TPTX, [$^3$H]25-OH-D$_3$-fed animals were precipitated with cold trichloroacetic acid (final concentration, 5%). The precipitate was sedimented in a refrigerated centrifuge (RC-3, Ivan Sorvall, Inc., Newton, Conn.) at 1,000 *g* for 15 min. The inorganic phosphorus content in portions of the supernate was determined by the method of Fiske and SubbaRow (39).

*Statistical analysis.* Blood chemistries, electrical parameters, and $J_{ms}$ and $J_{sm}$ were analyzed for intergroup differences using one way analyses of variance (52). When the analysis of variance indicated a difference between groups, Student's *t* test was used to determine the probability level of the differences. Paired *t* tests were employed to evaluate the differences between $J_{ms}$ and $J_{sm}$ within groups.

## RESULTS

*Plasma chemistries.* Table I shows the effects of chronic TPTX and of vitamin D replacement (in the form of [$^3$H]25-OH-D$_3$) on plasma calcium, magnesium, and phosphorus concentrations in rats otherwise maintained on a vitamin D-deficient diet. Plasma calcium levels in the sham-operated animals were lower than normal despite replacement with 25-OH-D$_3$, probably because of the restricted dietary calcium intake. The lower plasma calcium levels and higher plasma phosphorus levels in the TPTX animals confirm the successful ablation of the parathyroid glands. The fact that plasma calcium levels were similar in both of the TPTX groups even though one group was maintained in a vitamin D-depleted state may be due to the greater dietary calcium intake in the D-deficient animals (0.8 vs. 0.02%). The lower plasma magnesium levels in the vitamin D-deficient TPTX group may be related to the combined effects of both vitamin D-depletion and dietary calcium intake on intestinal magnesium absorption, as well as the influence of vitamin D-depletion and parathyroidectomy on magnesium mobilization from bone.

*Transport studies.* Both vitamin D deficiency and the feeding of a high calcium diet containing the vitamin to intact animals are associated with decreases in the levels of 1,25-(OH)$_2$-D$_3$ production and duodenal calcium absorption (5, 26). To determine whether or not the feeding of a high calcium test diet to intact animals had effects on calcium transport comparable to vitamin D deficiency, intact animals were raised for 2 wk on a vitamin D-containing diet with either 2.0% (a high calcium diet, HCD) or 0.02% (LCD). Transport studies were conducted using the protocol described in Methods. The net absorption of calcium ($J_{net}$) in the HCD animals was 8.4 nmol of calcium/cm$^2$/h±1.3 SEM, $n = 5$; while LCD $J_{net}$ was 30.5±1.6, $n = 5$ (LCD > HCD, $P < 0.001$). The $J_{net}$ for the HCD animals was identical to the value we had previously reported for a vitamin D-deficient group in a prior study (vitamin D-deficient, saline control, $J_{net} = 8.5$ nmol·cm$^{-2}$·h$^{-1}$ [42]).

In the studies of the effects of chronic dietary cal-

TABLE I

*Effects of TPTX and [$^3$H]25-OH-D$_3$ Administration on Blood Chemistries and Duodenal Calcium Transport*

| Experimental condition (diet) | Number | Plasma calcium | Plasma magnesium | Plasma phosphorus | Calcium flux, nmol·cm$^{-2}$·h$^{-1}$ | | |
|---|---|---|---|---|---|---|---|
| | | | | | $J_{ms}$ | $J_{sm}$ | $J_{net}$* |
| | | *mg/100 ml* | | | | | |
| TPTX, no 25-OH-D$_3$ (0.8% calcium) | 5 | 5.33±0.38 | 1.64±0.04 | 14.28±1.02‖ | 17.1±1.8 | 5.3±0.9‡‡ | 11.8±2.1§§ |
| TPTX, +25-OH-D$_3$ (0.02% calcium) | 5 | 4.41±0.21 | 2.66±0.13§ | 16.23±0.95‖ | 25.1±1.4¶ | 4.5±0.6 | 20.6±1.4§§, ‖‖ |
| Sham, +25-OH-D$_3$ (0.02% calcium) | 8 | 8.24±0.22‡ | 2.49±0.08§ | 10.20±0.33 | 29.7±2.6¶, ** | 4.0±0.2 | 25.7±2.6§§, ‖‖, ¶¶ |

The preparation of animals, 25-OH-D$_3$ repletion and the procedures employed for measurement of plasma calcium, magnesium, phosphorus, and duodena calcium transport are described in Methods. All values are ±SEM.

* $J_{net} = J_{ms} - J_{sm}$.

‡ Sham, +25-OH-D$_3$ plasma calcium > both TPTX groups plasma calcium, $P < 0.001$.

§ Sham and TPTX +25-OH-D$_3$ plasma magnesium > TPTX, no 25-OH-D$_3$ plasma magnesium, $P < 0.001$.

‖ Both TPTX groups plasma phosphorus > sham +25-OH-D$_3$ plasma phosphorus, $P < 0.001$.

¶ Sham and TPTX +25-OH-D$_3$ calcium $J_{ms}$ > TPTX, no 25-OH-D$_3$ $J_{ms}$, $P < 0.005$ and 0.01, respectively.

** Sham and TPTX +25-OH-D$_3$ $J_{ms}$ are not different, $P > 0.10$.

‡‡ Calcium $J_{sm}$ is the not different between groups, $F_{2,15} = 1.31$, $P > 0.25$.

§§ There was active calcium absorption in all groups, $P < 0.005$ or greater.

‖‖ Sham and TPTX +25-OH-D$_3$ calcium $J_{net}$ > TPTX, no 25-OH-D$_3$ $J_{net}$, $P < 0.005$ and 0.01, respectively.

¶¶ Sham and TPTX +25-OH-D$_3$ $J_{net}$ are not different, $P > 0.10$.

cium deprivation and TPTX on calcium transport, TPTX, vitamin D-deficient animals were used as a control group to provide a depressed transport base line. As the data for the vitamin D-deficient TPTX animals indicate (Table I), calcium transport levels were comparable to those of the HCD sham animals mentioned above. Furthermore, the sham animals fed the vitamin D-deficient, 0.02% Ca diet and repleted with [$^3$H]25-OH-D$_3$ (Table I), had increased transport levels which were equivalent to the LCD sham group (see above), indicating that they had indeed adapted to dietary calcium restriction. Of great significance is the observation that both the TPTX and sham animals repleted with [$^3$H]25-OH-D$_3$ (equivalent to 312 pmol/day) adapted similarly to the dietary calcium deprivation (Table I). The enhanced active calcium absorption in these two groups resulted from increases in $J_{ms}$, rather than any accompanying changes in $J_{sm}$. This finding confirms the earlier results of Walling and Rothman (6, 53).

There were no differences in electrical parameters between groups. Short-circuit current ranged from 74.4 to 116.3 $\mu$amps·cm$^{-2}$, the transmural PD ranged from 5.3 to 7.1 mV, and the resistance of the intestine ranged from 88.2 to 97.5 $\Omega$·cm$^2$.

*Distribution of radioactivity at steady state.* The animals were fed a vitamin D-deficient diet for the duration of the experiments and they were given physiologic doses of [$^3$H]25-OH-D$_3$ after surgery. This protocol ensured the establishment of steady-state conditions in the labeling of pools of 25-OH-D$_3$ and its subsequent metabolites. Table II shows the effects of TPTX on the tissue localization of radioactivity and on the distribution of radioactivity between the aqueous and chloroform phases after this chronic administration of [$^3$H]25-OH-D$_3$. More radioactivity was present in the tissues of the TPTX group than in those of the sham group. Such differences may in part be related to the fact that both groups received the same amount of [$^3$H]25-OH-D$_3$ (equivalent to 312 pmol daily for 20 days) despite the fact that the TPTX group gained less weight during the experiment (mean weight at sacrifice was sham = 199 g vs. TPTX = 130 g). It is unlikely however, that the two- to fourfold increment in tissue specific activity in the TPTX group is due to this factor alone. The data from plasma and kidney suggests that, in addition, there may have been a relative decrease in the rate of degradation of [$^3$H]25-OH-D$_3$ and its metabolites to more readily excretable water-soluble compounds in the TPTX animals.

*Vitamin D metabolites.* Periodic acid oxidation was performed on pooled portions of those chromatographic fractions which composed the peaks of eluted radioactivity (see Methods). Oxidation of peak V*a* from plasma and intestinal mucosa produced a 76–90% loss of radioactivity in both experimental groups. Oxidation of peaks IV and V from the same tissues produced less than a 20% reduction in radioactivity. Based on the elution volume as well as the results of the

TABLE II

*Plasma and Tissue Tritium Content and Sephadex LH-20 Chromatographic Fractions Obtained from Sham and TPTX Rats Fed an LCD and Repleted with [$^3$H]25-OH-D$_3$ for 20 Days*

| | Plasma | | | | Intestinal mucosa | | | | Kidney | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sham | | TPTX | | Sham | | TPTX | | Sham | | TPTX | |
| Tissue radioactivity | 21,900* | | 65,538 | | 672 | | 2,706 | | 222 | | 401 | |
| Chloroform soluble, % | 77.5 | | 92.4 | | 69.6 | | 53.6 | | 58.8 | | 88.7 | |
| Aqueous, % | 22.5 | | 7.6 | | 30.4 | | 46.4 | | 41.2 | | 11.3 | |
| Peaks | *pmol/ml*§ | (%)‡ | *pmol/ml* | (%) | *pmol/g DNA* | (%) | *pmol/g DNA* | (%) | *pmol/g DNA* | (%) | *pmol/g DNA* | (%) |
| I + I*a* (esters of 25-OH-D$_3$) | — | (0.9) | — | (0.3) | — | (10.8) | — | (9.9) | — | (19.3) | — | (5.5) |
| IV (25-OH-D$_3$) | 5.4 | (34.5) | 20.7 | (49.8) | 58.4 | (23.3) | 235.5 | (29.4) | 333.7 | (34.7) | 999.0 | (45.9) |
| V*a* (24,25-(OH)$_2$-D$_3$) | 2.9 | (21.3) | 18.3 | (44.1) | 28.0 | (11.2) | 156.0 | (19.5) | 178.0 | (18.1) | 777.0 | (36.7) |
| V (1,25-(OH)$_2$-D$_3$) | 1.4 | (10.0) | 0.6 | (2.0) | 87.0 | (34.6) | 89.0 | (11.3) | 113.7 | (11.5) | 73.3 | (3.4 |
| Chloroform:hexane 70:30 strip | — | (3.9) | — | (1.4) | — | (1.3) | — | (1.1) | — | (0.6) | — | (0.5) |
| Methanol strip | — | (24.4) | — | (2.4) | — | (18.8) | — | (28.8) | — | (15.8) | — | (7.5) |

* Tissue radioactivity expressed as disintegrations per minute per milliliter (plasma) or disintegrations per minute per gram DNA (intestinal mucosa, kidney). The data represent results obtained from pooled samples from 10 sham and 5 TPTX animals (see Methods). Material from animals not meeting the TPTX criteria at sacrifice (see Methods) was discarded.

‡ Radioactivity in each peak expressed as percent of total disintegrations per minute off column.

§ Expressed as picomoles per milliliter (plasma) or picomoles per gram DNA (intestinal mucosa, kidney). Peak I and I*a* are esters of 25-OH-D$_3$; peak IV is 25-OH-D$_3$; peak V*a* is predominantly 24,25-(OH)$_2$-D$_3$; peak V is predominantly 1,25-(OH)$_2$-D$_3$ as per Holick and DeLuca (49). Radioactivity in chloroform:hexane 70:30 and methanol strips are unidentified metabolites of 25-OH-D$_3$.

periodate reaction, it can therefore be assumed that peak IV represents 25-OH-D$_3$, peak V*a* represents predominantly 24,25-(OH)$_2$-D$_3$, and peak V represents predominantly 1,25-(OH)$_2$-D$_3$ in both sham and TPTX tissue extracts.

The severalfold greater localization of radioactivity in the tissues of the TPTX group can largely be accounted for in plasma and kidney by the greater accumulation of 25-OH-D$_3$ and 24,25-(OH)$_2$-D$_3$; whereas in the intestine, these compounds together with the more polar metabolites in the methanol strip material may contribute to the higher level (Table II). Also, the ratio of 25-OH-D$_3$ in the plasma of TPTX vs. sham animals (3.8) is similar to that noted in the mucosa (4.0); likewise, the ratio of 24,25-(OH)$_2$-D$_3$ in the plasma of TPTX vs. sham animals (6.3) is similar to that noted in mucosa (5.6). Of great interest is the remarkably similar concentration of 1,25-(OH)$_2$-D$_3$ localizing in the intestinal mucosa of both groups. The amounts of 1,25-(OH)$_2$-D$_3$ were similar when expressed as picomoles per gram DNA (Table II) or per gram wet weight mucosa (sham = 0.58 pmol/g wet weight vs. TPTX = 0.59). Although many tissues were not examined, the lower concentrations of 1,25-(OH)$_2$-D$_3$ in the plasma and kidney of the TPTX group suggest that the total body pool size of this dihydroxylated metabolite may be smaller than that of the sham group.

Radioactivity eluted from the Sephadex LH-20 columns with chloroform:hexane 65:35 (vol/vol) followed by chloroform:hexane 70:30 (vol:vol) (strip in Table II) accounted for only 70–80% of the radioactivity applied to the columns. Most of the remaining radioactivity was subsequently eluted from the Sephadex LH-20 with 300 ml of methanol. Metabolites more polar than those eluted with chloroform:hexane 70:30 (vol:vol) have recently been described by Frolik and DeLuca (23) and small amounts of these metabolites appeared in tissues obtained from rats 12–24 h after a single injection of [$^3$H]1,25-(OH)$_2$-D$_3$. The larger percentage of more polar metabolites in tissues from both sham and TPTX groups in the present study is no doubt related to the degree of labeling achieved by the chronic administration of [$^3$H]25-OH-D$_3$.

*Kidney phosphorus concentration.* Phosphorus levels were determined in pooled homogenates of whole kidney obtained from sham and TPTX animals fed [$^3$H]-25-OH-D$_3$ as described in Methods. Despite the significant elevation in serum phosphorus concentration in the TPTX, + 25-OH-D$_3$ group, the kidney phosphorus content was quite similar in both groups (sham, + 25-OH-D$_3$ group = 0.27 μmol of phosphorus/mg protein vs. TPTX, + 25-OH-D$_3$ group = 0.25).

## DISCUSSION

Earlier studies by Kimberg, Schachter, and Schenker (5) demonstrated that the adaptive increase in intes-

tinal calcium transport, which occurs in response to dietary calcium restriction, is not dependent upon the presence of the adrenal, parathyroid, pituitary, or thyroid glands. Furthermore, the results of the present study confirm the fact that adaptation of intestinal calcium transport to chronic dietary calcium restriction can indeed occur in the absence of the parathyroid glands. In recent studies however, PTH, secreted in response to serum calcium levels, has been proposed as an important regulator of 1,25-$(OH)_2$-$D_3$ synthesis (31–33). Thus, within hours after TPTX, 1,25-$(OH)_2$-$D_3$ disappears from blood and intestinal mucosa of rats on LCD; under these circumstances injection of PTH restores 1,25-$(OH)_2$-$D_3$ levels to normal (31). As a matter of fact, we have shown in acute studies that TPTX can indeed diminish intestinal calcium transport assessed 72 h after surgery.[2] In addition, the administration of moderate doses of parathyroid extract to chicks is followed by an increase in 25-OH-$D_3$-1-hydroxylase activity in subsequently prepared kidney homogenates (32). Finally, Rasmussen and his co-workers Wong, Bikle, and Goodman (33) have shown that the addition of "physiologic" concentrations of PTH or cyclic AMP to isolated chick kidney tubules stimulates the in-vitro production of 1,25-$(OH)_2$-$D_3$, whereas the addition of calcitonin (CT) may decrease the production of this metabolite. Neither the stimulatory effect of PTH nor the inhibitory effect of CT were observed at high concentrations of the hormones in vitro.

Recently, Galante and co-workers (34–37) have taken issue with the concept that changes in PTH and/or CT secretion can provide the explanation for changes in vitamin D metabolism and calcium transport seen in response to alterations in serum calcium levels and in response to variations in dietary calcium intake. Thus, they have demonstrated that the repeated administration of parathyroid extract to intact vitamin D-deficient rats on a high calcium intake may actually decrease the production of 1,25-$(OH)_2$-$D_3$ from 25-OH-$D_3$ (34), whereas repeated large doses of CT may enhance 1,25-$(OH)_2$-$D_3$ production by the kidney (35). In interpreting these studies performed on intact animals, it must be remembered that the nature of the effects of PTH and of CT on 25-OH-$D_3$ metabolism may be dose dependent (38), and also that compensatory adjustments in endogenous hormone secretion may modify the results obtained in intact animals. These workers also found that removal of the parathyroid and ultimobranchial glands from vitamin D-deficient chicks on an adequate calcium intake does not affect the enhanced 25-OH-$D_3$-1-hydroxylase activity present in the kidneys (36). In addition, Tanaka and DeLuca (54) have found that dietary phosphorus deficiency may also lead to enhanced 1,25-$(OH)_2$-$D_3$ synthesis both in the presence and absence of the parathyroid glands.

Although the precise role of PTH and/or CT in modulating 1,25-$(OH)_2$-$D_3$ synthesis under physiologic circumstances remains uncertain, based on the results of the present study as well as others (5, 37), it is clear that neither hormone is absolutely essential for the adaptation of intestinal calcium absorption to chronic dietary calcium restriction. Initially, it might seem difficult to reconcile the findings of the present study and the earlier transport studies of Kimberg and his co-workers (5) with the results of recent experiments by Garabedian, Holick, DeLuca, and Boyle (31) concerned with the acute effects of TPTX on 25-OH-$D_3$ metabolism and our own studies dealing with the acute effects of TPTX on intestinal active calcium absorption.[2] One must keep in mind however, that the earlier studies of Kimberg et al. (5) were performed 18 days after TPTX, and the present studies were undertaken 21 days after TPTX, whereas the other studies referred to above were performed 72 h after TPTX (31).[2]

The present relatively long-term studies confirm earlier observations (5) and indicate that adaptation of active calcium absorption to a low calcium intake can occur similarly in both sham-operated and TPTX animals (Table I). These studies further demonstrate that chronic TPTX with thyroid replacement does indeed lead to alterations in the metabolism of 25-OH-$D_3$ (Table II). The tissue-specific radioactivity in plasma, intestinal mucosa, and kidney is higher in the TPTX animals, and it is unlikely that this increment is solely caused by the somewhat different dose-animal weight relationships between the two groups. The results presented in Table II suggest that in the absence of the parathyroid glands there may be a decrease in the rate at which water-soluble, readily excretable metabolites are formed. Furthermore, as one might anticipate from the results of the more acute studies (31), there is a relative increase in the amount of 24,25-$(OH)_2$-$D_3$ and a relative decrease in the amount of 1,25-$(OH)_2$-$D_3$ formed after TPTX. Although many tissues were not examined, it is not at all unlikely that the total body pool size of 1,25-$(OH)_2$-$D_3$ was diminished in the TPTX animals.

Of interest, with respect to the transport data, is that chronically TPTX animals on a LCD retain a reduced but nonetheless continued capacity to convert 25-OH-$D_3$ to 1,25-$(OH)_2$-$D_3$. Especially notable is the finding that in the TPTX animals on a LCD, the small intestinal mucosa, a vitamin D target tissue with spe-

[2] Walling, M. W., M. J. Favus, and D. V. Kimberg. Manuscript in preparation.

cific receptors and affinity for 1,25-$(OH)_2$-$D_3$ (24), selectively accumulates this active metabolite. As a matter of fact, the concentration of 1,25-$(OH)_2$-$D_3$ in the mucosa is nearly identical in both experimental groups. Indeed, the intestinal mucosal concentration of 1,25-$(OH)_2$-$D_3$ found in this study is similar to that previously reported by Favus, Kimberg, Millar, and Gershon (51) in rapidly growing vitamin D-deficient rats given one dose of [$^3$H]25-OH-$D_3$ (73.4 pmol/g mucosal DNA or 0.39 pmol/g wet weight) and by Garabedian et al. (31) in rats fed a LCD (0.02%) followed by a single injection of [$^3$H]25-OH-$D_3$ (0.36 pmol of 1,25-$(OH)_2$-$D_3$/g mucosal wet weight). Since we found similar levels of active calcium absorption and intestinal 1,25-$(OH)_2$-$D_3$ content in both the [$^3$H]-25-OH-$D_3$-repleted sham and TPTX animals, an intestinal response to 1,25-$(OH)_2$-$D_3$ would appear to be a plausible explanation for the results. Thus, continued production of 1,25-$(OH)_2$-$D_3$, perhaps a slower rate of metabolism of 1,25-$(OH)_2$-$D_3$ and selective accumulation in the intestinal mucosa could all have contributed to the observed adaptive response.

An alternative possibility must be considered, for it has been suggested that 1,25-$(OH)_2$-$D_3$ may not be a mandatory metabolite for the intestine to respond to vitamin D (29, 30). Thus, Boyle and his co-workers Omdahl, Gray, and DeLuca have recently reported that 24,25-$(OH)_2$-$D_3$ can enhance active intestinal calcium absorption under conditions which are more chronic than those required to demonstrate 1,25-$(OH)_2$-$D_3$ activity (29). Holick et al. have further shown that the active form of 24,25-$(OH)_2$-$D_3$ may actually be a more polar trihydroxylated metabolite (1,24,25-$(OH)_3$-$D_3$) (30). Hydroxylation in a 1$\alpha$ position may well be a major determinant in the response of the intestinal receptor. In the present experiments in which there was chronic repletion with labeled 25-OH-$D_3$, an appreciable amount of polar material was eluted in the methanol strip fraction. Unlike plasma or kidney, more radioactivity in this fraction localized in intestinal mucosa from the TPTX animals than in the same tissue from the sham-operated animals. Whereas we did not identify the precise nature of the metabolites in this fraction, it is possible that a biologically active metabolite of 24,25-$(OH)_2$-$D_3$, such as that described by Boyle et al. (29) and by Holick et al. (30), was at least in part responsible for eliciting the transport adaptation in the TPTX animals. Even if this were the case, the results of the present study indicate that PTH is not the exclusive agent controlling the renal 25-OH-$D_3$-1-hydroxylase since 1,25-$(OH)_2$-$D_3$ was also present.

The results of the present study are of interest with regard to other reports in the literature in certain additional respects. Larkins et al. (37) for example, recently reported that TPTX rats maintained on a low calcium, low phosphate diet and supplemented with unlabeled 25-OH-$D_3$ were capable of increasing the production of 1,25-$(OH)_2$-$D_3$ as assessed by measuring the percentage of a pulse-labeling dose of [$^3$H]25-OH-$D_3$ appearing as [$^3$H]1,25-$(OH)_2$-$D_3$ in plasma and intestinal mucosa. Since, by virtue of the experimental design the pool sizes of unlabeled metabolites were unknown, and since transport studies which might corroborate pool size by differences in absorption were not performed, it is impossible to conclude from these studies that there was an actual increase in the rate of production of 1,25-$(OH)_2$-$D_3$ or an increase in the *amount* of this metabolite localizing in the mucosa of TPTX animals maintained on a LCD. In chronic repletion experiments of the type presented in the present report and by Larkins et al. (37), it is essential to label the metabolite pools to constant specific activity. The effects of an unlabeled metabolite pool on the subsequent binding of $^3$H-labeled 1,25-$(OH)_2$-$D_3$ in the mucosa have been clearly demonstrated by the studies of Chen and DeLuca (55). The results of the present studies also emphasize the importance of examining the levels of intestinal mucosal metabolites. Thus, the plasma metabolite data reported in the present study reflect the results of Tanaka and DeLuca (54) and others (26, 31), in which TPTX resulted in a lowering of the percentage of 1,25-$(OH)_2$-$D_3$ and an increase in the percentage of 24,25-$(OH)_2$-$D_3$ in the plasma. As emphasized by our results, plasma metabolite data need not necessarily be indicative of the actual amount of 1,25-$(OH)_2$-$D_3$ localizing in target organs such as the small intestinal mucosa.

In all studies involving parathyroidectomy or TPTX, one must consider the possibility that ablation of the glands may be incomplete. Thus, the production of small quantities of 1,25-$(OH)_2$-$D_3$ after TPTX in the present studies may conceivably have been due to incomplete removal of the parathytroid glands. While this is unlikely in view of the plasma calcium and phosphorus levels (Table I) it would not detract from the basic observation that chronically parathyroid-deficient animals can indeed adapt intestinal transport to meet the stresses of dietary calcium deprivation as described above. Furthermore, despite conflicting evidence (33, 35), CT may play a role in regulating renal 25-OH-$D_3$-1-hydroxylase activity. In the present studies it is unlikely that CT was a significant factor in modulating 1,25-$(OH)_2$-$D_3$ production or calcium transport in either group of animals. This hormone was absent because of total thyroidectomy in one group, and its secretion was probably suppressed by the LCD and low normal plasma calcium levels in the sham-operated animals (Table I).

There is still uncertainty with respect to the nature of the effects of PTH and CT on renal 25-OH-$D_3$-1-hydroxylase activity in the acute experimental setting (31–

36), and there is also uncertainty regarding the importance of these hormones as modulators of 25-OH-$D_3$-1-hydroxylase activity under more physiologic circumstances. The results of the present studies indicate that chronic absence of PTH can indeed exert a profound influence on the metabolism of 25-OH-$D_3$ (Table II), but the present studies and others (36, 37, 54) indicate that neither PTH nor CT is absolutely essential for the continued production of 1,25-$(OH)_2$-$D_3$. It is clear then, as suggested by Tanaka and DeLuca (54) and by Galante et al. and others (34, 36, 37, 56), that mechanisms other than one requiring the direct participation of PTH probably play a role in the control of 1,25-$(OH)_2$-$D_3$ synthesis. Dietary phosphorus deprivation and other measures that lower the serum inorganic phosphorus level apparently can increase 1,25-$(OH)_2$-$D_3$ production independently of any effects of PTH per se (54). These observations as well as others have led to the hypothesis that renal cortical cell inorganic phosphorus levels, whether modulated by PTH action or by dietary manipulation, may be critical in regulating 25-OH-$D_3$-1-hydroxylase activity (54). The TPTX animals in the present study as well as those in the earlier experiments performed by Kimberg and his co-workers (5) were maintained on an adequate dietary phosphorus intake and had the marked rise in plasma phosphorus characteristic of the parathyroidectomized state. Furthermore, in the present study, plasma phosphorus levels in TPTX animals (16.2±1.0 mg/100 ml, Table I), are in considerable excess of the phosphorus level at which acute studies have suggested that 1,25-$(OH)_2$-$D_3$ production ceases (54). Our determinations of renal phosphorus content were performed on homogenates of whole kidney rather than on homogenates of cortex as described by Tanaka and DeLuca (54). Nonetheless, despite marked differences in plasma phosphorus concentrations and in rates of 1,25-$(OH)_2$-$D_3$ production, we could not detect any differences in renal phosphorus content when comparing the sham and TPTX animals. Renal inorganic phosphorus content may simply represent a crude marker for some other more important parameter which regulates 25-OH-$D_3$-1-hydroxylase activity (such as the calcium ion activity within mitochondria). Since renal 25-OH-$D_3$-1-hydroxylase activity seems to be inhibited by the addition of calcium ions in vitro (32, 56), it seems quite likely that the mitochondrial calcium ion activity may indeed play an important role in modulating the activity of this key Mg-dependent enzyme (34, 36, 37, 56). The precise role, if any, of the calcium ion activity in the renal cell mitochondrion in accounting for the continued activity of 25-OH-$D_3$-1-hydroxylase in the chronically TPTX animals maintained on an LCD remains to be established.

In addition to confirming the observation that chronically TPTX rats can adapt intestinal calcium absorption to help meet the stresses of dietary calcium deprivation even though plasma phosphorus levels are elevated, the present studies also provide some insights into the basis for this adaptive response. Furthermore, these studies emphasize the fact that results obtained in the acute experimental situation should only be extrapolated to the more chronic setting with great caution. Finally, these studies suggest that the factors regulating the synthesis of 1,25-$(OH)_2$-$D_3$ and possibly 1,24,25-$(OH)_3$-$D_3$ may be more complex than previously appreciated.

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