Decrease in Serum Immunoreactive Parathyroid Hormone in Rats and in Parathyroid Hormone Secretion In Vitro by 1,25-Dihydroxycholecalciferol

B. S. Chertow, D. J. Baylink, J. E. Wergedal, M. H. H. Su, and
A. W. Norman

From the Departments of Medicine and Nuclear Medicine, Veterans
Administration West Side Hospital, and the University of Illinois College of
Medicine, Chicago, Illinois 60680; Department of Medicine, Seattle Veterans
Administration Hospital and University of Washington, Seattle, Washington
98108; and Department of Biochemistry, University of California,
Riverside, California 92502

ABSTRACT The present study determined the effects of 1,25-dihydroxycholecalciferol on serum immunoreactive parathyroid hormone and on parathyroid hormone secretion in vitro. Rats injected i.p. with 1,25-dihydroxycholecalciferol, 130 pmol (2 U)/140 g body wt, which is probably a physiologic dose, had a significant 43% decrease in serum immunoreactive parathyroid hormone at 4 h. In addition, this dose of 1,25-dihydroxycholecalciferol inhibited the serum immunoreactive parathyroid hormone response to hypocalcemia induced by phosphate injection. Because the increment in serum immunoreactive parathyroid hormone was less but the decrement in serum calcium more in phosphate plus 1,25-dihydroxycholecalciferol-treated than in phosphate plus vehicle-treated rats, the impaired serum immunoreactive parathyroid hormone response to 1,25-dihydroxycholecalciferol could not be attributed to the change in serum calcium. In studies of parathyroid hormone secretion from bovine parathyroid tissue in vitro, the concentration of 1,25-dihydroxycholecalciferol used for most experiments was 1 nM, which is in the range found in rat serum. 1,25-Dihydroxycholecalciferol at 1 or 100 nM significantly inhibited parathyroid hormone secretion when medium calcium concentration was normal (1.5 mM), high (3.0 mM), and low (1.0 mM). Maximum inhibition ranged from 19 to 74%;

inhibition was generally seen after 2 h of incubation; and inhibition was sustained or progressive thereafter. Vitamin A, 0.1 μM, caused a marked stimulation of parathyroid hormone secretion. 1,25-Dihydroxycholecalciferol at 1 nM markedly reduced (44%) the effect of vitamin A to stimulate parathyroid hormone secretion. This effect of 1,25-dihydroxycholecalciferol was maximal at 1 h and persisted thereafter. Another steroid, hydrocortisone, 10 μM, did not inhibit parathyroid hormone secretion, suggesting that the 1,25-dihydroxycholecalciferol effect was not a nonspecific inhibitory effect on parathyroid cells. Because other workers have shown that parathyroid hormone directly stimulates 1,25-dihydroxycholecalciferol secretion, our results are consistent with the concept that there is a feedback loop where parathyroid hormone directly stimulates secretion of 1,25-dihydroxycholecalciferol, which in turn directly inhibits secretion of parathyroid hormone.

INTRODUCTION

Recent studies showing that vitamin D must undergo chemical transformations before it is metabolically active (1–4) have aroused new interest in the target actions of vitamin D. 1,25-Dihydroxycholecalciferol (1,25-(OH)$_2$D$_3$) [1] is an important biologically active

[1] Abbreviations used in this paper: % B/F, percent bound/free; ECF, extracellular fluid; iPTH, immunoreactive parathyroid hormone; 1,25-(OH)$_2$D$_3$, 1,25-dihydroxycholecalciferol; 25-OHD$_3$, 25-hydroxycholecalciferol; PTH, parathyroid hormone; TPTX, thyroparathyroidectomized.

metabolite of vitamin D (5, 6). The classical target organs of vitamin D metabolites are gut, bone, and kidney (7, 8). Additionally, Lumb and Stanbury suggested from a number of clinical observations in patients with vitamin D deficiency (viz, serum immunoreactive parathyroid hormone [iPTH] higher than expected for the level of serum calcium) that vitamin D metabolites may directly inhibit parathyroid hormone (PTH) secretion (9). Moreover, Oldham, Arnaud, and Jowsey found that in vitamin D-deficient puppies treated with 25-hydroxycholecalciferol (25-OHD₃) there was a decrease in serum iPTH that could not be attributed to an increase in serum calcium (10). Oldham and 'co-workers also isolated a calcium-binding protein from normal procine parathyroid tissue (11). Finally, Arnaud suggested that a deficiency of 1,25-(OH)₂D₃ in renal failure might result in an inability of extracellular fluid (ECF) calcium to suppress PTH secretion (12). These findings raise the possibility that active vitamin D metabolites have a direct action on parathyroid gland. To test this possibility, iPTH levels were measured in serum from rats injected with 1,25-(OH)₂D₃ and in media from cultured bovine parathyroid tissue treated with 1,25-(OH)₂D₃.

METHODS

The 1,25-(OH)₂D₃ used in this work was prepared from 25-OHD₃ (generously supplied by Dr. J. Babcock, The Upjohn Company, Kalamazoo, Mich.) via incubations in vitro with mitochondria from kidneys obtained from rachitic chicks as previously described (13).

Rat studies. Male Holtzman rats, weighing about 140 g, were fed a diet containing 0.6% calcium, 0.6% phosphorus, and 2 IU of vitamin D₃/g of diet. Four experiments were done to evaluate the effects of 1,25-(OH)₂D₃ and/or i.p. phosphate and i.v. calcium on serum iPTH: (Exp. 1) Rats were given 130 pmol (2 U) of 1,25-(OH)₂D₃ or vehicle (95% ethanol, 0.2 ml) i.p. Four h later, blood was obtained by tail vein for measurement of serum calcium (14) and serum iPTH. (Exp. 2) At zero time one group of rats was injected i.p. with vehicle and another group with 130 pmol of 1,25-(OH)₂D₃. Immediately thereafter, both groups were given 1.5 meq of phosphate (Na₂-HPO₄ and KH₂PO₄, pH 7.4) i.p. A control group was injected with saline and vehicle but not phosphate. Blood was obtained by tail vein 4 and 8 h later for serum calcium and iPTH analyses. (Exp. 3) The effect of a larger dose of phosphate, i.p., 2.0 meq, on serum calcium and iPTH was evaluated. (Exp. 4) The acute effect of i.v. calcium on serum iPTH was evaluated as follows. The test group was infused with calcium i.v. as CaCl₂, 0.2 mg Ca/min per 150 g, for 8 min. The control group was infused with i.v. saline over the same time interval. In both groups, blood was obtained by cardiac puncture at 2, 4, 6, and 8 min for measurement of serum calcium (14) and serum iPTH. Total volume infused per rat in both groups was 0.4 ml. The animals were under light ether anesthesia during the 8-min experiment.

Rat serum iPTH assay. The method used to perform this assay was similar to that described by Arnaud, Tsao, and Littledike for human serum iPTH (15). Highly puri-

fied bovine PTH, generously supplied by Dr. J. Hamilton, Kansas City, Mo., was used to prepare the iodinated tracer and as the standard for the assay. Iodination of the hormone with ¹³¹I of high specific activity (Industrial Nuclear Co., Inc., St. Louis, Mo.) was accomplished by the Berson and Yalow modification of the method of Greenwood and associates (16). The iodinated PTH was purified with QUSO G-32 (Philadelphia Quartz Co., Chester, Pa.) as described by Yalow and Berson (17). The antiserum used, generously supplied by Dr. C. Arnaud, Mayo Clinic, Rochester, Minn., was chicken-12 anti-bovine antiserum, which predominantly detects the region of bovine PTH that is carboxyl-terminal to amino acid residue 34.[a]

Incubation mixtures contained diluent, chicken-12 antibovine antiserum at a final dilution of 1:2,200, different amounts of standard bovine PTH, and 100 μl of "unknown" rat serum. Diluent contained 9 parts 0.1 M barbital buffer, pH 8.6, with 500 U/ml of Trasylol (FBA Pharmaceuticals, Inc., New York) and 1 part hypoparathyroid serum. After the mixtures were incubated on a shaker at 4°C for 3 days, 200 μl of tracer in barbital buffer containing approximately 10,000 cpm was added to the above incubation mixtures and incubation was then carried out for an additional 2 days. Bound ¹³¹I-PTH and free ¹³¹I-PTH were separated by the dextran-coated charcoal method (15). Supernates and precipitates were then counted in a Nuclear Chicago 1185 automatic gamma counter (Searle Analytic Inc., Des Plaines, Ill.). Corrections were made for nonspecific incubation damage, which ranged from 4.6 to 9%. Also, the final results were corrected for blank values obtained with hypoparathyroid serum by a procedure similar to that described by Arnaud and associates (15).

Curves of percent bound/free (% B/F) as a function of bovine PTH and of rat serum containing a high level of iPTH (obtained from calcium-deficient and vitamin D-deficient rats) were superimposable between about 90 and 30% (Fig. 1). Accordingly, this curve was used to determine rat serum iPTH in bovine PTH equivalents. Control and test serum samples from individual experiments were measured in the same assay. The intra- and interassay coefficients of variation of serum iPTH were ±8 and ±15%, respectively. With this assay, we have demonstrated changes in serum iPTH in the appropriate direction under conditions known to alter serum PTH. Accordingly, serum iPTH was detectable in about 99% of control rats; undetectable in severely hypocalcemic (serum calcium about 5.5 mg/100 ml) thyroparathyroidectomized (TPTX) rats on a control diet; decreased 38% in intact rats during i.v. infusion of calcium (Fig. 2); increased 118% when serum calcium was decreased 8% (Fig. 4); increased 301% when serum calcium was decreased 34% (Table I); and increased more than 10-fold in chronically hypocalcemic rats (serum calcium 5.1 mg/100 ml at time of assay; see Fig. 1).

In vitro studies of the iPTH secretion rate. Because the technique used to measure the rate of bovine PTH secretion in vitro and the application of this technique to study PTH secretion have been described previously (18–20), only a brief description of this technique will be given. In each flask, freshly collected bovine parathyroid tissue, weighing about 20 mg, was incubated at 37°C in Eagle's medium with 10% calf serum (total volume 2 ml) under an atmosphere of 95% O₂:5% CO₂. There were four flasks per group. Initial calcium concentration of the medium was 1.5 mM. The 1st h of incubation served as
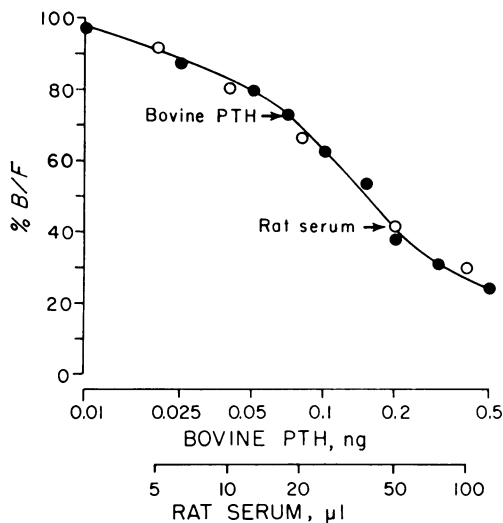
FIGURE 1 Standard curve for rat serum iPTH assay. % B/F are shown as a function of the amount of purified bovine PTH and of rat serum containing a high level of iPTH. The serum was obtained from rats fed a diet containing 0.01% calcium and no vitamin D for 4 wk. Serum calcium was 5.1 mg/100 ml and serum iPTH was > 3,000 pg/ml compared with about 200 pg/ml in rats fed a normal diet. The two sets of data give superimposable curves. These results were duplicated in a second study (not shown). Also, for the bovine PTH standard curve, the coefficients of variation (interassay variance from eight different assays) ranged from ±3 to ±12% for % B/F values between 90 and 30% B/F.

the equilibration period and the corresponding incubation medium was not analyzed. Medium collected during the 2nd h of incubation was used to determine the basal secretion rate. The parathyroid tissue samples were then incubated in media containing normal calcium (1.5 mM), low calcium (1.0 mM), or high calcium (3.0 mM), and the following agents alone or in combination were added: 1,25-(OH)₂D₃, final concentration 1 or 100 nM; retinol (vitamin A), final concentration 0.1 µM; hydrocortisone, final concentration 10 µM; and vehicle (ethanol, final concentration 0.2% except in the hydrocortisone experiment in which it was 0.1%). (Ethanol was used to dissolve 1,25-(OH)₂D₃, vitamin A, and hydrocortisone.) Time-matched control tissues were run in every experiment. To determine PTH secretion rate, medium iPTH was analyzed at 30- or 60-min intervals (at which time medium was changed) by a slight modification of the PTH radioimmunoassay method described by Arnaud and associates (15), and the rates were expressed as picograms of PTH/milligram wet wt per hour.

The antiserum used in this assay, GP-8, was developed by Dr. G. A. Williams. The portions of the bovine PTH molecule recognized by this antiserum were assessed by comparing the ability of two PTH standards (synthetic bovine PTH 1-34 [Beckman Instruments Inc., BioProducts Dept., Palo Alto, Calif.] which contains only the aminoterminal end of the PTH molecule, and purified bovine PTH 1-34 [Wilson Laboratories, Chicago, Ill.] which contains both amino and carboxyl terminals of the molecule) to compete with tracer in the assay system. The slope of % B/F as a function of molar concentration of bovine PTH

1-34 was significantly greater than zero, and the slope of % B/F as a function of molar concentration of bovine PTH 1-84 was significantly greater than the slope with bovine PTH 1-34. Therefore, because both standards exhibited competition with tracer and because bovine PTH 1-84 exhibited greater competition than bovine PTH 1-34, this antiserum detects both amino and carboxyl terminals of bovine PTH. However, based on the data from this experiment, the sensitivity of this antiserum was greater for the carboxyl than for the amino terminal of bovine PTH. The final results were corrected for blank values obtained with unused medium in a manner similar to that described by Arnaud and associates for serum (15). In the bovine PTH assay, the intra- and interassay coefficients of variation were ±8 and ±13%, respectively. Media from individual experiments were analyzed in the same assay. Analysis of variance or Student's t test was used to determine statistical significance (21).
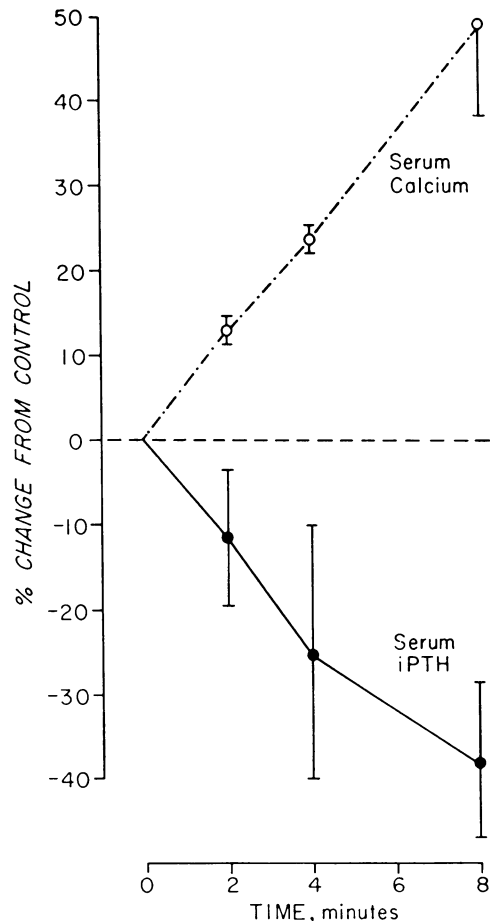

FIGURE 2 Serum calcium and iPTH in normal rats in response to i.v. calcium infusion. Test group was infused i.v. with calcium as CaCl₂, 0.2 mg/min between 0 and 8 min. The control group was infused i.v. with saline over the same time interval. Mean±SE of 11 rats per group. Serum calcium was significantly increased (P < 0.001) at 2 min and thereafter. Serum iPTH was significantly decreased (P < 0.02) at 8 min. Data are plotted as a percentage of control values.

TABLE I

Serum Calcium and iPTH 4 h after Injection of Phosphate (PO₄) or PO₄ Plus 1,25-(OH)₂D₃

	Serum calcium			Serum iPTH		
Agents injected	Concentration	Percent change from control	P	Concentration	Percent change from control	P
	$mg/100\ ml$			$pg/100\ ml$		
Exp. 2*						
Saline plus vehicle (control)	11.1±0.3‡			224±103		
PO₄, 1.5 meq, plus vehicle	10.4±0.7	−8	<0.02	489±140	118	<0.001
PO₄, 1.5 meq, plus 1,25-(OH)₂D₃, 2 U	9.0±0.9	−19	<0.001	285±109	27	NS
Exp. 3						
Saline (control)§	10.7±0.1			140±109		
PO₄, 2.0 meq	7.1±1.0	−34	<0.001	562±137	301	<0.001

* Data at the 4-h time interval from exp. 2; also see Fig. 4.
‡ Mean±SD.
§ Control data for the PO₄, 2.0 meq. This experiment was done at a different time than the experiment comprising the remainder of the results in this table.

In this in vitro assay system, PTH secretion from bovine parathyroid tissue into medium changed in the appropriate direction in response to changes in medium calcium concentration. Accordingly, PTH secretion increased when the medium contained a low calcium concentration and decreased when the medium contained a high calcium concentration (18) (Figs. 5 and 6).

RESULTS

Effects of 1,25-(OH)₂D₃ on serum iPTH in rats. In normal rats 4 h after i.p. injection of 1,25-(OH)₂D₃, as compared with rats injected with vehicle, there was no change in serum calcium but a statistically significant 43% decrease in serum iPTH (Fig. 3).* Thus, 1,25-(OH)₂D₃ treatment caused a decrease in basal serum iPTH that could not be attributed to an increase in serum calcium.

In another experiment the effect of 1,25-(OH)₂D₃ treatment on the serum iPTH response to hypocalcemia induced by phosphate injection was evaluated (Fig. 4). In the vehicle-treated group, compared with the control group (which received vehicle plus saline but not phosphate), phosphate injection caused a significant decrease in serum calcium at 4 h (P < 0.02), but the decrement at 8 h was not statistically significant. In the 1,25-(OH)₂D₃-treated group, phosphate injection caused a marked and significant decrease in serum calcium at 4 h (P < 0.001), and the decrement at 8 h was still significant (P < 0.01). The decrement in serum calcium was greater in the 1,25-(OH)₂D₃ than in the vehicle-treated group at 4 h (P < 0.005) and at 8 h, but at the latter time interval the difference was not statistically

* Earlier we found the opposite effect (1974. Clin. Res. 22: 479A); we now feel that those results were invalid.

significant. Compared with the control group, serum iPTH was markedly increased in the vehicle-treated group at both 4 h (P < 0.001) and 8 h (P < 0.001) after phosphate injection. Although there was some increase in serum iPTH at both 4 and 8 h after phosphate injection in the 1,25-(OH)₂D₃-treated group, the increment was not statistically significant at either time interval. The difference in serum iPTH between vehicle- and 1,25-(OH)₂D₃-treated groups was significant at both 4 h (P < 0.005) and 8 h (P < 0.05). Thus,
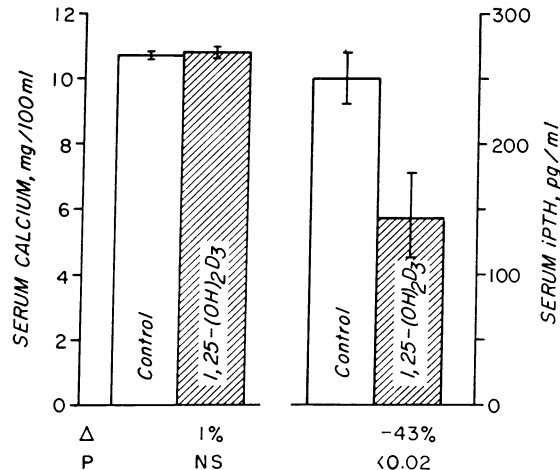

FIGURE 3 Effect of 1,25-(OH)₂D₃ treatment on serum calcium and iPTH in normal rats. The control rats were injected i.p. with vehicle (0.2 ml, 95% ethanol) and the test rats with 130 pmol of 1,25-(OH)₂D₃ dissolved in 95% ethanol, and 4 h later blood was obtained for serum calcium and iPTH. 10 rats per group; mean±SE.
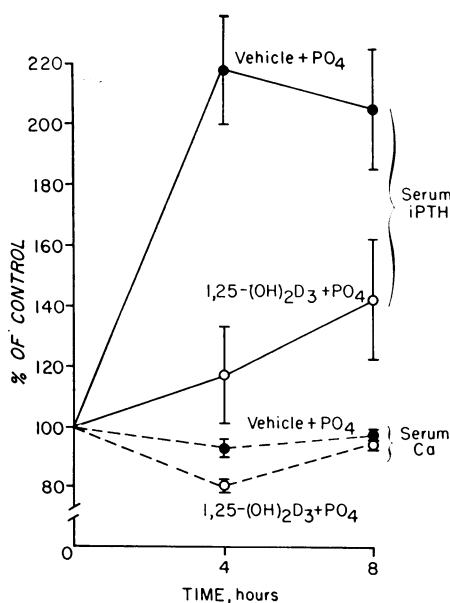
FIGURE 4 Effect of 1,25-(OH)$_2$D$_3$ treatment on serum iPTH response to hypocalcemia induced by phosphate (PO$_4$) injection. At zero time, one group of rats was injected i.p. with vehicle and another group with 130 pmol 1,25-(OH)$_2$D$_3$. Immediately thereafter, both groups were given 1.5 meq of PO$_4$ i.p. A control group was injected with vehicle plus saline. Serum calcium (Ca) and iPTH were measured in blood samples obtained 4-8 h later. Data are plotted as a percentage of control values. 10 rats per group; mean±SE. See Results for statistical analyses.

phosphate injection caused, after 4 h, a significantly greater decrement in serum calcium but a significantly lesser increment in serum iPTH in the 1,25-(OH)$_2$D$_3$-treated than in the vehicle-treated group (Fig. 4).

In animals injected with different doses of phosphate, there was an inverse relationship between serum calcium and iPTH (Table I). Thus, because serum calcium was lower in the group treated with phosphate plus 1,25-(OH)$_2$D$_3$ than in the group treated with phosphate plus vehicle, the decreased serum iPTH response as a result of 1,25-(OH)$_2$D$_3$ treatment is particularly noteworthy (Fig. 4).

Effect of 1,25-(OH)$_2$D$_3$ on the rate of PTH secretion in vitro. To determine if the above in vivo changes in serum iPTH could have resulted from a direct action of 1,25-(OH)$_2$D$_3$ on parathyroid cells to inhibit PTH secretion, in vitro experiments were done. (All PTH released into media, regardless of the mechanism of release, is considered to be secreted PTH.) In bovine parathyroid tissue incubated in media with a physiological calcium concentration (1.5 mM), 1,25-(OH)$_2$D$_3$ treatment caused a significant decrease in the cumulative amount of PTH secreted over 240 min (Table II). Two different doses of 1,25-(OH)$_2$D$_3$ were

used (1 and 100 nM), and although the higher dose appeared to have a greater effect, no significant difference in cumulative response between the two doses was found, perhaps because of the small number of observations. In this experiment, although not shown in Table I, PTH secretion rates were measured hourly from 1 to 4 h. Compared with controls, 1,25-(OH)$_2$D$_3$ at 100 and 1 nM decreased PTH secretion rate throughout the experiment and the decrement was consistently greater at 100 than at 1 nM; however, the only decrement in PTH secretion rate that was statistically different from the control rate was at 1 h with 100 nM 1,25-(OH)$_2$D$_3$.

In bovine parathyroid tissue incubated in a low-calcium medium (1.0 mM), the PTH secretion rate, as compared with basal PTH secretion rate (i.e., when medium calcium was 1.5 mM), was significantly increased at 1-4 h (Fig. 5). In bovine parathyroid tissue incubated in a high-calcium medium (3.0 mM), the PTH secretion rate, compared with the basal secretion rate, was significantly decreased at 1-4 h (Fig. 6). Though a control group with a medium calcium of 1.5 mM was not included in the present study, previous work with this in vitro system indicates that throughout the incubation period a low-calcium medium (0.75 mM) causes an increase in the PTH secretion rate and that a high-calcium medium (3.0 mM) depresses PTH secretion (18). 1,25-(OH)$_2$D$_3$ at 1 nM significantly decreased the effect of a low-calcium medium to increase PTH secretion rate; this effect was evident (statistically significant) at 2 h and persisted or perhaps was progressive thereafter (Fig. 5). Additionally,

TABLE II

Effect of 1,25-(OH)$_2$D$_3$ on Cumulative PTH Secretion Over 4 h from Bovine Parathyroid Tissue Incubated in Media Containing a Physiologic Concentration of Calcium (1.5 mM)

	PTH secretion	
	Basal secretion (−60 to 0 min)*	Cumulative secretion after addition of test agents (0 to 240 min)*
	pg/mg wet wt	
Vehicle (control)	393±37‡	1,516±171‡
1,25-(OH)$_2$D$_3$, 1 nM	339±34	1,222±135§
1,25-(OH)$_2$D$_3$, 100 nM	348±54	1,104±97§

* Zero time is considered to be the time at the end of the 60-min basal secretion period.
‡ Mean±SE of four samples per group.
§ By analysis of variance, cumulative PTH secretion was significantly less in the 1,25-(OH)$_2$D$_3$-treated groups than in the control group, $P < 0.05$.

in the high-calcium medium, 1,25-(OH)₂D₃ treatment produced a further decrement in the PTH secretion rate which was statistically significant at 3 and 4 h (Fig. 6).

In recent work, Chertow and co-workers showed that vitamin A stimulates the PTH secretion rate in vitro (19). This effect was confirmed in the present study where vitamin A at 0.1 μM caused a marked stimulation of the PTH secretion rate (Fig. 7). The maximum increment in the PTH secretion rate was seen at 1 h, and thereafter the effect decreased with time (Fig. 7). 1,25-(OH)₂D₃ at 1 nM inhibited the effect of vitamin A to increase the PTH secretion rate; the inhibitory effect of 1,25-(OH)₂D₃ was evident (statistically significant) at 1 h and persisted throughout the remainder of the experiment (Fig. 7). When the PTH secretion rates were plotted as a percentage of control, the curves for vitamin A and vitamin A plus 1,25-(OH)₂D₃ were quite similar in shape (Fig. 7). These curves would be consistent with either an effect of 1,25-(OH)₂D₃ to decrease the basal level of secretion or to inhibit specifically the action of vitamin A on PTH secretion.

Effect of hydrocortisone on the rate of PTH secretion in vitro. In order to evaluate the specificity of the inhibitory effect of 1,25-(OH)₂D₃ on PTH secretion, the effect of another steroid, hydrocortisone, on
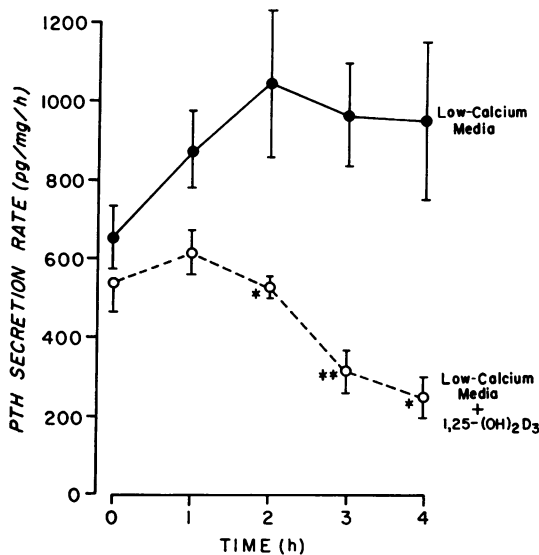
FIGURE 5 Effect of 1,25-(OH)₂D₃ on PTH secretion rate of bovine parathyroid tissue incubated in media containing a low calcium concentration (1.0 mM). Zero time data represent basal PTH secretion rates of parathyroid tissue incubated in media containing 1.5 mM calcium. At zero time, media were changed to contain 1.0 mM calcium and either vehicle (ethanol, final concentration 0.2%) or 1,25-(OH)₂D₃, 1 nM. Four samples per group; mean±SE. * P < 0.05; ** P < 0.01.
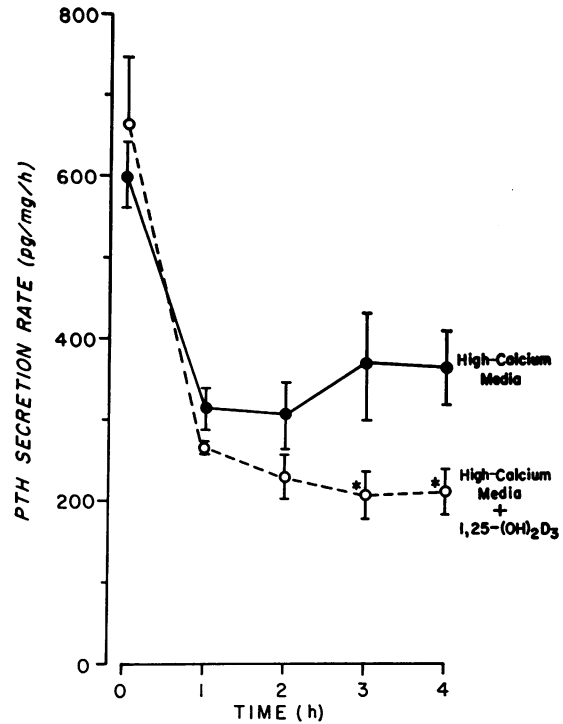
FIGURE 6 Effect of 1,25-(OH)₂D₃ on PTH secretion rate of bovine parathyroid tissue incubated in media containing a high calcium concentration (3.0 mM). Zero time data represent basal PTH secretion rates of parathyroid tissue incubated in media containing 1.5 mM calcium. At zero time, media were changed to contain 3.0 mM calcium and either vehicle or 1,25-(OH)₂D₃, 1 nM. Four samples per group; mean±SE. * P < 0.05.

PTH secretion was evaluated (Table III). Hydrocortisone, 10 μM, had no consistent or statistically significant effect on PTH secretion from bovine parathyroid tissue incubated in medium containing a physiologic concentration of calcium (1.5 mM) or a low concentration of calcium (0.75 mM) during 3-h incubation periods.

DISCUSSION

Our results support and extend work by other investigators (9–12), cited in the Introduction, on the relationship between vitamin D and PTH secretion. There are three important issues that must be considered before the significance of our results can be properly evaluated: (a) the validity of the PTH assays used in this work, (b) what molecular species of PTH are secreted and the fate of secreted PTH, and (c) whether our results were obtained with physiologic doses of 1,25-(OH)₂D₃. Rat serum iPTH is difficult to measure, largely because most antisera to bovine PTH do not cross-react with rat PTH. In our rat serum iPTH assay, the % B/F curve for dilutions of rat serum
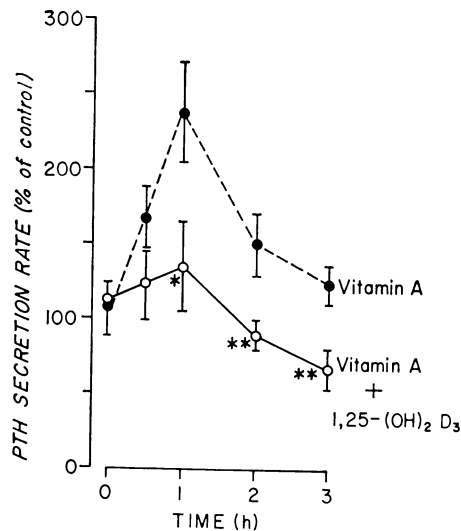
FIGURE 7 Effect of 1,25-(OH)$_2$D$_3$ on vitamin A-stimulated PTH secretion rate of bovine parathyroid tissue in vitro. Zero time data represent basal PTH secretion rates of parathyroid tissue incubated in media containing 1.5 mM calcium. At zero time, the media were changed to contain vehicle (control), vitamin A, 0.1 μM, or vitamin A, 0.1 μM, plus 1,25-(OH)$_2$D$_3$, 1 nM. Data are plotted as a percentage of control values. At 1, 2, and 3 h, PTH secretion rate was significantly greater in the vitamin A-treated than in the vitamin A plus 1,25-(OH)$_2$D$_3$ group. Four samples per group; mean$\pm$SE. * $P < 0.01$.

containing a high level of iPTH were superimposable on the bovine PTH standard curve, suggesting good immunological cross-reactivity with the antiserum used in our assay. Because of this and our finding of: (a) progressive increments in serum iPTH in response to decrements in serum calcium, and (b) progressive

decrements in serum iPTH in response to increments in serum calcium, we feel that our assay detects decreases as well as increases in serum iPTH. The iPTH assay used to measure PTH secretion rate of bovine parathyroid tissue in vitro has been validated previously (18, 19). Additionally, the validity of these assays is supported by the internal consistency of our in vivo and in vitro data.

The molecular species (i.e., native PTH, pro-PTH, or PTH fragments) and fate of secreted PTH are also pertinent to the interpretation of the effect of 1,25-(OH)$_2$D$_3$ in terms of PTH secretion. With respect to in vitro work, Sherwood, Rodman, and Lundberg obtained evidence from bovine parathyroid tissue that glandular or native PTH (mol wt 9,500) was a precursor which was converted to a smaller mol wt (mol wt 7,000) and then apparently secreted into medium (22). Fischer, Oldham, Sizemore, and Arnaud (23) and Arnaud and co-workers (24), working with porcine and human parathyroid tissue, respectively, also found evidence that the predominant secreted species in vitro was smaller than native hormone but biologically active. In contrast, other laboratories have obtained evidence that bovine pro-PTH is a larger mol wt species than native PTH, that pro-PTH is not secreted in vitro, and that the predominant species secreted in vitro is native hormone (25-27). Our in vitro results tend to agree with the conclusion that the hormone secreted is native hormone, in that secreted PTH was immunologically indistinguishable from native PTH (18). In any case, the majority if not all of the above studies are consistent with the concept that the predominant species secreted in vitro is not pro-PTH and is biologically active.

TABLE III

Effect of Hydrocortisone on PTH Secretion over 3 h from Bovine Parathyroid Tissue Incubated in Medium Containing a Physiologic Concentration of Calcium (1.5 mM) or a Low Concentration of Calcium (0.75 mM)

Vehicle	PTH secretion rate			
	Time...0*	1 h	2 h	3 h
	pg/mg wet wt per h			
1.5 mM calcium in medium				
Control	375$\pm$53‡	416$\pm$18	395$\pm$20	428$\pm$48
Hydrocortisone, 10 μM	371$\pm$54	413$\pm$46	400$\pm$28	406$\pm$36
0.75 mM calcium in medium				
Control	391$\pm$40	1,027$\pm$92	1,354$\pm$87	1,310$\pm$141
Hydrocortisone, 10 μM	443$\pm$22	1,312$\pm$202	1,632$\pm$131	1,473$\pm$109

* Basal secretion rate over the 1-h period before addition of test agent.
‡ Mean$\pm$SE of eight samples (1.5 mM calcium in medium) or four samples (0.75 mM calcium in medium). No significant differences between control and hydrocortisone groups were found at any time points or in cumulative secretion (not shown).

674   Chertow, Baylink, Wergedal, Su, and Norman

Regarding the fate of secreted PTH, some (27) but not all workers (22) found that when radioactive PTH was added to the medium of parathyroid tissue slices there was degradation of the labeled hormone. Because our antiserum has greater sensitivity for the carboxyl than for the amino terminal of PTH, it is possible that in vitro degradation of native PTH could spuriously affect the quantitation, based on medium iPTH analyses, of the amount of hormone secreted. This seems unlikely, however, because the amount of degradation of labeled native hormone in vitro in 1 h is negligible (27) and because our medium was changed hourly or more frequently. Thus, if, as seems likely, the major species secreted was native PTH and since PTH degradation in medium under our conditions was negligible, our in vitro results indicate that 1,25-(OH)$_2$D$_3$ decreases secretion of native PTH.

Our in vivo serum iPTH results, while not a direct measure of PTH secretion, may reflect changes in secretion. Although there is indirect evidence in humans that PTH fragments can be secreted (28), other studies suggest that in both humans and cows, secreted hormone is largely native PTH (29, 30). However, the domnant form of circulating PTH in humans and cows appears to be small mol wt fragments (30, 31). Nevertheless, changes in serum iPTH tend to reflect changes in the secretion of native hormone (12, 15, 31). Although rat parathyroid tissue has been shown to synthesize pro-PTH (32), neither the secreted nor circulating species of PTH in rats has been studied. Interpreted in the light of the above data and our in vitro data, our finding that 1,25-(OH)$_2$D$_3$ decreases serum iPTH in rats strongly suggests but does not establish that 1,25-(OH)$_2$D$_3$ decreases PTH secretion in vivo.

Concerning the issue of 1,25-(OH)$_2$D$_3$ dosage, the concentration of 1,25-(OH)$_2$D$_3$ in bovine serum is unknown; however, in rats fed vitamin D$_3$, the basal level of serum 1,25-(OH)$_2$D$_3$ was 17 ng/100 ml or 0.41 nM and the level in response to a calcium-deficient diet was about 100 ng/100 ml or 2.5 nM (32a). Thus, the concentration of 1,25-(OH)$_2$D$_3$ used in our in vitro work, 1 nM, is within the range of that found in rat serum. However, because 1,25-(OH)$_2$D$_3$, like cholecalciferol and 25-hydroxycholecalciferol (33), may be largely bound in serum to carrier proteins and because our incubation medium contained only 10% calf serum, it is possible that the level of free, and presumably active, 1,25-(OH)$_2$D$_3$ was greater in our incubation medium than in serum.

In any case, the dose we used is similar to that used by Raisz, Trummel, Holick, and DeLuca, who found by means of their in vitro resorbing system that a resorptive response to 1,25-(OH)$_2$D$_3$ was detectable at about 0.1 nM and that there was a log dose response between about 0.1 and 10 nM (34). 1,25-(OH)$_2$D$_3$ at 10 nM also stimulates calcium-45 uptake by intestine in vitro (35). Thus, apart from the uncertainty of the issue of free 1,25-(OH)$_2$D$_3$ concentration, it would seem that the concentration of 1,25-(OH)$_2$D$_3$ we used in our in vitro work is probably in the physiological range.

The physiologic dose of 1,25-(OH)$_2$D$_3$ in vivo has not been established. In this study we used 130 pmol (2 U) of 1,25-(OH)$_2$D$_3$, a dose that did not increase serum calcium. Other workers have used between 1 and 10 U of 1,25-(OH)$_2$D$_3$ for in vivo work with rats (36–38). In our rats, dietary intake of vitamin D$_3$ was about 26 IU per day. Although the comparative actions of 1,25-(OH)$_2$D$_3$ and vitamin D$_3$ vary considerably depending upon the response measured, 1,25-(OH)$_2$D$_3$ is about five times more active than vitamin D$_3$ in maintaining serum calcium (39). On this basis, one can estimate that the dose of 1,25-(OH)$_2$D$_3$ used in this study was equivalent to about 40% of the daily oral dose of vitamin D. Although it is difficult from the above observations to be certain of what constitutes a physiologic dose of 1,25-(OH)$_2$D$_3$, we feel that 2 U of 1,25-(OH)$_2$D$_3$ in a 140-g rat is probably in the physiological range.

With the above qualifications in mind, we feel that the results of this study indicate that 1,25-(OH)$_2$D$_3$, probably at physiologic doses, decreases PTH secretion in vitro and serum iPTH in vivo. The inhibition of PTH secretion by 1,25-(OH)$_2$D$_3$ in vitro was probably not due to a nonspecific inhibition of parathyroid cell function, because 1,25-(OH)$_2$D$_3$ at similar concentrations stimulates rather than inhibits both bone resorption and calcium-45 uptake by intestine in vitro (34, 35) and because in this study another steroid, hydrocortisone, did not inhibit PTH secretion (Table III).

Although the only vitamin D metabolite evaluated in this work was 1,25-(OH)$_2$D$_3$, recently Henry and Norman showed in vitamin D-deficient chicks that the concentration of radioactive 1,25-(OH)$_2$D$_3$, after injection of radioactive 1,25-(OH)$_2$D$_3$ or radioactive 25-OHD$_3$, was higher in the parathyroid glands than in blood (40). However, no evidence was found that either 25-OHD$_3$ or 24,25-(OH)$_2$D$_3$ was concentrated by parathyroid glands (40). This raises the possibility that 1,25-(OH)$_2$D$_3$ is the predominant vitamin D metabolite that acts on the parathyroid glands. Moreover this observation strengthens the conclusion that 1,25-(OH)$_2$D$_3$ plays a direct role in PTH secretion.

Our in vivo studies, in addition to being consistent with our in vitro work, disclosed an unexpected effect of 1,25-(OH)$_2$D$_3$ on serum calcium. The degree of

hypocalcemia in the rats given phosphate plus 1,25-(OH)$_2$D$_3$ was greater than that in rats given phosphate alone (Fig. 4). Of the various possible explanations for this change, the simplest one is that the greater degree of hypocalcemia was due to inhibition of PTH secretion by 1,25-(OH)$_2$D$_3$. If so, a dose of 1,25-(OH)$_2$D$_3$ that decreases serum iPTH does not appear to replace the effect of PTH to maintain serum calcium. If this explanation were correct, one would expect to find hypocalcemia in rats given sufficient 1,25-(OH)$_2$D$_3$ to decrease serum iPTH; however, in the present study no decrease in serum calcium was found when serum iPTH was significantly depressed, at least when evaluated at the one time interval of 4 h (Fig. 3). Further work will be required to resolve the interrelationship between 1,25-(OH)$_2$D$_3$ and PTH on calcium metabolism.

The mechanism of PTH secretion differs from that of many hormones in that low external (i.e., medium or ECF) calcium stimulates rather than inhibits PTH secretion (18, 41). The mechanism of PTH secretion is similar to that of many hormones in that it is probably mediated by cyclic AMP (18, 42). The importance of cyclic AMP is emphasized by the fact that dibutyryl cyclic AMP increases PTH secretion whether medium calcium is high or low (18, 42). If PTH secretion were primarily mediated by cyclic AMP, 1,25-(OH)$_2$D$_3$ could inhibit PTH secretion by acting on the net production or subsequent effects of cyclic AMP. One of the most physiologically significant effects of 1,25-(OH)$_2$D$_3$, at least in intestinal epithelial cells, is to increase transcellular calcium transport, and this is accompanied by an increase in cytoplasmic calcium (43). With respect to the latter, it is of interest that an increase in cytoplasmic calcium can in some in vitro systems inhibit adenyl cyclase activity (41, 44) and in others inhibit cyclic AMP-mediated changes in enzyme activity (45). Either of these two changes, if it occurred in parathyroid cells, could inhibit cyclic AMP-mediated PTH secretion. However, whether the effect of 1,25-(OH)$_2$D$_3$ on parathyroid cells is mediated by changes in either cellular calcium metabolism or cyclic AMP remains to be determined. Also unknown is the mechanism whereby vitamin A stimulates PTH secretion. Inasmuch as 1,25-(OH)$_2$D$_3$ inhibits this effect of vitamin A, it is of interest that vitamin A causes changes in parathyroid cell membranes (20) and that vitamins A and D have direct and sometimes antagonistic effects on cell membranes (46–49). Our results with vitamin A indicate that whatever the mechanism of 1,25-(OH)$_2$D$_3$ inhibition of PTH secretion, it is not specific for PTH secretion mediated by external calcium.

The observed effect of 1,25-(OH)$_2$D$_3$ to inhibit PTH secretion could be of physiological significance. There is evidence from both in vitro and in vivo studies that PTH stimulates 1,25-(OH)$_2$D$_3$ synthesis (50, 51). Thus, if, as seems likely from the results of this study, physiologic levels of 1,25-(OH)$_2$D$_3$ inhibit PTH secretion, this would be indicative of a feedback loop where PTH stimulates the secretion of 1,25-(OH)$_2$D$_3$, which in turn inhibits PTH secretion. Accordingly, 1,25-(OH)$_2$D$_3$ as well as ECF calcium would control PTH secretion by feedback inhibition. This redundance could result in a more exact and sustained control of serum calcium under diverse conditions.

REFERENCES

1. Ponchon, G., and H. F. DeLuca. 1969. The role of the liver in the metabolism of vitamin D. *J. Clin. Invest.* **48:** 1273–1279.
2. Fraser, D. R., and E. Kodicek. 1970. Unique biosynthesis by kidney of a biologically active vitamin D metabolite. *Nature (Lond.).* **228:** 764–766.
3. Gray, R., I. Boyle, and H. F. DeLuca. 1971. Vitamin D metabolism: the role of kidney tissue. *Science (Wash. D. C.).* **172:** 1232–1234.
4. Norman, A. W., R. J. Midgett, J. F. Myrtle, and H. G. Nowicki. 1971. Studies on calciferol metabolism. I. Production of vitamin D metabolite 4B from 25-OH-cholecalciferol by kidney homogenates. *Biochem. Biophys. Res. Commun.* **42:** 1082–1087.
5. Holick, M. F., H. K. Schnoes, and H. F. DeLuca. 1971. Identification of 1,25-dihydroxycholecalciferol, a form of vitamin D$_3$ metabolically active in the intestine. *Proc. Natl. Acad. Sci. U. S. A.* **68:** 803–804.
6. Wong, R. G., J. F. Myrtle, H. C. Tsai, and A. W. Norman. 1972. Studies on calciferol metabolism. V. The occurrence and biological activity of 1,25-dihydroxyvitamin D$_3$ in bone. *J. Biol. Chem.* **247:** 5728–5735.
7. Omdahl, J. L., and H. F. DeLuca. 1973. Regulation of vitamin D metabolism and function. *Physiol. Rev.* **53:** 327–372.
8. Norman, A. W., and H. Henry. 1974. The role of the kidney and vitamin D metabolism in health and disease. *Clin. Orthop. Relat. Res.* **98:** 258–287.
9. Lumb, G. A., and S. W. Stanbury. 1974. Parathyroid function in human vitamin D deficiency and vitamin D deficiency in primary hyperparathyroidism. *Am. J. Med.* **56:** 833–839.
10. Oldham, S. B., C. D. Arnaud, and J. Jowsey. 1973. Influence of vitamin D on canine parathyroid glands (PTG's). *Clin. Res.* **21:** 254. (Abstr.)
11. Oldham, S. B., J. A. Fischer, and C. D. Arnaud. 1972. Isolation and properties of a calcium binding protein (CaBP) from normal porcine parathyroid glands

(pPTGs). *Excerpta Med. Int. Congr. Ser. No. 256.* 95. (Abstr.)

12. Arnaud, C. D. 1973. Hyperparathyroidism and renal failure. *Kidney Int.* **4**: 89–95.

13. Norman, A. W., R. J. Midgett, J. F. Myrtle, and H. G. Nowicki. 1971. Studies on calciferol metabolism. I. Production of vitamin D metabolite 4B from 25-OH-cholecalciferol by kidney homogenates. *Biochem. Biophys. Res. Commun.* **42**: 1082–1087.

14. Baylink, D., M. Stauffer, J. Wergedal, and C. Rich. 1970. Formation, mineralization, and resorption of bone in vitamin D-deficient rats. *J. Clin. Invest.* **49**: 1122–1134.

15. Arnaud, C. D., H. S. Tsao, and T. Littledike. 1971. Radioimmunoassay of human parathyroid hormone in serum. *J. Clin. Invest.* **50**: 21–34.

16. Berson, S. A., and R. S. Yalow. 1966. Iodoinsulin used to determine specific activity of iodine-131. *Science (Wash. D. C.).* **152**: 205–207.

17. Yalow, R. S., and S. A. Berson. 1960. Immunoassay of endogenous plasma insulin in man. *J. Clin. Invest.* **39**: 1157–1175.

18. Williams, G. A., G. K. Hargis, E. N. Bowser, W. J. Henderson, and N. J. Martinez. 1973. Evidence for a role of adenosine 3',5'-monophosphate in parathyroid hormone release. *Endocrinology.* **92**: 687–691.

19. Chertow, B. S., G. A. Williams, R. Kiani, K. L. Stewart, G. K. Hargis, and R. L. Flayter. 1974. The interactions between vitamin A, vinblastine, and cytochalasin B in parathyroid hormone secretion. *Proc. Soc. Exp. Biol. Med.* **147**: 16–19.

20. Chertow, B. S., R. J. Buschmann, and W. J. Henderson. 1975. Subcellular mechanism of parathyroid hormone secretion: ultrastructural changes in response to calcium, vitamin A, vinblastine and cytochalasin B. *Lab. Invest.* **32**: 190–200.

21. Edwards, A. L. 1954. Statistical Methods for the Behavioral Sciences. Holt, Rinehart and Winston, Inc., New York. 1st edition. 542 pp.

22. Sherwood, L. M., J. S. Rodman, and W. B. Lundberg. 1970. Evidence for a precursor to circulating parathyroid hormone. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 1631–1638.

23. Fischer, J. A., S. B. Oldham, G. W. Sizemore, and C. D. Arnaud. 1972. Calcium-regulated parathyroid hormone peptidase. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 2341–2345.

24. Arnaud, C. D., G. W. Sizemore, S. B. Oldham, J. A. Fischer, H. S. Tsao, and E. T. Littledike. 1971. Human parathyroid hormone: glandular and secreted molecular species. *Am. J. Med.* **50**: 630–638.

25. Habener, J. F., B. W. Kemper, J. T. Potts, Jr., and A. Rich. 1974. Calcium-independent intracellular conversion of proparathyroid hormone to parathyroid hormone. *Endocr. Res. Commun.* **1**: 239–246.

26. Martin, T. J., P. B. Greenberg, and R. A. Melick. 1972. Nature of human parathyroid hormone secreted by monolayer cell cultures. *J. Clin. Endocrinol. Metab.* **34**: 437–440.

27. Hamilton, J. W., F. W. Spierto, R. R. MacGregor, and D. V. Cohn. 1971. Studies on the biosynthesis *in vitro* of parathyroid hormone. II. The effect of calcium and magnesium on synthesis of parathyroid hormone isolated from bovine parathyroid tissue and incubation medium. *J. Biol. Chem.* **246**: 3224–3233.

28. Silverman, R., and R. S. Yalow. 1973. Heterogeneity of parathyroid hormone. Clinical and physiological implications. *J. Clin. Invest.* **52**: 1958–1971.

29. Habener, J. F., G. W. Tregear, T. D. Stevens, P. C. Dee, and J. T. Potts, Jr. 1974. Radioimmunoassay for proparathyroid hormone. *Endocr. Res. Commun.* **1**: 1–17.

30. Habener, J. F., D. Powell, T. M. Murray, G. P. Mayer, and J. T. Potts, Jr. 1971. Parathyroid hormone: secretion and metabolism *in vivo. Proc. Natl. Acad. Sci. U. S. A.* **68**: 2986–2991.

31. Segre, G. V., J. F. Habener, D. Powell, G. W. Tregear, and J. T. Potts, Jr. 1972. Parathyroid hormone in human plasma. Immunochemical characterization and biological implications. *J. Clin. Invest.* **51**: 3163–3172.

32. Chu, L. L. H., R. R. MacGregor, C. S. Anast, J. W. Hamilton, and D. V. Cohn. 1973. Studies on the biosynthesis of rat parathyroid hormone and proparathyroid hormone: adaptation of the parathyroid gland to dietary restriction of calcium. *Endocrinology.* **93**: 915–924.

32a. Hughes, M. R., P. F. Brumbaugh, M. R. Haussler, J. E. Wergedal, and D. J. Baylink. Regulation of serum 1α,25-dihydroxyvitamin D₃ by calcium and phosphate in the rat. *Science (Wash. D. C.).* In press.

33. Smith, J. E., and D. S. Goodman. 1971. The turnover and transport of vitamin D and of a polar metabolite with the properties of 25-hydroxycholecalciferol in human plasma. *J. Clin. Invest.* **50**: 2159–2167.

34. Raisz, L. G., C. L. Trummel, M F. Holick, and H. F. DeLuca. 1972. 1,25-Dihydroxycholecalciferol: a potent stimulator of bone resorption in tissue culture. *Science (Wash. D. C.).* **175**: 768–769.

35. Corradino, R. A. 1973. Embryonic chick intestine in organ culture. A unique system for the study of the intestinal calcium absorptive mechanism. *J. Cell Biol.* **58**: 64–78.

36. Frolik, C. A., and H. F. DeLuca. 1972. Metabolism of 1,25-dihydroxycholecalciferol in the rat. *J. Clin. Invest.* **51**: 2900–2906.

37. Wong, R. G., A. W. Norman, C. R. Reddy, and J. W. Coburn. 1972. Biologic effects of 1,25-dihydroxycholecalciferol (a highly active vitamin D metabolite) in acutely uremic rats. *J. Clin. Invest.* **51**: 1287–1291.

38. Tanaka, Y., and H. F. DeLuca. 1974. Role of 1,25-dihydroxyvitamin D₃ in maintaining serum phosphorus and curing rickets. *Proc. Natl. Acad. Sci. U. S. A.* **71**: 1040–1044.

39. Tanaka, Y., H. Frank, and H. F. DeLuca. 1973. Biological activity of 1,25-dihydroxyvitamin D₃ in the rat. *Endocrinology.* **92**: 417–422.

40. Henry, H. L., and A. W. Norman. 1975. Studies on the mechanism of action of calciferol. VII. Localization of 1,25-dihydroxy-vitamin D₃ in chick parathyroid glands. *Biochem. Biophys. Res. Commun.* **62**: 781–788.

41. Rasmussen, H., D. B. P. Goodman, and A. Tenenhouse. 1972. The role of cyclic AMP and calcium in cell activation. *CRC Crit. Rev. Biochem.* **1**: 95–148.

42. Abe, M., and L. M. Sherwood. 1972. Regulation of parathyroid hormone secretion by adenyl cyclase. *Biochem. Biophys. Res. Commun.* **48**: 396–401.

43. Borle, A. B. 1973. Calcium metabolism at the cellular level. *Fed. Proc.* **32**: 1944–1950.

44. Marcus, R., and G. D. Aurbach. 1971. Adenyl cyclase from renal cortex. *Biochim. Biophys. Acta.* **242**: 410–421.

45. Kissebah, A. H., B. R. Tulloch, H. Hope-Gill, P. V. Clarke, N. Vydelingum, and T. R. Fraser. 1975. Mode of insulin action. *Lancet.* **1**: 144–147.

46. Lucy, J. A., and J. T. Dingle. 1964. Fat-soluble vitamins and biological membranes. *Nature (Lond.).* **204**: 156–160.

47. Billitteri, A., and Y. Raoul. 1969. Antagonism between vitamin A and D at the level of membranes. Nutritional aspects of the development of bone and connective tissue [in French]. *Bibl. Nutr. Dieta.* **13**: 162–170.

48. Billitteri, A., and Y. Raoul. 1968. Antagonism of vitamin A and D at the level of the lysosomes. Study in mice *in vivo* [in French]. *C. R. Seances Soc. Biol.* **162**: 657–661.

49. DeLuca, H. F., G. W. Engstrom, and H. Rasmussen. 1962. The action of vitamin D and parathyroid hormone in vitro on calcium uptake and release by kidney mitochondria. *Proc. Natl. Acad. Sci. U. S. A.* **48**: 1604–1609.

50. Rasmussen, H., M. Wong, D. Bikle, and D. B. P. Goodman. 1972. Hormonal control of the renal conversion of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. *J. Clin. Invest.* **51**: 2502–2504.

51. Garabedian, M., M. F. Holick, H. F. DeLuca, and I. T. Boyle. 1972. Control of 25-hydroxycholecalciferol metabolism by parathyroid glands. *Proc. Natl. Acad. Sci. U. S. A.* **69**: 1673–1676.