ing doses of warfarin might be followed by enhanced pharmacological responses and toxicity. To investigate this possibility the pharmacokinetic properties of warfarin administered in vivo would have to be compared in individuals of different albumin phenotype who are receiving warfarin therapeutically. Other drugs may differ from warfarin in showing either no change or enhanced binding in vitro to variant albumins.

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25-Hydroxycholecalciferol to 1,25-Dihydroxycholecalciferol: Conversion Impaired by Systemic Metabolic Acidosis

Abstract. *An acute systemic acidosis in vitamin D depleted rats that was induced by ammonium chloride feeding resulted in defective biological hydroxylation of 25-hydroxycholecalciferol to 1,25-dihydroxycholecalciferol. Impaired enzymatic hydroxylation occurred despite the presence of either hypophosphatemia or hypocalcemia. The data suggest that acidosis interferes with the adaptive enzymatic control of 25-hydroxycholecalciferol metabolism in the vitamin D depleted state.*

Renal tubular acidosis is a syndrome characterized by abnormalities in tubular function, although the glomerular filtration rate remains normal. Hypophosphatemia, hypercalciuria, secondary hyperparathyroidism, metabolic acidosis, calcium malabsorption, and growth retardation accompany the disease, as well as skeletal abnormalities which are diagnosed radiologically as rickets in children and osteomalacia in adults (*1–3*). Although the skeletal lesions and calcium malabsorption do respond to pharmacological doses of vitamin D (*3*), they are also reversed by alkali therapy alone (*2, 4, 5*). 1,25-Dihydroxycholecalciferol [1,25-$(OH)_2D_3$], the most potent biologically active metabolite of vitamin D_3, is enzymatically formed from 25-hydroxycholecalciferol (25-OH-D_3) within the mitochondria of renal cortical cells (*6, 7*). Although it has been established that 1,25-$(OH)_2D_3$ synthesis is defective in end-stage renal disease (*8*), is suppressed by hyperphosphatemia (*9*), and is stimulated by parathyroid hormone (PTH) and hypophosphatemia (*9, 10*), the effect of a systemic acidosis on 1,25-$(OH)_2D_3$ production is still unknown. Our data herein support the hypothesis that the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ is defective in acidotic nonuremic vitamin D depleted animals despite an associated hypophosphatemia or hypocalcemia, both of which are reported to lead to stimulated 1-hydroxylase activity.

Vitamin D deficiency was produced in weanling male rats by feeding them a vitamin D deficient diet for 7 weeks. During this interval the animals were divided into two separate groups: group 1 received a vitamin D deficient diet containing 0.2 percent phosphorus and 0.06 percent calcium; group 2 received a vitamin D deficient diet containing 0.2 percent phosphorus and 1.3 percent calcium. After the rats became vitamin D deficient, both groups were subdivided. For groups 1B and 2B, distilled water containing 1.8 percent NH_4Cl was substituted for the regular distilled drinking water. Groups 1A and 2A continued to receive the regular distilled water. Three days later, 62.5 pmole of 25-OH-[26,27-^{3}H]D_3 (specific activity, 9.3 c/mmole) dissolved in 0.1 ml of ethanol was injected intrajugularly into all the animals; 18 hours after the injection all animals were killed; blood was obtained from the abdominal aorta of each animal and the calcium and phosphorus content and the *p*H were measured. Measured portions of serum as well as portions of small intestinal mucosa with identical protein concentration were then pooled for each of the four groups. The pooled samples were homogenized and extracted as reported previously (*11*), and were chromatographed on columns of Sephadex LH-20 according to the method of Holick *et al.* (*12*). The radioactivity recovered as 1,25-$(OH)_2$-[26,27-^{3}H]D_3 in the eluates was rechromatographed by means of Celite liquid-liquid partition chromatography as described by Haussler and Rasmussen (*13*). Portions of individual fractions obtained from either the LH-20 or Celite chromatographic procedures were dissolved in a solution containing 3.0 g of PPO (2,5-diphenyloxazole) and 100 mg of diethyl POPOP (1,4-bis-[5-phenyl-2-oxazolyl]-benzene) per liter of toluene and counted in a Tri-Carb liquid scintillation counter with a 2 percent counting error.

As shown in Table 1, NH_4Cl decreased the *p*H of the blood in both group 1B and group 2B. The serum calcium concentrations of the rats in groups 1A and 1B were lower and the phosphorus higher than the values obtained from groups 2A and 2B. Whereas acidosis resulted in a decrease in the serum calcium and an increase in phosphorus in group 2B, it produced an increase in serum calcium and a fall in phosphorus in group 1B animals. As noted in Table 2, serum and intestinal concentrations of ^{3}H-labeled 1,25-$(OH)_2D_3$ in the hypocalcemic animals of group 1A was greater than that observed in the normocalcemic animals of group 2A despite a significantly higher amount of circulating phosphorus in group 1A (Table 1). Since hypocalcemia is a potent secretagogue

for parathyroid hormone in animals (*14, 15*) as well as man, we attribute the higher tissue levels of ^{3}H-labeled 1,25-$(OH)_2D_3$ in group 1A to the hypocalcemia, resulting in secondary hyperparathyroidism and a stimulated renal conversion of ^{3}H-labeled 25-OH-D_3 to ^{3}H-labeled 1,25-$(OH)_2D_3$, a phenomenon documented by others (*10, 15–17*). The facts that the concentrations of ^{3}H-labeled 1,25-$(OH)_2D_3$ in the serum and intestinal homogenates of group 1A were greater than those observed for the relatively hypophosphatemic group 2A is also consistent with previous observations of Baxter and DeLuca (*16*) and Henry *et al.* (*18*). Studying the effects of high- and low-dietary concentrations of calcium and phosphorus on the enzymatic regulation of vitamin D_3 metabolism, Baxter and DeLuca noted a threefold increment of 1,25-$(OH)_2D_3$ production in hypocalcemic animals (that is, a mean calcium concentration of 7.2 mg/dl) when compared to hypophosphatemic animals (that is, a mean phosphorus concentration of 1.5 mg/dl). Similarly, Henry *et al.* (*18*) noted that renal 25-OH-D_3-1-hydroxylase activity was more sensitive to induced decrements in circulating calcium levels than to similar perturbations in phosphorus.

We are aware of the fact that in vivo, the tissue accumulation of ^{3}H-labeled 1,25-$(OH)_2D_3$ after the systemic administration of ^{3}H-labeled 25-OH-D_3 need not necessarily reflect the renal conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$. However, the observations that serum and intestinal concentrations of ^{3}H-labeled 1,25-$(OH)_2D_3$ were higher and ^{3}H-labeled 25-OH-D_3 lower in group 1A compared to group 2A (Table 2) are consistent with this assumption.

Groups 1A and 2A compared to their acidotic counterparts groups 1B and 2B demonstrated decreased concentrations of ^{3}H-labeled 1,25-$(OH)_2D_3$ in blood and intestine and comparable increments in ^{3}H-labeled 25-OH-D_3 (Table 2). Alterations in serum and intestinal ^{3}H-labeled 1,25-$(OH)_2D_3$ concentrations observed in the acidotic animals were not attended by significant changes in ^{3}H-labeled 24,25-$(OH)_2D_3$ in these tissues. This observation may appear at first glance inappropriate since changes in renal 1-hydroxylase activity are characteristically associated with reciprocal changes in 24-hydroxylase activity (*19*). The discrepancy may reflect the fact that intestinal and serum concentrations of ^{3}H-labeled 24,25-$(OH)_2D_3$ inadequately reflect renal 24-hydroxylase activity, or that the response of 1- and 24-renal hydroxylase systems to a systemic acidosis is mediated by factors other than those which control the enzymatic response to PTH, Ca^{2+}, or inorganic phosphate (*7, 20*). The conversion of ^{3}H-labeled 25-OH-D_3 to ^{3}H-labeled 1,25-$(OH)_2D_3$ was suppressed in group 1B despite hypocalcemia (and presumably secondary hyperparathyroidism) and a serum phosphorus concentration (2.9 ± 0.3 mg/dl) that was lower than that in group 1A (4.8 ± 0.5 mg/dl). The data are consistent with the hypothesis that, in the vitamin D deficient state, a fall in systemic *p*H impairs the renal mitochondrial 1- and 24-hydroxylase response to factors (that is, PTH and hypophosphatemia) which normally condition enzymatic adaptation and subsequent 25-OH-D_3 metabolism. They are reminiscent of studies in vivo demonstrating in the renal cortex a blunted response of cyclic adenosine monophosphate to PTH in the acidotic animal (*21*), and experiments in vitro defining inhibition of the renal mitochondrial 25-OH-D_3-1-hydroxylase by low *p*H's (*7, 20*).

Our results might bear some relation to observations of vitamin D resistance in nonuremic acidotic states (*3*) and to other data illustrating a reversal of the intestinal malabsorption of calcium (*5*) and the healing of rachitic and osteomalacic lesions in patients with renal tubular acidosis by the administration of alkali alone (*2, 4*). This would imply that the skeletal and intestinal responses to the ingestion of alkali are due (at least in part) to a reactivation of the renal enzymatic control system which is programmed primarily for the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ in vitamin D deficient states characterized by hypophosphatemia and secondary hyperparathyroidism.

Table 1. Effect of diet and NH_4Cl on the arterial concentration of calcium (Ca), phosphorus (P_i), and *p*H in vitamin D deficient animals. Individual values represent the mean ± standard error.

Group	Number of animals	Ca (mg/dl)	P_i (mg/dl)	*p*H
1A	10	4.5 ± 0.2*	4.8 ± 0.5*	7.28 ± 0.07
1B	9	6.0 ± 0.2†	2.9 ± 0.3†	7.08 ± 0.12†
2A	10	9.3 ± 0.2	1.6 ± 0.2	7.29 ± 0.05
2B	11	8.2 ± 0.3†	2.4 ± 0.3†	7.12 ± 0.07†

*Significantly different from group 2A with $P < .01$. †Significantly different from animals on identical diets without NH_4Cl supplementation (groups 1A and 2A, respectively) with $P < .01$.

Table 2. Effect of diet and NH_4Cl on the metabolic fate of [26,27-^{3}H]-25-OHD_3 in vitamin D deficient animals. The values represent the mean ± standard error of three separate experiments; three or four portions of pooled serum and intestinal homogenates were analyzed separately in each experiment. All data are expressed as percentages of total lipid soluble radioactivity obtained during initial extraction of serum and intestinal mucosa. Fractions containing the vitamin D compounds were identified by means of cochromatography with crystalline preparations on LH-20 and Celite columns and by high-pressure liquid chromatography (*22*).

Tissue	Group 1A	Group 1B	Group 2A	Group 2B
		^{3}H-labeled 25-OH-D_3		
Serum	72.0 ± 0.7*	78.3 ± 1.4†	80.8 ± 2.0	87.1 ± 1.0*
Intestine	16.7 ± 0.6*	23.6 ± 0.8‡	23.4 ± 2.3	31.8 ± 0.6*
		^{3}H-labeled 1,25-$(OH)_2D_3$		
Serum	21.7 ± 0.5*	15.0 ± 1.0‡	14.7 ± 1.9	9.0 ± 0.9*
Intestine	64.4 ± 0.7*	54.9 ± 3.0§	56.5 ± 2.6	41.0 ± 2.1*
		^{3}H-labeled 24,24$(OH)_2D_3$		
Serum	2.3 ± 0.2	1.6 ± 0.3	1.1 ± 0.1	1.1 ± 0.0
Intestine	3.0 ± 0.3	2.6 ± 0.4	2.9 ± 0.3	3.6 ± 0.3

*Significantly different from group 2A ($P < .05$). †Significantly different from group 1A ($P < .025$). ‡Significantly different from group 1A ($P < .005$). §Significantly different from group 1A ($P < .05$).

To a reactivation of the renal enzymatic control system which is programmed primarily for the conversion of 25-OHD_3 to 1,25-$(OH)_2D_3$ in vitamin D deficient states characterized by hypophosphatemia and secondary hyperparathyroidism.

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Aequorin Luminescence: Relation of Light Emission to Calcium Concentration—A Calcium-Independent Component

Abstract. *Light emission from the calcium-sensitive bioluminescent protein aequorin was measured at calcium ion concentrations of 10^{-9} to 10^{-2} molar. At very low Ca^{2+} concentrations, light emission is independent of calcium ion concentration. The maximum slope of the log-log plot of light as a function of calcium ion concentration is about 2.5. The complete relation is well described by a two-state model involving three calcium-binding sites.*

Aequorin, the photoprotein extracted from the jellyfish *Aequorea* (*1*), emits light when it is exposed to calcium ions. This unusual property has generated considerable interest in the mechanism of the light-emitting reaction (*2*) and has led to the use of aequorin as an indicator of the concentration of free calcium ions $[Ca^{2+}]$ in a variety of cells (*3*). The precise nature of the relation between $[Ca^{2+}]$ and light emission is of importance to both of these subjects. Previous studies of this relation have been restricted to limited ranges of $[Ca^{2+}]$ and have generally led to the conclusion that the rate of the luminescent reaction is proportional to $[Ca^{2+}]^2$ (*4*). We report here a study of the relation between $[Ca^{2+}]$ and light emission over a range of $[Ca^{2+}]$ from less than 10^{-9} to $10^{-2}M$ which reveals two new findings: (i) at very low $[Ca^{2+}]$ there is a light emission that is independent of $[Ca^{2+}]$ and (ii) at intermediate $[Ca^{2+}]$ the relation between light emission (L) and $[Ca^{2+}]$ is not adequately described by an equation of the form $L \propto [Ca^{2+}]^2$.

Aequorin was extracted and purified (*4*) from *Aequorea* collected at Friday Harbor, Washington. For these experiments highly purified salt-free lyophilized aequorin was dissolved in distilled water in quartz. Ten microliter volumes of this solution were forcefully injected into a reaction cuvette containing 1 ml of test solution (legend to Fig. 1), giving a final aequorin concentration of about $5 \times 10^{-8}M$. The reaction cuvette was situated above a photomultiplier, and, by the combined use of variable electronic amplification and neutral density filters, light could be measured linearly over eight orders of magnitude. The peak light intensity (before significant aequorin consumption occurred) was used as the index of reaction rate.

Figure 1 shows the result of a representative experiment in which $[Ca^{2+}]$ was varied with dilutions of $CaCl_2$ and by the use of three different calcium buffers. The points for each buffer have been shifted horizontally as a group to give the best fit to the points obtained by calcium dilution. This is equivalent to a determination of the apparent association constant for the calcium buffer and has the advantage that the constant is appropriate for the conditions of pH, temperature, and ionic strength used in the experiment. The log of the apparent association constant (M^{-1}) for EGTA determined in this way at pH 7.0 is 6.45—a value between the widely accepted one of 6.65 (*5*) and others reported to be around 6.1 (*6*). It is worth noting that the conclusions which follow are equally valid if reported association constants are used, the advantage of the above procedure being only that it reduces the horizontal scatter of points in Fig. 1.

Under the conditions of our experiments, the concentration-effect curve is essentially flat at $[Ca^{2+}]$ below $10^{-8}M$. The tendency toward flattening is apparent in the calcium dilution series although the lowest points of this series must be regarded as the least reliable because they would be most influenced by calcium contamination or by EDTA contamination in the aequorin solution. The finding, however, was confirmed with all three of the calcium buffers, most convincingly with CDTA, which buffers $[Ca^{2+}]$ well over the lowest range that we explored. Furthermore, when no calcium was added, all three chelators gave the same low light level. In this case the $[Ca^{2+}]$ cannot be accurately calculated; atomic absorption spectroscopy showed that the total calcium in these solutions (no ingredients of which were passed through the Chelex column; see legend to Fig. 1) was 1×10^{-6} to $2 \times 10^{-6}M$, and on this basis the $[Ca^{2+}]$ for these points was calculated to lie between 3×10^{-11} and $6 \times 10^{-10}M$ (depending on chelator). In seven experiments with aequorin collected in five different years, the magnitude of this light (as the $\log_{10}$ of the fraction of maximal light emission) was -6.40 ± 0.16 (mean ± standard deviation, $n = 7$).

As the $[Ca^{2+}]$ is raised from low levels, the light increases so that the slope of the log-log plot increases from zero to a maximum of about 2.5 (at $[Ca^{2+}] \cong 10^{-6}M$) and then falls again to zero at saturating $[Ca^{2+}]$ levels. Between 3×10^{-7} and $3 \times 10^{-6}M$, the points lie close to a straight line with a slope of 2.52 in this experiment (determined by linear regression of the points on the log-log plot over the stated range). In eight experiments, the mean slope determined in this way from calcium dilutions was 2.46 ± 0.033 (mean ± standard error, $n = 8$). The EGTA-buffered points extend over this range and confirm this finding (slope 2.54 ± 0.039, mean ± S.E., $n = 7$). The slopes from the calcium dilution and the EGTA-buffered series are not significantly different ($P > .1$), but both are significantly greater than 2.0 ($P < .001$).

Some properties of the Ca^{2+}-independent light were studied. It is absent when the aequorin has been discharged by Ca^{2+}. When pH was varied, light intensity was least at pH 7 and was increased by a factor of 2 to 3 at pH 5 and 9. When the KCl concentration in the test solution was increased from 0.01 to $0.50M$, the Ca^{2+}-independent light was reduced by a factor of 2. As temperature was increased from 0° to 40°C, the Ca^{2+}-independent light increased by a factor of 67. An attempt was made to see whether the Ca^{2+}-independent light was dependent on free oxygen. We could detect no difference in the light emission of solu-