are packaged together in the secretion granules (*13*), the enhanced ChTg secretion observed in these experiments did not result from secretion of the entire contents of the zymogen granule. Rather, the present observations fit with other examples of nonparallel and enzyme-selective transport reported during the past several years (*2, 11, 14*) and provide a potential physiological link between a natural stimulant and the enzyme-specific secretory response (*15*).

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14 November 1973

## Radioreceptor Assay for 1α,25-Dihydroxyvitamin $D_3$

Abstract. *A competitive protein binding assay with a sensitivity of 80 picograms has been developed for 1α,25-dihydroxyvitamin $D_3$, the hormonal form of vitamin $D_3$. 1α,25-Dihydroxyvitamin $D_3$ displaced tritiated hormone from a cytosol-chromatin receptor preparation isolated from chick small intestine, providing a simple assay for the hormone. The concentration of 1α,25-dihydroxyvitamin $D_3$ in human plasma, as determined by this assay, is approximately 6 nanograms per 100 milliliters; in patients with renal disease the concentration of this kidney-produced hormone is significantly lower.*

Vitamin $D_3$ is metabolized to 25-hydroxyvitamin $D_3$ (25-OH-$D_3$) (*1*) and subsequently to 1α,25-dihydroxyvitamin $D_3$ [1α,25-$(OH)_2$-$D_3$] (*2, 3*). The latter conversion occurs exclusively in the kidney (*4*) and produces what is considered to be the hormonal form of the vitamin. 1α,25-$(OH)_2$-$D_3$ is the most active and fastest acting metabolite of vitamin D in stimulating both intestinal calcium transport and bone mineral mobilization (*5*).

Previous detection of 1α,25-$(OH)_2$-$D_3$ has been carried out only in experimental animals, and there is little information available on the occurrence of this hormone in humans. Defects in the production of this hormone may explain abnormal calcium metabolism in diseases such as chronic renal failure. There is also evidence that parathyroid hormone is involved in the regulation of the renal synthesis of 1α,25-$(OH)_2$-$D_3$ (*6*), suggesting that abnormal production of parathyroid hormone may affect calcium homeostasis by altering the circulating level of 1α,25-$(OH)_2$-$D_3$. In order to study the role of 1α,25-$(OH)_2$-$D_3$ in calcium metabolism in normal humans and to study its possible relation to metabolic bone disease, we have developed a sensitive, competitive protein binding assay capable of detecting circulating amounts of the hormone.

The assay depends upon the binding of 1α,25-$(OH)_2$-$D_3$ to its chromatin receptor in the small intestine (*7*). This receptor is saturable and binds 1α,25-$(OH)_2$-$D_3$ with high affinity ($K_d \approx 10^{-9}M$). The receptor is specific for 1α,25-$(OH)_2$-$D_3$, and the radioactive hormone is displaced from its binding site only by much higher concentrations of 25-hydroxyvitamin $D_3$ (150-fold excess) and vitamin $D_3$ (> 20,000-fold excess) (*8*). Moreover, 1α,25-$(OH)_2$-$D_3$ binds initially to a cytosol receptor protein, and this sterol receptor complex migrates into the nucleus, via a temperature-dependent process, where it associates with the chromatin (*8*). In practice the receptor system is prepared by isolating Triton X-100–washed chromatin from intestines of rachitic chicks (*7*) and reconstituting it with the 100,000*g* supernatant from a centrifuged homogenate of the same intestine. A 200-μl portion of the receptor system (containing 100 μg of DNA) was added to a small tube containing nitrogen-dried sterol or

Table 1. Concentration of 1α,25$(OH)_2$-$D_3$ in plasma of normal individuals and in patients with disorders in calcium metabolism; S.D., standard deviation.

| Group | Patient (treatment) | Calcium (mg/100 ml) | 1α,25-$(OH)_2$-$D_3$* (ng/100 ml ± S.D.) | Average of 1α,25-$(OH)_2$-$D_3$ (ng/100 ml ± S.D.) |
|---|---|---|---|---|
| Normal | 20 (none) | 10.5–11.7† | 4.1–8.5† | 6.4 ± 1.2 |
| Renal disease | 1 (no dialysis) | 6.5 | 3.1 ± 0.4 | |
| | 2 (dialysis 1.3 yr) | 12.2 | 2.9 ± 0.4 | |
| | 3 (dialysis 3.5 yr) | 8.5 | 2.3 ± 0.6 | 2.6 ± 0.5‡ |
| | 4 (dialysis 1.7 yr) | 11.8 | 1.9 ± 0.3 | |
| | 5 (dialysis 0.7 yr) | 11.0 | 2.6 ± 0.2 | |
| Postsurgical hypoparathyroid§ | 1 (surgery 1970) | 9.5 | 3.3 ± 0.5 | |
| | 2 (surgery 6/1973) | 8.6 | 6.7 ± 0.4 | 4.7 ± 1.8‖ |
| | 3 (surgery 1968) | 10.1 | 4.1 ± 0.2 | |
| Presumed primary hyperparathyroid | 1 (no surgery) | 12.6 | 12.3 ± 0.6 | |
| | 2 (surgically confirmed adenoma) | 15.5 | 11.4 ± 0.7 | |
| Rachitic chicken | | 5.3 | 1.8 ± 0.6¶ | |
| Normal chicken | | 11.4 | 10.1 ± 1.2¶ | |

* Represents the average of four assays; corrected for losses occurring in purification sequence. † Range for 20 normal volunteers. ‡ Significantly different from normal, $P < .005$. § All patients received 100,000 international units of vitamin $D_2$ daily. ‖ Significantly different from normal, $P < .10$. ¶ Represents two separate assays on pooled plasma from 14 chickens each.

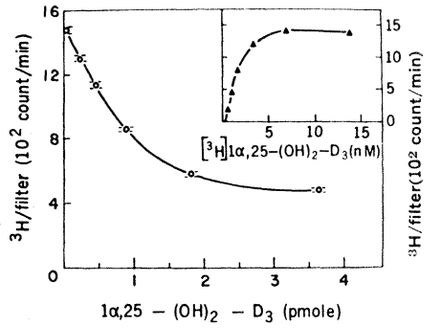


Fig. 1. Standard curve for 1α,25-$(OH)_2$-$D_3$. A sample of 5 n*M* [$^3$H]1α,25-$(OH)_2$-$D_3$ was incubated with increasing amounts of nonradioactive 1α,25-$(OH)_2$-$D_3$ and reconstituted cytosol-chromatin receptor system. The amount of bound tritrated compound is plotted as a function of the amount of nonradioactive hormone in the incubation mixture. [$^3$H]1α,25-$(OH)_2$-$D_3$ (9.8 c/mmole) was produced, in vitro, by a modification of the method of Lawson *et al.* (*3*). Homogenates of kidney from 4-week-old rachitic chicks were prepared and mixed with a phosphate buffer and a reduced nicotinamide adenine dinucleotide phosphate–generating system (*9*). [$^3$H]25-Hydroxyvitamin $D_3$ (10 nmole) (Amersham/Searle) was added to this reaction mixture in 1 ml of ethanol, and incubation was carried out under air at 37°C with gentle shaking for 1 hour. Lipids were extracted with a mixture of chloroform and methanol (1 : 2, by volume) (*9*). The [$^3$H]1α,25-$(OH)_2$-$D_3$ was purified by column chromatography on silicic acid, Sephadex LH-20, and Celite. The final radiochemical purity of the [$^3$H]1α,25-$(OH)_2$-$D_3$ as determined by chromatography on Celite was greater than 98 percent. Nonradioactive 1α,25-$(OH)_2$-$D_3$ was prepared as described (*9*). Each point on the standard curve represents the average ± standard error of the mean of the three assays. (Inset) Saturation of reconstituted cytosol-chromatin receptor system with [$^3$H]1α,25-$(OH)_2$-$D_3$. Increasing amounts of [$^3$H]1α,25-$(OH)_2$-$D_3$ (9.8 c/mmole) were incubated with the receptor system for 20 minutes at 25°C. The amount of bound [$^3$H]1α,25-$(OH)_2$-$D_3$ per filter was then determined. Each point represents the average of three determinations.

sterol extract and incubated for 20 minutes at 25°C. The chromatin was then harvested on glass fiber filters (Gelman type A), with the use of a 30-place Millipore manifold. The filters were washed with five portions (8 ml) of 1 percent Triton X-100 in 0.01*M* tris-HCl, *p*H 7.5, to remove unbound sterol. Control experiments without chromatin indicated that this procedure results in greater than 98 percent removal of free sterol from the filters. The specifically bound [$^3$H]1α,25-$(OH)_2$-$D_3$ was then extracted from the filters with methanol and chloroform (2:1), and the radioactivity was counted (38 percent efficiency) in a standard liquid scintillation counting solution (*8*).

In the competitive binding assay (Fig. 1) the receptor becomes filled when the concentration of hormone is 5 n*M*. This concentration of radioactive 1α,25-$(OH)_2$-$D_3$ is then used in the presence of increasing concentrations of nonradioactive 1α,25-$(OH)_2$-$D_3$ to create an isotope dilution-standard curve for the amount of nonradioactive hormone. Nearly linear competition results in the range of 0 to 1 pmole of added sterol, and the assay is sensitive enough to detect 0.20 pmole (83 pg).

The assay was then used to determine the concentration of 1α,25-$(OH)_2$-$D_3$ in human plasma. Heparinized blood was collected and lipids were extracted in methanol and chloroform (2:1) (*9*). 1α,25-$(OH)_2$-$D_3$ was isolated from the lipid extract by successive chromatography on a silicic acid column and two Sephadex LH-20 columns (*10*). The recovery of 1α,25-$(OH)_2$-$D_3$ after plasma extraction and chromatography was determined by initial addition of [$^3$H]1α,25-$(OH)_2$-$D_3$ (2500 count/min) to each plasma sample, and the assay results were corrected for losses of tritium. Recoveries ranged from 50 to 75 percent. Purified extracts of plasma were assayed as described.

Concentrations of 1α,25-$(OH)_2$-$D_3$ in plasma, as measured by the above assay, are shown in Table 1. The average concentration from 20 normal humans (6.4 ± 1.2 ng/100 ml) corresponds to $1.5 \times 10^{-10}$*M* 1α,25-$(OH)_2$-$D_3$, and is 1/300 that of its immediate precursor, 25-hydroxyvitamin $D_3$ (*11*). The concentration of 1α,25-$(OH)_2$-$D_3$ in the plasma of patients with chronic renal failure is less than 1/2 the normal concentration. This finding suggests that decreased production of the hormone may play a role in the osteodystrophy and calcium imbalance associated with kidney disease.

Hypoparathyroid patients appear to have slightly lower levels of 1α,25-$(OH)_2$-$D_3$ than normal in spite of the fact that they are under treatment with large doses of vitamin $D_2$ (Table 1). Presumably, in these patients the primary circulating vitamin D metabolites are derivatives of vitamin $D_2$, rather than vitamin $D_3$ as in normal individuals (*12*). Therefore, the results on patients receiving large supplements of vitamin $D_2$ must be viewed with caution because the relative affinity of the chick intestinal receptor for 1α,25-$(OH)_2$-$D_2$ and 1α,25-$(OH)_2$-$D_3$ is not known at present. Two hyperparathyroid patients had approximately twice the normal concentration of 1α,25-$(OH)_2$-$D_3$. These results suggest that parathyroid hormone may be involved in production of 1α,25-$(OH)_2$-$D_3$ in humans.

As a control for the human study, plasma from rachitic and normal chickens was assayed. The apparent concentration of 1α,25-$(OH)_2$-$D_3$ in the rachitic chick was 1.8 ng per 100 ml of plasma which is 1/6 that in the normal chicken (Table 1). The value in the rachitic chick either represents a true concentration of 1α,25-$(OH)_2$-$D_3$ in this vitamin D state or is a measure of nonspecific competition in the binding assay. In order to further confirm that displacement of the tritiated hormone was caused by 1α,25-$(OH)_2$-$D_3$, human plasma extracts which had been purified by successive silicic acid and two Sephadex LH-20 columns were treated with periodic acid (*13*) or were further purified by Celite chromatography. These procedures eliminate possible interference by 25,26-dihydroxyvitamin $D_3$, a circulating metabolite of vitamin $D_3$ which is not resolved from 1α,25-$(OH)_2$-$D_3$ by silicic acid or Sephadex LH-20 chromatography (*10*). Portions of plasma extracts were assayed after each step of the purification scheme (Table 2). Essentially identical plasma concentrations of 1α,25-$(OH)_2$-$D_3$ were determined with these additional purification

Table 2. Plasma 1α,25-$(OH)_2$-$D_3$ concentrations (nanograms per 100 ml) at various stages of purification as determined by radioreceptor assay. The values shown are corrected for losses occurring in each purification step.

| Sample | Purification scheme | | | |
|---|---|---|---|---|
| | A* | B† | C‡ | D§ |
| 1 | 8.3 | 7.3 | | |
| 2 | 10.9 | 7.4 | | |
| 3 | 8.2 | 7.2 | | |
| 4 | 5.0 | 4.2 | 4.3 | |
| 5 | 11.9 | 8.4 | 8.3 | |
| 6 | 7.5 | 6.0 | 6.4 | |
| 7 | 6.5 | 1.9 | | 2.1 |
| 8 | 7.7 | 6.0 | | 6.0 |
| 9 | 6.8 | 6.5 | | 6.2 |

* Silicic acid column and one Sephadex LH-20 column. † A plus additional Sephadex LH-20 column. ‡ B plus Celite liquid-liquid partition column. § B plus successive treatment with periodic acid and final Sephadex LH-20 column.

steps. However, an interfering substance was removed by the second Sephadex LH-20 column (Table 2, scheme B), demonstrating that at least three successive column purifications are required before assaying 1$\alpha$,25-(OH)$_2$-D$_3$ from human plasma by this method.

This radioreceptor assay has shown for the first time that the hormonal form of vitamin D$_3$, 1$\alpha$,25-(OH)$_2$-D$_3$, is detectable in humans. The use of the intestinal chromatin receptor system to detect very low levels of 1$\alpha$,25-(OH)$_2$-D$_3$ further confirms the high affinity, specificity, and physiologic importance of this receptor for 1$\alpha$,25-(OH)$_2$-D$_3$. Application of the nuclear hormone receptor complex to the measurement of 1$\alpha$,25-(OH)$_2$-D$_3$ represents a unique approach to the assay of sterol hormones. Moreover, the use of filters to adsorb the chromatin associated receptor during the washing away of unbound sterol is a novel method of separating free sterol from that which is bound to macromolecular components. This assay may be used to study the regulation of the metabolism of 25-OH-D$_3$ to 1$\alpha$,25-(OH)$_2$-D$_3$ in humans and experimental animals. Finally, the utility of the assay for diagnosis of disorders in calcium homeostasis, such as hypoparathyroidism and renal osteodystrophy, prior to possible treatment with 1$\alpha$,25-(OH)$_2$-D$_3$, is clear. Involvement of the hormone in hyperparathyroidism and neoplastic hypercalcemia may also be determined.

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13. A purified plasma sample was treated for 10 minutes with 10 ml of periodic acid (1 mg/ml) in a mixture of water and ethanol (67 : 33). This procedure converts 24,25-dihydroxyvitamin D$_3$ and 25,26-dihydroxyvitamin D$_3$ to the corresponding aldo- and keto- derivatives (unpublished observations).
14. Supported by PHS grant AM 15781, PHS training grant GM 01982, contract 71-2301 from the National Institute of Child Health and Human Development, and PHS grant HL 13636. We thank Ms. Kristine Bursac for expert assistance in performing the numerous assays.

27 August 1973; revised 6 November 1973 ■

## Evidence that Enzyme Polymorphisms Are Selectively Neutral, but Blood Group Polymorphisms Are Not

Abstract. *Data on enzyme polymorphism and blood group polymorphism were examined with special reference to molecular evolution, by using a statistic that depends on neither population structure nor other ecological factors. The data of the former are consistent with the hypothesis of neutral mutant and random genetic drift, whereas the latter are in accord with the hypothesis of balanced selection.*

In our previous report (*1*), published polymorphism data were used in conjunction with an invariant principle to conclude that the available data are consistent with the "neutral" theory of Kimura and Ohta (*2*). The principle is that when we assess the amount of heterozygosity, given that the gene frequency of the whole population is specified, the total amount is independent of the population structure (*3*). The data then available were some 400 proteins. We compared the data with four distinct hypotheses: all mutants are (i) neutral, (ii) advantageous, (ii) deleterious, and (iv) overdominant. They were consistent with (i) and (ii). The report had its reverberation in population genetics and molecular evolution (*4, 5*). Since then more data have been published, and therefore we made the same survey of all available data, which amount to 1045 proteins (*6*). The present result turned out to confirm the previous conclusion, and therefore our claim was greatly reinforced. The result is presented in Fig. 1A. We note that the distribution of the data presented in Fig. 1A is considerably flatter than that in our previous report (*1*). The flatness of the distribution is characteristic of neutral or advantageous mutants.

We have applied the same analysis to polymorphism data of human blood groups alone (*7*). Although the num-

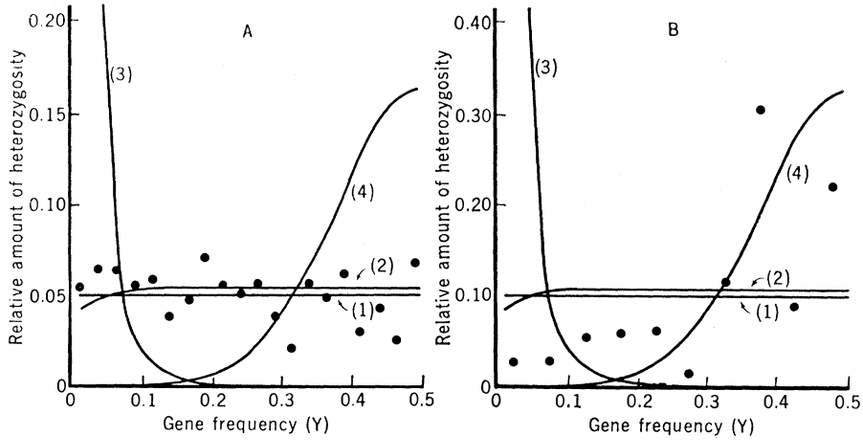


Fig. 1. Distribution patterns of heterozygosity. (A) Enzyme polymorphisms; (B) blood group polymorphisms. The curves indicate the theoretical expectations: (1) neutral, (2) advantageous, (3) deleterious, and (4) overdominance. The dots indicate the observed results. (The total area under each curve and the dots is unity.) $Y$ is the global gene frequency.