

THE BIOLOGICAL ACTIVITY OF 25-HYDROXYCHOLECALCIFEROL, A METABOLITE OF VITAMIN D₃*

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In 1966 Lund and DeLuca¹ demonstrated unequivocally the existence of a major polar metabolite fraction of vitamin D in many tissues of rats given ³H-vitamin D₃. This metabolite, which is able to cure rickets in rats at least as well as vitamin D, has since been demonstrated in human plasma,² porcine plasma,³ and chicks.⁴ Further study revealed that this metabolite fraction, like vitamin D, initiates bone mobilization and intestinal transport of calcium,⁵ and its effect on calcium transport is more rapid than that of vitamin D. Stohs and DeLuca⁶ demonstrated that this metabolite fraction is virtually the exclusive form of vitamin D in intestinal nuclei prior to the initiation of biochemical events leading to the rise in calcium transport.

Recently, the major if not the sole biologically active component of this fraction was isolated in pure form from the plasma of hogs fed vitamin D₃, and its structure was unequivocally established as 25-hydroxycholecalciferol (25-OH D₃).^{3, 7} The biological properties of this pure compound have now been examined in a number of systems, and the results are reported in this communication.

Materials and Methods.—*Vitamin D:* The crystalline vitamin D₃ was obtained from General Biochemicals, Inc., Chagrin Falls, Ohio. 25-Hydroxycholecalciferol (25-OH D₃) was isolated in pure form from the plasma of hogs, as described previously.³ The exact concentrations of these substances were determined by UV absorption at 265 m μ with a molar extinction coefficient of 18,200. The substances were dissolved in cottonseed oil for oral administration or in ethanol for intravenous administration.

Preparation of rats: Weanling male albino rats were obtained from the Sprague-Dawley Co., or from the Badger Research Corporation, both of Madison, Wisconsin. They were housed individually in hanging wire cages and given food and water *ad libitum*.

For the rickets cure test, we used the rachitogenic diet of Steenbock and Black⁸ as modified by the addition of crystalline vitamins at the level described by DeLuca *et al.*⁹ After the rats had been on this diet for 21 days, they were given either 25-OH D₃ or vitamin D₃ orally in 0.1 ml cottonseed oil or intrajugularly in 0.02 ml ethanol. Controls were given the appropriate carrier alone. The rats were killed 7 days later, their radii removed and stained with AgNO₃ solution, and the degree of new calcification was scored visually as described in the *U.S. Pharmacopoeia*.¹⁰

In intestinal transport experiments, rats were fed for 5 weeks the adequate Ca and P diet described earlier,⁹ after which the animals exhibited a serum calcium concentration of 4.0–4.5 mg/100 ml. The 25-OH D₃ or vitamin D₃ was administered intravenously in 0.02 ml ethanol. At the indicated times, the ability of the duodenal section of intestine to transport calcium against a concentration gradient was assessed by the everted gut sac technique described earlier,¹¹ except that the medium consisted of 125 mM NaCl, 10 mM fructose, 0.25 mM CaCl₂ containing ⁴⁵Ca, and 30 mM tris(hydroxymethyl)aminomethane buffer (Tris buffer), pH 7.4.¹² The incubations were at 37°C under a stream of O₂. Samples from inside and outside the sacs were taken after 1.5 hr and counted in a model 3000 Packard Tri-Carb liquid scintillation counter. The scintillator solution¹² consisted of 3 liters dioxane, 300 gm naphthalene, 14 gm 2,5-diphenyloxazole, 600 mg 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene, to which was added a solution of 36 mg Na₂·EDTA in

500 ml H₂O. The data are expressed as ratios of ⁴⁵Ca inside the sac to that in the incubation medium.

Experiments on the mobilization of bone: Rats were fed the purified diet described earlier,⁹ except that calcium was omitted. After 2-3 weeks on this low-calcium diet they were severely deficient, with serum calcium concentrations of 4.0-4.5 mg/100 ml. At this time the rats were given 2.5 μg of either vitamin D₃ or 25-OH D₃. At the indicated times thereafter, the rats were killed and the serum calcium concentration was determined by Webster's method.¹³ The rise in serum calcium in rats on this diet reflects increased mobilization of bone.

Rickets prevention test in chicks: Day-old chicks obtained from the Sunny-Side Hatchery of Oregon, Wisconsin, were fed a rachitogenic diet¹⁴ with graded levels of 25-OH D₃ or vitamin D₃ dispersed in the diet. Twenty chicks were used at each dosage level. After 21 days the chicks were sacrificed and the ash content of tibiae pooled from each dosage level was determined according to standard AOAC procedures.¹⁴

Results.—In many repeated "line test" assays of antirachitic activity with rats, the 25-hydroxycholecalciferol was always 1.3-1.5 times more active than crystalline vitamin D₃. Independent assays by the Wisconsin Alumni Research Foundation Laboratory gave the same result. Examples of such results are given in Table 1. The assays also show that intravenous administration of the 25-OH D₃ is just as effective as oral administration, thus demonstrating that passage through the intestinal mucosa is not essential to 25-OH D₃ activity.

The results shown in Table 2 demonstrate that 25-OH D₃ is extremely effective in initiating calcium transport in the duodenum of deficient rats. It is of great interest that even when administered intravenously, it initiates calcium transport much more rapidly than does vitamin D itself. A significant stimulation by 0.25 μg vitamin D₃ is observed only after six to ten hours, whereas 0.25 μg 25-OH D₃ produces a response within two to three hours after its administration. These

TABLE 1. *Comparative effectiveness of 25-hydroxycholecalciferol and vitamin D₃ in the cure of rickets in rats.*

| Type of dosage | 25-OH D ₃ (IU/μg) | Vitamin D (IU/μg) |
|-----------------|------------------------------|-------------------|
| Oral (8) | 58 ± 5* | 40 ± 4* |
| Oral (7) | 52 ± 3 | 38 ± 5 |
| Intravenous (7) | 56 ± 2 | 41 ± 3 |

The standard line test assay for vitamin D activity was carried out as described in *U.S. Pharmacopoeia*.¹⁰ The figures in parentheses represent number of rats per group.

* Standard deviation.

TABLE 2. *Effect of intrajugular administration of 0.25 μg of 25-hydroxycholecalciferol or vitamin D₃ on calcium transport by everted intestinal sacs.*

| Hours after administration | ⁴⁵ Ca Serosal/ ⁴⁵ Ca Mucosal | |
|----------------------------|--|---------------------------|
| | Vitamin D ₃ | 25-Hydroxycholecalciferol |
| Control | 1.25 ± 0.05 (12) | |
| 2 | | 1.24 ± 0.13* (4) |
| 3 | | 1.74† ± 0.13 (4) |
| 4 | 1.01 ± 0.12* (4) | 1.66† ± 0.13 (4) |
| 6 | 1.32 ± 0.10 (4) | 2.6 ± 0.4 (4) |
| 10 | 2.0 ± 0.3 (3) | 2.3 ± 0.6 (4) |

* Plus or minus the standard error of the mean. Numbers in parentheses show the numbers of animals in each group.

† *P* < 0.01 above control.

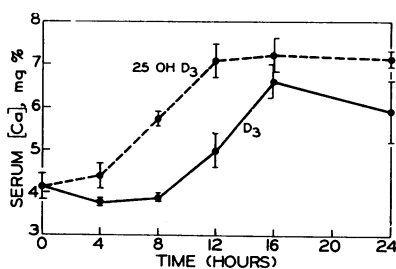


FIG. 1.—Serum calcium response to 2.5- μ g intravenous doses of 25-hydroxycholecalciferol (---) and vitamin D₃ (—) in vitamin D-deficient rats on a low calcium diet. Each point is the mean (\pm SD) of duplicate assays of serial samples from three to five rats.

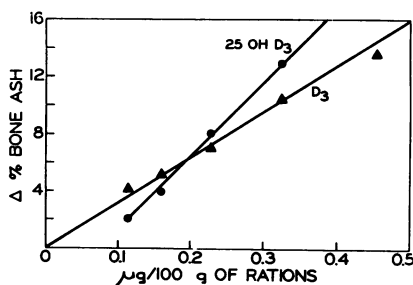


FIG. 2.—The increase in per cent bone ash over the controls for graded doses of 25-hydroxycholecalciferol (●) and vitamin D₃ (▲). Each point represents the pooled tibiae of 20 chicks.

TABLE 3. Activity of 25-hydroxycholecalciferol as measured by the chick bone ash assay.

| Weight (μ g)/100 gm of Rations | Body Weight during Assay Period (gm) | | Bone ash |
|-------------------------------------|--------------------------------------|-------|----------|
| | Initial | Final | |
| D ₃ | | | |
| Oil Only | 35 | 116 | 28.3* |
| 0.113 | 35 | 142 | 32.4 |
| 0.159 | 35 | 145 | 33.4 |
| 0.225 | 35 | 151 | 35.3 |
| 0.319 | 35 | 166 | 38.7 |
| 0.450 | 35 | 152 | 41.8 |
| 25-OH D ₃ | | | |
| 0.159 | 35 | 140 | 32.2 |
| 0.225 | 35 | 151 | 36.3 |
| 0.319 | 35 | 162 | 41.2 |

* Each assay is for the pooled tibiae from 20 chicks receiving each dose.

results suggest that at least a part of the lag in vitamin D action may result from the necessity for its conversion to 25-OH D₃ before it can act.

25-Hydroxycholecalciferol was also highly effective in eliciting a rise in serum calcium at the expense of bone in rats on a low calcium diet (Fig. 1). Again it is apparent that a significant elevation of serum calcium occurred 4–6 hours after administration of 2.5 μ g of 25-OH D₃, whereas 8–12 hours was required for a similar response to vitamin D₃. The rise in serum calcium in response to vitamin D also can be shown with as little as 0.25 μ g of 25-OH D₃.

In chicks the 25-OH D₃ is also more active than vitamin D₃. Exactly how much more active is not clear because at the higher dosage levels 25-OH D₃ was considerably more active than vitamin D₃, whereas at the two lowest dosage levels it appeared less active (Table 3 and Fig. 2). This may be due to instability of the 25-OH D₃ when dispersed in small concentrations in the diets. Other explanations are possible and additional investigations with chicks will be necessary to clarify this point.

Discussion.—It has been generally accepted that cholecalciferol or vitamin D₃ is the most potent antirachitic substance known. This report demonstrates that a metabolite of vitamin D₃, namely, 25-hydroxycholecalciferol, is even more potent than cholecalciferol. Its superior antirachitic potency is easily demonstrated in both rats and chicks, animals which differ markedly in their response to

various D vitamins of different side-chain structure. Previous modifications of the side-chain structure have been shown to decrease antirachitic potency in one or both species.

Of great interest is the high probability that the 25-OH D₃ represents the metabolically active form of vitamin D₃. Not only is this metabolite more active than vitamin D₃, but it acts much more rapidly in both intestine and bone, the prime targets of vitamin D action. As little as 0.25 μg of 25-OH D₃ elicits an intestinal calcium transport response within three hours, while about eight to ten hours is required for the same amount of vitamin D₃ to exert its action. Thus, much of the lag in vitamin D action on intestinal calcium transport and on bone mobilization can be accounted for by its conversion to 25-OH D₃. For a 0.25-μg dose, approximately five to six hours of the lag in vitamin D action probably can be attributed to the conversion of D₃ to 25-OH D₃. Although the present results provide strong evidence that 25-OH D₃ is the metabolically active form of vitamin D, final proof must rest with isolated systems incapable of converting vitamin D₃ to 25-OH D₃, which respond to 25-OH D₃ and not to vitamin D₃ itself. Investigations with 25-OH D₃ are thus continuing to clarify this point.

Summary.—The biological activity of 25-hydroxycholecalciferol, a metabolite of vitamin D₃ isolated from porcine plasma, is established. The metabolite is approximately 1.4 times more active than vitamin D₃ in curing rickets in rats, and is also more active than the vitamin in chicks as determined by the bone ash assay. In vitamin D-deficient rats, intravenous administration of 0.25 μg of the metabolite initiates calcium transport across the intestine much earlier than a similar dose of the vitamin. A 2.5-μg dose of the metabolite administered intravenously to deficient rats also causes an earlier rise in serum calcium concentration resulting from bone resorption than does a similar dose of vitamin D₃. These data, together with those obtained earlier with impure preparations of the metabolite, suggest that 25-hydroxycholecalciferol is the metabolically active form of vitamin D₃.

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