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Importance of the Stereochemical Position of the 24-Hydroxyl to Biological Activity of 24-Hydroxyvitamin D_3†

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ABSTRACT: Both stereoisomers of 24-hydroxyvitamin D_3, i.e., 24(*S*)-hydroxyvitamin D_3 and 24(*R*)-hydroxyvitamin D_3, stimulate intestinal calcium transport almost equally well in the rat. The duration of effect is somewhat shorter for the 24(*S*)-hydroxyvitamin D_3 than for the 24(*R*)-hydroxyvitamin D_3. However, the 24(*S*)-hydroxyvitamin D_3 has little or no activity in the mobilization of calcium from bone, in the growth of rats on a low calcium diet, in the elevation of serum phosphorus of rachitic rats, or in the calcification of bone. On the other hand, the 24(*R*)-hydroxyvitamin D_3 is almost as active as 25-hydroxyvitamin D_3 in all of these systems, although its activity is not always of equal duration to that of 25-hydroxyvitamin D_3. The selectivity of these systems for only one of the 24-hydroxy stereoisomers supports the idea that in vivo 24-hydroxylation of vitamin D compounds is of functional importance.

Although it is well established that vitamin D_3 must be hydroxylated on C-25 in the liver and subsequently on C-1 in the kidney before it can carry out its well-known functions in intestine and bone (DeLuca, 1974), the biological significance of 24-hydroxylation of vitamin D_3 metabolites remains unknown. Yet an abundant amount of 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$)[1] is found in normal rats (Boyle et al., 1971). Additionally, when 1-hydroxylation of 25-hydroxyvitamin D_3 (25-OH-D_3) is suppressed, 24-hydroxylation is stimulated, illustrating that its synthesis is regulated (Boyle et al., 1971, 1972; Omdahl et al., 1972; Holick et al., 1972). The 24,25-$(OH)_2D_3$ can undergo 1-hydroxylation to form 1,24,25-trihydroxyvitamin D_3 (1,24,25-$(OH)_3D_3$) (Holick et al., 1973), an active metabolite on intestinal calcium transport, but so far only trace amounts of this substance have been found in normal animals (Holick et al., 1973; Kleiner-Bossaller and DeLuca, 1974). The possibility that 24,25-$(OH)_2D_3$ is an intermediate in the elimination of vitamin D compounds cannot be excluded, although its persistence in animals would not support this belief (Boyle et al., 1973). There remains, therefore, the lingering idea that 24-hydroxylation of vitamin D_3 compounds has physiological or functional significance especially since the many functions of the vitamin have not been elucidated.

During the course of our search for a function for this metabolite of vitamin D, the two isomeric forms of 24-hydroxyvitamin D_3 (24-OH-D_3) were synthesized, namely 24(*S*)-OH-D_3 and 24(*R*)-OH-D_3 (Ikekawa et al., 1975). In studying their biological activity we were impressed with the marked selectivity for one of these isomers, 24(*R*)-OH-D_3, by all the biological systems known to be responsive to vitamin D_3 except intestinal calcium transport. In this system both isomers are almost equally effective. These surprising results strongly suggest that 24-hydroxylation of vitamin D_3 compounds is functionally important.

Materials and Methods

Animals. Weanling male rats (Holtzman Co., Madison, Wis.) were fed either a low calcium, adequate phosphorus, vitamin D deficient diet (Suda et al., 1970) or a high calcium, low phosphorus, vitamin D deficient diet (Tanaka and DeLuca, 1974). The low calcium diet produced a severe hypocalcemia and retarded growth while the low phosphorus diet produced hypophosphatemia and severe rickets within 2 weeks (Tanaka and DeLuca, 1974). After this 2-week period, the rats were used to examine the effectiveness of the vitamin D analogs. The analogs were administered at various doses either intravenously or intraperitoneally in 0.05 ml of ethanol. In each case, controls received the appropriate volume of vehicle.

Analogs of Vitamin D_3. The 25-OH-D_3 was a gift from the Philips-Duphar Company of Weesp, The Netherlands. Both stereoisomers of 24-OH-D_3 were chemically synthe-

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[1] Abbreviations used are: 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 24-OH-D_3, 24-hydroxyvitamin D_3.

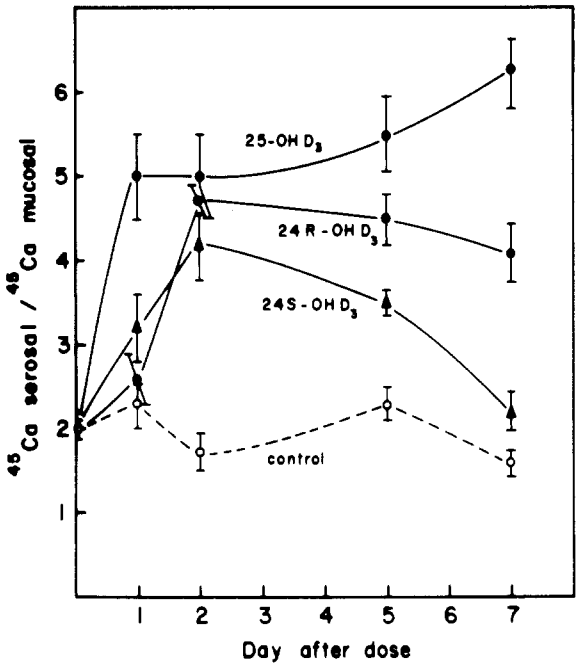

FIGURE 1: Intestinal calcium transport in response to the 24-hydroxy isomers of vitamin D_3. The rats which had been fed the low calcium–vitamin D deficient diet for 2 weeks were given 650 pmol of either compound dissolved in 0.05 ml of 95% ethanol intrajugularly. Rats in the control group received the ethanol vehicle only. Rats were killed at various times after dose and their intestinal calcium transport activity was measured. Each point is expressed as mean ± standard error of the mean. There were six rats in each group. Their serum calcium concentration and increase of body weight are shown in Figure 2 and Table I, respectively.

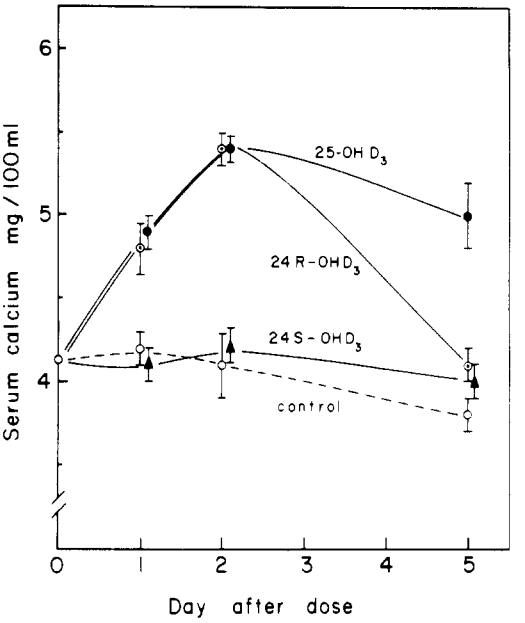

FIGURE 2: The effect of 24-OH-D_3 isomers on bone calcium mobilization of rats. The rats were fed the low calcium–vitamin D deficient diet for 2 weeks. They were then given 650 pmol of either compound dissolved in 0.05 ml of ethanol intrajugularly. At the indicated times their serum calcium concentration was measured as described in the text. Each group had six rats. Data are expressed as mean ± standard error of the mean.

sized as described by Ikekawa et al. (1975), and their stereochemical configuration was determined chemically (Koizumi et al., 1975).

Calcium Transport Measurement. Immediately after killing the animals by decapitation, the duodenum was removed and everted for intestinal calcium transport measurement as described by Martin and DeLuca (1969).

Measurements of Serum Calcium and Phosphorus. Serum calcium concentration was determined in the presence of 0.1% lanthanum chloride by means of a Perkin-Elmer Model 403 atomic absorption spectrometer. A 10% trichloroacetic acid solution was added to an aliquot of serum and the supernatant obtained by centrifugation was analyzed for inorganic phosphorus as described by Chen et al. (1956).

Measurement of Antirachitic Activity. The rachitic rats were given either a single dose or a small daily dose of the compound being tested and 7 days after the first dose they were killed and their radii and ulnae were removed, split lengthwise, and stained in 1.5% silver nitrate. The new calcification was scored as described in the U.S. Pharmacopoeia (1955). Femurs were also removed from the carcasses, cleaned of connective tissue, and extracted in a Soxhlet extracter with 100% ethanol for 24 hr and then with 100% diethyl ether for an additional 24 hr. The extracted femurs were dried to constant weight and ashed at 650° for 24 hr for determination of mineral content.

Results

Figure 1 shows that both isomers of 24-OH-D_3 stimulate intestinal calcium transport equally well in 48 hr in agreement with our previous report. Both isomers give a much slower response than 25-OH-D_3 and their effectiveness is shorter lived especially for the 24S-OH-D_3 isomer. Some selectivity is shown for the 24(R)-OH-D_3 inasmuch as its activity is sustained for a much longer period in this system.

Table I: Growth of Vitamin D Deficient Rats on a Low Calcium Diet in Response to 24-OH-D_3 Isomers.[a]

Compound	Increase of Body Weight 5 Days after Dose
Ethanol vehicle	9 ± 1
24(S)-OH-D_3	10 ± 1
24(R)-OH-D_3	19 ± 2[b]
25-OH-D_3	19 ± 2[b]

[a] The compounds (650 pmol) were dissolved in 0.05 ml of 95% ethanol and dosed to rats that had been fed the low calcium–vitamin D deficient diet for 2 weeks. Data are expressed as mean of increase in body weight ± standard error of the mean 5 days after dose. Each group had six rats. Intestinal calcium transport response and serum calcium concentration are shown in Figures 1 and 2, respectively. [b] Significantly different from control $p < 0.001$.

The bone calcium mobilization system on the other hand shows great preference for the 24(R)-OH-D_3 (Figure 2). The 24(R)-OH-D_3 gives a response in this system which is almost identical with that elicited by 25-OH-D_3, while 24(S)-OH-D_3 showed no activity at the same dose level. It is of some interest that the 24(R)-OH-D_3 response was not sustained to the extent shown by 25-OH-D_3. Table I shows that the growth of these rats on the low calcium diet shows an equal response to 25-OH-D_3 and 24(R)-OH-D_3 but no response to the 24(S)-OH-D_3.

A single dose of 650 pmol of 24(R)-OH-D_3 to rachitic hypophosphatemic rats causes a rapid elevation of serum phosphorus (Figure 3) and marked mineralization of epiphyseal plate (Table II) similar to 25-OH-D_3 while the 24(S)-OH-D_3 produced little or no response. If small daily

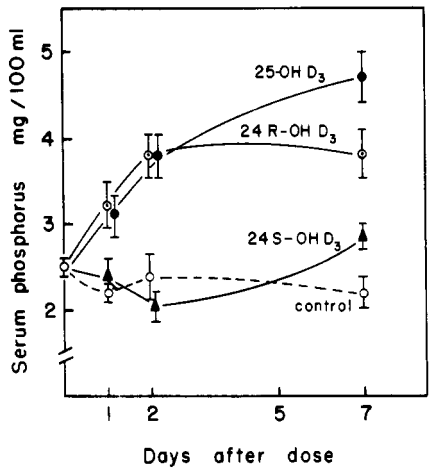

FIGURE 3: The elevation of serum phosphorus of rachitic rats in response to the 24-OH-D_3 isomers. Rats were fed the low phosphorus–vitamin D deficient diet for 2 weeks. They were given 650 pmol of either compound dissolved in 0.05 ml of 95% ethanol intrajugularly. The control group received ethanol alone. At various times, their serum inorganic phosphorus concentration was measured as described in the text. Data are expressed as mean ± standard error of the mean. Each group had six rats.

Table II: Antirachitic Activity of 24-OH-D_3 Isomers.[a]

Compound	Serum Calcium (mg/100 ml)	Epiphyseal Plate Calcification Score[b]
Ethanol vehicle	9.1 ± 0.3	0 ± 0
24(*S*)-OH-D_3	8.6 ± 0.2	0 ± 0
24(*R*)-OH-D_3	9.0 ± 0.2	4.8 ± 0.9
25-OH-D_3	9.9 ± 0.3	>5

[a] Rats were fed the low phosphorus–vitamin D deficient diet for 2 weeks and then given 650 pmol of either compound dissolved in 0.05 ml of ethanol intrajugularly. Seven days later their radii and ulnae were used for line test assay as described in the text. Each group had six rats. Data are expressed as mean ± standard error of the mean. Their serum phosphorus concentrations are shown in Figure 3. [b] A single 100-ng dose of vitamin D_3 gives a calcification score of 4 (U. S. Pharmacopoeia, 1955).

doses of the isomers are given each day for 7 days to rachitic rats, the selectivity for the 24(*R*)-OH-D_3 isomer is clearly shown (Table III). The 24(*R*)-OH-D_3 gives a response almost identical with that of 25-OH-D_3 while the 24(*S*)-OH-D_3 has a slight effect on serum phosphorus, but if anything depressed mineralization of bone as revealed by the ash content.

Discussion

This report shows the marked specificity for the 24(*R*)-hydroxyl of 24-OH-D_3 for expression of vitamin D activity in a variety of systems. It is clear that for mineralization of bone, the elevation of serum phosphorus and the mobilization of calcium from bone, the 24(*R*)-OH-D_3 is the isomer that can approach 25-OH-D_3 in its activity while its enantiomorph has little or no activity in these systems. Although it is tempting to speculate that for these functions in vivo, stereospecific 24-hydroxylation of either 25-OH-D_3 or 1,25-$(OH)_2D_3$ or both must take place, other explanations for the results are possible. For example, the *S* isomer may be rapidly degraded and excreted. However, this would seem unlikely since this isomer will initiate intestinal calcium transport almost as well as the *R* isomer. It is possible that the 24(*S*)-hydroxyl interferes with or does not permit binding to a receptor or functional site and that the discrimination is not necessarily for 24(*R*)-OH-D_3 but against the 24(*S*)-OH-D_3. This possibility also seems less likely since the vitamin D_2 series of compounds have a methyl group in the stereo position occupied by the hydroxyl of 24(*S*)-OH-D_3, yet this compound is not discriminated against in the rat (Jones and DeLuca, unpublished results). However, since the groupings are not equivalent, this argument is not compelling. In any case the possibility that 24-*R*-hydroxylation is essential to functions other than intestinal calcium transport must now be considered.

It is of some interest that the intestinal calcium transport system responds equally well to the 24(*S*)-OH-D_3 and 24(*R*)-OH-D_3. Since nephrectomy prevents their response (Ikekawa et al., 1975), it seems likely that 1-hydroxylation is essential to their effectiveness in this system. Since both are active it seems likely that the 25-OH-D_3-1-hydroxylase does not discriminate between these two forms of vitamin D_3.

The exact relationship between these results and those obtained with biologically generated and chemically synthesized 24,25-$(OH)_2D_3$ is unknown at the present time. It is certain that the synthetic 24,25-$(OH)_2D_3$ used previously is a mixture of isomers, which might account for its lack of activity in the systems shown to be responsive to the 24(*R*)-OH-D_3. Biologically generated material was rarely used and that was isolated from pigs given large amounts of vitamin D_3 (Boyle et al., 1973). The configuration of the 24-hydroxyl of this isolated material is also unknown and must be reexamined before the relationship to the present results can be discussed.

The remarkable selectivity for the *R* isomer illustrates the potential importance of the 24-hydroxyl to the functions of vitamin D in all systems except possibly the intestinal calcium transport system. At the very least, these results suggest that the correct side chain structure of vitamin D is of vital importance to most of its functions.

Table III: Ash Accumulation in Bone in Response to 24-OH-D_3 Isomers.[a]

Compound	Serum Phosphorus (mg/100 ml)	Serum Calcium (mg/100 ml)	Epiphyseal Calcification Score[b]	Bone Ash (g)	Bone Ash (%)
Ethanol vehicle	2.1 ± 0.2	8.8 ± 0.3	0	0.03572 ± 0.00188	27 ± 0.5
24(*S*)-OH-D_3	3.0 ± 0.2	8.7 ± 0.1	0.4 ± 0.4	0.03046 ± 0.00180	25 ± 1
24(*R*)-OH-D_3	4.0 ± 0.2[c]	9.9 ± 0.2	5	0.04260 ± 0.00175	31 ± 1[c]
25-OH-D_3	4.0 ± 0.3[c]	10.2 ± 0.3	5	0.04139 ± 0.00097	31 ± 1[c]

[a] Rats were fed the low phosphorus–vitamin D deficient diet for 2 weeks and then were given 130 pmol dissolved in 0.05 ml of ethanol intraperitoneally daily for 5 days. Seven days after the first dose, rats were killed and their femurs were used for bone ash measurement as described in the text. Data are expressed as mean ± standard error of the mean and each group had six rats. [b] A single 100-ng dose of vitamin D_3 gives a calcification score of 4 (U. S. Pharmacopoeia, 1955). [c] Significantly different from control $p < 0.005$.

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Conformational Changes in the Progesterone Binding Globulin–Progesterone Complex†

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ABSTRACT: An improved purification procedure for the progesterone-binding globulin (PBG) of the pregnant guinea pig has been developed utilizing sulfopropyl Sephadex, a strong cation exchanger, in the first step. The method exploits the low p*I* (2.8) and favorable acid stability of the glycoprotein. Subsequent chromatographies on DEAE-cellulose and Sephadex G-200 afford a highly purified PBG that exhibits the previously observed polydispersity (R. M. Burton et al. (1974), *Biochemistry 13,* 3554–3561). Circular dichroism, optical rotatory dispersion, and difference uv spectra all indicate the purified protein to undergo a conformational transition upon forming a complex with a steroid ligand. The CD and ORD spectra cannot be interpreted in terms of tertiary structure probably due to carbohydrate contributions. However, the difference spectra indicate strong perturbation of both a tryptophan residue and the steroid chromophore in the complex.

Progesterone-binding globulin (PBG)[1] isolated from the blood of pregnant guinea pigs is a glycoprotein of an unusually high carbohydrate content. It binds progestogens and androgens with high specificity whereas the affinity to corticoids is lower. The protein is found at relatively high levels during late pregnancy. The binding specificity and relative abundance make PBG well suitable for the study of high affinity steroid binders.

Several laboratories have reported the purification of PBG (Milgrom et al., 1973; Lea, 1973; Burton et al., 1974), but all reported methods are rather involved and none of them takes advantage of PBG's acid stability and extremely low isoelectric pH of 2.8 (Harding et al., 1974). The present communication reports on the use of a strong cation exchanger to separate the acidic PBG molecule from the bulk of plasma proteins in one step. A preliminary account of this method has been given (Stroupe and Westphal, 1974).

Many reports on the interaction of proteins with steroids have emphasized the influence of temperature, pH, ionic strength, specific salts, and steroid structure on the affinity of binding (Westphal, 1971). There appear to be almost no observations of changes in conformation in high affinity binders upon forming steroid complexes. To explore the conformational aspects of the PBG-steroid complex, measurements of circular dichroism (CD), optical rotatory dispersion (ORD), and uv difference spectra were performed with the PBG purified by the improved method.

Materials and Methods

Pooled pregnant guinea pig serum was obtained from Grand Island Biologicals. Sulfopropyl (SP) Sephadex C-50

† From the Department of Biochemistry, University of Louisville School of Medicine, Health Sciences Center, Louisville, Kentucky 40201. *Received February 24, 1975.* This is communication No. 32 in the series Steroid-Protein Interactions; for the preceding paper see Stroupe et al. (1975). This work was supported by a grant from the National Institute of Arthritis, Metabolism, and Digestive Diseases (AM-06369) and a research career award (U.W.) from the Division of General Medical Sciences (GM-K6-14, 138) of the U.S. Public Health Service.

[1] Abbreviations used are: PBG, progesterone-binding globulin; CBG, corticosteroid-binding globulin; pregnenolone, 5-pregnen-3β-ol-20-one; SP-Sephadex, sulfopropyl Sephadex; PAS, periodic acid–Schiff base.