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Bone-Resorbing Activity of Analogues of 25-Hydroxycholecalciferol and 1,25-Dihydroxycholecalciferol: Effects of Side Chain Modification and Stereoisomerization on Responses of Fetal Rat Bones *in Vitro*

PAULA H. STERN[1] AND THALIA MAVREAS

Department of Pharmacology, Northwestern University Medical School, Chicago, Illinois 60611

CLARENCE L. TRUMMEL[2]

Department of Pharmacology and Toxicology, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14620

HEINRICH K. SCHNOES AND HECTOR F. DELUCA

Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin – Madison, Madison, Wisconsin 53706

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SUMMARY

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Organ cultures of fetal rat bone were used to test the effects of molecular modification on the bone-resorbing activity of the vitamin D_3 metabolites 25-OH-D_3 and 1,25-$(OH)_2D_3$. Addition of 26-hydroxyl group to the 25-OH-D_3 side chain reduced the activity more than 10-fold. Shortening the 25-OH-D_3 side chain by 1 carbon atom reduced the activity by at least two orders of magnitude. Removal of the 26- and 27-methyl groups diminished the bone-resorbing activity still further. 1,25-$(OH)_2D_3$ was likewise more active than the vitamin D_3 derivatives with which it was compared. The 3α-hydroxy epimer of 1,25-$(OH)_2D_3$ [1,25-$(OH)_2D_3$ (3α)] was more than three orders of magnitude less active than 1,25-$(OH)_2D_3$. 1,24(*R*),25-$(OH)_3D_3$ was approximately $^1/_{10}$ as active as 1,25-$(OH)_2D_3$. When the 24-hydroxyl was in the *S* configuration, the trihydroxy derivative was even less effective. 1,25-$(OH)_2D_3$ was approximately equiactive with 1,25-$(OH)_2D_2$. The results illustrate the importance of precise structural characteristics at a number of sites on the molecule for optimal bone-resorbing activity. The data also show that in terms of direct effects on bone, no known naturally occurring vitamin D_3 metabolite or synthetic congener surpasses 1,25-$(OH)_2D_3$ in activity. A striking correlation exists between the

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[2] Present address, Department of Oral Biology, School of Dentistry, University of Colorado Medical Center, Denver, Colorado 80222.

structure-activity relationships shown here and published studies on the binding of vitamin D analogues to subcellular "receptors." Good correlations also can be demonstrated between effects of the 1-hydroxylated derivatives on bone resorption *in vivo* and *in vitro*. Greater inconsistencies between results *in vitro* and *in vivo* are found with compounds lacking a 1-hydroxyl group.

INTRODUCTION

It is now known that vitamin D must be metabolized in order to exert its characteristic effects on calcium metabolism (1, 2). Hydroxylation in the liver produces 25-OH-D_3[3] (3, 4), the metabolite present in largest quantities in the circulation (5). This substance is subsequently hydroxylated to either 24,25-$(OH)_2D_3$ or 1,25-$(OH)_2D_3$ (6). 1α-Hydroxylation, which appears to be limited to the kidney, results in the production of 1,25-$(OH)_2D_3$, the most rapidly acting and potent metabolite described to date (7–13). 25-OH-D_3, 24,25-$(OH)_2D_3$, and 1,25-$(OH)_2D_3$ have previously been shown to cause resorption when added to cultures of fetal rat bone *in vitro* (11–17). The present study examines the effects of several molecular modifications on the bone-resorbing activities of 25-OH-D_3 and 1,25-$(OH)_2D_3$. These alterations include modification of the length of the side chain, addition of hydroxyl groups on the side chain, and substitution of stereoisomers on the side chain and elsewhere. Studies of the effects of vitamin D analogues in bone organ cultures can give information on structure-activity relationships not provided by administration of the compounds *in vivo* or binding studies *in vitro*. Responses *in vivo* reflect distribution, metabolism, and elimination, as well as indirect effects due to actions at other sites, such as the intestine, in addition to the actual interaction with the bone cell "receptor." Effects in culture compared with responses *in vivo* can help to identify pharmacokinetic factors which modify the response *in vivo*. Binding studies, although they theoretically represent the purest form of interaction, are often carried out under nonphysiological conditions of temperature, pH, and concentration and do not incorporate a characteristic end organ response. Available evidence suggests that neither 25- nor 1-hydroxylation occurs in the fetal rat bone organ cultures (11, 14). If other metabolic steps are likewise lacking, the relative effects of different analogues in organ culture and as competitors for subcellular receptors should be proportional. Comparisons of responses in the two systems should help to evaluate the validity of binding studies.

[3] The abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1α, 25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 24-nor-25-OH-D_3; 24-nor-25-hydroxyvitamin D_3; 26,27-bisnor-25-OH-D_3, 26,27-bisnor-25-hydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1α,24,25-trihydroxyvitamin D_3; 1α,25-$(OH)_2D_3$ (3α), 3-epi-1α-dihydroxyvitamin D_3; 1,25-$(OH)_2D_2$, 1α,25-hydroxyvitamin D_2; 1α-OH-3-deoxy-D_3, 1α-hydroxy-3-deoxyvitamin D_3; 1α-OH-3-deoxy-25-OH-D_3, 1α-hydroxy-3-deoxy-25-hydroxyvitamin-D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3.

METHODS

Compounds. 1,25-$(OH)_2D_3$, 25,26-$(OH)_2D_3$, 24-nor-25-OH-D_3, 26,27-bisnor-25-OH-D_3, and 1,25-$(OH)_2D_3$ (3α) were synthetic compounds, prepared as described previously (18–20). 1,25-$(OH)_2D_2$ was prepared enzymatically (21). The *R* and *S* isomers of 1,24,25-$(OH)_3D_3$ were gifts of Hoffmann-La Roche, and 25-OH-D_3 was a gift of the Upjohn Company. Compounds were stored at $-20°$ under N_2 in 95% ethanol.

Cultures. Details of the methods have been published previously (13, 22–24). Paired fetal rat radii and ulnae labeled with ^{45}Ca were incubated for 48 or 72 hr in a 5% CO_2 atmosphere with a modified BGJ medium (23) containing 1 mg/ml of bovine serum albumin. Vitamin D analogues were added in 95% ethanol. In some experiments bones were first cultured for 8 or 24 hr without vitamin D analogues to reduce exchangeable ^{45}Ca content. The regular culture medium was used for 24-hr initial cultures. Eight-hour initial cultures were carried out in culture medium without al-

bumin or sodium bicarbonate and in an air atmosphere.

RESULTS

Modifications of carbon backbone of side chain. 25-OH-D_3, 24-nor-25-OH-D_3, and 26,27-bisnor-25-OH-D_3 were compared, as were 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2D_2$. Shortening of the 25-OH-D_3 side chain by 1 carbon atom resulted in the compound 24-nor-25-OH-D_3. This analogue had less bone-resorbing activity than 25-OH-D_3 (Fig. 1). Although the responses to 25-OH-D_3 were maximal at 0.1 μM, no significant effects of the 24-nor compound were noted at 0.75 μM. The 24-nor analogue was active at 7.5 μM. The bisnor compound with carbons 26 and 27 deleted was inactive at concentrations as high as 7.5 μM.

The side chain modifications involved in the change from 1,25-$(OH)_2D_3$ to 1,25-$(OH)_2D_2$ did not profoundly affect bone-resorbing activity (Fig. 2). The D_2 derivative was slightly, but not significantly, less active.

Addition of hydroxyl groups to side chain. 25-OH-D_3 and 25,26-$(OH)_2D_3$ were compared, as were 1,25-$(OH)_2D_3$ and 1,24(R),25-$(OH)_3D_3$. 25,26-$(OH)_2D_3$ was less active than 25-OH-D_3 when the bone-resorbing activities of the two compounds were compared (Table 1). Only a small effect was obtained with 1 μM 25,26-$(OH)_2D_3$, whereas 25-OH-D_3 had maximal activity at 0.1 μM.

Addition of a 24-hydroxyl group in the R configuration to 1,25-$(OH)_2D_3$ results in a biologically occurring substance, 1,24(R),25-$(OH)_3D_3$ (25).[4] This material was somewhat less than 1/10 as active as 1,25-$(OH)_2D_3$ in this system (Fig. 3).

Stereochemistry of hydroxyl groups. 1,24(R),25-$(OH)_3D_3$ was compared with 1,24(S),25-$(OH)_3D_3$, and 1,25-$(OH)_2D_3$, with 1,25-$(OH)_2D_3$ (3α). 1,24(S),25-$OH)_3D_3$ (Fig. 2) and 1,25-$(OH)_2D_3$ (3α) represent stereoisomers of the naturally occurring compounds 1,24(R),25-$(OH)_3D_3$ and 1,25-$(OH)_2D_3$. In both cases the unnatural stereoisomer was much less active than the naturally occurring compound (Figs. 4 and 5). The difference between the activities of 1,24,25-$(OH)_3D_3$ R and S compounds was more than an order of magnitude, whereas modification in the 3-hydroxyl position decreased the response by more than three orders of magnitude.

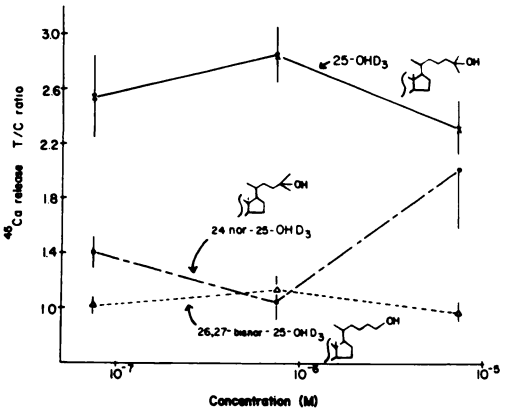

FIG. 1. *Comparison of responses of cultured fetal rat bones to 25-OH-D_3, 24-nor-25-OH-D_3, and 26,27-bisnor-25-OH-D_3*

Bones were cultured for 24 hr without the vitamin D analogues and then transferred to medium containing the compounds for an additional 48-hr incubation. Each point represents the mean treated to control (T/C) ratio for four bone pairs. Bars denote standard errors.

DISCUSSION

The search for more potent or more selective analogues of the naturally occurring vitamin D metabolites has yielded a variety of derivatives. Thus far none has been found which is more potent *in vivo* than 1,25-$(OH)_2D_3$. The studies presented here indicate that with the exception of conversion of the D_3 side chain to a D_2 side chain, all the modifications tested, whether representing natural modifications [i.e., 1,24(R),25-$(OH)_3D_3$ formation] or synthetic alterations (substitution of a 3α-hydroxyl group for the 3β-hydroxyl), diminish the bone-resorbing activity of 1,25-$(OH)_2D_3$ *in vitro*. Likewise, modifications of 25-OH-D_3 such as shortening the side chain or addition of a hydroxyl group in position 24 (15) or 26 only diminish the bone-resorbing activity of the compound *in vitro*. Only 1-hydroxylation, giving 1,25-

[4] Y. Tanaka and H. F. DeLuca, unpublished observations.

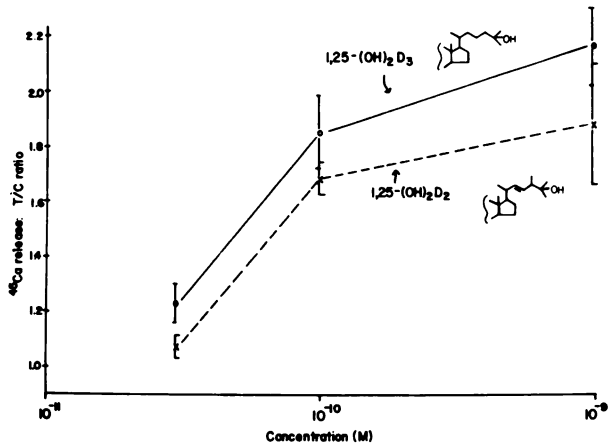

FIG. 2. *Comparison of responses of cultured fetal rat bones to 1,25-(OH)$_2$D$_3$ and 1,25-(OH)$_2$D$_2$*
Bones were cultured for 8 hr without vitamin D analogues and then transferred to medium containing the compounds for an additional 48-hr incubation. Each point represents the mean treated to control (T/C) ratio for five bone pairs. Bars denote standard errors.

TABLE 1

Comparison of bone-resorbing activities of 25,26-(OH)$_2$D$_3$ and 25-OH-D$_3$ in vitro

Values are treated to control ratios of ^{45}Ca released from paired fetal rat bones and are the means ± standard errors of the responses from the indicated numbers of bone pairs. Bones were incubated for 8 hr without the vitamin D analogues and then transferred to medium containing the compounds for an additional 72-hr incubation.

Concentration	25,26-(OH)$_2$D$_3$	25-OH-D$_3$
μM		
0.1	1.20 ± 0.13 (3)	2.23, 1.92
1.0	1.31 ± 0.07 (4)	2.20 ± 0.30 (4)

(OH)$_2$D$_3$, enhances the activity of 25-OH-D$_3$ (11–13, 16, 17). Although direct comparisons of 24,25-(OH)$_2$D$_3$ and 1,24,25-(OH)$_2$D$_3$ were not made in these studies, cross-comparisons indicated that 1α-hydroxylation enhances the activity *in vitro* of 24(*R*),25-(OH)$_2$D$_3$ (15) to about the same extent as it increases the activity of 25-OH-D$_3$ (13), approximately two orders of magnitude.

Deletion of hydrophobic portions of the side chain to give 24-nor-25-OH-D$_3$ or 25,26-bisnor-25-OH-D$_3$ diminished the activity of 25-OH-D$_3$. These observations are consistent with our earlier studies *in vitro* with the 1α-hydroxylated vitamin D$_3$ derivative lacking a side chain, 1α-hydroxypregnacalciferol (26). This analogue was considerably less active than 1α-OH-D$_3$. In the case of 26,27-bisnor-25-OH-D$_3$, the oxidation state of the hydroxyl substituent was modified from a tertiary to a primary alcohol. This could conceivably also contribute to the altered activity. 26,27-Bisnor-25-OH-D$_3$ and 24-nor-25-OH-D$_3$ were found to be ineffective in producing resorption of cultured calvariae from newborn mice (26). The concentration of 24-nor-25-OH-D$_3$ used in this study, 50 ng/ml (approximately 0.1 μM), would have been inactive in our system as well. In view of our previous studies *in vitro*, in which 24(*S*)-OH-D$_3$ and 24(*S*),25-(OH)$_2$D$_3$ were much less active than their naturally occurring 24(*R*)enantiomers (15), it was not surprising that 1,24(*R*),25-(OH)$_3$D$_3$ was more active than the 1,24(*S*),25-(OH)$_3$D$_3$ analogue. Another unnatural stereoisomer, the 3α-hydroxy derivative of 1,25-(OH)$_2$D$_3$, was more than 1000 times less active than the natural compound *in vitro*. This marked decrease in activity may be the result of steric hindrance from the 3-hydroxyl group being in the α configuration, since Mahgoub[5] has found that deletion of the 3β-hydroxyl group, giving 1α-OH-3-deoxy-25-OH-D$_3$, only diminishes the activity of 1,25-(OH)$_2$D$_3$ about 50-fold.

It is of interest to compare the effects of

[5] A. Mahgoub, personal communication.

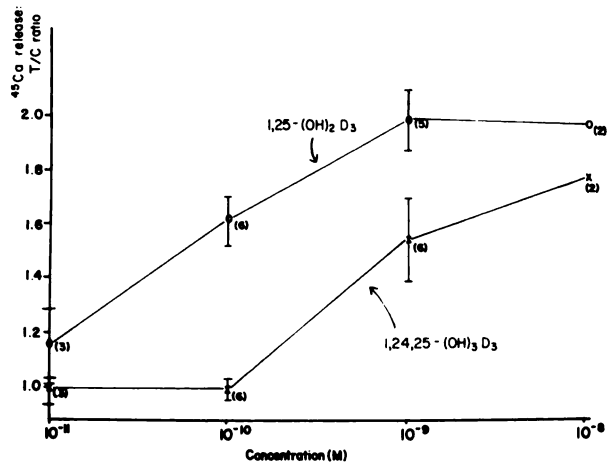

FIG. 3. *Comparison of responses of cultured fetal rat bones to 1,25-(OH)$_2$D$_3$ and 1,24(R),25-(OH)$_3$D$_3$*
Bones were cultured for 8 hr without vitamin D analogues and then transferred to medium containing the compounds for an additional 48-hr incubation. Values are means ± standard errors of the numbers of bone pairs shown in parentheses.

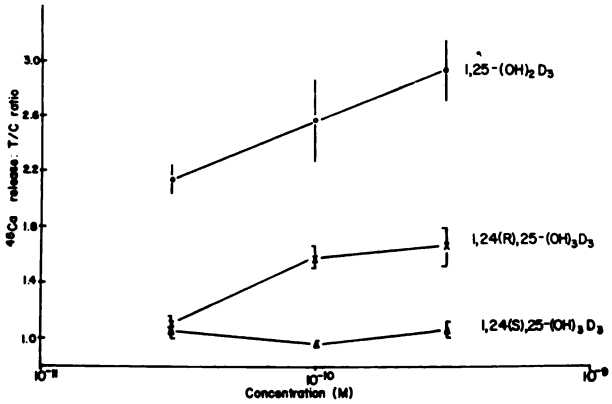

FIG. 4. *Comparison of responses of cultured fetal rat bones to 1,25-(OH)$_2$D$_3$ and R and S isomers of 1,24,25-(OH)$_3$D$_3$*
Bones were cultured for 8 hr without vitamin D analogues and then transferred to medium containing the compounds for an additional 48-hr incubation. Each point represents the mean treated to control (T/C) ratio for seven bone pairs. Bars denote standard errors.

administration *in vitro* and *in vivo* on the bone-resorbing activity of these compounds. Upon administration to the rat *in vivo*, vitamin D_2 and D_3 gave roughly equivalent effects on bone calcium mobilization (27, 28). 25-OH-D_2 and 25-OH-D_3 were likewise equiactive on this parameter (27, 28). 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ had similar antirachitic activities in the rat, with 1,25-$(OH)_2D_3$ possibly being slightly more active (21). Thus the observed similarities between the bone-resorbing activities of 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ *in vitro* are consistent with the observations *in vivo* and suggest that these two compounds do not undergo further modification before they act upon bone *in vivo*.

Comparison of the activities of the 1α-hydroxylated vitamin D_3 compounds on bone *in vitro* and *in vivo* also reveals a qualitative correspondence. Since extensive dose-response studies were not carried out *in vivo*, quantitative comparisons are

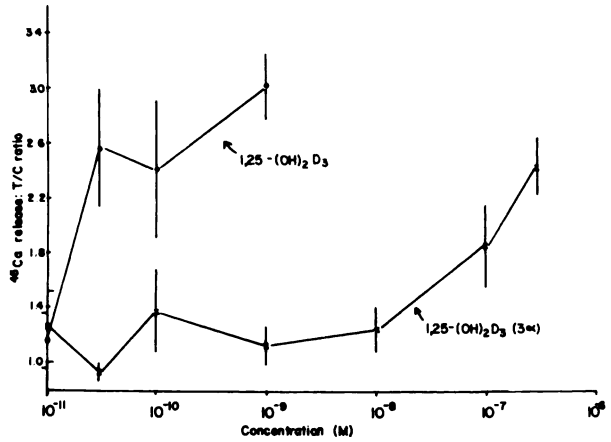

FIG. 5. *Comparison of responses of cultured fetal rat bones to 1,25-(OH)$_2$D$_3$ and 1,25-(OH)$_2$D$_3$ (3α)* Bones were cultured for 8 hr without vitamin D analogues and then transferred to medium containing the compounds for an additional 48-hr incubation. Each point represents the mean treated to control (T/C) ratio for four bone pairs. Bars denote standard errors.

more difficult. The trihydroxy metabolite 1,24(*R*),25-(OH)$_3$D$_3$ was slightly less active than the dihydroxy compound 1,25-(OH)$_2$D$_3$ in the rat (29). Since the trihydroxy metabolite seems to be a normal metabolite of 1,25-(OH)$_2$D$_3$ (30), it may very well represent a physiological inactivation step. All the identified naturally occurring analogues of 1,25-(OH)$_2$D$_3$ have now been shown to be less active on bone than 1,25-(OH)$_2$D$_3$ *in vitro* as well as *in vivo*. Since significant 1-hydroxylation does not seem to occur under these conditions (11), at least some of the differences seen *in vivo* may be due to differences in sensitivity of the responding tissues to the compounds rather than to pharmacokinetic factors. Preliminary studies indicated that substitution of a 3α-hydroxyl for the normal 3β-hydroxyl markedly reduced intestinal calcium transport and bone calcium mobilization (18), and the effects on bone resorption are again qualitatively consistent with this.

In the group of compounds lacking a 1α-hydroxyl group, correlations between the current results and the reported activities *in vivo* (19, 20, 31–33) are less consistent. *In vitro*, both 24-nor-25-OH-D$_3$ and 25,26-(OH)$_2$D$_3$ were more active than 26,27-bisnor-25-OH-D$_3$. 25-OH-D$_3$ was more than one order of magnitude more potent than 25,26-(OH)$_2$D$_3$ and two orders of magnitude more potent than 24-nor-25-OH-D$_3$. *In vivo* in the rat, 2.5 μg of 25,26-(OH)$_2$D$_3$ administered intrajugularly failed to stimulate bone calcium mobilization significantly (19), whereas 2.5 μg of the 26,27-bisnor compound did elicit a moderate response and 25 μg produced a larger effect (20). 25-OH-D$_3$ was active at 0.25 μg. In other studies, no difference was found between the bone-mobilizing activities of 25-OH-D$_3$ and 24-nor-25-OH-D$_3$, although both compounds were tested at 25 μg, which might have been a supramaximal amount (32). Interestingly, 37.5 μg of 24-nor-25-OH-D$_3$ given intraperitoneally to chicks was inactive, wheras the same dose of 25-OH-D$_3$ elicited a significant response (33). Thus a number of the results *in vivo* seem quantitatively inconsistent with the findings *in vitro*. It must be noted that the data *in vivo* on these compounds were obtained from vitamin D-deficient animals with intact kidneys. Since the activities observed *in vivo* are thus probably produced by 1-hydroxylated derivatives rather than the parent compounds (19), it is not unexpected that inconsistencies exist. The responses *in vitro* may be more comparable to the bone mobilization observed with high concentrations of 25-OH-D$_3$ in vitamin D-replete (i.e., with 1-hy-

droxylase presumably suppressed) intact or nephrectomized animals (34). Differences in absorption, distribution, and excretion of the analogues could also contribute to the disparities.

At the other end of the spectrum, we would like to compare the culture results with those obtained in simpler systems *in vitro*, such as binding studies. Interpretations are made somewhat tenuous because the published binding studies have been done with intestinal tissue of vitamin D-deficient chickens (35–37). Despite this obvious limitation, the results obtained in the above cytosol-chromatin binding systems are in striking agreement in most cases with data from fetal bone organ cultures. Vitamin D_3 itself is inactive both in culture and in binding assays (14, 36, 38). 25-OH-D_3 and 1α-OH-D_3 were 100–1000 times less active than 1,25-$(OH)_2D_3$ both in organ culture (11–13) and in binding assays (35–37). 1,24,25-$(OH)_3D_3$ was one-eighth as active as 1,25-$(OH)_2D_3$ in binding to chick intestinal cytosol (37), which corresponds reasonably well with the relative activities in the current studies. 24,25-$(OH)_2D_3$ was 5000 times less active than 1,25-$(OH)_2D_3$ in competing for 1,25-$(OH)_2D_3$ binding sites (37). In fetal rat bone organ cultures, 1,25-$(OH)_2D_3$ was generally active over a 0.001–1 nM range (11, 13), whereas more than 10 nM 24,25-$(OH)_2D_3$ was required for activity (15). Thus, in the case of six analogues for which published data are available, the correlation between effects in the two systems is striking. The only exception to date is 1α-OH-3-deoxy-D_3, which was inactive *in vitro* on fetal rat bone at concentrations up to 100 μM (13).[5, 6] This compound, which was less active on bone than on intestine *in vivo* (39), had weak binding activity on chick intestinal cytosol, approximately a 5000-fold excess being required to produce 50% displacement of 1,25-$(OH)_2D_3$. On the basis of the correspondence between binding and bone resorption noted with the other analogues, the cultured bones should have responded to the concentrations used. This single disparity between the chick intestinal binding system and the bone organ culture is certainly not a major one and may reflect the fact that bone calcium mobilization tends to be more sensitive to structural modifications of 1,25-$(OH)_2D_3$ than the intestinal response (39).

The good correlation between the structure-activity relationships for binding and for activity in organ culture supports the concept that the vitamin D_3 analogues are probably not extensively metabolized in culture. This would permit the further conclusion that the entire 1,25-$(OH)_2D_3$ molecule, all three hydroxyl groups, and the intact side chain are essential for optimal direct bone-resorbing activity.

[6] P. H. Stern, unpublished observations.

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