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STUDIES ON THE MODE OF ACTION OF VITAMIN D—XIV. QUANTITATIVE ASSESSMENT OF THE STRUCTURAL REQUIREMENTS FOR THE INTERACTION OF 1α,25-DIHYDROXYVITAMIN D_3 WITH ITS CHICK INTESTINAL MUCOSA RECEPTOR SYSTEM

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SUMMARY

The structural requirements for the interaction of the 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] molecule with its chick intestinal mucosa receptor system have been quantitatively evaluated. This analysis was carried out using structural analogs of 1α,25-dihydroxyvitamin D_3 in a competitive binding assay with a reconstituted chromatin–cytosol system from rachitic chick intestinal mucosa. In this assay the steroid–cytosol receptor complex binds avidly to the chromatin. The results for the metabolites and analogs were expressed on a linear scale of relative competitive index (RCI) where the RCI of 1α,25$(OH)_2D_3$ is defined as 100. These studies demonstrated that the 1α- and 25-hydroxyl functions were the most critical groups to the binding process: their absence reduced the RCI to 0.5 and 0.4 respectively. The 3β-hydroxyl, although important, is somewhat less critical since the RCI was reduced to only 5.7. When all three of these hydroxyls are present, the receptor will tolerate alterations in the side chain at carbon-24. Thus shortening the side chain of 1α,25$(OH)_2D_3$ by only one methylene group reduced the RCI to 46. When the 25-hydroxylated side chain is present, modification of the A-ring at C-1, C-3, C-4, C-10 and C-19 all markedly reduce the RCI. Competitors with both A-ring and side chain modifications were found to have almost no ability to interact with the receptor system. Collectively these results support the view that the intestinal cytosol–chromatin receptor system has a very high degree of specificity for its hormonal ligand which reflect bonding-interactions at multiple (carbons 1, 3, 4, 10, 19, 24, and 25) sites on the 1α,25$(OH)_2D_3$ molecule. A model is presented for this interaction which emphasizes the key role of a standard length side chain with a 25-OH functional group.

INTRODUCTION

The ability of a steroid hormone to affect a biological response in a given tissue is dependent on the presence in that tissue of a specific receptor system for the hormone. Studies on the mode of action of such steroid hormones as estradiol [1–3], progesterone [4, 5], testosterone [6, 7], aldosterone [8] and glucocorticoids [9–11] have led to a delineation of the steps required for a physiological response. The steroid enters the cell and binds with high affinity and stereostructural specificity to a cytoplasmic receptor. This steroid–receptor complex undergoes some type of activation process that allows it to bind to the nuclear or chromatin fraction of the cell. This apparently alters transcriptional events leading to the *de novo* synthesis of new proteins which are believed to function in the biological response.

In recent years it has been found that vitamin D_3‡, which can either be taken in through the diet or

‡ Vitamin D_3 or cholecalciferol is officially described as a seco-steroid. The chemical nomenclature of the IUPAC and IUB for this molecule is 9,10-seco-5,7,10(19)-cholestatrien-3β-ol.

Abbreviations used in this paper for the various vitamin D compounds are: 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 24-nor-1α,25$(OH)_2D_3$, 24-nor-1α,25-dihydroxyvitamin D_3; 1α,24R,25$(OH)_3D_3$, 1α,24R,25-trihydroxyvitamin D_3; 1α,24S,25$(OH)_3D_3$, 1α,24S,25-trihydroxyvitamin D_3; 1α-OH-D_3, 1α-hydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; 24R,25$(OH)_2D_3$, 24R,25-dihydroxyvitamin D_3; 24S,25$(OH)_2D_3$, 24S,25-dihydroxyvitamin D_3; 24-nor-25-OH-D_3, 24-nor-25-hydroxyvitamin D_3; 24-homo-25-OH-D_3, 24-homo-25-hydroxyvitamin D_3; 23,24-dinor-25-OH-D_3, 23,24-dinor-25-hydroxyvitamin D_3; 3-deoxy-1α,25$(OH)_2D_3$, 3-deoxy-1α,25-dihydroxyvitamin D_3; 19,25-$(OH)_2DHV_3$-II, 19,25-dihydroxy-10S(19)-dihydrovitamin D_3; 19,25$(OH)_2DHV_3$-III, 19,25-dihydroxy-10R(19)-dihydrovitamin D_3; 25-OH-DHT_3, 25-hydroxydihydrotachysterol$_3$; 25-OH-5,6-*trans*-D_3, 25-hydroxy-5,6-*trans*-vitamin D_3; 3-deoxy-1α-OH-D_3, 3-deoxy-1α-hydroxyvitamin D_3; 1α-OH-3-epi-D_3, 1α-hydroxy-3-epi-vitamin D_3; 3-deoxy-3α-methyl-1α-OH-D_3, 3-deoxy-3α-methy-1α-hydroxyvitamin D_3; 19-OH-DHV_3-II, 19-hydroxy-10S(19)-dihydrovitamin D_3; 19-OH-DHV_3-III, 19-hydroxy-10R(19)-dihydrovitamin D_3; 5,6-*trans*-D_3, 5,6-*trans*-vitamin D_3; DHT_3, dihydrotachysterol$_3$; 24-nor-25-OH-5,6-*trans* D_3, 24-nor-25-hydroxy-5,6-*trans*-vitamin D_3; 24-homo-25-OH-5,6-*trans*-D_3, 24-homo-25-hydroxy-5,6-*trans*-vitamin D_3. RCI, relative competitive index.

biosynthesized in the skin by U.V.-irradiation of the pro-vitamin (7-dehydrocholesterol), must be metabolized to have biological activity. The steroid is first hydroxylated by the liver at the 25-position on the side chain [12] to form 25-hydroxyvitamin D_3 and then hydroxylated at the 1α-position on the A-ring to form 1α,25-dihydroxyvitamin D_3 [13–15]. This metabolite is the hormonally active molecule [16, 17] which acts on intestinal mucosa, bone and kidney to facilitate the uptake of calcium into the serum.

Since its structure was determined in 1971 [18–20], it has been demonstrated that, in the intestinal mucosa, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] acts in a manner entirely analogous to other steroid hormones. Upon entering the cell, 1α,25$(OH)_2D_3$ first binds to a cytoplasmic receptor [21, 22]. This complex then translocates to the chromatin fraction [23–26] where it alters transcriptional events [27, 28] and leads to the *de novo* synthesis of a vitamin D-dependent calcium binding protein [29–31].

The chick intestinal cytoplasmic receptor has been found to be a protein [21, 22] of approximately 47,000 dalton MW [25] with a sedimentation coefficient 3–3.7S [22, 26]. It is specific for 1α,25$(OH)_2D_3$ [21, 24, 32] which it binds with high affinity [22, 26], and low capacity [22, 32]. The receptor, thus, satisfies the classical criteria for a steroid hormone receptor.

A unique structural aspect to the seco-steroid vitamin D is that the A-ring is conformationally mobile [33–35]. Since the molecule does not have a fused A/B ring system, the A-ring is free to undergo chair–chair interconversions in much the same way as cyclohexane. An intriguing problem is to identify the advantages/disadvantages of this fact upon any ligand–receptor interactions.

In this paper we wish to report the newest developments in our understanding of the structural requirements for the interaction of 1α,25$(OH)_2D_3$ with its chick intestinal mucosa receptor system. This analysis has been carried out utilizing a competitive binding assay in which the ability of increasing amounts of nonradioactive analog to compete with a standardized amount of tritiated 1α,25$(OH)_2D_3$ for its interaction with its receptor system is measured. The extent of the competition can be quantitatively related to the structural differences between the analog and the tritiated 1α,25$(OH)_2D_3$.

MATERIALS AND METHODS

Animals. White Leghorn cockerels were obtained on the day of hatching and were raised on a rachitogenic diet [36] for four weeks. During this time the animals became severely rachitic and were killed by decapitation.

Preparation of subcellular fractions. Intestines were fractionated into chromatin and cytosol fractions exactly as described previously in detail [32]. These two fractions are recombined by homogenization to form the reconstituted chromatin–cytosol preparation. This mixture was prepared fresh each time the assay was run.

Receptor assay. Aliquots (0.5 ml) of the reconstituted chromatin and cytosol were incubated for 45 min at 23°C in 1.5 × 9.5 cm polypropylene centrifuge tubes with 10 pmol of [^{3}H]-1α,25$(OH)_2D_3$ and the analog of choice. After the incubation, chromatin was harvested by centrifugation and washed with 10 mM Tris–Cl, 0.5% Triton X-100, pH 8.5 as previously described [32]. The final washed chromatin pellets were extracted for [^{3}H]-1α,25$(OH)_2D_3$ with 4 ml of 2:1 methanol–chloroform. The extract was dried in a scintillation vial and 9 ml of butyl-PBD cocktail [5.25 g 2-(4′-tert-butylphenyl-5-[4′-biphenyl, 3,4-oxadiaxole])/liter toluene] was added. The samples were counted to 3% counting error in a Beckman model LS233 liquid scintillation counter. Tritium efficiency was 45% as determined by external standard.

Competitors were examined at multiple concentrations and their ability to compete with the [^{3}H]-1α,25$(OH)_2D_3$ for chromatin binding was assessed. A standard curve for nonradioactive 1α,25$(OH)_2D_3$ was generated with each assay and results of the analog competition were normalized to this value. The ability of a competitor to decrease the amount of chromatin bound [^{3}H]-1α,25$(OH)_2D_3$ can be described by the following equation [37].

$$\text{Percent Maximum Bound} = \frac{[^3\text{H} - 1\alpha,25(OH)_2D_3]}{[^3\text{H} - 1\alpha,25(OH)_2D_3] + \alpha[\text{competitor}]}.$$

In this equation α is the competitive index for the analog. For 1α,25$(OH)_2D_3$ α equals 1.0 and the equation simply describes a dilution curve of the specific activity of the tritiated 1α,25$(OH)_2D_3$. Taking the reciprocal of the equation leads to a linear relationship between

$$\frac{1}{\% \text{ Max}}$$

and

$$\frac{[\text{competitor}]}{[^3\text{H} - 1\alpha,25(OH)_2D_3]}$$

$$\frac{1}{\% \text{ Max Bound } ^3\text{H} - 1\alpha,25(OH)_2D_3}$$

$$= 1 + \frac{\alpha[\text{competitor}]}{[^3\text{H} - 1\alpha,25(OH)_2D_3]}.$$

To normalize day to day variations in the assay, the competitive index for the analog is compared to the competitive index for 1α,25$(OH)_2D_3$ obtained on that day to yield a relative competitive index (RCI).

$$\text{RCI} = \frac{\alpha_{\text{competitor}}}{\alpha_{1\alpha,25(OH)_2D_3}} \times 100.$$

For 1α,25$(OH)_2D_3$ the RCI is defined as 100.

For the weakest competitors (RCI < 0.05) it is necessary to use micromolar (0.1–20 μM) concentrations of analog to affect significant competition. At these concentrations the solubility of some of the analogs is likely to be near its limit. The question of whether the steroid is in true solution, bound to hydrophobic regions on protein, aggregated into micelles or some combination of all of these possibilities becomes very important. Therefore, although it is possible to obtain RCI values at or below 0.01, we have set this as the lower limit of the assay and assigned an RCI value of zero to competitors that are weaker than this value.

Chemicals. Radioactive [26,27-methyl-^{3}H]-25-hydroxyvitamin D_3 (9–12 Ci/mmol) was obtained from Amersham/Searle and converted *in vitro*, as previously described [36], to $1\alpha,25(OH)_2D_3$. The specific activity of the tritiated $1\alpha,25(OH)_2D_3$ was diluted to the desired level with synthetic $1\alpha,25(OH)_2D_3$. Nonradioactive crystalline $1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,24S,25(OH)_3D_3$, $24R,25(OH)_2D_3$, $24S,25(OH)_2D_3$ and 25-OH-5,6-*trans*-D_3 were generous gifts of Dr. M. Uskoković of Hoffmann–LaRoche. Vitamin D_3 and dihydrotachysterol$_3$ were obtained from Philips–Duphar (Weesp, The Netherlands), 25-OH-D_3 was kindly supplied by Dr. J. Babcock of Upjohn and 25-OH-DHT_3 was a gift of Dr. P. Bell, Cardiff, Wales. The other analogs (24-nor-$1\alpha,25$-$(OH)_2D_3$, 24-nor-25-OH-D_3, 24-homo-25-OH-D_3, 23,24-dinor-25-OH-D_3, 24-nor-25-OH-5,6-*trans*-D_3, 24-homo-25-OH-5,6-*trans*-D_3, 3-deoxy-$1\alpha,25(OH)_2D_3$, 3-deoxy-1α-OH-D_3, $19,25$-$(OH)_2DHV_3$-II, $19,25(OH)_2DHV_3$-III, 19-OH-DHV_3-II, 19-OH-DHV_3-III, 1α-OH-D_3, 1α-OH-3-epi-D_3, 3-deoxy-3α-methyl-1α-OH-D_3 and 5,6-*trans*-D_3 were chemically synthesized in our laboratories. The purity of each analog was established by analytical thin-layer chromatography in several systems prior to assay and the UV-spectrum of each analog corresponded to that previously determined for the compound. The purity of most of the analogs from this laboratory was further assessed by running the assay on separately synthesized and purified synthetic batches.

RESULTS

In recent years a number of structural analogs of $1\alpha,25(OH)_2D_3$ have become available to us for the assessment of their ability to compete with [^{3}H]-$1\alpha,25(OH)_2D_3$ for interaction with the intestinal receptor system for this steroid. These compounds have generally fallen into one of three categories: analogs of $1\alpha,25(OH)_2D_3$ possessing (a) side chain modifications, (b) A-ring modifications, or (c) both side chain and A-ring modifications.

The results of typical competition binding assays for $1\alpha,25(OH)_2D_3$, 24-nor-$1\alpha,25(OH)_2D_3$, 25-OH-D_3 and 1α-OH-D_3 are shown in Fig. 1. Samples were run in duplicate and plotted in linearized form. The

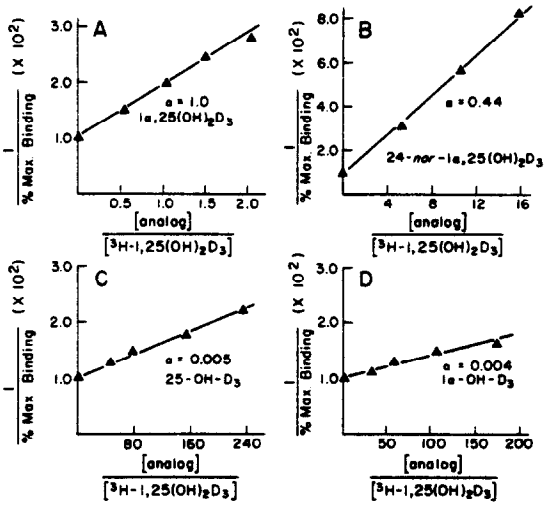

Fig. 1. Results of competitive binding assays for selected analogs. (A) Standard curve for $1\alpha,25(OH)_2D_3$. This curve is generated during each assay day and is used to normalize day to day variations in the assay. (B) Binding curve for 24-*nor*-$1\alpha,25(OH)_2D_3$. (C) Binding curve for 25-OH-D_3. (D) Binding curve for 1α-OH-D_3. Assays were carried out as described in Materials and Methods. Results are expressed in linearized form. Competitive indices (a) are equal to the slope of the line and were determined by linear regression analysis. Each data point represents the mean of duplicate samples.

slope of the line is determined by a least squares linear regression analysis and is equal to the competitive index. Analogs are assayed, when possible, on separate days at least two times each from two different synthetic batches. Results from 4–10 individual determinations are pooled and the coefficient of variation in the RCI is between 5% for the best competitors, and approximately 50% for the poorest competitors; i.e. those with RCI less than 0.5.

Figure 2 shows the structure and the relative competitive index (RCI) of the side chain analogs of $1\alpha,25(OH)_2D_3$ that we have examined to date. It has been known for several years [24, 32] that the removal of the 25-OH group as in 1α-OH-D_3 results in a very large reduction in the ability of the steroid to interact with the receptor system; this is reflected by its very low RCI (0.4). We have also evaluated three side chain analogs which still retain the 25-OH function (Fig. 2). All three of these analogs 24-nor-$1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$ and $1\alpha,24S,25$-$(OH)_3D_3$ are good competitors, and further emphasize the key importance of the 25-hydroxyl group. The addition of a 24-OH (either R or S configuration) or even the deletion of carbon-24 altogether only reduced the RCI to 41, 33 or 46 respectively. Each of these analogs is a 100× better competitor than 1α-OH-D_3.

We have also been able to examine a number of side chain analogs of 25-OH-D_3. These compounds, shown in Fig. 3, must be compared with 25-OH-D_3 as a reference, instead of $1\alpha,25(OH)_2D_3$ (we have chosen to include these analogs in the first category

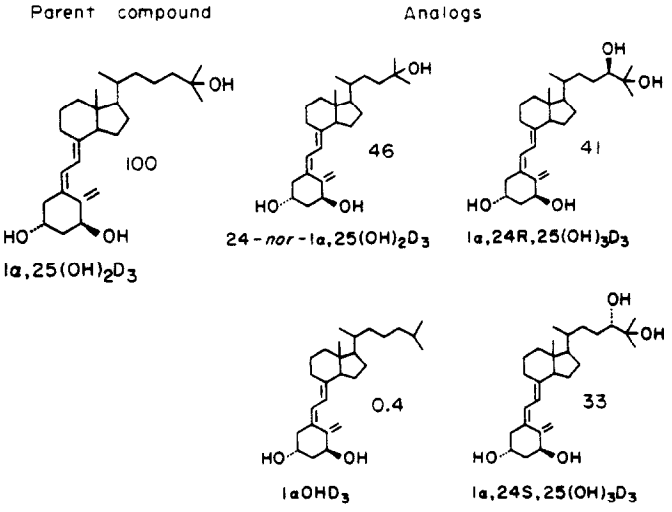

Fig. 2. Side chain analogs of 1α,25-Dihydroxyvitamin D_3. The parent compound, 1α,25$(OH)_2D_3$, has a Relative Competitive Index (RCI) of 100 by definition [37]. 24-nor-1α,25$(OH)_2D_3$ has a side chain that is one carbon shorter than 1α,25$(OH)_2D_3$. The 24-hydroxylated analogs (1α,24R,25$(OH)_3D_3$ and 1α,24S,25$(OH)_3D_3$) only differ by the stereochemical orientation of the hydroxyl on carbon-24. 1α-OH-D_3 has the critical 25-OH missing. The numbers beside the analog structure are average relative competitive index values.

although technically they are analogs with both A-ring and side chain-modifications). Once again, modification of the side chain hinders the interaction of the steroid with its receptor system. Introduction of a 24-OH group to this side chain (R- or S-epimer) resulted in an approximate 94% reduction in the RCI to about 0.03. Shortening or lengthening the side chain had a similar effect. 24-Nor-25-OH-D_3 and 24-homo-25-D_3 were only 2.4% as effective a competitor as 25-OH-D_3. Shortening the side chain by 2 carbons (23,24-dinor-25-OH-D_3) completely abolished binding.

In addition to the side chain analogs, we have examined some of the structural requirements in the

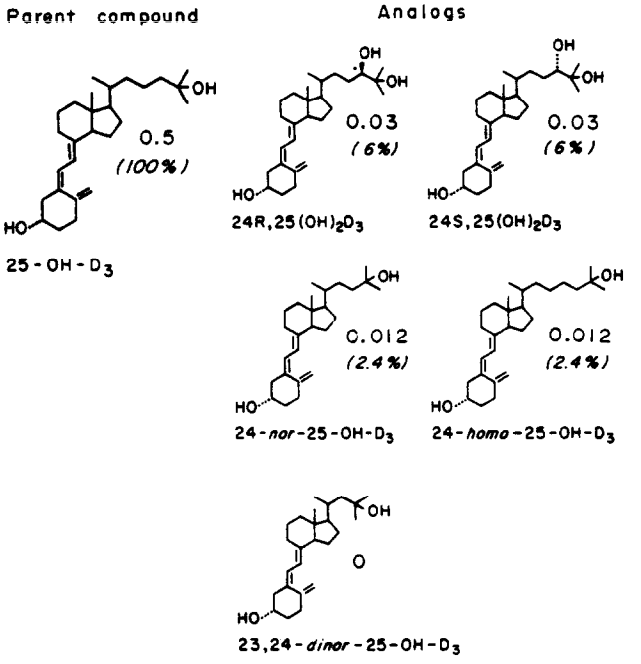

Fig. 3. Side chain analogs of 25-hydroxyvitamin D_3. The parent compound of this series is 25-OH-D_3: its RCI was determined to be 0.5. Side chains are modified by insertion of an hydroxyl at C-24 (24R,25$(OH)_2D_3$ and 24S,25$(OH)_2D_3$), lengthening the side chain (24-homo-25-OH-D_3) or shortening the side chain (24-nor-25-OH-D_3 and 23,24-dinor-25-OH-D_3). The italicized percentages allow comparison between the RCIs for the analogs and 25-OH-D_3, the parent compound of this series.

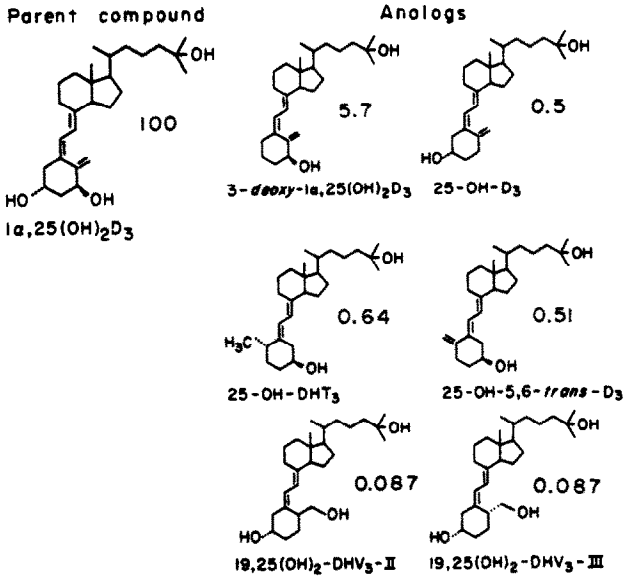

Fig. 4. A-ring analogs possessing a 25-hydroxylated side chain. In the 5,6-*cis* series analogs have either the 3β-OH (3-deoxy-1α,25(OH)$_2$D$_3$) or 1α-OH (25-OH-D$_3$) removed or the 10(19) double bond reduced and hydroxylated (19,25(OH)$_2$DHV$_3$-II and 19,25(OH)$_2$DHV$_3$-III). In the 5,6-*trans* series compounds with the normal (25-OH-5,6-*trans*-D$_3$) or reduced (25-OH-DHT$_3$) 10(19) double bond can be compared with 3-deoxy-1α,25(OH)$_2$D$_3$ to ascertain effects of the C-10 group.

A-ring that allow efficient ligand-receptor interaction (Fig. 4). The most striking observation is that the 1α-OH is 10 fold more important for binding than the 3β-OH, i.e. the RCI for 3-deoxy-1α,25(OH)$_2$D$_3$ and 25-OH-D$_3$ are 5.7 and 0.5 respectively. By comparing the two 5,6-*trans* analogs [25-OH-DHT$_3$ and 25-OH-5.6-*trans*-D$_3$] with 3-deoxy-1α,25(OH)$_2$D$_3$ it appears that the inversion of the A ring to the 5,6-*trans* orientation with a concomittant change of the C-19 carbon effects a 10× decrease in competition.

Two other A-ring analogs of 25-OH-D$_3$ were synthesized to assess whether a 19-OH function might, to some extent, satisfy the 1α-OH requirement for binding. Both the R- and S-epimer of 19,25-(OH)$_2$-DHV$_3$ (Fig. 4) were found to be only 0.09% as effective as 1α,25(OH)$_2$D$_3$ and 6% as effective as 25-OH-D$_3$.

When the side chain and the A-ring are simultaneously modified there is very little interaction of the analog with the 1α,25(OH)$_2$D$_3$ receptor system (Fig. 5). Those analogs without a 25-OH group that

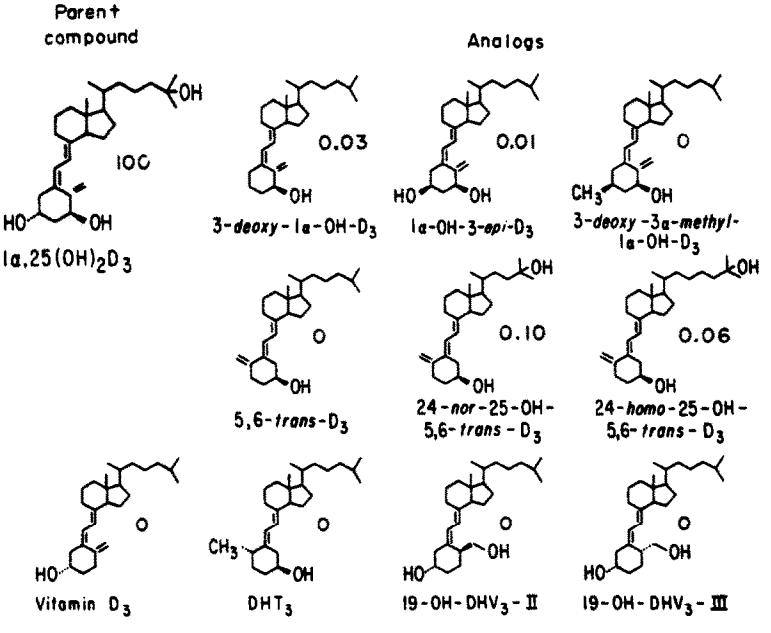

Fig. 5. Analogs with modified A-ring and side chain in the same molecule.

were in any way modified at C-1, C-3, C-4, C-10 or C-19 were nearly devoid of their ability to compete with $1\alpha,25(OH)_2D_3$. When the 3β-OH was removed or epimerized to the 3α-position there was a very slight amount of competition (3-deoxy-1α-OH-D_3 and 1α-OH-3-epi-D_3). When the 1α-OH was removed (vitamin D_3) or the C-19 methylene was converted to an hydroxymethyl group (19-OH-DHV_3-II and 19-OH-DHV_3-III) or when a methyl group was added at the 3α-position on 3-deoxy-1α-OH-D_3 to give 3-deoxy-3α-methyl-1α-OH-D_3 there was no competition*. Analogs of the 5,6-*trans* series with either no 25-OH (5,6-*trans*-D_3 and DHT_3) or an altered side chain that does possess a 25-OH (24-nor-25-OH-5,6-*trans*-D_3 and 24-homo-25-OH-5,6-*trans*-D_3) were also examined. Although the former compounds had no ability to compete, the presence of an altered side chain possessing the 25-OH group did result in a minimal detectable level of competition.

DISCUSSION

The availability of new structural analogs of $1\alpha,25(OH)_2D_3$ has extended our ability to probe the molecular requirement for the interaction of $1\alpha,25(OH)_2D_3$ with its chick intestinal mucosa receptor system. We have utilized a competitive binding assay in a reconstituted chromatin-cytosol system to relate steroid structural modifications to differences in competition. This type of assay is mechanistically more complex than a simple binding assay (ligand binding to cytosol receptor) but it reflects more accurately the situation that an analog would face *in vivo*. In order to elicit a biological response, the analog must not only bind to the cytosol receptor but be translocated to the nucleus as well. We have chosen to treat the competition data as a direct effect on ligand binding. The possibility that some analogs may demonstrate apparent competition through a mechanism other than ligand binding is currently being investigated. Selected analogs have been examined in a simple competition binding assay with mucosa cytosol receptor. The results suggest that these compounds compete for binding to the receptor to a similar extent as presented in the present study (manuscript in preparation).

Previously [32], we had reported that the structural elements that most affected binding were the three hydroxyl groups on the molecule (1α, 3β- and 25-hydroxyl functions). In our present study it was found that by removing any of these groups (comparing 3-deoxy-$1\alpha,25(OH)_2D_3$, 25-OH-D_3 and 1α-OH-D_3) there was a reduction of the RCI to 5.7, 0.5 and 0.4 respectively. Thus the presence of a 1α-OH or 25-OH are approximately 10-fold more contributive to tight ligand-receptor binding than the 3β-OH.

Three new analogs have been examined which clearly define the side chain requirements of the $1\alpha,25(OH)_2D_3$ molecule. 24-Nor-$1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$ and $1\alpha,24S,25(OH)_3D_3$ all possess the three critical hydroxyls (1α-, 3β- and 25-OH). The nor analog has a side chain one carbon shorter while the two 24-hydroxyl analogs have an added functional group at C-24. All three of these compounds were very good competitors (RCI 46, 41, and 33 respectively) and considerably better than 3-deoxy-$1\alpha,25(OH)_2D_3$ (RCI 5.7), the previously best known competitor. However, it is noteworthy that as subtle a modification as shortening the side chain of $1\alpha,25(OH)_2D_3$ by one carbon effected a 50% reduction in the RCI (see discussion below). In addition, $1\alpha,25(OH)_2D_2$ which has a C-24S methyl and $\Delta^{22,23}$ double bond on the side chain also appears to be a very good competitor [38]. With all three critical hydroxyl groups present, then, it appears that certain side chain modifications may be reasonably well tolerated by the receptor.

A somewhat different picture of the consequence of side chain modification emerges when the series of analogs without a 1α-OH group are compared (see Fig. 3). Side chain analogs of 25-OH-D_3 are less effective competitors than would be predicted on the basis of the results with the side chain analogs of $1\alpha,25(OH)_2D_3$. With the presence of a 24-hydroxyl group on the 25-OH-D_3 skeleton there is a 94% decrease in the RCI as compared to 25-OH-D_3 while in the presence of a 1α-OH this change only caused a 59% reduction in RCI. Shortening or lengthening the side chain seemed to have an even greater effect. The RCI for 24-nor-25-OH-D_3 is about 52% lower than what might be expected from the results with 24-nor-$1\alpha,25(OH)_2D_3$ (2.4% vs. 54%). Thus the presence of the 1α-OH is a ligand stabilizing factor which permits the receptor to tolerate greater perturbations of the side chain. But it is also apparent that the intestinal receptor has only a limited capacity to accommodate changes in the length of the side chain.

When A-ring analogs possessing the normal 25-hydroxyl side chain were examined, some additional features of the interaction were noted. When 3-deoxy-1α-$25(OH)_2D_3$ is compared to 25-OH-5,6-*trans*-D_3, the importance of the C-19 methylene group can be quantitated. In the *trans* compound carbon-19 is on the opposite side of the ring to its normal orientation and, in addition, the 3β-hydroxyl is in a pseudo-1α structural equivalent position. Here there is a 10X decrease in the RCI upon displacing the C-10(19) double bond from the one o'clock to the eleven o'clock position in the A-ring.

Interestingly, the C-10(19) reduction product of 25-OH-5,6-*trans*-D_3, 25-OH-DHT_3, which possess a C-10 methyl group which has more steric bulk on one face of the A-ring competes slightly better than the methylene compound (0.64 vs 0.51). We found that by substituting an R or S C-10 hydroxymethyl function for the C-10 methylene group there is about

* The analog 3-deoxy-3α-methyl-1α,25-dihydroxyvitamin D_3 (W. H. Okamura, M. N. Mitra, M. R. Pirio, A. Mourino, S. C. Carey and A. W. Norman, *J. Org. Chem.*, in press) exhibits 50% of the RCI of 3-deoxy-$1\alpha,25$-$(OH)_2D_3$.

a 4× reduction in competibility when compared to 25-OH-D_3 (which possess the C-10 methylene). These $19,25(OH)_2$-dihydrovitamins were completely devoid of both binding and biological activity although it was theoretically possible that the C-19 hydroxyl might, to some extent, satisfy the polarity effects needed in the region of C-1. Clearly when an hydroxyl group was introduced onto the C-19 carbon it in no way functioned as a pseudo 1α-OH. That it actually reduced the ligand–receptor interaction may reflect either hydrophobic or steric repulsion interactions with the C-10 methylene group–ligand binding site of the receptor.

In analogs where there were both side chain (no 25-hydroxyl or altered chain length with a 25-hydroxyl present) and A-ring modifications present the RCIs were very nearly zero.

From these studies it is possible to further refine the topological features of the $1\alpha,25(OH)_2D_3$ molecule that enable it to interact efficiently with its receptor system. The main features of this interaction elucidated to date are (a) the very strong requirement for the 1α and 25-hydroxyl groups and to a lesser extent the 3β-hydroxyl, (b) the slight tolerance for modifications of the side chain at carbon-24 when the three key hydroxyl groups are present, (c) the steric problems imposed by a 180° rotation of the A-ring or the reduction of the C-10 methylene group, and (d) the decrease in competition when the 3β-OH is epimerized to the 3α-configuration or substituted with a 3α-methyl group. It, thus, appears that there are combinations of polar and steric requirements at at least carbons 1, 3, 4, 10, 19, 24, and 25 that all contribute to the interaction of $1\alpha,25(OH)_2D_3$ with its chick intestinal mucosa receptor system.

A further feature of the $1\alpha,25(OH)_2D_3$ molecule, which is not readily apparent upon inspection of its structure in two dimensions, is that the A-ring pos-

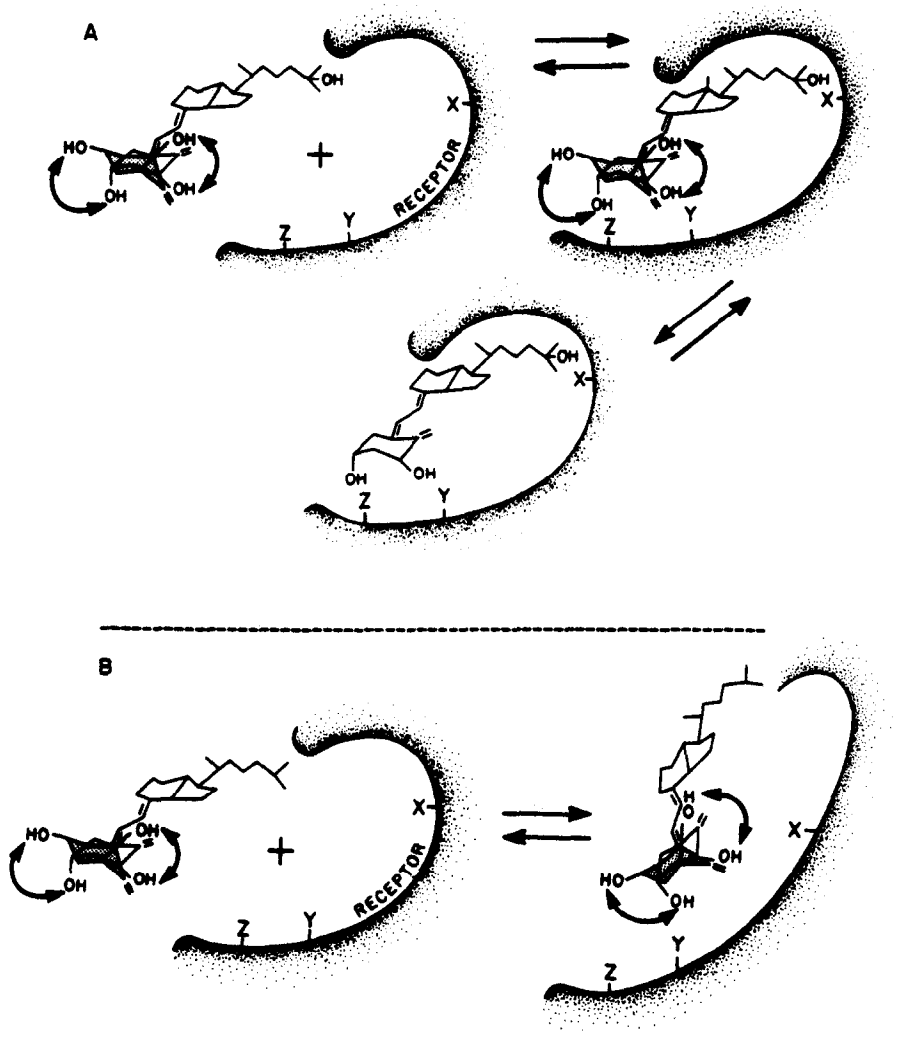

Fig. 6. Model for binding of vitamin D seco-steroids to receptors illustrating the key contribution of the 25-hydroxyl group. (A) Preliminary association of the 25-OH containing side chain of an analog facilitates the subsequent "capture" or retards the "release" of the conformationally mobile A-ring. (B) The absence of a 25-OH group or when the 25-OH-containing side chain is shortened prevents "capture" or enhances "release" of the A-ring: this is reflected by lower RCI values in Figs 2–4.

sesses a great deal of conformational freedom not found in other steroid hormone systems. Since the molecule no longer has a *trans*-fused A/B/C ring system, the A-ring is free to undergo chair–chair interconversions in much the same way as a cyclohexane molecule. This phenomenon which we have discussed previously in some detail [34, 35, 39, 40] is completely unique to the vitamin D system. It is known from detailed studies from our laboratories [35, 41] that changing the substituent groups on the A-ring of vitamin D steroids alters the equilibrium population ratio of the two chair conformers. This in turn changes the fraction of the 1α-OH or 3β-OH groups which may be axial or equatorial. This has important implications for the structural specificity of the A-ring. In one chair conformer the 1α-OH will be equatorially oriented while in the other conformer the same 1α-OH will be axially oriented. In view of the high degree of stereostructural specificity exhibited by A-ring analogs it seems unlikely that the A-ring continues to oscillate while bound to the receptor or that both conformers bind with equal affinity. Stated simply, the stringent structural requirements the $1\alpha,25(OH)_2D_3$ molecule make it very likely that the receptor recognizes only one of the conformers.

This then suggests why the presence of the 25-OH group and a side chain of standard length are so important for optimal binding of the $1\alpha,25(OH)_2D_3$ seco-steroid to its intestinal receptor and 25-OH-D_3 to its serum binding protein [43]. In both of these systems an analog with a standard 8-carbon side-chain without a 25-OH group or with a lengthened or shortened side chain with a 25-OH group binds much less effectively to their receptors*. These facts then support the model for receptor–seco-steroid binding given in Fig. 6. In this model a standard-length side chain with a 25-OH group play a critical role in permitting the receptor to optimally interact with the conformationally mobile A-ring.† In addition, the relationship of this model to the phenomenon of the activation of steroid–receptor complexes would be an important one in understanding the molecular mechanism of the subsequent nuclear events.

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* Our laboratory has shown that 24-nor-25-OH-D_3 is devoid of biological activity and that 24-*homo*-25-OH-D_3 has only 50% of the biological activity of vitamin D_3 in the chick [42]. In addition, it is known that both of these analogs and the parent vitamin D_3 bind with much lower affinity to the serum binding protein for 25-OH-D_3 [43].

† The RCI values for a steroid represent quantitation of an apparent equilibrium condition. But such numerical values of necessity reflect ratios of "k_{on}" and "k_{off}" thus any model system based on comparison of RCI values must focus on both "on" and "off" processes for the ligand–receptor interaction. In another steroid–receptor systems it has in fact been reported for a series of related steroids with differing K_{eq} that "k_{off}" was the more important kinetic parameter [44].

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