Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 461–467. Pergamon Press. Printed in Great Britain

STRUCTURE-FUNCTION STUDIES OF THE INTERACTION OF THE HORMONALLY ACTIVE FORM OF VITAMIN D_3, 1α,25-DIHYDROXY-VITAMIN D_3, WITH THE INTESTINE

ANTHONY W. NORMAN,* DANA A. PROCSAL,* WILLIAM H. OKAMURA† and RICHARD M. WING†
Departments of *Biochemistry and †Chemistry, University of California, Riverside, California 92502, U.S.A.

SUMMARY

The steroid cholecalciferol or vitamin D_3 is known to undergo a successive two-step hydroxylation, first in the liver to give 25-OH-D_3 and then in the kidney to produce 1α,25-$(OH)_2$-D_3. The kidney is postulated to be the endocrine gland which produces the biologically active form of vitamin D.

The biological response to 1α,25-$(OH)_2$-D_3 in the target intestine is believed to occur as a consequence of association with cytoplasmic and nuclear receptors, as is the case for other classical steroid hormones. A steroid competition assay has been devised using receptors present in the intestinal chromatin. A comparison of the relative competition in the assay and biological activity of a variety of analogs has defined some of the essential structural elements required for vitamin D activity.

These structure–function relationships of analogs of 1α,25-$(OH)_2$-D_3 are also discussed with particular emphasis on the A-ring conformation. It is emphasized that the A-ring of these seco-steroids consist of a pair of rapidly equilibrating chair conformers. As a consequence, different chair conformations produce different orientations of substituent groups in the A-ring. It is proposed that the 1α-hydroxyl of 1α,25-dihydroxyvitamin D_3 or its geometric equivalent in analogs must occupy the equatorial as opposed to the axial orientation for optimization of biological activity.

The last decade has produced remarkable advances in our understanding of the many parameters concerned with calcium and phosphorous homeostasis. Three of the most important of these biological regulators are calcitonin, parthyroid hormone and vitamin D. It is the purpose of this article to review some of the recent developments that have occurred specifically with regard to our understanding of the chemistry, metabolism and mechanism of action of vitamin D.

Perhaps the most striking advance has been the recognition that the mode of action of vitamin D, which may chemically be classified as a steroid* is highly similar to that of many other classical steroid hormones, such as estrogen, aldosterone, hydrocortisone or testosterone [1]. Norman and Henry [2, 3] have elaborated on the thesis that 1α,25-dihydroxyvitamin D_3 [1α,25-$(OH)_2$-D_3] is a steroid hormone produced by the kidney in response to various physiological signals. With this concept in mind, it is possible to identify new relationships between the molecular topology of vitamin D_3 (the hormone), its secretory organ (the kidney), its target tissues (the intestine, skeleton and perhaps kidney), and various disease states related to vitamin D.

It is now firmly established that vitamin D_3 undergoes an obligatory two step metabolism prior to the production of the biologically active species, 1α,25-$(OH)_2$-D_3. First vitamin D_3 is transported to the liver where it becomes hydroxylated at the end of its side chain on carbon-25. After hydroxylation by the liver, 25-OH-D_3 is transported to the kidney where it undergoes a second hydroxylation at carbon 1 to produce 1α,25-$(OH)_2$-D_3. The 1α,25-$(OH)_2$-D_3 then is transported *via* the plasma to the target tissues—the intestine, bone and possibly the kidney—where the biological responses attributable to vitamin D occur.

Our laboratory has proposed [4, 5] that the mode of action of 1α,25-dihydroxyvitamin D_3 is very much akin to that of other classical steroid hormones. That is, it produces its biological response by association with first a cytoplasmic and then nuclear receptor in the target cell where it stimulates a "read-out" of genetic information which is essential to the development of the characteristic physiological response. As documented in Fig. 1, it has been possible to correlate changes in the renal output of 1α,25-$(OH)_2$-D_3 and its localization in the tissue receptors in the target intestine with coordinate changes in biological response. The controlling signal appears to be related to the calcium-demand of the organism. Thus, under circumstances of a high calcium demand, there is an increased activity of the kidney enzyme which produces 1α,25-$(OH)_2$-D_3, an increased localization in the intestine and a larger biological response.

A key point of control in this endocrine system is the regulation of the output by the kidney of 1α,25-$(OH)_2$-D_3. The enzyme responsible for this hydroxylation, the 25-OH-vitamin D_3-1α-hydroxylase, is known to be structurally as well as functionally related to other mixed-function oxidases present in

* According to the International Union of Pure and Applied Chemistry Commission on the nomenclature of biological chemistry, vitamin D_3 (cholecalciferol) may be defined as a steroid, in particular a seco-steroid. The chemical name is 9,10-secocholesta-5,7,10(19)-trien-3β-ol. A *seco*-steroid is one where one of the rings has undergone fission. In the case of vitamin D_3 this is the B ring.

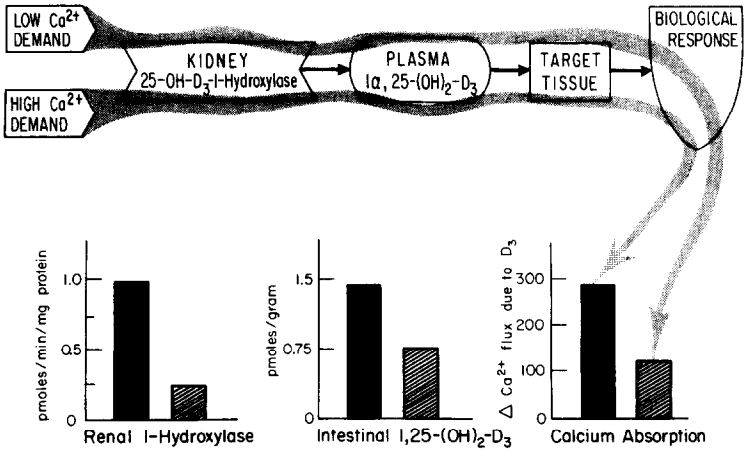

Fig. 1. Endocrine system for the production of 1α,25-$(OH)_2$-vitamin D_3. The production of 1α,25-$(OH)_2$-D_3 by the kidney is known to be regulated in a manner related to the calcium demand of the organism [8]. Thus under conditions of high Ca demand, the specific activity of the renal 25-OH-vitamin D_3-1α-hydroxylase is elevated and the production of 1α,25-$(OH)_2$-D_3 is increased. Consequently more 1α,25-$(OH)_2$-D_3 localizes in the target intestine resulting in an increased rate of intestinal calcium absorption.

steroidogenic tissues [6]. Such enzymes, including the 1α-hydroxylase, are composed of a flavoprotein, iron-sulfer protein (adrenodoxin) and cytochrome P-450 [6]. The oxygen of the hydroxyl is known to be derived from molecular oxygen [7]. Further the concentration of the renal 25-OH-vitamin D_3-1α-hydroxylase is itself believed to be related to the "calcium demand" of the organism [8]. The calcium demand is transmitted to the kidney through changes in the ratio of (PTH status)/(vitamin D status). As this ratio becomes elevated, the rate of biosynthesis of the enzyme increases, resulting in a higher steady-state concentration of the 1α-hydroxylase and a higher rate of output of 1α,25-$(OH)_2$-D_3 [8]. In addition, Ca^{2+}, phosphate and 1α,25-$(OH)_2$-D_3 can act directly on the 1α-hydroxylase as inhibitory modulators all capable of reducing the output of 1α,25-$(OH)_2$-D_3 [6]. Thus the output of the steroid can be regulated in accordance with the calcium demand of the organisms.

All data obtained to date support the view that 1α,25-$(OH)_2$-D_3 is the molecular form of vitamin D responsible for production of the physiological responses characteristic of this vitamin. The following observations support this contention: (a) 1α,25-$(OH)_2$-D_3 localizes primarily in the target intestine [4, 9] and bone [10], but not in presumed non-target tissues such as the liver or spleen; (b) it is the predominant form of vitamin D_3 found in the chick intestine after a physiological dose of the vitamin [4]; (c) it has a biological activity 5–10 times greater than the parent vitamin D_3 in terms of stimulating intestinal calcium transport [11] and mobilizing calcium from bone [10]; (d) in the intestine the 1α,25-$(OH)_2$-D_3 is associated exclusively with the nucleus and its chromatin [4, 5, 9]; (e) the intestinal nuclear binding sites for 1α,25-$(OH)_2$-D_3 are selective and finite [4, 9]; (f) the appearance of 1α,25-$(OH)_2$-D_3 in the intestine correlates well with the subsequent development of the physiological response of an increased intestinal calcium transport [4]; (g) actinomycin D, an inhibitor of DNA directed RNA synthesis, blocks development of the physiological response which normally ensues after administration of either vitamin D_3 or 1α,25-$(OH)_2$-D_3 [12]; and (h) 1α,25-$(OH)_2$-D_3 stimulates the synthesis of both RNA [13] and new species of protein in the target intestine.

In the remainder of this article we will review the relationship and significance of two very recent developments in the vitamin D field to the foregoing proposed steroid hormone mechanism of action of vitamin D. These are: (a) elucidation of the molecular topology of vitamin D and analogs in solution; and (b) development of a steroid receptor assay for 1α,25-$(OH)_2$-D_3. Together these two advances permit, for the first time in the vitamin D field, a detailed structure–function analysis.

The recognition of 1α,25-$(OH)_2$-D_3 as the active form of vitamin D prompted this and many other laboratories, to begin intensive efforts to chemically synthesize this new metabolite. During the course of our investigations, we obtained access to a 300 MHz proton magnetic resonance [p.m.r.] spectrometer, which, in conjunction with lanthanide induced shift (LIS) reagent studies [14], vastly simplified structural proofs of rather complex synthetic intermediates. The p.m.r.–LIS reagent studies at 300 MHz also made it possible to determine the dynamic molecular structure of vitamin D_3 and analogs of 1α,25-$(OH)_2$-D_3 in solution [15], which was of direct relevance to our parallel structure-function studies. The 300 MHz p.m.r.–LIS reagent method enabled us to analyze all of the resonances attributable to the A-ring protons and the 6,7,9β,19Z and 19E and methyl protons of vitamin D_3. What turned out to be of especial interest was that detailed information on the dynamic solution conformation of the A-ring and seco-B ring portions of vitamin D_3 evolved. As shown in Fig. 2, vitamin D was originally represented as I on the basis of the original chemical structural studies to

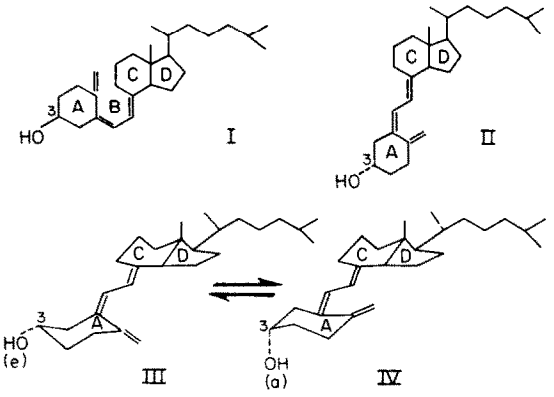

Fig. 2. Evolution of conformation representations of vitamin D. Representation I resulted from the original chemical structure determination. The first X-ray crystallographic analysis indicated the presence of a single A-ring chair conformation as shown in III, but this was normally simplified to that shown in II. Our recent report [14] and this paper emphasize that in solution there is a rapid equilibration between the two A-ring chair conformations, as shown in III and IV. In structures III and IV, the (e) and (a) refer respectively to the equatorial and axial orientations of the 3-hydroxyl which in both instances is geometrically β.

relate it to its precursor steroids. The X-ray crystallographic analysis of Hodgkins *et al.* [16] indicated the presence of a single A-ring chair conformation with the *seco*-B part extended by a 180° rotation about the single bond linking C-6 and C-7 in a "non-steroid" like topology as shown in III. The formula III was quickly simplified by workers in the field to the planar perspective formula II. This simplification unfortunately placed less emphasis on the fact that the A-ring should be expected to exist as a pair of chair conformers, rapidly equilibrating with one another. The p.m.r.–LIS results, recently reported from our laboratories, can only be interpreted if such a dynamic equilibrium is occurring between an approximately equimolar mixture of conformers, III and IV. The spectra of the side chain analogs, vitamin D_2 and octanorvitamin D_3 [17] revealed no change in chemical shifts or fine structure of the resonances assigned to the A-ring, the seco-B-ring, and the 9β protons. Thus, the side chain has no effect on the conformation of the A- and *seco*-B rings.

During the course of these biochemical studies, certain structure–function relationships between the steroid, 1α,25-$(OH)_2$-D_3 and its interaction with its biological system became apparent. Okamura *et al.* [18] recently reviewed and discussed possible relationships between the molecular topology and biological function for vitamin D. The exceptional importance of the 1α-hydroxyl group of 1α,25-$(OH)_2$-D_3 to the active form of D_3, was emphasized. By way of review, this emphasis seemed justified on the basis of the following observations.

i. 1α,25-$(OH)_2$-D_3 is fully active in its ability to produce stimulation of intestinal calcium transport in nephrectomized rats while the parent D_3 and its metabolite 25-OH-D_3 are inactive [19].

ii. 1α-OH-D_3, a chemically synthesized analog of 1α,25-$(OH)_2$-D_3 lacking the 25-OH-group, is active in nephrectomized rats [20].

iii. 3-D-1α-OH-D_3, another synthetic relative of 1α,25-$(OH)_2$-D_3 lacking both the 3β-and 25-OH groups, is able to stimulate a greater maximal intestinal calcium response than can saturating dose levels of 1α,25-$(OH)_2$-D_3 [21].

iv. Significant biological activity is exhibited by several pseudo-1α-hydroxyl containing analogs, which possess carbon frameworks brought about by a formal 180° rotation about the 5,6-bond of D_3 [22]. This fortuitously places the 3β-OH in the same geometric orientation as the 1α-OH of 1α,25-$(OH)_2$-D_3, and 3-D-1α-OH-D_3. The pseudo-1α-hydroxyl containing analogs include DHT_3, 25-OH-DHT_3, 5,6-*trans*-D_3, 25-OH-5,6-*trans*-D_3, and isotachysterol$_3$ (see Fig. 3).

The four points just enumerated suggested a minimal planar blueprint necessary for biological activity which incorporates a 1α-OH [or pseudo-1α-OH],* a side chain, R, on a seco-steroid backbone, but does not include a 3β-OH or C-19 carbon atom. The side chain, R, requirements have clearly not been defined, but it is definite that the nature of the side chain is important (R. Johnson, Riverside-Unpublished observations).

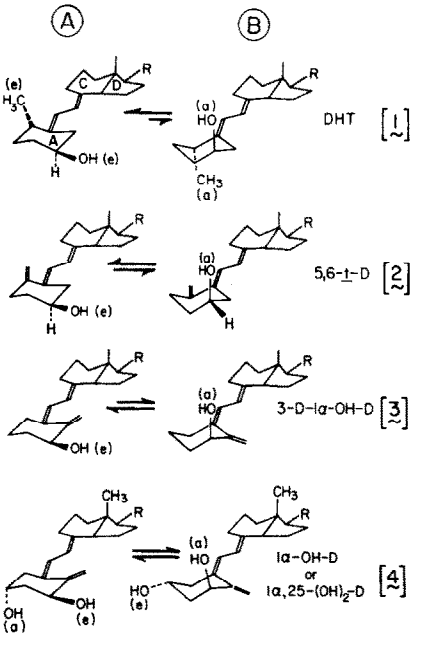

Fig. 3. Conformations of equilibrating chair forms of dihydrotachysterol [DHT] (top row, 5,6-*trans*-D[5,6-*t*-D_3] (second row), 3-deoxy-1α-OH-vitamin D_3 [3-D-1α-OH-D_3] (third row), and 1α-OH-D_3 or 1α,25-$(OH)_2$-D_3 (bottom row).

* It is convenient to class vitamin D-like molecules as analogs of 1α,25-$(OH)_2$-D_3 if they possess a hydroxyl in a position geometrically similar to the 1α-OH in 1α,25-$(OH)_2$-D_3. Such a hydroxyl group can be of a 1α-OH or some other hydroxyl such as the 3β-OH when the A-ring has undergone 180° rotation.

The usefulness of the p.m.r.–LIS studies for determining dynamic 3-dimensional structures in solution suggested studies of other vitamin D-like molecules. Our attention focused first on dihydrotachysterol$_3$, because of its high biological activity [23]. Our recently reported p.m.r.-LIS results established that the A-ring exists nearly completely in the single chair form (Fig. 3) with both the C-10 methyl and C-3 hydroxyl equatorially oriented as expected. A semi-quantitative conformational analysis of the two chair conformers of DHT_3 (or DHT_2) indicates that the observed conformer should be favored over its diaxial counterpart by *ca.* 2·2 kcal. Thus the equilibrium mixture should contain $\approx 97\%$ of the observed equatorial conformer at 25°C. The presence of the pseudo-1α-hydroxyl group in DHT_3 appears essential for biological activity and now, it has been shown that this hydroxyl in DHT_3 is equatorially oriented.

Another analog, 5,6-*trans*-D_3, differs from DHT_3 only in the oxidation state of the C-10–C-19 bond. In DHT_3, the C-10–C-19 bond is an α-oriented single bond and in 5,6-*t*-D_3 the C-10–C-19 bond is a double bond. How should this structural difference affect the orientation of the pseudo-1α-hydroxy of 5,6-*t*-D_3? The two chair-like conformers (2a and 2b, Fig. 3) of the latter should be more nearly equal in energy and again a semi-quantitative analysis of 2a and 2b suggests that their free energy difference should be *ca.* 0·4 kcal with the e-form preferred (66%).

A close relative of DHT_3 is a compound known as DHV-IV. It differs from DHT_3 only in that it has the opposite configuration at C-10, that is its methyl group is β. This has the consequence that the predominant A-ring conformer in solution should exist with its pseudo-1α-hydroxyl *ca.* 91% in the axial configuration. It is also significant that DHV-IV has little or no biological activity [24].

In summary, of the three psuedo-1α-hydroxyl containing analogs (DHT_3,5,6-*t*-D_3 and DHV-IV), DHT_3 appears to be the most biologically active. The A-ring of each member of this triad can exist as a pair of dynamically interconverting chair conformers. In one of these, the pseudo-1α-hydroxyl assumes an equatorial orientation and in the other it is axial. The relative proportions of these conformers depend upon the nature and/or sterochemistry of the C-10–C-19 bond as described above. We recently proposed [18] that the conformer with the 1α-hydroxyl or pseudo-1α-hydroxyl equatorial is the preferred biologically active form. Thus, it is predicted that the biological activities and the relative amount of the conformer with the pseudo-1α-hydroxyl equatorial should follow the same order for the trio of compounds discussed above: $DHT_3 > 5,6\text{-}t\text{-}D_3 >$ DHV-IV.

With these initial considerations in mind, it was instructive to analyze the A-ring conformations available to 1α,25-$(OH)_2$-D_3 and other putative analogs (see Fig. 3). It seems possible to design analogs which would possess high biological potency and which would provide further critical tests of our new theory. For our initial exercise, we considered 1α-OH-D_3 and the molecule produced by removing its 3β-OH, which is named 3-deoxy-1α-hydroxyvitamin D_3 (3-D-1α-OH-D_3). For 1α-OH-D_3, the A-ring should exist as a nearly equimolar mixture of the two conformers. There can only be an equatorial-axial or axial-equatorial combination of the two hydroxyls in this molecule and the environment of the axial hydroxyl component is nearly identical in each conformer (the free energy difference should be *ca.* 0 kcal/mol). By removing the 3β-OH of 1α-OH-D_3 to give 3-D-1α-OH-D_3, it is readily predicted on the basis of our new theory that the latter should be more active. In the A-ring of 3-D-1α-OH-D_3, which contains only the single hydroxyl, the equatorial form should be favored by about *ca.* 0·4 kcal ($\sim 66\%$ equatorial). We have in fact synthesized 3-D-1α-OH-D_3 and have compared its biological activity with D_3, 1α-OH-D_3, and 1α,25-$(OH)_2$-D_3. At saturating dose levels of the latter three substances, they were able to elicit the same maximum stimulation of intestinal calcium transport [21]. This is reasonable since the first two substances are apparently metabolized to the third so their identical biological activities are expected.

Our conformational model as embodied in the 3-dimensional blueprint has certain implications for several facets of the multi-stepped action of vitamin D. It is possible to envision that the active site of the renal 25-OH-vitamin D_3-1α-hydroxylase forms the covalent bond between C-1 and the oxygen of the incoming hydroxyl so that it is inserted not only with the necessary 1α-configuration, but also with only an equatorial or axial orientation. Whichever conformer is formed would then rapidly equilibrate once the product was released from the 1α-hydroxylase.

A more important problem concerns the consequences of our new hypothesis in terms of the interaction that must occur between the steroid hormone, 1α-25-$(OH)_2$-D_3 or its analog, and the target intestine with its receptors. It is known that steroid hormones [25, 26], including 1α, 25-$(OH)_2$-D_3 [5], elicit biological response by multi-stepped processes. First the steroids bind to a cytoplasmic receptor protein which then becomes "activated" so that the steroid–receptor complex may move to the nucleus and associate in a specific fashion with the chromatin. Then, in some as yet undefined processes, the production of new RNA and protein ensues leading to the development of the biological response. Shown in Fig. 4 are some data supporting the existence of such a system for the interaction of 1α,25-$(OH)_2$-D_3 in the intestine.

The results of a dose–response study in which increasing quantities of [^{3}H]-1α,25-$(OH)_2$-D_3 were administered intracardially to rachitic chicks are presented in Fig. 4a. Three hours after receiving the indicated doses of steroid, the birds were sacrificed and intestinal chromatin fractions prepared by our standard procedures [5]. A nonionic detergent, Triton X-100, was used to wash the chromatin fraction free of nonspecifically bound steroid. These data provide

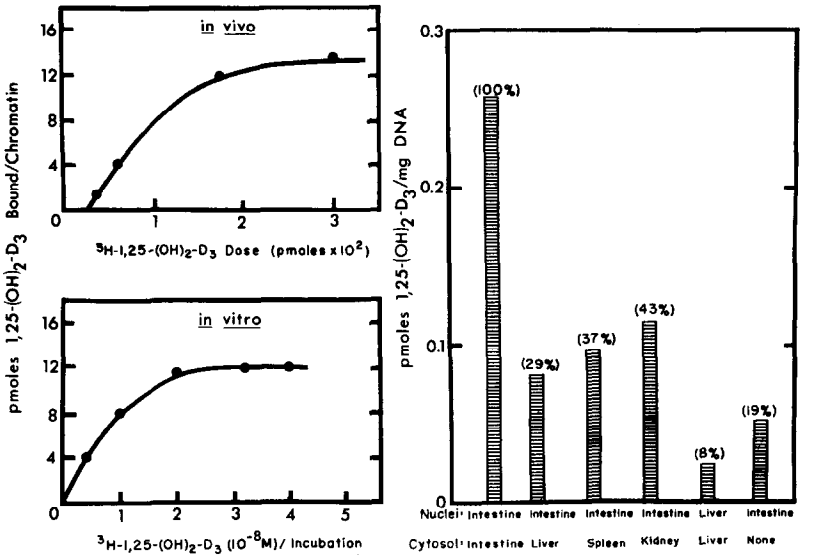

Fig. 4. Evidence for the existence of a cytoplasmic receptor and saturable chromatin receptor for 1α,25-$(OH)_2$-D_3 in the intestine. (A) Saturation of intestinal chromatin receptor *in vivo* 3 h after intracardial administration of doses of [^{3}H]-1α,25-$(OH)_2$-D_3. (B) Saturation of intestinal chromatin after incubation *in vitro* of [^{3}H]-1α,25-$(OH)_2$-D_3 with homogenates of intestinal mucosal (from vitamin D-deficient chicks), followed by subsequent isolation of the chromatin. (C) Tissue specificity of cytoplasmic and chromatin binding of 1α,25-$(OH)_2$-D_3. Cytosol protein (100,000 *g* supernatant) was obtained from different chick tissues and preincubated with [^{3}H]-1α,25-$(OH)_2$-D_3. An intestinal crude nuclear fraction (600 *g* pellet) was then added to the "labelled cytosol" during a second incubation. Next the Triton-X-100-washed chromatin was prepared. In 4a, b and c the chromatin was isolated by the procedure of Tsai *et al.*[5].

evidence for a finite number of binding sites for 1α,25-$(OH)_2$-D_3 in the chromatin fraction.

In an effort to duplicate under *in vitro* circumstances the results obtained from doses of 1α,25-$(OH)_2$-D_3 given *in vivo*, the radioactive steroid was initially incubated with homogenates of intestinal mucosa [5] at 25° for 60 min. The purified intestinal chromatin fraction was then isolated and the radioactivity determined. As shown in Fig. 4b, saturation of the chromatin fraction was achieved at a level of *ca.* 11-12 pmol of specifically bound steroid per chick intestinal chromatin. This result is in excellent agreement with previous results obtained under *in vivo* conditions where *ca.* 14 pmol of steroid was found in the chromatin fraction following the administration of a saturating dose of [^{3}H]-1α,25-$(OH)_2$-D_3.

Studies of the tissue specificity of the cytoplasmic receptor for 1α,25-$(OH)_2$-D_3 were performed and the results summarized in Fig. 4c. These data indicate that there is a discrete requirement for the presence of the intestinal cytoplasm fraction for transfer of the 1α,25-$(OH)_2$-$D_3$to the chromatin. As shown in the histogram (Fig. 4c), only 19% of the maximum chromatin localization occurs when 1α,25-$(OH)_2$-D_3 is incubated with purified intestinal nuclei alone. Further, the cytosol of the target tissue, the intestine, is the best mediator for the transfer of 1α,25-$(OH)_2$-D_3 to the nuclei of intestinal mucosa.

In toto, these data support the conclusion that 1α,25-$(OH)_2$-D_3 must first bind with a specific receptor protein present in the cytoplasm of the target intestinal mucosa cells, and then this is followed by movement of the steroid into the nuclei where it associates with a finite number of specific chromatin receptor sites.

The ability to duplicate, *in vitro*, the conditions which permit the binding of 1α,25-$(OH)_2$-D_3 to its specific chromatin receptor has enabled us to develop a receptor binding assay for the steroid. The experimental protocol includes preparing the receptor system by isolating Triton-X-100-washed chromatin from a homogenate prepared from the intestines of rachitic chicks, and then recombining it with the cytosol fraction of the same intestinal homogenate (see the legend to Fig. 5).

As shown in Fig. 5, the addition of cold 1α,25-$(OH)_2$-D_3 to the assay mixture results in a proportional decrease in the maximal amount of labelled steroid bound to the chromatin. This decrease in radioactivity bound is due to a dilution of the pool of radioactive steroid such that 50% of the maximal radioactivity bound occurs at a ratio of [cold 1α,25-$(OH)_2$-D_3]/[labelled 1α,25-$(OH)_2$-D_3] equal to 1. Thus, this assay provides a very useful tool for assaying 1α,25-$(OH)_2$-D_3.

With the development of a receptor assay for 1α,25-$(OH)_2$-D_3, it became possible to screen various structural analogs for their ability to compete with 1α,25-$(OH)_2$-D_3 for its receptor system. Studies of this nature afford an excellent opportunity to obtain data which help define the essential structural elements required for the association of 1α,25-$(OH)_2$-D_3 with its chromatin receptor. The results of these studies, as shown in Fig. 5, indicate that both 25-OH-D_3

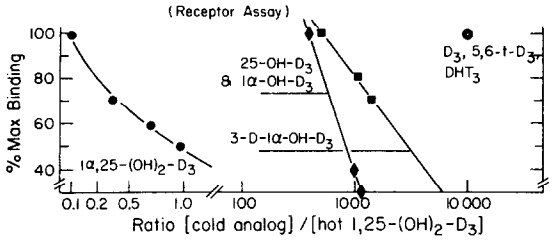

Fig. 5. Competition curves for structural analogs of 1α,25-$(OH)_2$-D_3 binding to the intestinal chromatin receptor. Chromatin and the cytosol fraction prepared from the same 40% homogenate of intestinal mucosa obtained from rachitic chicks were recombined such that there was 0·05 chick chromatin per ml of cytosol. One ml volumes of the reconstituted mixture were then added to incubation tubes containing 20 pmol of [^{3}H]-1,25-$(OH)_2$-D_3 and cold analog dissolved in 100 μl of ethanol. Following an incubation for 60 min at 25°C, the chromatin was isolated and washed twice with 0·5% Triton-X-100 in 0·01 M Tris, pH 8·5. Specifically bound 1,25-$(OH)_2$-D_3 was extracted with a 2:1 mixture of methanol and chloroform and the radioactivity determined. The value 100% maximal radioactivity bound is defined as that amount of radioactivity bound to the chromatin in the absence of cold analog.

and 1α-OH-D_3 and 1α-OH-D_3 are the most effective of the analogs tested in competing with 1α,25-$(OH)_2$-D_3 for the receptor system, but that a 900-fold excess concentration of these analogs is required for a 50% decrease in the maximal binding of 1α,25-$(OH)_2$-D_3. The compound, 3-D-1α-OH-D_3 is required in a 5000-fold greater concentration than 1α,25-$(OH)_2$-D_3 to decrease the binding of the steroid by 50%. The pseudo-1α-hydroxyl analogs 5,6-*t*-D_3 and DHT_3 as well as D_3 do not effectively compete with 1α,25-$(OH)_2$-D_3 even at concentrations 10,000 times greater than that of 1α,25-$(OH)_2$-D_3.

The results from these competition studies support the conclusion that the receptor system is highly specific for 1α,25-$(OH)_2$-D_3. In terms of the structural elements required for interaction with the receptor system, it is apparent that hydroxyl groups at both the 1α and 25 positions play an important role. Competition studies employing 25-OH-D_3 and 1α-OH-D_3 indicate that the loss of an hydroxyl group at either the 1α or 25 position, respectively, results in an analog with a greatly diminished ability to compete with 1α,25-$(OH)_2$-D_3. The inability of D_3 to successfully compete with 1α,25-$(OH)_2$-D_3 even at excessively great concentrations further emphasizes the importance of hydroxyl groups at the 1α and 25 positions. It can also be argued that a hydroxyl group at the 3β position is not as important as a hydroxyl at the 1α-position since 3-D-1α-OH-D_3 will compete with 1α,25-$(OH)_2$-D_3 at a concentration that D_3 will not. In addition, the inability of either DHT_3 or 5,6-*t*-D_3 to compete with 1α,25-$(OH)_2D_3$ at a concentration that 3-D-1α-OH-D_3 will compete suggests that a *cis* geometry of the A-ring is preferred over a *trans* geometry.

Summarized in Table 1 are the results of the competition studies and also the relative biological activities of the analogs compared to 1α,25-$(OH)_2$-D_3. The much greater relative amounts of these analogs required to cause a 50% decrease in the binding of 1α,25-$(OH)_2$-D_3 to its chromatin receptor, *in vitro*, than that required for 50% of the maximal biological response, suggests that these analogs probably do not interact directly with the chromatin receptor under *in vivo* conditions. Rather, it seems likely that these analogs are further metabolized to compounds with a greater ability to bind to the chromatin receptor. The well known metabolism of D_3 and 25-OH-D_3 to 1α,25-$(OH)_2$-D_3 certainly is consistent with this proposal. By the same token, it seems reasonable that 1α-OH-D_3 may be further metabolized *via* a hydroxylation at the 25 position in the liver to give the active metabolite 1α,25-$(OH)_2$-D_3. In the case of 3-D-1α-OH-D_3, which lacks hydroxyls at both the 3β and 25 positions, this analog may be further hydroxylated in the liver to yield 3-D-1α,25-$(OH)_2$-D_3. This compound, by virtue of now possessing hydroxyl groups at the two most critical positions, namely the 1α and 25 positions, should have a greater ability to bind to the chromatin receptor. The pseudo-1α-hydroxyl analogs 5,6-*t*-D_3 and DHT_3 may also be further hydroxylated at the 25 position to generate products which should be better able to interact with the 1α,25-$(OH)_2$-D_3 receptor system.

From the foregoing it is apparent that there is a requirement for both the intestinal cytosal and nucleus for transfer of 1α,25-$(OH)_2$-D_3 to the highly spe-

Table 1. Structure–Function relationships for analogs of 1α,25-$(OH)_2$-D_3

Compound	Missing hydroxyl	Relative activity* Intestinal chromatin receptor	Intestinal calcium transport†
1α,25-$(OH)_2$-D_3	None	1·0	1·0
1α-(OH)-D_3	25-OH	900	2·4
25-OH-D_3	1α-OH	900	4·8
3-D-1α-OH-D_3	3β-OH,25-OH	5000	34·7
5,6-trans-D_3	Pseudo-3β-OH,25-OH	> 10,000	7000
DHT_3	Pseudo-3β-OH,25-OH	> 10,000	194
D_3	1α-OH,25-OH	> 10,000	6·7

* Relative activity is defined as the concentration relative to 1α,25-$(OH)_2$-D_3 required to produce 50% of the maximum attainable biological response or competition in the intestinal steroid-receptor assay.

† Intestinal calcium transport was determined by the method of Hibberd and Norman[23].

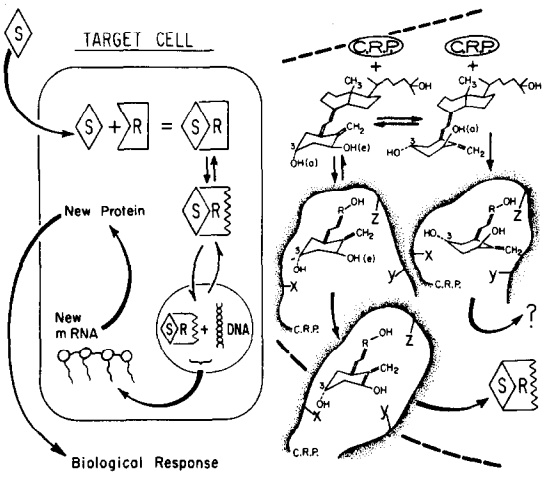

Fig. 6. Proposed two-step mode of action of $1\alpha,25\text{-}(OH)_2\text{-}D_3$ in the intestine. S = $1\alpha,25\text{-}(OH)_2\text{-}D_3$; R = CRP = cytoplasmic receptor protein.

cific receptors in the chromatin. A necessary consequence of this general model for steroid hormone action with regard to our proposed three-dimensional blueprint for biological activity is that some aspect or component of the system must discriminate or differentiate between the axial and equatorial conformers of this compound. An example of how this might occur in the intestinal system is presented in Fig. 6. This is necessary to explain the preferential biological activity of steroids which exist primarily in the equatorial form.

It remains to the future to delineate the detailed biochemical and chemical facets of the proposed hormonal mode of action of vitamin D. The concept of conformational optimization of biological activity of vitamin D-like compounds, as proposed in this paper, is clearly one which should be further examined.

Acknowledgements—This work was supported in part by USPHS Grants AM-09012, AM-14,750 and AM-16,595. AWN is the recipient of a Career Research Development Award 1-KD-AM-13,657.

REFERENCES

1. Norman A. W.: *Biol. Rev.* **43** (1968) 97–137.
2. Norman A. W. and Henry H.: *Clin. Orthop. Related Res.* **98** (1974) 257–287.
3. Norman A. W. and Henry H.: *Recent Prog. Horm. Res.* **30** (1974) 431–480.
4. Tsai H. C., Wong R. G. and Norman A. W.: *J. biol. Chem.* **247** (1972) 5511–5519.
5. Tsai H. C. and Norman A. W.: *J. biol. Chem.* **248** (1973) 5967–5975.
6. Henry H. and Norman A. W.: *J. biol. Chem.* **249** (1974) 7429–7535.
7. Ghazarian J. G., Schnoes H. K. and DeLuca H. F.: *Biochemistry* **12** (1973) 2555–2558.
8. Henry H., Midgett R. J. and Norman A. W.: *J. biol. Chem.* **249** (1974) 7584–7592.
9. Haussler M. R. and Norman A. W.: *Proc. natn. Acad. Sci. U.S.A.* **62** (1969) 155–162.
10. Wong R. G., Myrtle J. F., Tsai H. C. and Norman A. W.: *J. biol. Chem.* **247** (1972) 5728–5735.
11. Norman A. W. and Wong R. G.: *J. Nutrit.* **102** (1972) 1709–1718.
12. Tsai H. C., Midgett R. J. and Norman A. W.: *Archs Biochem. Biophys.* **157** (1973) 339–347.
13. Tsai H. C., Norman A. W.: *Biochem. biophys. Res. Commun.* **54** (1973) 622–627.
14. Wing R. M., Uebel J. J. and Anderson K. K.: *J. Am. chem. Soc.* **95** (1973) 6046–6056.
15. Wing R. M., Okamura W. H., Pirio M. R., Sine S. M. and Norman A. W.: *Science* **186** (1974) 939–941.
16. Hodgkin D. C., Rimmer B. M., Dunitz J. D. and Trueblood K. N.: *Chem. Soc.* **947** (1963) 4954–4956.
17. Velluz L. and Amiard J. B. S.: *C. r. hebd. Séanc. Acad. Sci., Paris* **244** (1957) 1794–1796.
18. Okamura W. H., Norman A. W. and Wing R. M.: *Proc. natn. Acad. Sci., U.S.A.* **71** (1974) 4194–4197.
19. Wong R. G., Norman A. W., Reddy C. R. and Coburn J. W.: *J. clin. Invest.* **51** (1972) 1287–1291.
20. Holick M. F., Semmler E. J., Schnoes H. K. and DeLuca H. F.: *Science* **180** (1973) 190–191.
21. Okamura W. H., Mitra M. N., Wing R. M. and Norman A. W.: *Biochem. biophys. Res. Commun.* **60** (1974) 179–184.
22. Holick M. F., Garabedian M., and DeLuca H. F.: *Biochemistry* **11** (1972) 2715–2719.
23. Hibberd K. and Norman A. W.: *Biochem. Pharmac.* **18** (1969) 2347–2355.
24. von Werder F.: *Z. physiol. Chem.* **260** (1939) 119–134.
25. Jensen E. V., Numata M., Brecher P. I. and DeSombre E. R.: *In The Biochemistry of Steroid Hormone Action* (Edited by R. M. S. Smellie). Academic Press, New York (1971) pp. 133–159.
26. O'Malley B. W., McGuire W. L., Kohler P. O. and Korenman S. G.: *Recent Prog. Horm. Res.* **25** (1969) 105–160.