*CHROMOSOMAL RECEPTOR FOR A VITAMIN D METABOLITE**

By Mark R. Haussler and Anthony W. Norman

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CALIFORNIA (RIVERSIDE)

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Abstract and Summary.—Evidence has been presented for the existence of an acidic protein(s) or protein portion of a more complex molecule which has a high affinity for binding noncovalently a biologically active metabolite of vitamin D. This molecule could be solubilized from the residual chromatin via treatment with either 0.3 *M* KCl or high pH and has been purified 167-fold over the crude mucosa homogenate. Characterization of the still crude receptor fraction showed that it contains significant amounts of RNA and that it may exist in multiple forms, i.e., a 50,000–70,000 and a >200,000 molecular weight species. The binding capacity of the receptor fraction for the metabolite is saturated after administration of a physiological dose of the parent vitamin D.

The presence of vitamin D is essential to the development and continued operation of a mechanism for the absorption of calcium by the intestinal mucosa.[1–3] It has been proposed that vitamin D may generate this characteristic physiological response via its ability to activate or stimulate the biochemical expression of genetic information to induce the synthesis of enzymes or the alteration of membrane structure necessary for calcium absorption.[1, 2, 4–6] This hypothesis was based primarily on the observation that actinomycin D treatment completely blocked the intestinal response to vitamin D.[2, 4, 6] Subsequently, it was found that vitamin D could stimulate the incorporation of ^{3}H-uridine or ^{3}H-orotic acid into intestinal mucosa RNA.[7, 8]

Specific information as to how the vitamin might be initiating this proposed series of biochemical events requires a knowledge of the subcellular location of physiological amounts† of radioactive vitamin D_3 and its metabolites. We have previously reported that, after a physiological dose of ^{3}H-vitamin D_3, the radioactivity isolated from the target intestinal mucosa of rachitic chicks was located primarily in the nuclear fraction[9] and, more specifically, in the nuclear chromatin fraction(s). Organic solvent extraction and chromatography of the radioactivity present in the entire intestinal mucosa indicated the presence of at least three metabolites of vitamin D. However, 85–90 per cent of only one of these metabolites was associated exclusively with the chromatin fraction.[5] This metabolite, which is more polar than vitamin D, was found to have biological activity equivalent to that of the parent vitamin D.

In this communication, we wish to report the existence and isolation of a macromolecular receptor for the biologically active polar metabolite of vitamin D from the chromatin fraction of the intestinal mucosa.

Materials and Methods.—Animals: One-day-old white Leghorn cockerels, generously provided by H and N of California, Inc., were raised on a vitamin D–deficient diet[2] for approximately 4 weeks. By this time their growth had ceased and they exhibited the classical symptoms of rickets.[10]

Chemicals: 4–^{14}C-vitamin D_3 (Philips-Duphar, Amsterdam), specific activity 20.8

mc/mmole, and 1,2-^{3}H-vitamin D_3 (New England Nuclear Corp., Boston), specific activity 38.8 mc/mmole, were employed in most experiments. Generally labeled ^{3}H-vitamin D_3 (4.9 mc/mmole), ^{3}H-vitamin D_2 (5.1 mc/mmole), and ^{3}H-dihydrotachysterol$_2$ (2.5 mc/mmole), all prepared by the procedure of Wilzbach,[11] were utilized in the experiment which involved higher levels of the D− vitamins. The radiochemical purity of these labeled steroids was assessed by a reversed-phase chromatography system which is capable of separating vitamin D_2 from vitamin D_3,[12] and all were found to have a radiochemical purity of greater than 95%. Nonradioactive vitamin D_3 was obtained from the Philips-Duphar Co. The digestive enzymes pronase, ribonuclease (RNase), and deoxyribonuclease (DNase) were all obtained from Calbiochem, Los Angeles. Cesium chloride (99.9%) was purchased from General Biochemicals, Chagrin Falls, Ohio.

Preparation of subcellular fractions: All chicks were injected intracardially with the appropriate steroid dissolved in 0.20 ml of 1,2-propanediol. At the prescribed time interval, the chick was sacrificed and the small intestine removed, rinsed with 0.25 *M* sucrose in 0.05 *M* Tris-HCl, pH 7.5, 0.025 *M* KCl, and 0.005 *M* $MgCl_2$ (0.25 *M* sucrose-TKM), and then the mucosa was scraped free from the serosa with a microscope slide. After preparation of a 10% homogenate in 0.25 *M* sucrose-TKM, the crude nuclei were isolated by centrifugation at 800 × *g* for 10 min in a Sorvall model RC-2 centrifuge. Purified chromatin was prepared from the crude nuclei essentially by the procedure of Haussler, Myrtle, and Norman.[5]

The receptor fraction was routinely prepared by homogenizing the purified chromatin in a solution of 0.30 *M* KCl and 0.01 *M* Tris HCl, pH 7.5, with the aid of a Potter-Elvehjem homogenizer equipped with a Teflon pestle. This solution was then centrifuged at 27,000 × *g* for 1 hr to sediment the residual chromatin. The resulting supernatant fluid was then dialyzed against saturated $(NH_4)_2SO_4$ at 0°C for 5 hr to precipitate the receptor fraction. The precipitate which could be harvested via centrifugation at 27,000 × *g* for 1 hr was termed the crude receptor fraction.

Radioactivity measurements: All samples which were to be assayed for radioactivity content were extracted according to a slight modification of the procedure of Bligh and Dyer[13] as previously reported.[5] The resulting chloroform layer was evaporated to dryness with air in a liquid scintillation vial, and 10 ml of a counting solution consisting of 5 gm of phenylbiphenyl oxadiazole-1,3,4 (PBD) per liter of toluene was added. The sample was counted to 2% error in a Beckman CPM-200 liquid scintillation counter. The number of dpm of tritium and carbon-14 was determined by the use of subsequently added internal standards of ^{3}H-toluene or ^{14}C-benzene. The usual efficiency for carbon-14 was 65–70%, and for tritium it was 35–45%.

Chemical assays: The diphenylamine method of Dische[14] was utilized for DNA analysis, and the orcinol procedure[15] was employed for RNA analysis. In samples containing both RNA and DNA, the RNA data were corrected for the amount of DNA present. Protein was determined by the method of Lowry.[16]

Results.—That the active metabolite of vitamin D is bound to a macromolecular receptor in the intestinal chromatin fraction is shown in Figure 1. Pictured is the equilibrium centrifugation pattern in 4.0 *M* CsCl of mucosal chromatin obtained from rachitic chicks 15 hours after an intracardial dose of 20 IU (1.3 nmoles) of 4-^{14}C-vitamin D_3 (−D). Also shown is the pattern of mucosal chromatin obtained from normal chicks that had been dosed orally with 5000 IU (325 nmoles) of nonradioactive vitamin D_3 with the last dose coming three hours prior to an intracardial dose of 20 IU of 4-^{14}C-vitamin D_3. Under the conditions of the CsCl gradient generated, the DNA migrates to the bottom of the tube while the bulk of the protein floats to the top of the tube and forms a skin. The majority of the radioactivity associated with the −D chromatin assumed a position near the top of the tube. In contrast, the residual amount of

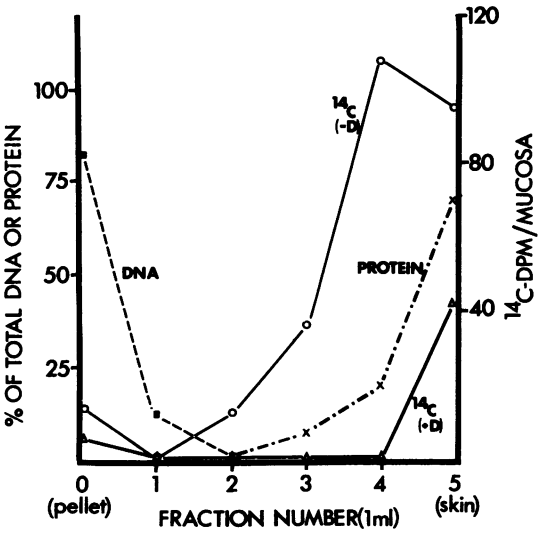

FIG. 1.—Centrifugation of intestinal mucosa chromatin through 4 *M* CsCl. Chromatin was prepared from intestinal mucosa of rachitic chicks (−*D*) or chicks which had been pretreated with an excess of nonradioactive vitamin D_3 (+*D*). Both groups of chicks received 20 IU of ^{14}C-vitamin D_3 15 hr prior to sacrifice. The chromatin was mixed into CsCl via homogenization to a final concentration of 4 *M* CsCl and then centrifuged at 165,000 × *g* in an SW-50L swinging bucket rotor at 3°C for 48 hr. Aliquots of the fractions were then analyzed for ^{14}C-radioactivity, DNA, and protein.

radioactivity associated with the +D chromatin was present only in the protein skin.

The results of Figure 1 prompted an examination of the chemical conditions or environment that might be utilized to dissociate the receptor + metabolite from the chromatin. A summary of the results obtained is shown in Table 1. In all these studies the end point followed was the removal from the chromatin of the radioactive metabolite. Acid conditions were not capable of significantly removing radioactivity, which suggests that the metabolite is not associated with a histone fraction. High pH conditions, on the other hand, were found to remove about 70 per cent of the chromatin-bound radioactivity. When the pH 10 or pH 12 extract was treated with trichloroacetic acid (TCA), the ^{14}C-metabolite was precipitated, an indication that it had been extracted while bound to a TCA-precipitable macromolecule. Successive treatment of the chromatin with 1 per cent Triton X-100 in 0.01 *M* Tris-HCl, pH 7.5, was incapable of removing

TABLE 1. *Exposure of chromatin to various chemical conditions.*

Treatment	Per cent removal of ^{14}C from chromatin*
Distilled H_2O	11
0.15 *N* H_2SO_4	8
0.03 *M* citrate, pH 4	14
0.03 *M* citrate, pH 6	0
0.01 *M* Tris, pH 7	6
0.01 *M* Tris, pH 8	12
0.01 *M* Tris, pH 10	67
0.02 *M* phosphate, pH 12	70
0.6 *M* KCl	83
1% Triton X-100 in 0.01 *M* Tris, pH 7.5 (3 washes)	10
1% Sodium lauryl sulfate	97

* Chromatin is isolated from intestinal mucosa of rachitic chicks which received 10 IU of ^{14}C-vitamin D_3. After treatment of the chromatin with the prescribed agent, it is reharvested by centrifugation and assayed for ^{14}C-radioactivity.

significant amounts of the ^{14}C-metabolite. This suggests that the ^{14}C-metabolite is tightly and specifically associated with some entity of the chromatin fraction, since treatment of subcellular fractions with this nonionic detergent has been shown to effect removal of nonspecifically bound lipids.[5]

By far the most gentle and effective means of solubilizing the metabolite while bound to a macromolecule was the treatment of the chromatin with moderate concentrations of KCl. The stepwise removal of proteins from the chromatin with increasing concentrations of KCl is shown in Figure 2. The removal of the ^{14}C-metabolite + receptor occurs over a narrow range of KCl concentration, from 0.15 to 0.30 *M* KCl. We have termed the resulting supernatant fraction, after removal of the residual chromatin, the crude receptor fraction.

A comparison of the chemical composition of the crude receptor fractions solubilized by 0.30 *M* KCl and 0.01 *M* Tris, pH 10, is given in Table 2. The KCl extraction method is used preferentially over the alkaline Tris extraction method since there is somewhat less total contaminating RNA and DNA. The KCl-extracted crude receptor has about 1.5 per cent of the total homogenate protein and 1.0 per cent of the homogenate RNA and 0.5 per cent of the homogenate DNA. It is presumed that the ^{14}C-metabolite is bound to a macromolecule in the crude receptor fraction, since prolonged dialysis results in no loss of radioactivity and because addition of $(NH_4)_2SO_4$ to 40 per cent saturation results

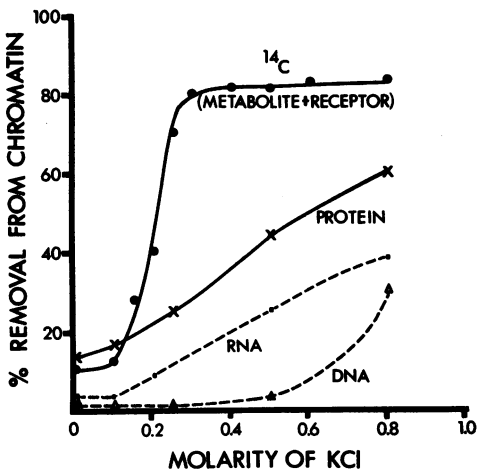

FIG. 2.—Selective solubilization of the chromatin-associated receptor with KCl. Chromatin, which had been prelabeled with ^{14}C-peak 4B, *in vivo*, was homogenized with various concentrations of KCl in 0.01 *M* Tris, pH 7.5 (25 ml). The residual chromatin was recovered by sedimentation at 27,000 × *g* for 1 hr in the RC-2 centrifuge. It was then assayed for ^{14}C-radioactivity, and the supernatant was assayed for DNA, RNA, and protein.

TABLE 2. *Chemical composition of receptor fractions solubilized from intestinal chromatin of one chick.*

Type of receptor fraction	Protein		RNA		DNA		Protein: RNA:DNA
	Mg	Per cent of homogenate	Mg	Per cent of homogenate	Mg	Per cent of homogenate	
0.3 *M* KCl*	11	1.5	0.25	1.0	0.07	0.5	157:3.5:1
0.01 *M* Tris, pH 10	8	1.1	0.32	1.3	0.11	0.8	73:11:1

* Chromatin was washed with 0.01 *M* Tris, pH 7.5, prior to solubilization with 0.3 *M* KCl in 0.01 *M* Tris, pH 7.5.

in precipitation of the ^{14}C-metabolite + receptor. The specific activity of the crude receptor fraction is 5.1 IU of metabolite/gm protein (0.331 pmole of metabolite/mg protein) as compared to a specific activity for the intestinal homogenate of 0.12 IU/gm protein (0.0078 pmole/mg protein). This represents a 42-fold purification. Further attempts at purification of the crude receptor fraction via classical fractionation techniques such as DEAE cellulose or Amberlite CG-50 ion exchange column chromatography have been unsuccessful owing to the apparent dissociation of the ^{14}C-metabolite from the receptor complex. Selective precipitation of the crude receptor with 15–30 per cent saturated $(NH_4)_2SO_4$ at 0°C followed by protamine sulfate precipitation of the redissolved precipitate produces an additional fourfold purification. Thus the highest specific activity obtained to date is 20 IU of metabolite/gm protein (1.30 pmoles/mg of protein) which is equivalent to an over-all purification of 167-fold.

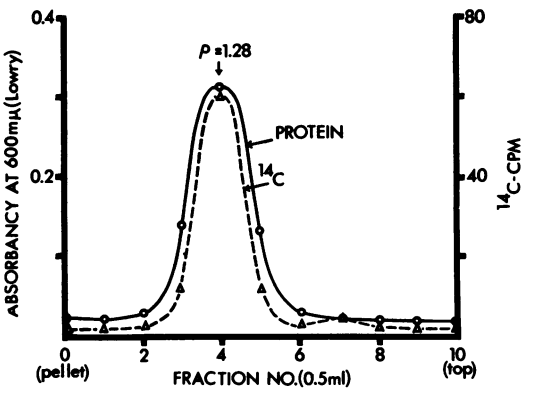

FIG. 3.—Equilibrium density gradient centrifugation of the receptor fraction in 2.0 *M* CsCl. Receptor fraction (^{14}C-metabolite-labeled) was prepared from four chicks and mixed into CsCl to a final concentration of 2.0 *M* CsCl. The samples were then centrifuged at 130,000 × *g* for 48 hr in a Spinco SW-50L rotor at 5°C. After the bottom of the tube was punctured, 0.5-ml fractions were collected. 20-μl portions were assayed for protein, and the balance of the fraction was extracted with $CHCl_3$-methanol (1:2) in order to determine the ^{14}C-steroid radioactivity. The density of the CsCl at the protein peak was not determined experimentally; the estimated value of $\rho = 1.28$ gm/ml was obtained from Maurer and Chalkley.[17]

As shown in Figure 3, the receptor and bound metabolite band together in a symmetrical peak at a density of 1.28 after centrifugation to equilibrium in 2.0 *M* CsCl. These results are somewhat analogous to that obtained by Maurer and Chalkley for a chromatin-bound receptor for estrogen.[17]

Further indications of the complex composition of the receptor fraction for the metabolite of vitamin D_3 are depicted in Figure 4. Chromatography of the crude receptor fraction + ^{14}C-metabolite on a 1.5 × 100-cm Sephadex G-200 column results in the appearance of two broad peaks of UV absorbance. The first emerges with the exclusion volume, while the second peak emerges at a point equivalent to a molecular weight of 50,000–70,000.[18] Both peaks contain the ^{14}C-metabolite, and the possibility of some sort of multimer is suggested by the high molecular weight of the first peak. We have obtained essentially analogous results after sucrose gradient centrifugation. As shown in Figure 4, incubation of the crude receptor fraction with RNase and DNase followed by Sephadex G-200 chromatography reduces the high-molecular-weight species and correspondingly increases the amount of the second peak. This perhaps indicates that

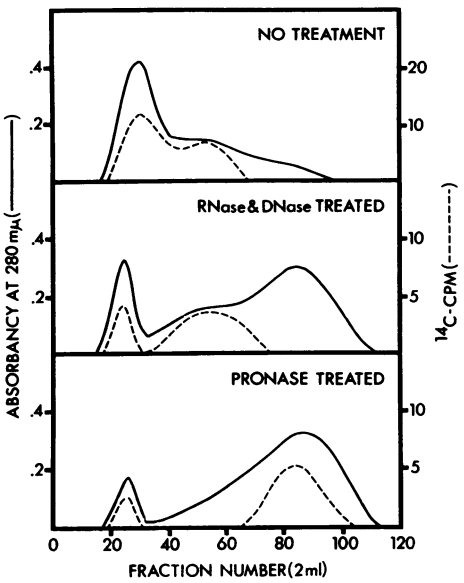

FIG. 4.—Sephadex G-200 column chromatography of untreated and enzyme-treated receptor fraction. Receptor fraction was prepared by 0.3 *M* KCl extraction of chromatin isolated from 12 chicks which each received 10 IU of ^{14}C-vitamin D_3 15 hr prior to killing. The fraction was concentrated to 6.0 ml via ammonium sulfate precipitation and divided into three equal parts. One aliquot was immediately chromatographed on Sephadex G-200. The other two portions were incubated for 15 hr at 20°C with either RNase (1 mg) and DNase (1 mg + 0.05 *M* Mg^{++}) or pronase (2 mg) and then chromatographed on Sephadex G-200.

nucleic acids may be involved in the formation of the multimer. In contrast, incubation of the crude receptor fraction with pronase for 15 hours at 20°C, followed by Sephadex G-200 chromatography, destroys the binding of the ^{14}C-metabolite to macromolecules, for the ^{14}C-metabolite now emerges from the column in the region of small molecules. Since the receptor molecule is destroyed by pronase, but not RNase or DNase, it is inferred that the metabolite is bound to a protein or protein portion of a more complex molecule.

As shown in Figure 5, the receptor has only a finite capacity to bind the vitamin D_3 metabolite. The binding capacity of the chromatin receptor is saturated after a dose of 25–30 IU (1.62–1.95 nmoles), and no increase is seen as the dose rises to 1000 IU (65 nmoles). This criterion of saturability is consistent with the concept that a specific site or molecule exists within the crude receptor fraction for the metabolite of vitamin D.

Also shown in Figure 5 are the results of administering to rachitic chicks increasing amounts of two vitamin D_3 analogs,† G-^{3}H-vitamin D_2 and G-^{3}H-dihydrotachysterol$_2$. Significantly less radioactivity from both these compounds is bound to the crude receptor fraction. Although a dose of 25–30 IU of G-^{3}H-vitamin D_2 saturates the receptor, it is pertinent to note that the plateau value of 0.02 IU (1.3 pmoles) per crude receptor from one chick is only 40 per cent of the plateau value observed for vitamin D_3. In the chick, vitamin D_2 has only one tenth the biological activity of D_3.

Discussion.—This paper provides evidence for the existence at the genome of a target tissue for vitamin D, the intestinal mucosa, of a macromolecular receptor for a biologically active metabolite of the vitamin. A previous report from this laboratory has documented that 45–55 per cent of the radioactivity present in the intestinal mucosa and 85–95 per cent of radioactivity present in the chromatin 8–15 hours after a dose of 10 IU (0.65 nmole) of radioactive vitamin D_3 exists as

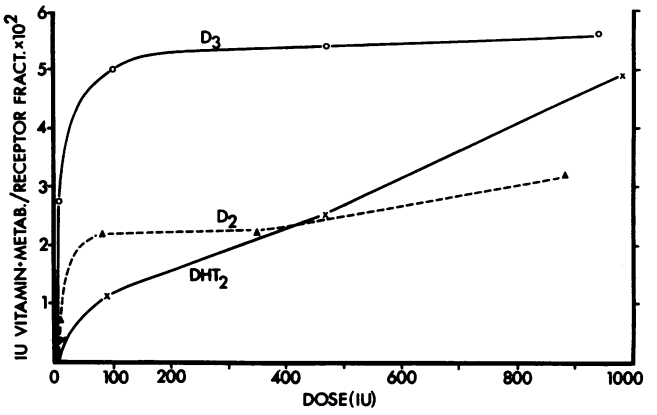

FIG. 5.—Saturation of the chromatin-associated receptor fraction as a function of dose of the various D vitamins. The indicated amount of G-^{3}H-vitamin D_3 (D_3), G-^{3}H-vitamin D_2 (D_2), or G-^{3}H-dihydrotachysterol$_2$ (DHT_2) was administered to rachitic chicks 15 hr prior to sacrifice. Intestinal chromatin (washed once with 1.0% Triton X-100) was then extracted with 0.3 *M* KCl and the solubilized receptor fraction assayed for tritium. Each number is the average of eight determinations on receptor fractions isolated from eight separate chicks.

a single polar metabolite which has biological activity equivalent to the parent vitamin.[5] The remainder of the whole mucosa radioactivity is divided between vitamin D and two other metabolites. Thus the macromolecule(s) present in the chromatin is binding the major biologically active form of the vitamin present in the target cell.

By making several simplifying assumptions, we can estimate the amount of receptor molecule that is present in the intestinal mucosa. From the plateau value of 0.05 IU (3.25 pmoles) of metabolite per one chick receptor fraction and an assumed 70 per cent yield of receptor, this can be corrected to 0.07 IU (4.5 pmoles) of metabolite per receptor from one chick intestinal mucosa (3 gm of tissue). Then with a value of 2×10^9 cells/gm chick intestinal mucosa[19] and Avogadro's number of 6.02×10^{23} molecules/mole (assuming that the molecular weight and specific activity of the metabolite are equivalent to that of the parent vitamin D employed), it can be calculated that there are 460 molecules of metabolite per intestinal mucosa cell or a metabolite concentration of 1.5×10^{-9} *M* in the entire intestinal mucosa. This value is comparable to either the 1700 or 2500 estrogen molecules per uterine cell nucleus estimated by Maurer and Chalkley[17] and Noteboom and Gorski.[20] Then on the assumption of one ^{3}H-metabolite molecule per receptor molecule, the concentration of the receptor is also 1.5×10^{-9} *M*. If the receptor has a molecular weight of 100,000, then there is, at most, approximately 0.5 μg of receptor per one chick intestinal mucosa. Since the entire mucosa contains 675 mg of protein, this means that approximately a 10^6-fold purification must be attained to isolate the receptor in homogeneous form. Obviously the 167-fold purification thus far achieved is only a small beginning in the isolation of the vitamin D receptor molecule.

The differing saturation plateaus in the receptor fraction (Fig. 5) resulting

after separate administration of radioactive vitamin D_2, D_3, and dihydrotachysterol$_2$ may provide a fundamental insight into the known differences in biological activity of these compounds.[10, †, ‡] It is possible that the lower plateau value for vitamin D_2 is a reflection of less efficient binding of the analogous vitamin D_2-polar metabolite to the receptor or, in turn, a less efficient association of this combination with the genome. Similarly a larger dose of G-^{3}H-dihydrotachysterol$_2$ is required to approach the saturation of the receptor. On the other hand, it is possible that compared to vitamin D_3, the active polar metabolites are less efficiently produced from the native D_2 vitamins. A detailed answer to this question must await the completion of a comparative study of all the vitamin D analogs and characterization of their metabolites.

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† 1 IU of vitamin D_2 (0.063 nmole) or vitamin D_3 (0.065 nmole) is equivalent to 0.025 μg. Vitamin D_2 and vitamin D_3 have an equal biological potency in the rat; in the chick, vitamin D_3 is approximately ten times more active than vitamin D_2.[10] The minimum daily physiological requirement for vitamin D_3 in the chick is 10–15 IU.[10]

‡ Vitamin D_2 differs from vitamin D_3 by having a 22–23 double bond and an additional methyl group on C-24 of its side chain. The dihydrotachysterols differ structurally from the vitamins D in that the C-10 methylene carbon is converted to a methyl group with an accompanying inversion of the A ring. Dihydrotachysterol$_2$ and dihydrotachysterol$_3$ have side chains identical to D_2 and D_3, respectively. On an equal weight basis D_3 is 100 times more active in the chick than dihydrotachysterol$_2$.[10]

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