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COMPARATIVE BIOLOGICAL EFFECTS OF VITAMINS D_2 AND D_3 AND DIHYDROTACHYSTEROL$_2$ AND DIHYDROTACHYSTEROL$_3$ IN THE CHICK*

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Abstract—The relative biological activities of vitamin D_3 (D_3), dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3) were assayed via oral feeding of different levels of the test compounds to chicks raised on a rachitogenic diet. After 28 days the relative growth, per cent of bone ash, and serum calcium levels were determined. The relative dose required to produce 50 per cent of the maximal D_3 response for DHT_2: DHT_3: D_3 was 96 : 20 : 1·0, where 1·0 is equivalent to 0·55 μg D_3.

Also, a comparison was made of the differing responses of the intestinal calcium absorption system (ICA) and the bone calcium resorption system (BCR) to varying doses of vitamin D_2, D_3, DHT_2 and DHT_3. It was found that, although D_3 was the most active of the compounds tested in both systems, compounds having the side chain of D_3 and DHT_3 act preferentially in the ICA system, whereas compounds having the side chain of D_2 and DHT_2 act preferentially in the BCR system. It is suggested that these results may be an indication of a different detailed mechanism of action of vitamin D in the ICA and BCR systems.

THE HALLMARK of vitamin D action is its intervention in calcium metabolism at the intestinal level to mediate calcium absorption[1] and at the skeletal level to increase bone resorption.[2] Much recent work from this laboratory[3–6] and other laboratories[7,8] concerning the basic mechanism of action of vitamin D in the intestinal mucosa supports the concept that the vitamin must be first metabolized to a new compound prior to the generation of its characteristic physiological response. The existence of several metabolites of vitamin D has been definitely shown,[5,7] and one of them has recently been characterized[9] as 25-OH-cholecalciferol. One of these metabolites then localizes in the nucleus of the target cell to activate the biochemical expression of genetic information and ultimately elicit a physiological response. Wasserman *et al.*[10] have proposed that a calcium-binding protein isolated in his laboratory may play a prominent role in this response.

One useful approach† to elucidating the details of this complex series of processes has been to examine the comparative metabolism and subcellular localization in the intestine and the skeleton of many closely related compounds of the vitamin D family,‡

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‡ The abbreviations used in this paper are: D_2 = vitamin D_2; D_3 = vitamin D_3; DHT_2 = dihydrotachysterol$_2$; DHT_3 = dihydrotachysterol$_3$; bone calcium resorption = BCR; intestinal calcium adsorption = ICA.

e.g. D_2, D_3, DHT_2 and DHT_3. This is a particularly useful experimental approach in the chick, where only a seemingly slight modification of the side chain of vitamin D has a profound effect on the biological activity.*

However, before any definite conclusion can be made concerning the significance of the comparative metabolism and subcellular localization of compounds of the vitamin D family, it is essential to know accurately the relative biological activities of these compounds and particularly if there is any difference in activity in the two target organs. As discussed by Norman,[11] it is not clear that the exact biochemical mechanism of action of vitamin D is identical in its two prime target organs, the skeletal system and the intestinal mucosa.

Chen and Bosmann[12] have suggested, on the basis of serum calcium levels, per cent bone ash and growth responses, that D_3 is approximately ten times more active than D_2 in the chick. On the basis of the structural differences in the side chains of these compounds (see footnote*), it might be anticipated that DHT_3 would be more potent than DHT_2 in the chick. However, contradictory results have been obtained.[13–16]

The primary purpose of this paper is to assess via conventional determinations the relative antirachitic effectiveness in chicks of D_2, D_3, DHT_2 and DHT_3. In addition the differential ability of these compounds to interact on bone calcium resorption (BCR) is compared with their ability to mediate intestinal calcium absorption (ICA).

METHODS

One-day-old White Leghorn cockerels (H & N of California, Inc.) were housed in electrically heated, tier brooders in an air-conditioned room from which sunlight was excluded. Two rachitogenic diets were utilized, one containing no added calcium and one containing 2·0% added $CaHPO_4 \cdot 2\ H_2O$ by weight. The basic diet contained the following (in per cent): ground wheat, 30·0; ground corn, 35·4; alcohol-extracted casein (General Biochemicals, Inc., Chagrin Falls, Ohio), 8·0; alfalfa, 6·0; soybean meal, 16·0; NaCl, 1·0; $CaHPO_4 \cdot 2\ H_2O$, 2·0; cottonseed oil, 1·0; charcoal, 0·5 $MnSO_4$, 20 mg/100 g diet; and all water-soluble vitamins, 0·10. The composition of the water-soluble vitamin preparation is given by Steenbock *et al.*[17] Vitamins A, E and K were added to the diet, three times weekly, as a solution in cottonseed oil. The low calcium diet was made with K_2HPO_4 in place of the $CaHPO_4$ in equal molar amounts. Both diets were chemically assayed for calcium and phosphorus. They contained (in per cent) 0·6 and 0·4 (rachitogenic diet) and $<0{\cdot}1$ and 0·4 (low calcium diet) calcium and phosphorus respectively.

Crystalline D_2, D_3, DHT_2 and DHT_3 (Philips–Duphar, Weesp, The Netherlands) were dissolved in a minimum of diethyl ether. The appropriate volume of 1, 2-propanediol was added, the ether was removed by warming and bubbling with nitrogen, and the ultraviolet spectrum and absolute concentration were determined by using a Beckman DB recording spectrophotometer. The compounds tested were given orally, usually three times weekly, beginning when the chicks were 2 days old. Control, vitamin D-deficient chicks received similar amounts of 1, 2-propanediol without added steroid.

* Vitamin D_2 differs from D_3 by having a 22–23 double bond and an additional methyl group on C–24 in 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. DHT_2 and DHT_3 have side chains identical to D_2 and D_3 respectively.

In the first experiment (Tables 1 and 2, and Fig. 1) the chicks received the rachitogenic diet containing the added $CaHPO_4$ and they were weighed twice weekly for 28 days. The chicks were sacrificed by decapitation, blood was collected, and one tibia and fibula were removed and frozen. Serum calcium levels were determined on 0·05 ml aliquots by the procedure of Spare[18] using murexide as an indicator. The absorbancy at 490 mμ followed Beer's law over a range of 2·0 to 15·0 mg per 100 ml (w/v). The per cent bone ash was determined by splitting the tibia and fibula lengthwise, removing the marrow, and then extracting the pooled bone samples in a Soxhlet extractor with diethyl ether for 8 hr. After drying at 100° overnight, the bones were weighed and then ashed in a muffle furnace for 16 hr at 700°. They were then reweighed. The results are expressed as per cent ash on a fat-free, dry basis.

In the second experiment (Table 3 and Fig. 2) the comparative effect of the test steroids on intestinal calcium absorption and bone mobilization was determined via a slight modification of the techniques of Carlsson and Lindquist.[19]

Chicks were first raised for 3 weeks on the standard rachitogenic diet with added $CaHPO_4$. They were next transferred to the rachitogenic diet with no added $CaHPO_4$ for 3 days. They then received one dose of the test steroid orally in 1, 2-propanediol. Twenty-four hr later the determination of intestinal calcium absorption was carried out by the procedure of Coates and Holdsworth.[20] In this procedure the chicks were anesthetized with diethyl ether and a small incision was made on the right side of the abdomen of each bird. The duodenal loop was lifted out, injected with 0·20 ml of a solution containing 1·6 mg $^{40}Ca^{++}$ as the chloride and 6×10^6 dpm $^{45}Ca^{++}$ (sp. act., 1·19 c/g) and then returned to the peritoneal cavity. The opening was closed with a wound clip. Thirty min after receiving the $^{45}Ca^{++}$, the chicks were sacrificed via decapitation and blood was collected. Serum was prepared from the whole blood by centrifugation; 0·25-ml aliquots were taken for total serum calcium as described above, and 0·25-ml aliquots were taken for $^{45}Ca^{++}$ determination in a thin-window, gas flow, Geiger–Muller counter. All samples were counted long enough to insure that the relative error was only $\pm$ 3 per cent. The counting efficiency for $^{45}Ca^{++}$ was approximately 25 per cent.

The simultaneous but independent determination of the BCR and the ICA response to a single dose of vitamin D may readily be determined in chicks which are receiving a diet essentially devoid of calcium. Twenty-four hr after administration of the vitamin D preparation to a chick, both the BCR and ICA systems will have responded. Since the chicks do not receive any calcium in the diet, an increase in serum calcium is a measure of the BCR system alone. Only a negligible portion of the 1·6 mg $^{40}Ca^{++}$ and $^{45}Ca^{++}$ placed in the duodenum is transferred into the blood in the 30-min test period.[20] However, the trace quantity of $^{45}Ca^{++}$ absorbed is a direct measurement of the animal's ability to transport calcium across the intestinal mucosa.

RESULTS

From a preliminary experiment and from the results of Bosmann and Chen,[13] it was possible to choose dose levels for the dihydrotachysterols that roughly correspond to 25–80 i.u.* of D_3 in antirachitic effects.

* 1 i.u. (International unit) of vitamin D_2 (0·063 nmole) or vitamin D_3 (0·065 nmole) is equivalent to 0·025 μg. There are no formal international unit definitions for the dihydrotachysterols.

Table 1 shows the growth response data for chicks which received varying levels of the vitamin D family for a period of 4 weeks. In the absence of vitamin D or a related compound, the rate of growth essentially plateaus by the fourth week, but it can be seen that 5–10 i.u. per/day or 50 i.u. per/week of D_3 is sufficient to maintain a maximal growth rate of the chick. These data confirm the earlier results of Chen and Bosmann.[12] However, neither 375 i.u. per week of D_2, 1200 i.u. of DHT_3 per week nor even 6000 i.u. per week of DHT_2 could maintain a maximal growth rate.

TABLE 1. GROWTH RESPONSE DATA

Groups*	Body wt. (g)			
	Day 1	Day 14	Day 21	Day 28
Rachitic controls	40	97	108	114 ± 18
Rachitogenic diet + D_3				
1	39	101	112	119 ± 14
25	38	134	185	225 ± 17
50	37	138	225	302 ± 34
75	38	148	210	295 ± 25
Rachitogenic diet + D_2				
375	39	110	179	220 ± 39
Rachitogenic diet + DHT_3				
75	39	110	135	147 ± 14
375	40	116	163	209 ± 43
1200	39	142	186	230 ± 33
Rachitogenic diet + DHT_2				
375	40	114	148	172 ± 17
3750	39	130	200	263 ± 18
6000	40	140	193	250 ± 22

*The level of the particular compound listed is the total amount in international units of the steroid that was orally administered (in equivalent doses) in 1, 2-propanediol to each chick/week. The body weights shown are the average ± the standard deviation for six to eight chicks.

In Fig. 1 the grams of growth response, the per cent bone ash, and the serum calcium levels are plotted versus the logarithm of the weekly administered dose of test steroid. Preliminary experiments indicated that 50–75 i.u. per week of D_3 also produced maximum responses in terms of per cent bone ash and serum calcium levels. Accordingly, the value attained at 75 i.u. per week was used to define the 100 per cent response. In rachitic chicks the serum calcium level is depressed to 6·4 mg per 100 ml and the per cent bone ash is lowered to 27 per cent as compared to 11·4 mg per 100 ml and 41 per cent respectively, for chicks which received 75 i.u. per week of D_3. These results are also comparable to those reported by Chen and Bosmann.[12]

Table 2 summarizes for DHT_2 and DHT_3 the dose equivalent to 50 per cent of the maximal D_3 response for each of the parameters reported in Fig. 1. Clearly, DHT_3 is significantly more active than DHT_2 in promoting growth, in increasing the per cent bone ash and in elevating serum calcium. The calculated average ratios for these three responses are $DHT_2 : DHT_3 : D_3 = 95 : 20 : 10$. On the average, DHT_3 is 5 times more active than DHT_2 but only 1/20 as active as D_3.

Figure 2 records the 24 hr effect of a single dose of D_2, D_3, DHT_2 and DHT_3 on bone calciums resorption and intestinal calcium absorption in chicks raised on a

rachitogenic diet, also essentially devoid of calcium. Of the compounds tested, D_3 is clearly the most active in both assays. However, the biological responses elicited by D_2, DHT_2 and DHT_3 are definitely not identical in the two systems.

This difference is more clearly shown in Table 3 where the dose of D_2, DHT_2 and DHT_3 equivalent to 50 per cent of the maximal D_3 response is recorded for BCR and

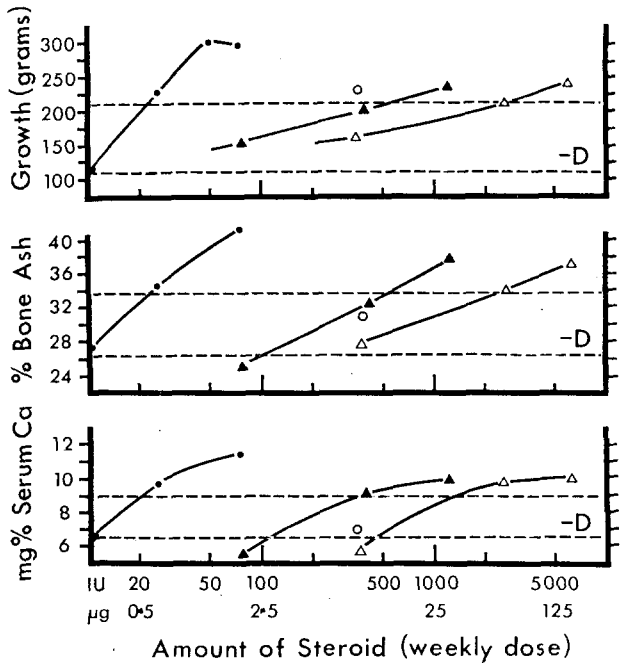

FIG. 1. Comparative effects of D_3, DHT_2 and DHT_3 on growth, per cent bone ash, and serum of chicks fed a normal calcium, vitamin D-deficient diet. The dose of the test compound in i.u. or μg (log scale) is plotted s. the particular response reported. ○——○ = D_2; ●——● = D_3; △——△ = DHT_2, ▲——▲ = DHT_3. The level of the particular compound listed is the total amount of steroid administered (in three equivalent doses) to each chick per week. The experiment was carried out for 28 days. Each number is the average value determined from samples obtained from 5–6 chicks. The standard errors for each determination did not exceed ± 6 per cent. The data for the growth response were taken from Table 1. The top dotted line represents the dose level equivalent to 50 per cent of the maximal D_3 response. The bottom dotted line represents the average response of ten rachitic chicks which received only 1, 2-propanediol and no vitamin D or analogue. The per cent bone ash data are expressed as the amount of ash × 100/g of fat-free, dried bone. The mg % is mg of Ca^{++}/100 ml or serum.

ICA. Although the dose of D_3 required for a 50 per cent maximum response is 2–5 times that reported in Table 2, this is not surprising. Only the response to a single dose of the vitamin is being measured in Table 3 as compared to the response to the steady state influx of D_3 reported in Table 2. The relative activity ratios of DHT_2 DHT_3: D_3 for the two assay systems are not widely divergent from the values reported in Table 2. It should be appreciated that the values reported in Table 3 are more clearly an indication of the specific response of ICA and BCR than are those of Table

2, where an interplay between the various vitamin D-responsive systems may occur to produce an "averaging" effect. No explanation can be given at this time for the low biological activity of D_2 in the ICA assay. However, this result has been obtained in two separate experiments.

DISCUSSION

The results presented in Table 2 and Fig. 1 clearly indicate that D_3, D_2, and DHT_3 and DHT_2 have markedly different biological activities in the chick. By using the conventional assays of growth, per cent bone ash and serum calcium levels, the average

TABLE 2. COMPARATIVE BIOLOGICAL ACTIVITIES OF COMPOUNDS OF THE VITAMIN D FAMILY*

	Dose equivalent to 50% of maximal vitamin D_3 response (μg)		
Assay	DHT_2	DHT_3	D_3
Serum Ca^{++}	32·5	9·4	0·55
% Bone ash	62·5	12·5	0·60
Growth	62·5	11·2	0·50
Average	52·5	10·7	0·55
	Relative activity†		
Serum Ca^{++}	59	17	1·0
% Bone ash	104	21	1·0
Growth	124	22	1·0
Average	95	20	1·0

*The data reported in the upper half of the table are obtained from Fig. 2 using the dotted line which indicates 50 per cent of the maximal response to vitamin D_3.

†The term "relative activity" is intended to indicate the amounts of each compound, relative to vitamin D_3, which must be administered to produce a response equivalent to 50 per cent of the maximal response attainable with D_3.

ratio of activities for DHT_2: DHT_3: D_3 is 95 : 20 : 1·0, or a ratio for DHT_2 : DHT_3 of 5 : 1. Also, as shown in Table 1 and Fig. 1, D_2 is slightly more active than an equivalent amount of DHT_3. This suggests an approximate activity ratio for D_2 : D_3 of 10 : 1 to 15 : 1, which is in agreement with the previous work of Chen and Bosmann.[12]

Each of the conventional assays measures a different aspect of the rachitic condition. Per cent bone ash reflects the end point in the development of rickets, i.e. the degree of calcification. Serum calcium levels are an average measurement of the vitamin D-mediated response of the ICA and BCR systems. The final body weight is an indirect measure of the general health of the animal. Thus it is not surprising that these assays should give somewhat differing activity ratios. It is important to note, however, that none of these assays measures directly and exclusively the response of any one of the vitamin D-responsive systems. Thus the results obtained from these conventional assays do not permit any direct or separate assessment to be made of the independent contributions of the ICA and BCR systems to calcium metabolism.

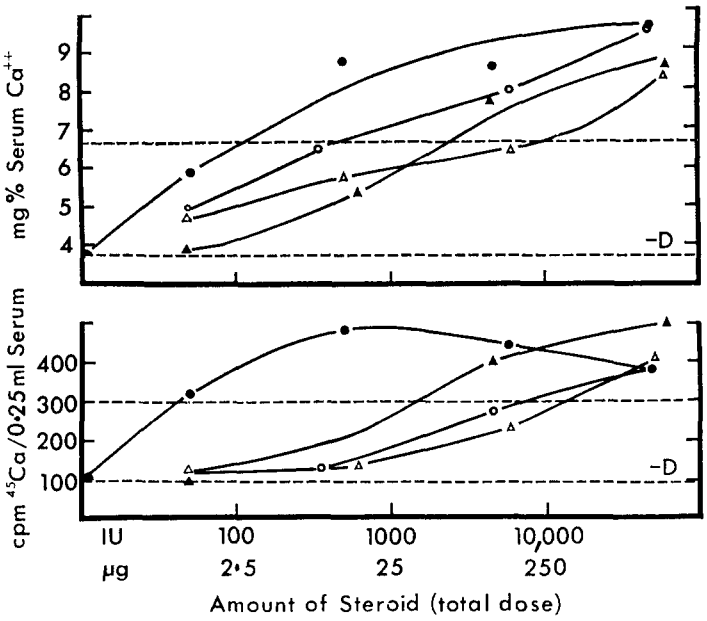

FIG. 2. Comparative effects of D_2, D_3, DHT_2 and DHT_3 on mediating bone resorption and intestinal calcium absorption.. The dose of the test compound in i.u.[2] or μg (log scale) is plotted vs. the particular response reported. ○ ——— ○ = D_2; ● ——— ● = D_3; △ ——— △ = DHT_2; ▲ ——— ▲ = DHT_3. All chicks were raised 3 weeks on the standard rachitogenic diet, then switched for 3 days to the low calcium rachitogenic diet. The chicks then received one oral dose of the test compound in 0·2 ml 1, 2-propanediol 24 hr later. The degree of bone resorption and the rate of intestinal calcium absorption were determined as described in the text. Each number is the average of four to five chicks. The standard errors for each determination did not exceed ± 5 per cent. The top dotted line represents the dose level equivalent to 50 per cent of the maximal D_3 response. The bottom dotted line is the average response of 10 rachitic chicks which received only 1, 2-propanediol and no vitamin D or analogue. The mg% is mg of Ca^{++}/100 ml of serum.

TABLE 3. COMPARISON OF ABILITY OF COMPOUNDS OF THE VITAMIN D FAMILY TO MEDIATE BONE CALCIUM RESORPTION AND INTESTINAL CALCIUM ABSORPTION*

	Dose equivalent to 50% of maximal vitamin D_3 response (μg)			
Assay	DHT_2	DHT_3	D_2	D_3
Bone calcium resorption (BCR)	225	50·0	10·7	2·5
Intestinal calcium absorption (ICA)	300	32·5	200	1·1
Ratio BCR/ICA	0·75	1·5	0·05	2·2
	Relative activity†			
BCR	90	20·0	4·3	1·0
ICA	270	29·0	180·0	1·0

* The data reported in the upper half of the table are obtained from Fig. 3 using the dotted line which indicates 50 per cent of the maximal response to vitamin D_3.

† The term "relative activity" is intended to indicate the amounts of each compound, relative to vitamin D_3, which must be administered to produce a response equivalent to 50 per cent of the maximal response attainable with D_3.

As might be anticipated from a consideration of the structural differences of the compounds tested (see footnote p. 2348) and of the results of Chen and Bosmann,[12] showing that the D_2 : D_3 activity ratio is 8 : 1 to 11 : 1, it is not surprising that DHT_3 is more biologically active than DHT_2 in the chick. These results are in contrast to those of Bosmann and Chen,[13] who found little difference in their activity in the rachitic chick. It is difficult to evaluate the earlier literature[14,15] on this subject, since the chemical composition of the compounds used was not clearly stated. DeMan and Roborgh[21,22] have recently studied the effects of DHT_2 and DHT_3 administered to rats. They reported that whereas the antirachitic activities of DHT_2 and DHT_3 were only 0·5 and 0·9 per cent, respectively, of that of D_3, the hypercalcemic activities of pharmacological doses of DHT_2 and DHT_3 were four and fifteen times, respectively, that of D_3. Their results are difficult to relate specifically to the data of this report because they used a different test organism. There is qualitative agreement, however, in that they did observe that DHT_3 was approximately two to four times more active than DHT_2 in both their hypercalcemic and their antirachitic assays.

A much more precise estimation of possible differing responses of the ICA and the BCR systems to the various compounds of the vitamin D family may be determined by utilizing rachitic chicks raised for several days on a diet devoid of calcium. This technique was originally utilized by Carlsson and Lindquist.[19] Under these conditions, any increase in total serum calcium can only be due to a response of the BCR system, while the appearance of radioactive calcium in the serum can be due only to the ICA system. In the 30-min period allowed for the absorption of the 1·6 mg $^{40}Ca^{++}$ + $^{45}Ca^{++}$, not enough of the dose would be absorbed to alter significantly the serum calcium level.[20]

The fact that the activity ratio for DHT_2 : DHT_3 : D_2 : D_3 calculated for the BCR system is lower than the ratio for the ICA system (Table 3) suggests that the conventional parameters (per cent bone ash, growth and serum calcium) used to evaluate the degree of vitamin D deficiency (Table 2) give activity ratios which are weighted in favor of the BCR system. The ratio of 96 : 20 : 1·0 (Table 2) is significantly closer to the BCR ratio of 90 : 20 : 1·0 than to the ICA ratio of 270 : 29 : 1·0 (Table 3).

As shown in Fig. 2 and Table 3, there is a striking difference in the interaction of D_2, D_3, DHT_2 and DHT_3 in the BCR and ICA systems. Those compounds with a side chain identical to that of cholesterol, i.e. D_3 and DHT_3 apparently can interact with the ICA system significantly more effectively than compounds with a side chain identical to that of ergosterol, i.e. D_2 and DHT_2. Conversely, D_2 and DHT_2 were more effective than D_3 and DHT_3 in interacting in the BCR system than in the ICA system. In each system, however, D_3 was the most active of the compounds tested. Clearly there are definite structural specificities in each of the vitamin D-dependent systems for compounds with different side chains and different B ring structures. However, this can only be clarified when a comparative study of the metabolism of all these compounds and the characterization of the metabolites has been completed.

The present data are suggestive that the mechanism of action of vitamin D in mediating intestinal calcium absorption and bone calcium resorption may not be identical. This was first tentatively postulated by Windaus and Auhagen,[23] who suggested that there was a *calcinosefakter* and an *antirachitischenfaktor* activity incorporated into the structure of vitamin D. This suggestion has received indirect support from the work of Carlsson and Lindquist[19] and Chen *et al.*[24] The difference in mechanisms

of the BCR and ICA system may be so fundamental as to require entirely structurally different initiators (i.e. different metabolites of vitamin D). Alternatively, both systems may respond most effectively to the same basic initiator (i.e. the same D_3 metabolite), but over a long period of time the general structural requirements of the two systems may have evolved in somewhat different directions. Clearly much further work remains to be done.

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