EVIDENCE FOR A 1,25-DIHYDROXYVITAMIN D-LIKE ACTIVITY IN TRISETUM FLAVESCENS: POSSIBLE CAUSE FOR CALCINOSIS IN GRAZING ANIMALS

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Summary

Trisetum flavescens, a grass occurring abundantly on Alpine pastures, induces vitamin D-dependent calcium-binding protein and stimulates intestinal calcium and phosphate absorption in rachitic chicks. Trisetum flavescens retains these biological activities when biotransformation of vitamin D to its active metabolite 1,25-dihydroxyvitamin D is blocked at the 1-hydroxylation step by feeding a diet high in strontium. This indicates that the plant does indeed contain a factor which requires no further metabolism for biological activity. Mimicking the action of 1,25-dihydroxyvitamin D would account for the severe calcinosis associated with ingestion of the grass by grazing animals.

Although a number of steroid hormones have been identified in several plants (1) there are only few accounts on vitamin D_3-related sterols occurring among them (2). Recently, the active metabolite of vitamin D_3, 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] (3), was shown to be present as a glycoside in Solanum malacoxylon (4, 5). Evidence was also obtained for a 1,25-$(OH)_2D_3$-like activity in another botanical species, Cestrum diurnum (6). In both instances, the presence of this hormonally acting sterol accounts for the severe calcinosis associated with the ingestion of the plants by grazing animals: Solanum malacoxylon causes a widespread disease in South America (called

[1]Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; T.f., Trisetum flavescens; CaBP, calcium-binding protein; P_i, inorganic phosphate.

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"Enteque seco" in Argentina or "Espichamento" in Brazil) (7), while Cestrum diurnum has been implicated in causing calcinosis in horses and cattle in Florida (8). A calcinosis of cattle in the Alpine region of Austria (9) and Germany (10) is characterized by chronic wasting, lameness, pain, ectopic calcifications of the cardiovascular system, lungs, kidney and tendons, ulceration of joint cartilage and extensive calcification of bones (9). This disease is produced by the ingestion of Trisetum flavescens (T.f.) (11), a grass abundant on pastures of this area. The similarity of symptoms to the other calcinotic diseases has stimulated the search for a 1,25-$(OH)_2D_3$-like substance as the toxic principle of T.f., but attempts to demonstrate such an activity have been unsuccessful so far (12). However, evidence presented in this paper clearly indicates the presence of a 1,25-$(OH)_2D_3$-like activity in T.f., fostering the idea that it might be the causative agent in T.f.-associated calcinosis also.

Experimental

Trisetum flavescens was grown on a pilot plot in Kremesberg (Lower Austria) and harvested in August 1976. The grass was stored at -20° C and lyophilized immediately before use.

Day-old White Leghorn cockerels were raised for 3 weeks on a rachitogenic (vitamin D-deficient) diet or on a commercial starter diet and then transferred to one of the experimental diets described below.

The rachitogenic diet was prepared according to Corradino and Wasserman (13), except that soy bean protein was substituted by casein. It contained 1.1 % Ca and 0.7 % P by direct analysis.

In the first set of experiments chicks were divided into groups of 5 to 6. One group remained on the rachitogenic diet serving as vitamin D-deficient control. The others received diets to which lyophilized T.f. had been added to a final content of 5, 9, and 13 %, respectively.

For a second line of experiments, a "normal diet" was prepared by addition of 1200 I.U./kg vitamin D_3 to the vitamin D-deficient diet. A "high strontium diet" had the same composition as the "normal diet" except that it contained 2.5 % Sr and 0.17 % Ca (14). For evaluation of its biological activity, lyophilized T.f. was added to the "high strontium diet" to a final content of 13 %. The same amount of T.f.-free hay was added to the "normal" and the "high strontium diet" serving as controls.

After 3 weeks on the starter diet chicks were divided into two groups. One was placed on the "normal diet" the other on the "high strontium diet". After one week when CaBP was undetectable in mucosal homogenates in the "high Sr" group, these chicks were again divided into two groups. One remained on the same diet, the other was fed the

TABLE 1: Induction of calcium-binding protein (CaBP) by T.f.*

Addition to vitamin D-deficient diet	Calcium-binding protein (CaBP)			
	Days on diet			
	2	4	8	10
5 % T.f.	-	-	+	+ +
9 % T.f.	-	+	+	+ +
13 % T.f.	-	+	+	- + +

*Each sign indicates positive or negative reaction (immunoprecipitation) in mucosal homogenate from the duodenum of one chick.

"high Sr diet" fortified with 13 % T.f. Two weeks later, intestinal CaBP, phosphate absorption and calcium transport were evaluated in all three groups containing 5 chicks each.

Intestinal Ca and phosphate (P_i) transport was determined in everted gut sacs. Segments of 4 cm lenghth were prepared from the jejunum and filled with 0.4 ml Krebs-Henseleit buffer (4.0 mM P_i, 0.6 mM Ca). The sacs were incubated (20 min, 32° C) in 5 ml of the same buffer with 1.2 mM P_i, 2.5 µCi ^{45}Ca and 1.0 µCi $^{32}P_i$. Calculations of calcium and phosphate movements were done according to Kowarski and Schachter (15).

Calcium-binding protein (CaBP) was detected in mucosal homogenates from duodenum by the Ouchterlony technique (16).

Results and Discussion

Chicks which had been raised for three weeks on the vitamin D-deficient diet are completely devoid of intestinal CaBP. Supplementation of their feed with lyophilized T.f. caused this strictly vitamin D-dependent protein to appear in duodenal mucosa (Table 1). With the exception of the lowest T.f. concentration used, CaBP could be detected 4 days after the chicks were placed on the T.f. containing diets.

Vitamin D-like effects of T.f. became also apparent when intestinal calcium and phosphate transport was determined in chicks fed 13 % T.f. in the diet and a comparison was made to age-matched vitamin D-deficient birds (Table 2); secretion of P_i in the luminal direction was

TABLE 2: Stimulation of intestinal phosphate and calcium transport by T.f.*

Diet	Phosphate transport			Calcium transport
	Net change	Uptake	Release	
Rachitogenic	-0.020 ±0.010	0.120 ±0.031	-0.142 ±0.024	0.005 ±0.001
Rachitogenic + 13 % T.f.	0.097 ±0.037	0.325 ±0.063	-0.229 ±0.086	0.023 ±0.009

*All data are expressed as μmoles/g tissue per min and represent means ± S.E.M. from 6 to 12 everted gut sacs. A negative sign indicates movement in luminal direction. All values except those for phosphate release are statistically different at $P < 0.025$ level.

TABLE 3: Reversal of strontium effects by Trisetum flavescens.*

Diet	CaBP n	Phosphate transport			Calcium transport
		Net change	Uptake	Release	
High Sr	0	-0.134 ±0.028	0.086 ±0.014	-0.220 ±0.026	0.004 ±0.002
High Sr + T.f.	4	-0.040[a] ±0.025	0.136[a] ±0.016	-0.176 ±0.018	0.017[a] ±0.004
Normal	5	-0.007[b] ±0.010	0.169[b] ±0.013	-0.175 ±0.010	0.035[b], [c] ±0.004

*n = number of chicks per group in which CaBP could be detected by immunoprecipitation. Transport rates are expressed as μmoles/g tissue per min. A negative sign indicates movement in luminal direction. Data represent means ± S.E.M. from 12 everted gut sacs. Superscripts indicate that values differ at least at $P < 0.05$ level(a, High Sr + T.f. vs. High Sr; b, High Sr vs. Normal; c, High Sr + T.f. vs. Normal).

reversed to net absorption. This effect is due mainly to stimulation of phosphate entry into the tissue. P_i movement in the opposite direction was unaffected by treatment with T.f. These effects on P_i movements across the mucosal surface are consistent with the reported action of vitamin D_3 and 1,25-$(OH)_2D_3$ (17). Under the conditions employ-

ed, mucosal calcium net flux was zero. In accordance with its vitamin D-like action, T.f. facilitates calcium exchange across the mucosal border of the jejunum (Table 3).

To establish whether this activity is the cause for the calcinogenic action of T.f., it is essential to demonstrate that T.f. retains its biological activity when vitamin D metabolism is blocked immediately prior to the formation of the active sterol, $1,25\text{-}(OH)_2D_3$. A diet high in strontium but otherwise normal in vitamin D_3, causes the disappearance of CaBP from intestinal mucosa and depresses calcium (13, 14) and phosphate transport (Peterlik and Wasserman, submitted for publication) (see also Table 3). Under this regimen, $1,25\text{-}(OH)_2D_3$ cannot be formed from its precursor 25-dihydroxyvitamin D_3 because of inhibition of the 1-hydroxylation step in the kidney (18). The results given in Table 3 clearly indicate that T.f. retains its biological activity under these conditions. The demonstration of CaBP induction by T.f. in chicks on a "high strontium diet" proves beyond doubt that the plant contains a substance which can mimick the action of $1,25\text{-}(OH)_2D_3$. Furthermore, everted gut sacs derived from those chicks display elevated levels of phosphate and calcium absorption when compared to "high strontium" controls though restoration of transport rates to normal values was not achieved (Table 3).

This suggests that T.f. contains a low $1,25\text{-}(OH)_2D_3$-like activity. From reported data on the ability to induce CaBP it would seem that the potency of Solanum malacoxylon is at least 25 times and of Cestrum diurnum 4 times greater than that of T.f. (14, 19). This bears on the question whether the $1,25\text{-}(OH)_2D_3$-like activity of T.f. is sufficient to account for the plant's calcinogenic effect. In this regard it would be important to know whether the molecular structure of the unknown substance is related to vitamin D_3 or D_2. If the latter were the case, this could explain the low activity encountered in chick

experiments and the high calcinogenic effect in grazing animals, since birds are by far less responsive to vitamin D_2 derivatives than mammals (20). In addition, the activity measured under experimental conditions may not fully resemble that of the living plant. A loss of activity might occur during processing of the grass and preparation of the diet. This is exemplified by the fact that dried T.f. is less potent in inducing CaBP than lyophilized material (unpublished results). This could probably account also for the failure to demonstrate a 1,25-$(OH)_2D_3$-like activity in other experiments (12). In light of the fact, that Trisetum flavescens is certainly ingested in large amounts by grazing animals, a weak 1,25-$(OH)_2D_3$-like activity effectively bypassing the kidney regulation of 1,25-$(OH)_2D_3$ synthesis must also seriously be considered as an important factor in the development of calcinosis.

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