FURTHER EVIDENCE FOR THE 1,25-DIHYDROXYVITAMIN D-LIKE ACTIVITY OF SOLANUM MALACOXYLON[+]

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Summary: Administration of an aqueous extract of the calcinogenic plant Solanum malacoxylon (S.m.) to vitamin D-deficient or strontium fed chicks produces significant plasma 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) activity within 6 hr. (via radioreceptor assay) and subsequently elicits the appearance of immunoreactive intestinal calcium binding protein. Studies of a purified aqueous extract of S.m. show that it does not compete effectively with radioactive 1,25-$(OH)_2D_3$ for binding to the sterol's intestinal receptor. However, treatment of the extract with β-glucosidase releases a biologically active substance which is soluble in organic solvents and efficiently competes with labeled sterol for the receptor. This factor migrates exactly with tritiated 1,25-$(OH)_2D_3$ on high resolution Celite liquid-liquid partition columns. Thus, S.m. contains a molecule very similar or identical to 1,25-$(OH)_2D_3$ which is combined with one or more carbohydrate moieties in the native plant. This glycoside is probably cleaved *in vivo* before biological activity is attained.

1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$)[1] is now considered to be the hormonal form of vitamin D, which has as its main action the increased absorption of calcium and phosphate from the intestine (1-3). The existence of 1,25-$(OH)_2D_3$ (4, 5) and its exclusive production by the kidney (6) were initially demonstrated in experimental animals and more recently in humans (7). It has also been shown that certain calcinogenic plants, such as Solanum malacoxylon (S.m.)[2], contain a substance or substances with biological activity similar to that of 1,25-$(OH)_2D_3$ (9). Ingestion of these botanical species by grazing animals causes calcinosis and pathologic features similar to those of hypervitaminosis D. Because the renal biosynthesis of 1,25-$(OH)_2D_3$ is strictly controlled by the calcium and phosphorus needs of animals (1, 10), it is likely that the etiology of the calcinosis involves excessive intake

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[1]Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; S.m., Solanum malacoxylon; CaBP, calcium binding protein.

[2]The name of preference is Solanum glaucophyllum according to D'Arcy (8), but Solanum malacoxylon (S.m.) is most frequently used in connection with the calcinogenic diseases.

of the 1,25-$(OH)_2D_3$-like activity in these plants which causes hyperabsorption of calcium and phosphorus even in the face of adequate serum levels of the ions.

The active factor in S.m. differs chemically from the sterol hormone in that it is soluble in water but not most organic solvents (9) and has an apparent molecular weight in excess of 1,000 (11). Yet aqueous extracts of S.m. have many 1,25-$(OH)_2D_3$-like actions, including enhancement of calcium binding protein (CaBP) synthesis and intestinal calcium absorption in chicks fed a high strontium diet (12). High strontium intake blocks the renal 1α-hydroxylase enzyme, allowing only 1α-hydroxylated-D-vitamins to function in such animals (13). S.m. is also active in the anephric (14) and diabetic rat (15), further demonstrating that it does not require 1α-hydroxylation for biologic potency. Finally S.m. is efficacious *in vitro*, and increases CaBP in embryonic duodenum in organ culture (16) as well as mobilizing calcium from cultured bone (17).

The present studies were designed to determine if the chick can metabolize S.m. to a lipid soluble form, monitoring such activity in plasma with a new radioreceptor assay for 1,25-$(OH)_2D_3$ (7, 18), and to reveal if a hydrolytic enzyme could similarly alter the solubility properties of the S.m. factor to resemble those of vitamin D sterols. We report here that either *in vivo* metabolism or incubation with β-glucosidase liberates a lipophilic substance strikingly similar to the 1,25-$(OH)_2D_3$ hormone.

MATERIALS AND METHODS

Materials. White Leghorn cockerels were made vitamin D-deficient by raising them for 4 weeks on rachitogenic diets (19). In the dietary strontium study, chicks were fed a commercial mash (Agway) for 2 weeks and then placed on the experimental diet for 7 days; this diet was identical to the rachitogenic diet but was supplemented with 1.2 IU vitamin D_3/gm and contained 2.56% Sr, 0.05% Ca and 0.76% P. S.m. plant was made available by Dr. H. R. Camberos, Ministry of Agriculture, Buenos Aires, Argentina and Dr. G. K. Davis, University of Florida at Gainsville. 1,25-$(OH)_2[^3H]D_3$ (6.5 Ci/m mole) was biosynthesized from 25-hydroxy[26(27)-methyl-$^3H_2$]D_3 (Amersham/Searle) as previously described (20). Crystalline nonradioactive 1,25-$(OH)_2D_3$ was obtained from Dr. M. Uskokovic of Hoffmann-La Roche, Inc. β-Glucosidase (almond) was purchased from Sigma Chemical Co. Chromatographic materials were obtained as described elsewhere (18) and all solvents used in chromatography were glass distilled.

Solanum malacoxylon Extraction and Hydrolysis. Aqueous extracts of dried S.m. leaf were prepared as previously described (21). The chloroform extracted leaf powder was extracted with water, the aqueous solution lyophilized and washed with methanol. The residue was dissolved in 75% ethanol in water and run on a silicic acid column

(Bio-Sil-HA, minus 325 mesh). The first solvent was n-butanol-acetic acid-H_2O (100:10:10) which removed impurities. The bioactive material was then eluted with n-propanol-H_2O (75:25). After removal of the solvent the active material was dissolved in 0.1 M acetate buffer (pH 5.0) and β-glucosidase (100 mg/50 ml) was added and incubation carried out at 35°C for 24 hr. After adding 50 ml methanol and 62.5 ml chloroform to the mixture, the lower chloroform phase was harvested and combined with 2 more chloroform washes. The final chloroform soluble fraction contained the active factor.

Purification of 1,25-$(OH)_2D_3$ Activity from Plasma. After adding 2,000 cpm of 1,25-$(OH)_2[^3H]D_3$ tracer (6.5 Ci/m mole) to each sample, lipid extraction was carried out as described elsewhere (18). Samples were purified by successive chromatography on Sephadex LH-20 (18), silicic acid (0.8 x 6.3 cm column, run with 10 ml diethyl ether and then 8 ml acetone to elute the 1,25-$(OH)_2D_3$ fraction) and micro-Celite (10) columns. Recovery was 40-80% and samples were virtually free of lipid residue.

Radioreceptor Assay for 1,25-$(OH)_2D_3$ Activity. Radioreceptor assay was performed as detailed by Brumbaugh et al (18) using chick intestinal receptor system except that 20 μl of ethanol was included in the incubations to aid in solubilizing the sterols (10).

Assay of Duodenal CaBP. CaBP was quantitated by radial-immunoassay (22) in supernatant fractions of homogenized duodenal mucosa. Protein was determined by the method of Lowry et al (23) and data expressed as μg CaBP/mg supernatant protein.

RESULTS AND DISCUSSION

Initially, an aqueous extract of S.m. was administered orally to chicks either deprived of vitamin D or incapable of biosynthesizing 1,25-$(OH)_2D_3$ because of the inhibitory effects of strontium. Table 1 summarizes plasma 1,25-$(OH)_2D_3$-like activity and intestinal CaBP as a function of time after S.m. Radioreceptor assay of purified plasma lipid-extracts shows that both vitamin D-deficient and strontium fed chicks have undetectable circulating levels of 1,25-$(OH)_2D_3$ activity prior to administration of S.m., but dramatic increases occur in plasma hormone activity within 6-12 hr of dosing. The 1,25-$(OH)_2D_3$ equivalent concentrations achieved are 5-10 times the normal circulating level of 1,25-$(OH)_2D_3$ in the chick (18). By 24 hr. plasma activity decreases markedly, suggesting that the active factor turns over rapidly, as does 1,25-$(OH)_2D_3$ (24). The fact that strontium inhibited chicks are able to produce the plasma 1,25-$(OH)_2D_3$-like substance is significant and substantiates the earlier conclusions that 1α-hydroxylation is not required for S.m. activity. Intestinal CaBP results correlate well with plasma hormone values, but there is a 12-18 hr lag between maximal 1,25-$(OH)_2D_3$ activity and maximal CaBP. These data are also consistent with the concept that 1,25-$(OH)_2D_3$ or its analogs function, at least in part, through new mRNA and CaBP synthesis in the chick.

We next determined the chromatographic mobility of the plasma 1,25-$(OH)_2D_3$-like

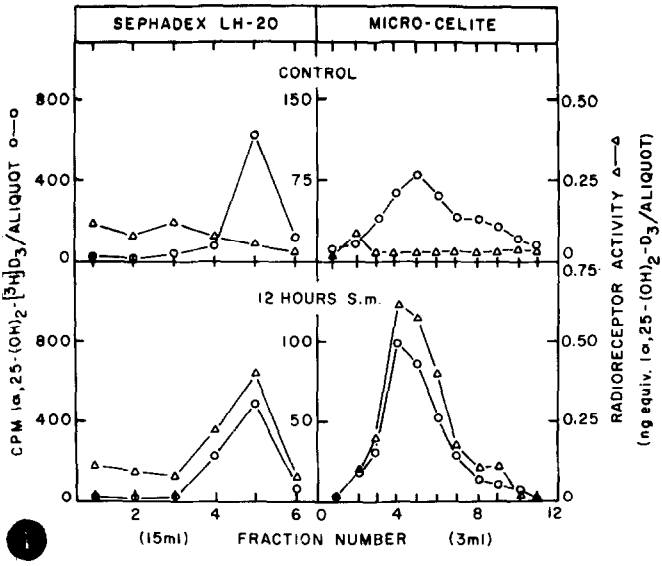
TABLE 1: Appearance of 1,25-$(OH)_2D_3$-like Activity in Plasma and Intestinal CaBP in Chicks Given an Oral Dose of Solanum malacoxylon.

Exp. No.	Addition to basal diet[a]	Time after dose (hrs)	Plasma Radioreceptor Activity[b] (ng equiv. 1,25-$(OH)_2D_3$/100 ml)	Intestinal CaBP (μg/mg protein)
1[c]	none	0	<1	0
	none	12	40	3.8
	none	24	7	6.1
2[d]	Vit. D_3 + Sr	0	<1	0
	Vit. D_3 + Sr	6	81	<0.5
	Vit. D_3 + Sr	12	75	1.5
	Vit. D_3 + Sr	24	14	6.7

[a]Basal diet = Cornell rachitogenic diet (19).
[b]Plasma was extracted with methanol-chloroform (2:1) and the extract purified by successive chromatography on Sephadex LH-20, silicic acid and micro-Celite as described in Materials and Methods.
[c]Each chick received an oral dose of aqueous S.m. extract equivalent to 50 IU vit. D_3; plasma was pooled from 10 chicks for receptor activity; CaBP represents the mean value of 4-5 chicks from the same group.
[d]Each chick received the oral dose of aqueous S.m. extract containing about 400 IU vit. D_3-equivalents; the values are the means of 5 chicks.

factor generated by S.m. treatment. Figure 1 illustrates the results of radioreceptor assays of individual column fractions during the purification of plasma from vitamin D-deficient (control) and chicks given S.m. extract 12 hr prior to sacrifice. Tracer 1,25-$(OH)_2[^3H]D_3$ migrates as expected on the columns, and no radioreceptor activity can be detected in control chicks. S.m. treated chicks exhibit plasma 1,25-$(OH)_2D_3$ activity which migrates with authentic 1,25-$(OH)_2[^3H]D_3$ on both Sephadex LH-20 and micro-Celite columns (Fig. 1, lower panels). Thus, the S.m. produced lipid which competes with radioactive 1,25-$(OH)_2D_3$ for the highly specific intestinal receptor (25), also migrates exactly with the marker hormone, suggesting that the assayable material in plasma is very similar in polarity to 1,25-$(OH)_2D_3$.

Previous work (21) indicated that the vitamin D-active factor in the native S.m. plant is probably a sterol glycoside and, after testing a series of available



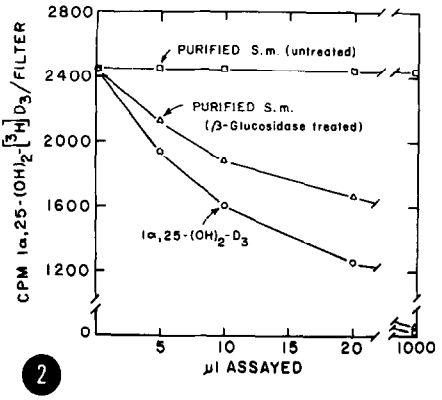


Fig. 1. Chromatographic migration of plasma 1,25-$(OH)_2D_3$-like factor generated by S.m. administration to vitamin D-deficient chicks. Control animals received no S.m., test animals were given S.m. 12 hrs before killing. Pooled plasma from 10 chicks per group was extracted as described in Materials and Methods and purified on successive 1 x 15 cm Sephadex LH-20 columns (18), silicic acid columns (see Methods) and 0.8 x 8.5 cm micro-Celite columns (10). 50% aliquots were counted to determine recovery and migration position of marker 1,25-$(OH)_2[^3H]D_3$ on both Sephadex LH-20 and micro-Celite columns. Radioreceptor assay was performed on 20% aliquots of Sephadex LH-20 fractions and 50% aliquots of micro-Celite fractions.

Fig. 2. Competition of S.m. with radioactive 1,25-$(OH)_2D_3$ for binding to the chick intestinal receptor system. Radioreceptor assays were carried out by filtration as described elsewhere (18). Nonradioactive 1,25-$(OH)_2D_3$ standard was utilized at a concentration of 0.65 IU/ml while S.m. solutions contained 5 IU of D_3 equivalents per ml. Silicic acid purified S.m. showed no activity while β-glucosidase treated S.m. exhibited significant radioreceptor activity (about 0.4 IU of 1,25-$(OH)_2D_3$ equivalents per ml). 1,25-$(OH)_2D_3$-like activity of the β-glucosidase incubated fraction is probably less than the vitamin D_3 equivalent activity because of incomplete hydrolysis.

glycosidases, we found that incubation of purified S.m. activity with β-glucosidase released an active substance which was soluble in chloroform. The chloroform extract exhibited marked vitamin D potency in strontium fed chicks (data not shown), demonstrating that it resembled its water soluble progenitor in biologic characteristics. Figure 2 shows its ability to compete effectively with labeled 1,25-$(OH)_2D_3$ for binding to the intestinal receptor. However, as is also illustrated in Fig. 2, untreated (purified) S.m. has no apparent activity in the radioreceptor assay. This strong contrast in radioreceptor activity between untreated and β-glucosidase in-

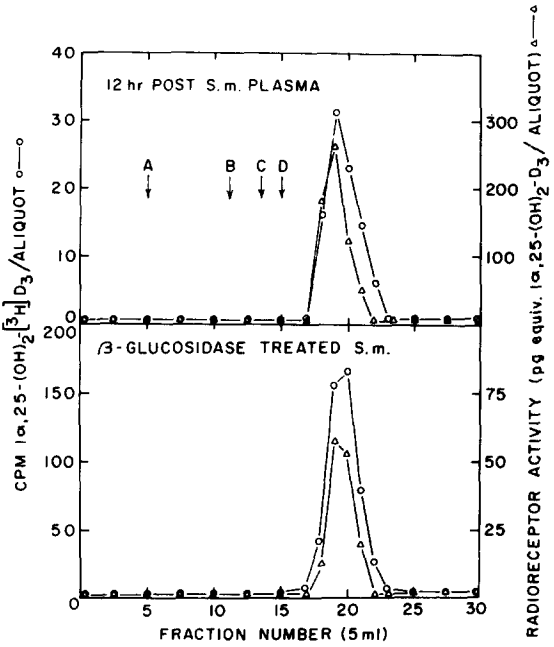


Fig. 3. 1 x 40 cm Celite liquid-liquid partition chromatography of lipid soluble S.m. factor. Chloroform soluble factor from either S.m. treated chick plasma (upper panel) or from β-glucosidase hydrolysis of purified S.m. (lower panel) was prepurified on Sephadex LH-20 and silicic acid columns (see Methods) and then analyzed on long Celite columns as described previously (27). Radioreceptor activity is compared to the migration position of the 1,25-$(OH)_2[^3H]D_3$ internal marker. Other vitamin D forms migrate as indicated: A, 25-hydroxyvitamin D_3; B, 24,25-dihydroxyvitamin D_3; C, 25,26-dihydroxyvitamin D_3; D, 1,25-dihydroxyvitamin D_2 (27, 28).

cubated S.m. suggests that the enzyme not only catalyzes the release of sugars to yield a lipid soluble factor, but also exposes a hydroxyl group(s) functional in the binding of 1,25-$(OH)_2D_3$-like sterols to the intestinal receptor. Our data are in opposition to a report by Walling et al (26) that a crude (desalted) water extract of S.m. competes with 1,25-$(OH)_2D_3$ for the intestinal receptor. Although we have found that crude water extracts of S.m. yield slight competition with 1,25-$(OH)_2D_3$ (data not shown), when the extract is purified by silicic acid chromatography (21), this apparent radioreceptor activity disappears (Fig. 2). Thus, the observation of Walling et al (26) is probably the result of nonspecific interference in the radioreceptor assay.

Finally, detailed chromatographic analysis was carried out on the lipid soluble

factor derived either from plasma of S.m. treated chicks or from the direct action of β-glucosidase on S.m. Celite liquid-liquid partition columns (1 x 40 cm) were utilized because these columns can resolve 1,25-$(OH)_2D_3$ from other dihydroxyvitamin D_3 forms (27) as well as from 1,25-dihydroxyvitamin D_2 (28). The data in Fig. 3 demonstrate that the factor or factors from both sources with 1,25-$(OH)_2D_3$-like activity migrate exactly with authentic labeled hormone. We therefore conclude that S.m. contains a molecule which is quite similar or identical to 1,25-$(OH)_2D_3$ which is linked to carbohydrate(s). Cleavage of this glycoside can be achieved (in part) with β-glucosidase and apparently occurs *in vivo* after ingestion of S.m. Further work will be required to detect tissue glycosidases capable of hydrolyzing S.m. as well as to characterize the structure of the carbohydrate portion of the plant factor. Yet the basic question of the molecular nature of the lipid soluble 1,25-$(OH)_2D_3$-like principle should be answered very soon when sufficient pure material is generated for physical and chemical identification.

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