Calcinogenic Factor in *Solanum malacoxylon*: Evidence That It Is 1,25-Dihydroxyvitamin D_3-Glycoside

Abstract. *After glycosidic cleavage of the water-soluble vitamin D-like principle of the calcinogenic plant* Solanum malacoxylon, *the active lipophilic portion was purified by column chromatography and analyzed by combined gas chromatography and mass spectrometry. It was identified as 1,25-dihydroxyvitamin D_3, the active form of vitamin D. Thus this active metabolite of vitamin D exists in the plant world, and its presence probably accounts for pathologic calcification in grazing animals ingesting* Solanum malacoxylon.

1,25-Dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] is the biologically active form of vitamin D, which is biosynthesized in the kidney and mediates calcium and phosphate absorption from the intestine (*1*). This active metabolite, found in experimental animals (*2, 3*) and in humans (*4, 5*), has not previously been detected in plants although cholesterol and cholesterol derivatives have been identified in some botanical species (*6*). The ingestion of certain plants by grazing animals causes calcinosis and pathologic features similar to hypervitaminosis D (*7*). These animals are usually hypercalcemic and hyperphosphatemic with extensive soft tissue calcification, resulting in stiffness of limbs, emaciation, and possibly death. One such calcinogenic plant species found in South American countries is *Solanum malacoxylon* (*7*).

An aqueous extract of *S. malacoxylon* has been shown to mimic the biological properties of 1,25-$(OH)_2D_3$ (*8*). Of particular significance was the observation that *S. malacoxylon* increases calcium absorption and induces calcium binding protein synthesis in chicks fed strontium to inhibit the kidney 1α-hydroxylase enzyme responsible for the formation of 1,25-$(OH)_2D_3$ (*9*). This and other findings (*8*) clearly demonstrate that the vitamin D–active substance in *S. malacoxylon* is at least a functional analog of 1,25-$(OH)_2D_3$, not requiring metabolic activation in the kidney. In contrast to these biological similarities is a marked chemical difference between the *S. malacoxylon* factor, which is water-soluble (*7*) and has an apparent molecular weight in excess of 1000 (*10*), and 1,25-$(OH)_2D_3$, which is preferentially soluble in organic solvents and has a molecular weight of 416 (*2, 3*). Therefore, the identification of the active *S. malacoxylon* principle and its comparison with the animal hormone was of considerable interest.

Preliminary characterization of the *S. malacoxylon* factor suggested that it is in part a sterol which is very similar or identical to 1,25-$(OH)_2D_3$ (*9*). Specific chemical detection reagents showed the presence of glycosidic moieties in the active factor and that the factor can be hydrolyzed in vitro (*11*) by β-glucosidase (E.C. 3.2.1.21) or in vivo by the chick to yield a biologically active compound which is soluble in organic solvents. This lipophilic substance was found to be strikingly similar to the 1,25-$(OH)_2D_3$ hormone, both in its chromatographic mobility and in its ability to compete with radioactive 1,25-$(OH)_2D_3$ for binding to the specific intestinal receptor protein (*12, 13*). Additional physical characterization included an ultraviolet absorption spectrum identical to 1,25-$(OH)_2D_3$ and a partial mass spectrum with a parent molecular ion at *m/e* 416 (*12*).

In the present study, the active lipophilic fragment of the *S. malacoxylon* factor was purified by column chromatography and identified by combined gas chromatography and mass spectrometry. Dried *S. malacoxylon* leaf (*14*) was extracted and the water-soluble active fraction was purified as described by Peterlik and Wasserman (*11*). Partial hydrolysis of this fraction, equivalent to 45 g of the original leaf powder, was accomplished with a mixed glycosidase preparation derived from the sea worm, *Charonia lampas* (*15*). Subsequent to hydrolytic cleavage, the biologically active fraction was soluble in a mixture of methanol and chloroform (2:1 by volume). After the addition of tritiated 1,25-$(OH)_2D_3$ (0.875 ng; 15,000 count/min) (*16*) to this methanol-chloroform–soluble fraction as an internal marker, purification was effected by chromatography on columns of Sephadex LH-20, silicic acid, and Celite (*17*). The purified hydrolyzed *S. malacoxylon* factor exhibited an ultraviolet absorption spectrum (*18*) identical to 1,25-$(OH)_2D_3$ (maximum absorbance at 264 nm, minimum at 228 nm). If an extinction coefficient equivalent to the native sterol hormone is assumed, 4 μg of active *S. malacoxylon* factor were isolated. Independent quantitation by radioreceptor assay (*4, 5*) verified the presence of 3 to 4 μg of 1,25-$(OH)_2D_3$–like activity. One microgram of this material was analyzed by direct probe mass spectrometry (*19*) to yield the spectrum shown in Fig. 1A. The compound displayed a parent molecular ion of *m/e* 416 as well as peaks at *m/e* 398, 380, and 362 which are characteristic of the parent minus one, two, and three H_2O, respectively. This suggests

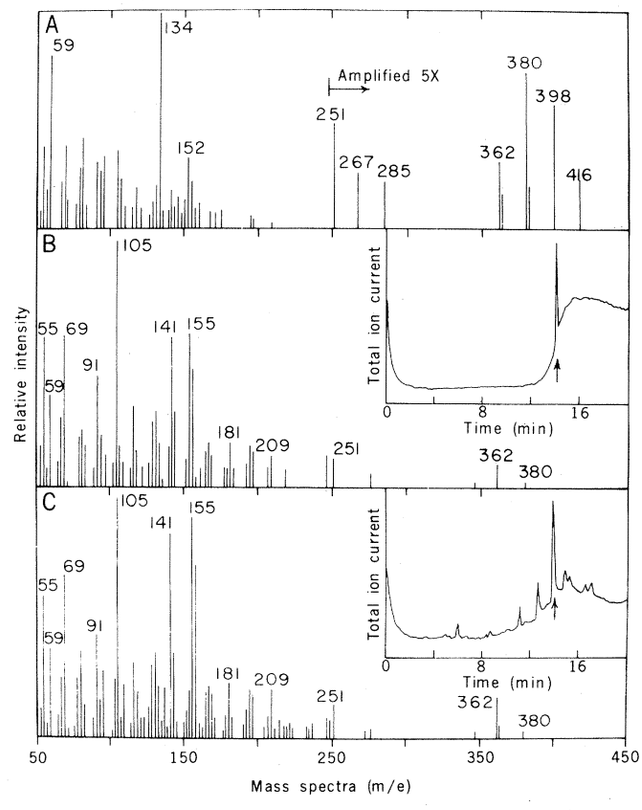

Fig. 1. Analysis of the lipophilic fragment of *S. malacoxylon* by gas chromatography and mass spectrometry. (A) Direct probe mass spectrum of 1 μg of purified hydrolyzed *S. malacoxylon*. (B) Gas chromatogram–mass spectrum of 1 μg of synthetic 1,25-$(OH)_2D_3$. The inset shows the gas chromatographic trace; the arrow shows the peak fraction which was characterized by mass spectrometry. (C) Gas chromatogram–mass spectrum of 2 μg of purified hydrolyzed *S. malacoxylon*. The inset shows the gas chromatographic trace; the arrow shows the peak fraction which was characterized by mass spectrometry.

that the *S. malacoxylon* factor, like 1,25-$(OH)_2D_3$, has three hydroxyl groups present in the molecule. Additional fragments at *m/e* 285, 267, 251, and 152 and 134 localize two of these hydroxyl groups in the A ring of the sterol molecule; and the strong signal at *m/e* 59 is consistent with the third hydroxyl being situated on C-25 in the side chain. This spectrum (Fig. 1A) is very similar to published mass spectra for authentic 1,25-$(OH)_2D_3$ (*2*, *3*). These data, in combination with the characteristic vitamin D ultraviolet spectrum and the high affinity of the hydrolyzed *S. malacoxylon* molecule for the specific intestinal receptor protein (*12*), support the conclusion that the active principle of *S. malacoxylon* is 1,25-$(OH)_2D_3$.

To further substantiate this conclusion, we analyzed the purified sample by combined gas chromatography and mass spectrometry (*20*) in the electron impact ionization mode and compared its mass spectrum with that of synthetic 1,25-$(OH)_2D_3$. Figure 1B shows the gas chromatographic trace obtained with 1 μg of synthetic 1,25-$(OH)_2D_3$ in the inset and the mass spectrum of the major peak of ion current emerging from the column. This peak emerges at a time of 14 minutes. The fragment pattern is distinct from that obtained by direct probe, except for fragments at *m/e* 251, 362, and 380, indicating thermal cyclization of 1,25-$(OH)_2D_3$ (*21*). The fragments with *m/e* 380, 362, and 251 represent the transformed parent molecular ion minus two H_2O, parent minus three H_2O, and the cyclized ABCD ring system minus side chain and two H_2O, respectively. The actual parent molecular ion is not seen because of the extreme dehydrating temperatures of the gas chromatograph–mass spectrometer combination.

Figure 1C illustrates the total ion current trace obtained from gas chromatography of 2 μg of purified hydrolyzed *S. malacoxylon* factor in the inset and the mass spectrum of the major peak of the ion current. Again, the major peak emerges at a time of 14 minutes, resolved from several minor contaminants present in the sample. Mass spectral analysis of this peak reveals a fragment pattern equivalent to that obtained for 1,25-$(OH)_2D_3$. The coincident migration of the active lipophilic fragment of *S. malacoxylon* factor with 1,25-$(OH)_2D_3$ on the gas chromatographic column and the identical mass spectra of the resulting peaks provides evidence that the structure of this calcinogenic factor is 1,25-$(OH)_2D_3$.

It must be emphasized that the *S. malacoxylon* factor occurs as a water-soluble substance in the native state and, after enzymatic cleavage with a mixed glycosidase preparation, becomes lipophilic. The same alteration of the solubility properties of the active substance by almond β-glucosidase (*12*) strongly suggests that the naturally occurring form of this principle is a 1,25-$(OH)_2D_3$-glycoside. More work is necessary to define the composition of the carbohydrates linked to the sterol and to determine the positions of conjugation. Even if there were a family of calcinogenic compounds with varying carbohydrate sequences, the common active unit would probably be the 1,25-$(OH)_2D_3$ hormone. This is supported by the fact that oral administration of crude *S. malacoxylon* extract to chicks rapidly produces significant plasma 1,25-$(OH)_2D_3$ (*12*), a finding which also implies that animals possess endogenous glycosidases for hydrolyzing the native factor or that hydrolysis is due to intestinal microbial action.

The discovery of 1,25-$(OH)_2D_3$-glycoside in *S. malacoxylon* raises the question of whether this sterol hormone is present in other calcinogenic plants, such as *Cestrum diurnum* which is indigenous to southeastern United States, Jamaica, and Hawaii (*5*). Also, the reason for the existence of such calcinogenic factors remains unknown. On one hand, they may play a role in the mineral metabolism of the plants, while on the other, they may serve some survival function in the plant's ecosystem. Recently 1,25-$(OH)_2D_3$ has been used in the treatment of numerous human disorders of calcium and phosphorus metabolism (*22*). The naturally occurring 1,25-$(OH)_2D_3$-glycoside, such as that found in *S. malacoxylon*, may offer therapeutic advantages over the synthetic sterol.

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14. *Solanum malacoxylon* plants were provided by H. R. Camberos, Ministry of Agriculture, Buenos Aires, Argentina, and G. K. Davis, University of Florida at Gainesville.
15. A mixed glycosidase preparation from the liver of the sea worm, *Charonia lampas*, was purchased from Miles Laboratories. Silicic acid purified *S. malacoxylon* factor (water-soluble) was dissolved in 50 ml of citrate-phosphate buffer (*p*H 5.0). To this was added 500 mg of mixed glycosidases derived from *Charonia lampas* and the mixture was incubated for 8 hours at 37°C under N_2. Next, 50 ml of methanol and 66 ml of chloroform were added and, after shaking, the phases were allowed to separate. The lower chloroform phase was combined with three chloroform washes of the upper phase and dried in a rotary evaporator under N_2, in a vacuum. A control experiment involving extraction of the glycosidase preparation alone showed that no 1,25-$(OH)_2D_3$-like activity was present and therefore all chloroform-soluble biological activity originated in the purified aqueous *S. malacoxylon* extract.
16. 1,25-$(OH)_2[^3H]D_3$ (6.5 c/mole) was biosynthesized from 25-hydroxy[26,27-methyl-^{3}H]vitamin D_3 (Amersham/Searle) as described by P. F. Brumbaugh and M. R. Haussler [*J. Biol. Chem.* **249**, 1251 (1974)]. The radioactive sterol was isolated by successive silicic acid, Sephadex LH-20, and Celite chromatography to yield a radiochemical purity of greater than 98 percent and its specific activity was verified by ultraviolet absorption spectrophotometry.
17. The following sequence of columns was employed: (i) Sephadex LH-20 column (1 by 15 cm); the column was eluted with 65 percent chloroform in hexane and the active fraction emerged between 40 and 95 ml. (ii) Silicic acid column (0.8 by 6.3 cm); after being passed through 10 ml of diethyl ether, the active fraction was eluted with 8 ml of acetone). (iii) Micro-Celite; the Celite columns were run as described by M. R. Haussler and H. Rasmussen [*J. Biol. Chem.* **247**, 2328 (1972)], with 10 percent ethyl acetate in hexane as the mobile phase and 45 percent water in ethanol as the stationary phase. The column was 0.8 by 8.5 cm and the active fraction emerged between elution volumes of 7 and 22 ml. (iv) Celite column (1 by 40 cm); run as described in (iii) except that 5-ml fractions were collected. The active fraction and the marker 1,25-$(OH)_2[^3H]D_3$ (yield of tritium was 88.7 percent) emerged in fraction numbers 20 to 27. Fractions 16 to 19 and 28 to 31 were

pooled and saved for use as a background blank in ultraviolet absorption spectrophotometry and mass spectrometry. 1,25-$(OH)_2D_3$-like activity was monitored on all columns by the radioreceptor assay of Brumbaugh *et al.* (*4*).

18. Ultraviolet absorption studies were performed on a dual-beam Beckman DB spectrophotometer fitted with a recorder. Because of the minimal absorbance present in the sample, a small amount of background absorbance from the Celite column or solvents, or both, was subtracted by reading the peak fractions off the last 1 by 40 cm Celite column (fractions 20 to 27) in 1 ml of distilled ethanol against an equal number of fractions obtained just prior to and after the peak (fractions 16 to 19 and 28 to 31), also in 1 ml of distilled ethanol.
19. A model 3200F-6103 Finnigan GC/MS system with quadrapole analyzer was utilized for mass spectrometry. Samples were directly introduced on the probe and continuous scanning was carried out while the samples were rapidly heated to 275°C above ambient temperature; ionization was accomplished by electron bombardment. Preliminary analysis was done on 500 ng of 1,25-$(OH)_2D_3$ and the synthetic hormone emerged from the probe at 225°C and produced the characteristic mass spectrum of this molecule, as published by Holick *et al* (*3*). One microgram of purified hydrolyzed *S. malacoxylon* was similarly analyzed, as was a comparable portion of the background blank saved from the final Celite column.
20. A 2-meter 3 percent QF1 (50 percent trifluoropropylmethyl silicone, Alltech, Ill.) column was employed for gas chromatography. The column was heated to 200°C above ambient temperature.
21. The differences noted in the mass spectral patterns between Fig. 1A and Fig. 1B suggest the formation of cyclized pyroderivatives during the gas chromatographic phase. Vitamin D and 1,25-$(OH)_2D_3$ are known to undergo a thermal cyclization reaction, yielding pyrocalciferols [H. Ziffer, W. J. A. Van den Heuvell, E. O. A. Haahti, E. C. Horning, *J. Am. Chem. Soc.* **82**, 6411 (1960); J. W. Blunt, H. F. DeLuca, H. K. Schnoes, *Biochemistry* **7**, 3317 (1968)]. The transformation apparently takes place in the "flash heating" zone of the gas chromatography column and, in the present case, produces 1α,25-dihydroxypyrocholecalciferol. Evidence for this cyclization is the striking diminution of the fragment at *m/e* 134, which in direct probe analysis is the major species and represents the ring A moiety plus C-6 and C-7 of the open triene system. Moreover, fragments at *m/e* 209 (*m/e* 251 minus 42) [H. Budzikiewicz, C. Djerassi, D. H. Williams, *Structure Elucidation of Natural Products by Mass Spectroscopy* (Holden-Day, San Francisco, 1964), vol. 2, p. 94] and 155, 141, 105, 69, and 55 are consistent with various cleavages of the fused ABCD ring array of classic steroids [G. R. Waller, *Biochemical Applications of Mass Spectroscopy* (Wiley-Interscience, New York, 1972), p. 278].
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Radioimmunoassay for Creatine Kinase Isoenzymes

Abstract. *Creatine kinase has three isoenzymes designated MM, MB, and BB, with BB being the brain form and MM the muscle form. Antibodies to BB creatine kinase were obtained by immunization of rabbits with human BB creatine kinase. The antibodies demonstrated specificity for BB and MB creatine kinase (myocardial isoenzyme), but no cross-reactivity with MM creatine kinase. With the use of this antibody, a highly sensitive radioimmunoassay capable of measuring picomolar amounts of MB creatine kinase has been developed. Clinical application of this method should provide a sensitive and specific test for the diagnosis of myocardial infarction.*

Creatine kinase (E.C. 2.7.3.2) is a dimeric molecule that exists in at least three isoenzyme combinations [each with a mass of approximately 82,000 daltons (*1*)] designated MM, MB, and BB on the basis of monomer composition, M being the predominant form in muscle and B being the predominant form in brain. Plasma from normal human subjects contains primarily MM, with less than 0.005 international unit (IU) of MB per milliliter and no detectable BB creatine kinase. The only human tissue containing appreciable amounts of MB creatine kinase is myocardium (*2*), and elevated MB creatine kinase activity in plasma is a remarkably sensitive and specific marker indicator of myocardial injury. Accordingly, analysis of serial changes in plasma MB creatine kinase activity has been utilized to estimate the extent of acute myocardial infarction in experimental animals and patients (*3, 4*). Results of such estimates are directly dependent on several parameters, one of which is the rate of disappearance of MB creatine kinase from plasma. Unfortunately, factors responsible for the disappearance of MB activity from the circulation in particular, and disappearance of activity of numerous other enzymes used as markers of organ injury in general, have not been well elucidated (*4*). Furthermore, it is not clear whether the disappearance of enzyme activity is rate-limited by inactivation, denaturation, or removal of intact enzyme molecules from the circulation. Despite the general similarity of individual creatine kinase isoenzymes, the rates of disappearance of their activities from the circulation are strikingly different and are not directly related to their lability in solution in vitro (*5*).

Ischemic heart disease is the most common cause of death in the Western world and in the United States alone it accounts for over 750,000 deaths per annum. The prevalence of myocardial infarction is high but the diagnosis is often difficult to confirm. Accordingly, the need for improved diagnostic tests with increased sensitivity and specificity exists. The present study was undertaken to develop a sensitive and specific assay for quantitative detection of isoenzymes of CK containing the B subunit based on physical properties of the enzyme molecules as well as isoenzyme activity. Analysis of profiles of creatine kinase isoenzymes in human tissue indicates that brain contains only BB (*2, 6*), skeletal muscle only MM (*2, 6, 7*), and heart, a combination of MM and MB (*2, 6*). Nevertheless, after brain injury, BB does not appear in the circulation, as shown by studies of patients with cerebral infarction, injury, or infection (*8, 9*). This may be due to the blood brain barrier, or lability of BB creatine kinase in cerebral spinal fluid, or blood itself, although the reason for the lack of appearance of BB has not been established. After cerebral damage, plasma MM is often elevated, presumably because it is released from skeletal muscle as a result of sympathetic stimulation (*10*). Analysis of creatine kinase isoenzyme activity in the plasma after intramuscular injections (*11*) and surgical procedures (*2*) demonstrates that plasma MM creatine kinase may be markedly elevated as a result of these procedures. However, neither BB nor MM increases in concentration. Since BB creatine kinase does not appear in human plasma after myocardial infarction (*7*), the availability of such an assay for the B subunit should be useful in characterizing the rate and nature of disappearance of MB creatine kinase isoenzyme activity from the circulation and in facilitating detection and quantification of myocardial infarction. Although several assays for MB creatine kinase activity have been developed (*6, 11, 12*), they are not ideally suited for quantitative analysis of large numbers of samples: their sensitivity is somewhat limited, and they rely exclusively on detection of enzyme activity rather than other physical properties of creatine kinase isoenzyme molecules.

MM and MB isoenzymes were prepared from human myocardium (obtained at necropsy within 3 hours of death) and BB was prepared from human brain as recently described (*13*). In brief, these tissues were homogenized in 0.05*M* tris-HCl (*p*H 7.4) containing 0.001*M* 2-mercaptoethanol, centrifuged at 31,000*g*, and extracted repetitively with ethanol. Individual isoenzymes were precipitated with 70 percent ethanol, resuspended, separated completely from each other by batch adsorption and column chromatography with diethylaminoethyl cellulose-