MOLECULAR PHARMACOLOGY, 14, 357-365

Responses of Fetal Rat Bones to *Solanum malacoxylon in Vitro*: a Possible Explanation of Previous Paradoxical Results

PAULA H. STERN[1] AND EARL M. NESS

Department of Pharmacology, Northwestern University, Chicago, Illinois 60611

HECTOR F. DELUCA

Department of Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

(Received June 14, 1977)
(Accepted November 16, 1977)

SUMMARY

STERN, PAULA H., NESS, EARL M. & DELUCA, HECTOR F. (1978) Responses of fetal rat bones to *Solanum malacoxylon in vitro*: a possible explanation of previous paradoxical results. *Mol. Pharmacol.*, 14, 357-365.

A partially purified extract of *Solanum malacoxylon* was tested for its actions on fetal rat bone *in vitro*. The extract had a biphasic effect on bone resorption *in vitro*, stimulating at low concentrations (0.3-0.3 mg/ml) and inhibiting at concentrations of 1 mg/ml and higher. The stimulatory effect could be antagonized with calcitonin or glucagon. At a concentration of 1.0 mg/ml, the *S. malacoxylon* extract antagonized the bone-resorbing effects of 1α,25-dihydroxyvitamin D_3[1,25-$(OH)_2D_3$] and parathyroid hormone. To determine whether effects *in vitro* could be due to 1,25-$(OH)_2D_3$ released into the medium during incubation, bones were cultured with equieffective concentrations of parathyroid hormone, 1,25-$(OH)_2D_3$, or *S. malacoxylon*, and the culture medium was extracted with organic solvents. Chloroform or benzene extracts from control or parathyroid hormone treated cultures were inactive, whereas those from 1,25-$(OH)_2D_3$-treated cultures retained most or all of the activity of the original media. Benzene extracts of *S. malacoxylon* culture media inhibited resorption. The inhibitory material could be removed by high-pressure liquid chromatography, but the benzene extract was still ineffective. The results would be consistent with *S. malacoxylon* acting *in vitro* as the unhydrolyzed glycoside conjugate. However, it is also possible that the bone cells hydrolyze the glycoside to release small amounts of 1,25-$(OH)_2D_3$, which then interacts with the receptor.

INTRODUCTION

Solanum malacoxylon, also known as *Solanum glaucophyllum*, is one of several toxic plants that produce symptoms in grazing animals similar to those elicited by excess vitamin D (1-3). Further evidence for the similarity between vitamin D and extracts from *S. malacoxylon* was obtained in experimental studies. *S. malacoxylon* administered *in vivo* stimulated intestinal calcium and phosphorus absorption (4-9), and intestinal calcium-binding protein was increased by treatment with *S. malacoxylon* extracts *in vitro* or *in vivo*

This work was supported by Grants AM 11262 and AM 14881 from the National Institutes of Health.

[1] Recipient of Research Career Development Award 5KO4 AM 70210 from the National Institutes of Health.

0026-895X/78/0142-0357$02.00/0

(7, 10, 11). In intact animals the extract produced a more rapid onset and termination than vitamin D_3 (4, 12). Humphreys (12) commented that the rapid effect "brings to mind the vitamin D metabolites," but that the solubility characteristics of the active principle (soluble in water, methanol, ethanol, and 2-propanol, not extractable from aqueous solution into ether, chloroform, or benzene) "are not consistent with it being any of the metabolites." Subsequently it has been shown that the *S. malacoxylon* principle can displace 1,25-$(OH)_2D_3$[2] from binding sites on intestinal chromatin (9), inhibit renal 25-hydroxyvitamin D_3 1-hydroxylase when administered *in vivo* (9), overcome the strontium-induced block in intestinal calcium transport (1), and stimulate calcium transport in vitamin D-deficient nephrectomized animals (8). The latter two actions would require a 1-hydroxylated vitamin D derivative (13–15). Attempts at chemical characterization indicated the presence of both carbohydrate and steroid components in the active principle (16). Identification of the active principle as 1,25-$(OH)_2D_3$ conjugated to a biologically hydrolyzable moiety was achieved in recent studies by Peterlik *et al.* (17) and Napoli *et al.* (18), in which *S. malacoxylon* extracts administered to animals increased blood levels of 1,25-$(OH)_2D_3$. In other recent studies (17, 19, 20), aqueous extracts of *S. malacoxylon* were incubated with glycosidase preparations. After chromatographic purification procedures a substance that co-chromatographed with 1,25-$(OH)_2D_3$ was obtained. Mass spectral analysis was consistent with the identification of the hydrolysis product as 1,25-$(OH)_2D_3$.

In view of the abundant chemical and physiological evidence for the identity of the active principle of *S. malacoxylon* with 1,25-$(OH)_2D_3$, the data obtained from studies of the effects of the extract on bone present something of a paradox. Mautalen's laboratory found evidence that the *S. malacoxylon* extract mobilized calcium from rat and rabbit bone (4, 21), whereas Uribe *et al.* (6) reported that a *S. malacoxylon* extract that was effective on the rat intestine when given *in vivo* had no bone calcium-mobilizing activity in these same animals. The results of studies *in vitro* on *S. malacoxylon*-induced bone resorption are likewise inconsistent. Lloyd *et al.* (22) and Simonite *et al.* (23) were able to demonstrate bone-resorbing effects of *S. malacoxylon* on mouse calvaria *in vitro*, whereas Moorhead *et al.* (24) were not. Puche and Locatto (25) reported stimulatory effects on calcium release and mitochondrial calcium uptake in chick bone. The inconsistent results are somewhat surprising, since the bone-resorbing effects *in vitro* of 1,25-$(OH)_2D_3$ have been reproduced in many laboratories (26–30), including our own. Since *S. malacoxylon* had not been studied *in vitro* in the rat despite the disparities in the findings *in vivo* in this species, we undertook the studies described below.

METHODS

Preparation of S. malacoxylon extract.[3] Fifty grams of dried *S. malacoxylon* leaves were extracted by stirring with diethyl ether for three 30-min periods. The residue was then extracted with methanol eight times in a Waring Blendor. A yield of 5.39 g of methanol-extractable material was obtained. Then 1.62 g of the methanol extract were applied to a 2 × 60 cm column containing 60 g of Sephadex LH-20, which was eluted with methanol. The first 20 ml were discarded, and the next 55 ml were collected and chromatographed on a 2 × 60 cm Sephadex G-100 column, which was eluted with deionized water. The void volume was discarded, and subsequent void volume equivalents were collected, lyophilized, and administered orally to rats in the amounts indicated. The collected fractions weighed as follows: fraction 1, 0.045 g; fraction 2, 0.016 g; fraction 3, 0.029 g; fraction 4, 0.013 g. Thus 1.93 mg of fraction 3 (the fraction of interest) were obtained from each gram of dried leaves.

Studies in vivo. Weanling male albino rats (Holtzman) were maintained on a

[2] The abbreviations used are: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PTH, parathyroid hormone.

[3] M. F. Holick, T. Tavela, H. K. Schnoes, and H. F. DeLuca, unpublished method.

vitamin D-deficient diet (31) for 4 weeks. For studies of calcium absorption, the diet contained 0.47% calcium and 0.3% phosphorus. For studies of calcium mobilization, the diet contained 0.02% calcium and 0.3% phosphorus. Blood was collected from the tail vein or from animals killed by decapitation. Serum was obtained from clotted blood by centrifugation.

Studies in vitro. Fetal rat radii and ulnae labeled with ^{45}Ca were cultured by methods described previously (32–35). For the studies reported here, the two bones from a given forelimb were cultured together as a radius-ulna pair. At the end of the culture, the bones were extracted with 0.1 N HCl. Results from this type of grouping are expressed as percentage of ^{45}Ca released, defined as

$$\frac{^{45}\text{Ca cpm/ml medium}}{^{45}\text{Ca cpm/ml medium} + {}^{45}\text{Ca cpm/ml extract}} \times 100$$

The *S. malacoxylon* extract was added in deionized water, as were calcitonin and glucagon. 1,25-$(OH)_2D_3$ was added in 95% ethanol, the final ethanol concentration being less than 0.005%. Chloroform or benzene extracts were prepared by adding an equal volume of the solvent to the culture medium, mixing well with a Pasteur pipette, and centrifuging to separate the phases. The organic phase was removed, evaporated, and reconstituted with culture medium. Bones were extracted with 1 ml of solvent. Benzene extracts were further purified in some experiments by back-extraction with 0.1 M sodium phosphate, pH 10, and high-pressure liquid chromatography. Each sample of medium was extracted three times with benzene and back-extracted twice with an equal volume of the phosphate buffer. The organic phase was separated by centrifugation, evaporated, redissolved in 13% 2-propanol in hexane, filtered through a Nucleopore membrane, and applied, in a 1-ml final volume, to a Waters μPorosil (silica) column. The column was eluted with 13% 2-propanol in hexane at 1 ml/min, and the fraction corresponding to 1,25-$(OH)_2D_3$ (12–16 min) was collected. Recovery of 1,25-$(OH)_2D_3$ was monitored by addition of [23,24-^{3}H]1,25-$(OH)_2D_3$, 78 Ci/mmole, to the medium at the start of the extraction. The benzene–phosphate–high-pressure liquid chromatographic method has been used for extraction of 1,25-$(OH)_2D_3$ from serum in preparation for bioassay (36).

Salmon calcitonin was a generous gift from Dr. J. Bastian, Armour Pharmaceutical Company. Glucagon, porcine-bovine, was obtained from Sigma. Synthetic 1,25-$(OH)_2D_3$ was prepared as described previously (37). High specific activity (78 Ci/mmol) [^{3}H]1,25-$(OH)_2D_3$ was synthesized by Dr. S. Yamada according to the procedure of Partridge *et al.* (38). *S. malacoxylon* leaves were a gift from Dr. Carlos Mautalen.

RESULTS

Organ culture studies were carried out with a partially purified *S. malacoxylon* extract (G-100 fraction 3; see METHODS). This material, administered *in vivo*, consistently stimulated intestinal calcium absorption but was ineffective in eliciting bone calcium mobilization in two experiments.[4] The effects of the partially purified *S. malacoxylon* extract on ^{45}Ca release from labeled fetal rat long bones are shown in Fig. 1a. A minimal concentration of 0.03 mg/ml of the crude extract was required to obtain any effect on bone resorption. Maximal stimulation of resorption was produced with a less than 10-fold greater concentration. At concentrations of *S. malacoxylon* above 0.3 mg/ml, bone resorption decreased. Responses to 1,25-$(OH)_2D_3$ in the same experiments are shown in Fig. 1b. The maximum response to the 1,25-$(OH)_2D_3$ was obtained at approximately 30 pg/ml. The effects of 41.6, 416, and 4160 pg/ml were not significantly different from those of 30 pg/ml, although in some experiments 4160 pg/ml gave a lesser effect. The concentrations of 1,25-$(OH)_2D_3$ and *S. malacoxylon* extract giving roughly equivalent effects in our studies were thus 30 pg/ml and 0.05–0.1 mg/ml, respectively. Since 1.93 mg of *S. malacoxylon* extract were obtained from each gram of dried leaves, our bone cultures

[4] M. F. Holick, T. Tavela, and H. F. DeLuca, unpublished observations.

detected 30 pg of 1,25-$(OH)_2D_3$-like activity in extract derived from 26 mg of dried leaves.

The release of ^{45}Ca from fetal rat bones was accompanied by readily observed gross morphological changes indicative of resorption. Further evidence that the ^{45}Ca release represented resorption is inhibition of the effects by either glucagon or calcitonin (Table 1). The diminished response noted at the higher *S. malacoxylon* concentration appeared to be a consequence of inhibition of bone resorption (Table 2). In experiment I, the higher (1.0 mg/ml) *S. malacoxylon* concentration did not by itself produce significantly less resorption than the lower (0.1 mg/ml) concentration. However, in combination with 1,25-$(OH)_2D_3$, the higher *S. malacoxylon* concentration produced a significantly diminished bone-resorbing effect. In experiment II, the *S. malacoxylon* extract at 1.0 mg/ml was shown to inhibit PTH-induced resorption as well as that stimulated by 1,25-$(OH)_2D_3$.

We attempted to determine whether the substance producing the bone resorption in the *S. malacoxylon*-treated cultures is the free vitamin D metabolite or the conjugate—i.e., whether the cultures hydrolyze the conjugate. We incubated bone cultures with *S. malacoxylon* extract and then attempted to isolate the active material from the bones or media with various solvent systems. For comparison, extracts were prepared from unincubated media and from media incubated under the same conditions, but without bones. Untreated

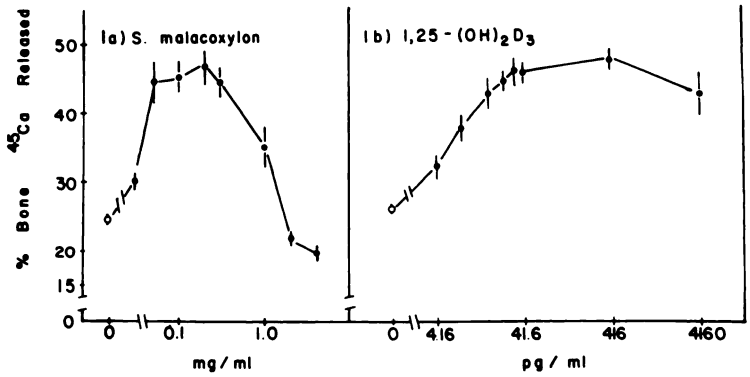

FIG. 1. *Dose-response curves of ^{45}Ca release from labeled fetal rat bones incubated with a partially purified extract of S. malacoxylon leaves (a) or 1,25-$(OH)_2D_3$ (b) in vitro*

Bones were cultured as radius-ulna pairs and extracted with 0.1 N HCl at the end of culture. Values are means ± standard errors of the percentage of ^{45}Ca released from the bones at each concentration of *S. malacoxylon* or 1,25-$(OH)_2D_3$.

TABLE 1

Inhibition of S. malacoxylon-induced bone resorption by glucagon or calcitonin

Bones were cultured for 48 hr with the indicated treatments. The concentration of glucagon was 1 μg/ml; that of calcitonin was 3 μg/ml. Values are means ± standard errors of five bone pairs for each treatment.

S. malacoxylon	Bone ^{45}Ca released			
	−Glucagon	+Glucagon	−Calcitonin	+Calcitonin
mg/ml	%	%	%	%
0[a]	21.6 ± 0.5	21.7 ± 0.9	19.2 ± 1.0	17.3 ± 0.9
0.025	25.7 ± 2.0	20.0 ± 0.9[b]	30.0 ± 6.0	19.4 ± 1.4
0.05	27.0 ± 1.7	23.5 ± 2.0	37.1 ± 8.3	19.2 ± 0.9
0.1	35.0 ± 3.1	25.3 ± 2.2[b]	41.7 ± 5.6	17.8 ± 1.3[b]

[a] Control.

[b] Significant inhibition of *S. malacoxylon* effect; $p < 0.05$.

TABLE 2

Inhibitory effect of a high concentration of S. malacoxylon extract on 1,25-$(OH)_2D_3$ or PTH-induced bone resorption

Bones were cultured for 48 hr with the indicated treatments. Values are means ± standard errors of four bone pairs for each treatment.

Treatment	Bone ^{45}Ca released
	%
Experiment	
None	17.0 ± 0.4
S. malacoxylon, 0.1 mg/ml	35.1 ± 2.5[a]
S. malacoxylon, 1.0 mg/ml	33.8 ± 4.8[a]
1,25-$(OH)_2D_3$, 80 pM	33.6 ± 3.6[a]
S. malacoxylon, 0.1 mg/ml, + 1,25-$(OH)_2D_3$	43.2 ± 3.3[a]
S. malacoxylon, 1.0 mg/ml, + 1,25-$(OH)_2D_3$	26.3 ± 2.0[a, b]
Experiment II	
None	17.7 ± 2.1
S. malacoxylon, 0.1 mg/ml	34.2 ± 5.9[a]
S. malacoxylon, 1.0 mg/ml	25.0 ± 1.8[a]
1,25-$(OH)_2D_3$, 50 pM	36.4 ± 2.7[a]
PTH, 300 ng/ml	40.7 ± 3.5[a]
S. malacoxylon, 1.0 mg/ml, + 1,25-$(OH)_2D_3$	29.1 ± 4.9[a]
S. malacoxylon, 1.0 mg/ml, + PTH	28.4 ± 2.0[a, c]

[a] Significant increase compared with control; $p < 0.05$.

[b] Significant inhibition compared with *S. malacoxylon*, 0.1 mg/ml, + 1,25-$(OH)_2D_3$; $p < 0.01$.

[c] Significant inhibition compared with PTH, 300 ng/ml; $p < 0.05$.

bones and bones treated with PTH served as additional controls. Table 3 shows the results obtained from such a pair of experiments, in which all media and tissues were extracted with chloroform as described in METHODS. Approximately equiactive concentrations of PTH, 1,25-$(OH)_2D_3$, and *S. malacoxylon* were used in the original media (Column A). Only extracts of media containing 1,25-$(OH)_2D_3$ caused significant resorption when added to subsequent cultures. Extracts of bones were ineffective. Since we have found benzene to be a more efficient solvent for extracting 1,25-$(OH)_2D_3$ from some biological fluids,[5] the studies were repeated with benzene extracts (Table 4). Again, all the 1,25-$(OH)_2D_3$ activity was recovered. Extracts of PTH-containing media were inactive, as were extracts of *S. malacoxylon*-containing media, even though these media were even more effective than 1,25-$(OH)_2D_3$ in producing resorption in the original experiments (column A). Since we had previously shown that the *S. malacoxylon* extract contained an inhibitor of bone resorption *in vitro*, we tested the benzene extract of the *S. malacoxylon* for possible inhibitory activity (Table 5). The results indicated that the benzene extract inhibited the bone-resorbing effect of added 1,25-$(OH)_2D_3$. We then applied a purification procedure that we have used to extract bioassayable 1,25-$(OH)_2D_3$ from normal human and rat serum (36) (see METHODS), in which the benzene extract was back-extracted with 0.1 M phosphate buffer, pH 10, and subjected to high-pressure liquid chromatography. The 1,25-$(OH)_2D_3$ region was collected. Extracts prepared by this method were no longer inhibitory (Table 6). However, the bone-resorbing activity of the added *S. malacoxylon* was not recovered in the 1,25-$(OH)_2D_3$ region in any of the three experiments (Tables 6 and 7). In other experiments (not shown) fractions of extracted *S. malacoxylon*-containing media or control media eluting between 0 and 22 min were tested for their effects *in vitro*. All fractions were devoid of both bone-resorbing and inhibitory activity. *S. malacoxylon* that had been extracted with benzene prior to being added to the culture medium still was inactive at a concentration of 2 mg/ml.

[5] T. E. Phillips, P. H. Stern, and S. V. Lucas, unpublished observations.

DISCUSSION

Our results indicate that a partially purified extract of *S. malacoxylon* elicits a biphasic response from cultured fetal rats bones. At concentrations of 0.03–0.3 mg/ml the *S. malacoxylon* extract produces bone resorption, whereas at concentrations of 1.0 mg/ml and above the bone-resorbing effect is inhibited. This dose-response curve is quite different from that of 1,25-$(OH)_2D_3$, which elicits maximal bone-resorbing activity over a concentra-

TABLE 3

Effect of chloroform extracts of PTH-, 1,25-(OH)$_2$D$_3$-, or S. malacoxylon-containing cultures on bone ^{45}Ca release

Values in column A are responses of bones to the original treatment. Values in other columns are from subsequent experiments, in which chloroform extracts of media from the original experiment were added to culture media. In experiment I, seven bone pairs were used for each treatment in column A, and four bone pairs for the extracts; in experiment II, eight bone pairs were used for each treatment in column A, and five bone pairs for the extracts.

Original treatment	Bone ^{45}Ca released (A)	Bone ^{45}Ca released by chloroform extract of:			
		Uncultured medium	Medium cultured without bones	Medium cultured with bones (A)	Bones
	%	%	%	%	%
Experiment I					
Control	21.3 ± 1.4		18.6 ± 2.6	21.5 ± 4.2	
PTH, 500 ng/ml	32.0 ± 2.3[a]		17.0 ± 1.0	14.8 ± 1.4	
1,25-(OH)$_2$D$_3$, 100 pM	38.9 ± 3.3[a]	35.3 ± 5.3	35.8 ± 3.7[a]	35.1 ± 4.8	
S. malacoxylon, 0.3 mg/ml	37.7 ± 3.6[a]		23.0 ± 2.5	22.4 ± 1.3	
Experiment II					
Control	20.1 ± 0.5				18.5 ± 0.8
1,25-(OH)$_2$D$_3$, 50 pM	36.0 ± 3.0[a]				18.0 ± 0.6
1,25-(OH)$_2$D$_3$, 100 pM	39.7 ± 1.2[a]				19.2 ± 0.6
S. malacoxylon, 0.15 mg/ml	43.2 ± 1.4[a]				17.5 ± 0.8
S. malacoxylon, 0.3 mg/ml	43.3 ± 2.8[a]				17.8 ± 0.6

[a] Significantly increased compared with controls; $p < 0.05$.

TABLE 4

Effect of benzene extracts of PTH-, 1,25-(OH)$_2$D$_3$-, or S. malacoxylon-containing culture media on bone ^{45}Ca release

Values in column A are responses of bones to the original treatment. Values in other columns are from subsequent experiments, in which benzene extracts of the media from the original experiment were added to culture media. Six bone pairs were used for each treatment in column A, and three bone pairs for the extracts.

Original treatment	Bond ^{45}Ca released (A)	Bone ^{45}Ca released by benzene extract of:	
		Medium cultured without bones	Medium cultured with bones (A)
	%	%	%
Control	22.6 ± 4.3	24.6 ± 0.5	20.1 ± 1.1
PTH, 500 ng/ml	38.9 ± 2.1[a]	18.4 ± 1.8	24.7 ± 0.7
1,25-(OH)$_2$D$_3$, 50 pM	30.0 ± 1.5	34.9 ± 3.0[a]	34.7 ± 2.1[a]
S. malacoxylon, 0.05 mg/ml	37.4 ± 4.6[a]	21.2 ± 1.1	18.6 ± 3.2
S. malacoxylon, 0.1 mg/ml	40.9 ± 3.1[a]	20.6 ± 3.6	21.2 ± 1.0
S. malacoxylon, 0.2 mg/ml	41.7 ± 4.5[a]	18.5 ± 2.3	21.7 ± 1.1

[a] Significantly increased compared with controls; $p < 0.05$.

tion range of at least an order of magnitude. At the present time we have little information on the nature of the inhibitor in the *S. malacoxylon* extracts. The chromatographic procedures used to purify the *S. malacoxylon* should remove inorganic salts, and we have determined that the addition of the *S. malacoxylon* extract does not increase the phosphate concentration of the culture media.[6] Conceivably

[6] P. H. Stern, E. M. Ness, and H. F. DeLuca, unpublished observations.

TABLE 5

Inhibition of 1,25-$(OH)_2D_3$-induced bone resorption by a benzene extract of partially purified S. malacoxylon

Partially purified *S. malacoxylon* was dissolved in tissue culture medium BGJ (GIBCO) to give a concentration of 0.3 mg/ml. An aliquot of this solution was extracted with an equal volume of benzene. The benzene extract was evaporated and reconstituted to its original volume with BGJ for assay. Each treatment was tested with three bone pairs.

Treatment	Bone ^{45}Ca released
	%
Control	17.8 ± 1.6
1,25-$(OH)_2D_3$, 60 pM	33.6 ± 3.9[a]
S. malacoxylon, 0.3 mg/ml, benzene extract	17.6 ± 1.2
S. malacoxylon, 0.3 mg/ml, benzene extract, + 1,25-$(OH)_2D_3$, 60 pM	16.9 ± 0.7[b]

[a] Significant increase compared with control; $p < 0.05$.

[b] Significant inhibition of effect of 1,25-$(OH)_2D_3$; $p < 0.01$.

the inhibitor could be either a nonspecific toxin or an analogue with antagonist properties. It is interesting that the inhibitory activity was not separated from the *S. malacoxylon* in four purification steps. Since the inhibitor also blocks the effects of PTH, it is not a specific antagonist of vitamin D analogues. The presence of an antagonist of bone resorption in some lots of *S. malacoxylon* could explain the disparity between the results of Uribe *et al.* (6) and those of Mautalen (4) and his colleagues (21). The original work of Uribe *et al.* was done with a crude aqueous extract of the same material used in the current study. Preliminary studies by Holick *et al.*[7] indicated that the *S. malacoxylon* material used in the current studies was likewise inactive on bone calcium mobilization. Bone mobilization, both in the original study by Uribe *et al.* and in the more recent work by Holick *et al.*, was tested at only one concentration of *S. malacoxylon*. Perhaps lower doses would have elicited an effect on bone. Because of the differences in purification procedures it is difficult to compare activities from various studies. It is of interest, however, that Corradino and Wasserman (11) and Procsal *et al.* (9) calculated that the leaves they used contained the equivalent of 10^{-6}–10^{-7} part of 1,25-$(OH)_2D_3$. Our data show only 30 pg of 1,25-$(OH)_2D_3$-like activity from 26 mg of dried leaves, or a content of 10^{-9} part of 1,25-$(OH)_2D_3$. This could be further evidence for the presence of an inhibitor in the material used in the current studies.

Our extraction studies were designed to determine whether free 1,25-$(OH)_2D_3$ was released from the *S. malacoxylon* conjugate during culture. There was no bone-resorbing activity in extracts of fresh *S. malacoxylon*-containing media or media incubated with or without bones. The studies do not rule out the possibility that conversion of the conjugate to 1,25-$(OH)_2D_3$ occurs in the bone cells. This is not improbable, since bone-resorbing cells

TABLE 6

Removal of inhibitor of bone resorption from benzene extracts of S. malacoxylon by aqueous back-extraction at pH 10 and high-pressure liquid chromatography

All treatments were carried out in a single experiment. Partially purified *S. malacoxylon* was dissolved in tissue culture medium BGJ to give a concentration of 0.3 mg/ml. An aliquot of this solution was extracted with an equal volume of benzene and back-extracted with an equal volume of 0.1 M phosphate buffer, pH 10, as described in METHODS. The organic phase was chromatographed on a μPorosil column with a 13% 2-propanol–87% hexane eluting solvent. The extracts in this experiment are the 12-16-min high-pressure liquid chromatographic eluates. Five bone pairs were used for each treatment.

Treatment	Bone ^{45}Ca released
	%
Control	25.1 ± 2.6
1,25-$(OH)_2D_3$, 60 pM	37.3 ± 2.6[a]
S. malacoxylon, 0.3 mg/ml	41.5 ± 2.3[a]
Control medium extract	20.2 ± 1.6
S. malacoxylon medium extract	21.2 ± 1.9
Control medium extract + 1,25-$(OH)_2D_3$, 60 pM	37.3 ± 2.6[a]
S. malacoxylon medium extract + 1,25-$(OH)_2D_3$, 60 pM	40.2 ± 2.9[a]

[a] Significant effect compared with control; $p < 0.01$.

[7] M. F. Holick *et al.*, unpublished observations.

TABLE 7

Recovery of 1,25-$(OH)_2D_3$ activity but not S. malacoxylon activity from culture media purified by benzene-pH 10-high-pressure liquid chromatographic procedure

Values in column A are responses of bones to the original treatment. Values in other columns are from subsequent experiments, in which the benzene-pH 10-high-pressure liquid chromatographic extracts of the media from the original experiment were added to the culture media. Six to eight bone pairs were used for each treatment in column A, and four bone pairs for the extracts. Amounts of extract added to the culture media were adjusted for recovery in experiments I and III. In experiment II recoveries from high-pressure liquid chromatography were 50–70%, except as noted below. These samples were not corrected for recovery.

Original treatment	Bone ^{45}Ca released (A)	Bone ^{45}Ca released by benzene-pH 10-chromatographic extract of:		
		Unincubated medium	Medium incubated without bones	Medium incubated with bones
	%	%	%	%
Experiment I				
Control	25.1 ± 2.6	22.2 ± 4.8		
1,25-$(OH)_2D_3$, 80 pM	38.5 ± 5.2[a]	43.2 ± 2.7[a]		
S. malacoxylon, 0.3 mg/ml	41.5 ± 2.3[a]	22.7 ± 2.9	19.9 ± 0.9	18.5 ± 1.0
Experiment II				
Control	18.4 ± 0.4		28.1 ± 2.8	22.3 ± 1.3
S. malacoxylon, 0.3 mg/ml	36.1 ± 3.1[a]		23.9 ± 4.5[b]	20.8 ± 0.5
Experiment III				
Control	16.1 ± 0.8	19.6 ± 3.2	16.1 ± 0.6	22.3 ± 3.1
S. malacoxylon, 0.3 mg/ml	37.8 ± 2.6[a]	17.4 ± 2.6	20.0 ± 4.1	21.7 ± 2.9

[a] Significantly different from control; $P < 0.05$. None of the extracts inhibited the effect of added 1,25-$(OH)_2D_3$.

[b] Recovery was only 19%.

appear to be rich in lysosomal enzymes (39, 40). A more sensitive system would be required to determine whether hydrolysis occurs at the tissue site, since extracts of bones treated with effective concentrations of 1,25-$(OH)_2D_3$ showed no resorbing activity. Thus at the present time we do not know whether the resorption is due to released free 1,25-$(OH)_2D_3$ or to the conjugate. Our previous studies on the bone-resorbing effects of a number of vitamin D analogues permit us to speculate on the possible nature of a *seco*-steroid glycoside conjugate that would retain bone-resorbing activity *in vitro*. Our results (28) and those of others (41) suggest that at least two hydroxyl groups must be free to obtain such activity. Vitamin D_3 and 1α-hydroxy-3-deoxyvitamin D_3, with single hydroxyl groups in either position 3 or 1 in the A ring, are both inactive at concentrations much higher than those of the *S. malacoxylon* extract used in the current studies. No work has been done with the compound containing only a 25-hydroxyl group, and thus it is still theoretically possible that a compound conjugated in both positions 1 and 3 but with a free 25-hydroxyl position could be active. This seems unlikely however, since the 25-hydroxyl and 1-hydroxyl seem to be equally important to the bone-resorbing activity (42) of vitamin D analogues. Analogues lacking one of the three hydroxyl groups retain substantial bone-resorbing activity (28, 30). It is not known whether conjugation to one or more sugar molecules would have an effect equivalent to deletion of a hydroxyl group.

ACKNOWLEDGMENTS

We would like to express our appreciation to Jan Miller and Thalia Mavreas for their excellent technical assistance, and to Lorraine Reeve and Michael Holick for providing the *S. malacoxylon* extract used in these studies.

REFERENCES

1. Worker, N. A., & Carrillo, B. J. (1967) *Nature*, 215, 72–74.
2. Sansom, B. F., Vagg, M. J. & Dobereiner, J.

(1971) *Res. Vet. Sci.*, 12, 604-605.
3. Wasserman, R. H. (1975) *Nutr. Rev.*, 33, 1-5.
4. Mautalen, C. A. (1972) *Endocrinology*, 90, 563-567.
5. O'Donnell, J. M. & Smith, M. W. (1973) *Nature* 244, 357-358.
6. Uribe, A., Holick, M. F., Jorgensen, N. A. & DeLuca, H. F. (1974) *Biochem. Biophys. Res. Commun.*, 58, 257-262.
7. Lawson, D. E. M., Smith, M. W. & Wilson, P. W. (1974) *FEBS Lett.*, 45, 122-125.
8. Walling, M. W. & Kimberg, D. V. (1975) *Endocrinology* 97, 1567-1575.
9. Procsal, D. A., Henry, H. L., Hendrickson, T. & Norman, A. W. (1976) *Endocrinology*, 99, 437-444.
10. Wasserman, R. H. (1974) *Science*, 183, 1092-1094.
11. Corradino, R. A. & Wasserman, R. H. (1974) *Nature*, 252, 716-717.
12. Humphreys, D. J. (1973) *Nat. New Biol.*, 246, 155-157.
13. Omdahl, J. L. & DeLuca, H. F. (1972) *J. Biol. Chem.*, 247, 5520-5526.
14. Fraser, D. R. & Kodicek, E. (1970) *Nature*, 228, 764-766.
15. Gray, R., Boyle, I. & DeLuca, H. F. (1971) *Science*, 172, 1232-1234.
16. Peterlik, M. & Wasserman, R. H. (1975) *FEBS Lett.*, 56, 16-19.
17. Peterlik, M., Bursac, K., Haussler, M. R., Hughes, M. R. & Wasserman, R. H. (1974) *Biochem. Biophys. Res. Commun.*, 70, 797-804.
18. Napoli, J. L., Reeve, L. E., Eisman, J. A., Schnoes, H. K. & DeLuca, H. F. (1977) *J. Biol. Chem.* 252, 2580-2583.
19. Wasserman, R. H., Henion, J. D., Haussler, M. R. & McCain, T. A. (1976) *Science*, 194, 853-855.
20. Haussler, M. R., Wasserman, R. H., McCain, T. A., Peterlik, M., Bursac, K. M. & Hughes, M. R. (1976) *Life Sci.*, 18, 1049-1056.
21. Campos, C., Ladizesky, M. & Mautalen, C. (1973) *Calcif. Tissue Res.*, 13, 245-248.
22. Lloyd, W., Wells, H., Walling, M. W. & Kimberg, D. V. (1975) *Endocrinol. Res. Commun.*, 2, 159-166.
23. Simonite, J. P., Morris, K. M. L. & Collins, J. C. (1976) *J. Endocrinol.*, 68, 18P-19P.
24. Moorhead, J. F., Humphreys, D. J., Varghese, Z., Basudde, C. D. K., Jenkins, M. V. & Wills, M. R. (1975) in *Proceedings of the Second Workshop on Vitamin D and Problems Related to Uremic Bone Disease* (Norman, A. W., Schaefer, K., Grigoleti, H. G. & Ritz, E., (eds.), pp. 725-730, de Gruyter, Berlin.
25. Puche, R. C. & Locatto, M. E. (1974) *Calcif. Tissue Res.*, 16, 219-226.
26. Raisz, L. G., Trummel, C. L., Holick, M. F. & DeLuca, H. F. (1972) *Science*, 175, 768.
27. Reynolds, J. J., Holick, M. F. & DeLuca, H. F. (1973) *Calcif. Tissue Res.*, 12, 295-301.
28. Stern, P. H., Trummel, C. L., Schnoes, H. K. & DeLuca, H. F. (1975) *Enocrinology*, 97, 1552-1558.
29. Atkins, D. & Peacock, M. (1974) *J. Endocrinol.*, 61, lxxix-lxxx.
30. Mahgoub, A. & Sheppard, H. (1977) *Endocrinology*, 100, 629-634.
31. Steenbock, H. & Herting, D. (1955) *J. Nutr.*, 57, 449-468.
32. Raisz, L. G. (1965) *J. Clin. Invest.* 44, 103-116.
33. Stern, P. H. (1969) *J. Pharmacol. Exp. Ther.*, 168, 211-217.
34. Stern, P. H. (1971) *Calcif. Tissue Res.*, 7, 67-75.
35. Stern, P. H., Mavreas, T., Trummel, C. L., Schnoes, H. K., & DeLuca, H. F. (1976) *Mol. Pharmacol.*, 12, 879-886.
36. Stern, P. H., Phillips, T. E., Lucas, S. V., Hamstra, A. J., DeLuca, H. F. & Bell, N. H. (1977) in *Proceedings of the Third Workshop on Vitamin D*, Norman, A. W., Schaefer, K., Coburn, J. W., Deluca, H. F., Fraser, D., Grigoleit, H. G. & Herrath, D. V. (eds.), pp. 531-540, de Gruyter, Berlin.
37. Semmler, E. J., Holick, M. F., Schnoes, H. K. & DeLuca, H. F. (1972) *Tetrahedron Lett.*, 40, 4147-4150.
38. Partridge, J. J., Faber, S. & Uskokovic, M. R. (1974) *Helv. Chim. Acta*, 57, 764-771.
39. Vaes, G. (1969) in *Lysosomes in Biology and Pathology* (Dingle, J. T. & Fell, H. B., eds.), Vol. 1, pp. 217-253, North-Holland, Amsterdam.
40. Lucht, U. (1972) *Z. Zellforsch*, 135, 229-244.
41. Trummel, C. L., Raisz, L. G., Hallick, R. B. & DeLuca, H. F. (1971) *Biochem. Biophys. Res. Commun.*, 44, 1096-1101.
42. Onisko, B. L., Schnoes, H. K., DeLuca, H. F. & Stern, P. H. (1976) *Fed. Proc.*, 35, 1718.