1,25-DIHYDROXYCHOLECALCIFEROL-LIKE ACTIVITY IN *SOLANUM MALACOXYLON*: PURIFICATION AND PARTIAL CHARACTERIZATION

M. PETERLIK and R. H. WASSERMAN

Department of Physical Biology, New York State Veterinary College, Cornell University, Ithaca, N. Y. 14853, USA

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1. Introduction

It is now well-established that the active principle in the calcinogenic plant, *Solanum malacoxylon (S.m.)*, has biological properties similar to 1,25-dihydroxycholecalciferol (1,25-$(OH)_2$-D_3) [1–3]. This plant, or an extract therefrom, is effective in the anephric rat [4] and can induce the synthesis of the vitamin D-dependent intestinal calcium-binding protein (CaBP) and increase calcium absorption in the chick under conditions in which the kidney 1α-hydroxylase enzyme system is blocked [1]. The possible use of the *S.m.* factor as therapeutic alternative to 1,25-$(OH)_2$-D_3 in cases of disturbed calcium metabolism due to kidney failure and other causes has stirred considerable interest in the isolation of the active substance.

Preliminary characterization of the *S.m.* principle revealed striking dissimilarities to vitamin D or to any of its biologically active metabolites and derivatives in two respects: high solubility in water and a molecular weight considerably higher than 1000 [2, 5]. These properties suggest a greater molecular complexity than vitamin D or its known metabolites. Previous attempts at isolation have been unsuccessful so far.

We wish to describe an isolation procedure which yields a highly purified, biologically active material that has allowed a more detailed, although preliminary, characterization of the 1,25-$(OH)_2$-D_3-like principle(s) in *Solanum malacoxylon*.

2. Methods and results

2.1. *Biological assay*

Determinations of the biopotency of purified fractions were based on the ability of *S.m.* to induce CaBP in the duodenal mucosa of rachitic chicks [2]. Four-week old rachitic chicks were fasted overnight and then dosed orally with *S.m.* fractions dissolved in 1.5 ml distilled water. The birds were fasted for additional 4 hr. The vitamin D-dependent CaBP in duodenal mucosa was determined 24 hr after dosing by the previously described procedure [6].

2.2. *Isolation procedure*

50 g of ground, dried leaf were extracted three times with 150 ml methanol: chloroform (2:1, v/v) by shaking for 2 hr. The mixture was filtered and the filtrate was discarded. The retained solid residue after drying at room temperature was shaken with 450 ml distilled water for 24 hr. The aqueous extract was separated from insoluble material by centrifugation and then lyophilized. The freeze-dried material (15 g) was extracted with methanol in a Soxhlet apparatus until nearly all methanol-soluble pigments were removed (usually 10 to 12 hr). The residue was then stirred for 3 hr in 100 ml ethanol: water (3:1, v/v) and the mixture centrifuged. The ethanol-insoluble residue was discarded. After evaporation of the extract at 37°C in vacuo, 1–2 g of an oily brown substance remained which contained up to 70% of the biological activity originally present in the aqueous extract. The remaining 30% was present in the previous methanol extract. Prolonged extraction with methanol did not change the relative amounts of activity in these fractions.

The oily substance obtained in the foregoing step was redissolved in 5 ml of 50% aqueous ethanol and applied to a 2 × 50 cm column packed with 100 g silicic acid (BIO-SIL HA-325 mesh, BIO-RAD Labor-

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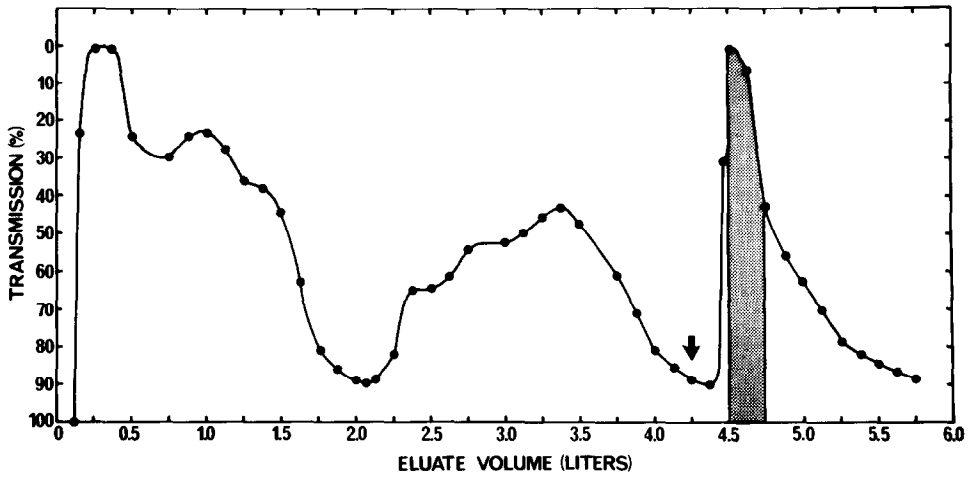

Fig.1. Chromatographic purification of a *S.m.* extract (for preparation see text) on a silicic acid column. Elution was started with *n*-butanol:acetic acid:water (100:10:10, v/v/v) followed by *n*-propanol:water (3:1, v/v) (arrow). The dotted bar relates to biologically active fractions.

atories, Richmond, Calif.) suspended in n-butanol: acetic acid:water 100:10:10 (v/v/v). The column was eluted with the same solvent system (1 ml/min, 4°C). With this solvent, biological activity remains in the column. The chromatographic profile of the eluent was followed by determining u.v. absorbancy at 250 nm (fig.1) and, after all elutable material was removed from the column, the solvent was changed to *n*-propanol:H_2O (3:1, v/v). Aliquots of the latter effluent were assayed for biological activity. From 30–50% of the activity of the ethanolic extract was recovered. The active fractions were combined and, after evaporation of solvent, the residue contained 30 mg of a brown semi-solid material. This was dissolved in 1.0 ml of 50% aqueous ethanol for further separation by preparative thin-layer chromatography (TLC) on Silica Gel G (500 μm). After development with *n*-propanol:water (3:1, v/v), the plate was allowed to dry at room temperature. The adsorbent was scraped off in 1.5 cm wide zones and extracted four times by gentle shaking with 3 ml distilled water for 2 hr. Biological activity was determined in the aqueous extract (recovery about 50%) and was found to reside at a distance of 4 to 9.5 cm from the origin (solvent front: 16 cm). The major achievement of this fractionation step was the separation of biologically active substances from almost all of the accompanying yellow colored inactive material.

Analytical TLC of the active fractions on Silica Gel G plates (250 μ) using the solvent described above showed a characteristic pattern of three spots with R_f values 0.35, 0.40, and 0.45 which were visible in u.v. light. No other substances could be detected by charring with conc. H_2SO_4. Whether the observed u.v. fluorescence is a constituent property of the active substance or is due to trace contaminants was not unequivocally determined. Rechromatography resulted in the separation of the fastest migrating substance (R_f = 0.45) from the two accompanying compounds with lower R_f values.

For further characterization of the unknown substances, the silica gel adsorbent was extracted with distilled water as described before. The aqueous extract was dried in vacuo at 37°C and the remaining solid matter was treated with 4.0 ml 50% aqueous ethanol and freed from insoluble material by centrifugation. A 0.1 ml sample was used for recording of u.v. spectra. Similar spectra were obtained from all active fractions. The most purified substance showed a λ_{max} = 274 nm.

The remaining ethanolic solution was concentrated in vacuo at 37°C to 400 μl. 25 μl samples were placed on TLC plates, and after development, subjected to chemical-spot analysis using specific reagents. Although only one of three substances present in the active fractions could be separated from the others, all three

Table 1
Reactivity of highly purified *S. malacoxylon* 1,25-$(OH)_2$-D_3-like factors with various group detection reagents on TLC plates

Reagent	Suitable for detection of:[a]	Reactivity of 1,25-$(OH)_2$-D_3-like factors
Dragendorff's reagent	Alkaloids; nitrogen containing compounds	Negative
Molybdophosphoric acid	Reducing compounds; sterols and steroids	Positive
Acetic anhydride-sulphuric acid	Δ^5-3-sterols	Positive
Perchloric acid	Δ^5 - and Δ^7 steriods	Positive
Antimony (III) chloride	Vitamins A and D; steroid glycosides	Positive
Anisaldehyde-sulphuric acid	Steroids; sugars	Positive
Naphthoresorcinol-phosphoric acid	Sugars	Positive

[a] From [7].

showed similar staining characteristics (results are given in table 1), suggesting a 3-hydroxy-seco-steroid glycoside as the common structure.

3. Discussion

The present investigation confirmed the presence of more than one 1,25-$(OH)_2$-D_3-like substance in *Solanum malacoxylon*. Humphreys [5] showed heterogeneity of the aqueous extract by chromatography on Sephadex G-15, and Cassels et al. [8] previously reported the separation of methanol-soluble from methanol-insoluble activity. In our study, there was also a distinctive methanol-soluble activity that accounted for about 30% of the activity. However, attention was given to the methanol-insoluble material since it displayed a higher specific activity (induction of CaBP/g dry material) and three biologically active substances could be distinguished in this fraction by their different migration rates on Silica Gel G.

Some speculations on the nature of these substances, based on their known effect in biological systems and their chemical properties, as reported herein on highly purified material, seem justified. *S.m.* is active in strontium-fed chicks and anephric rats [1, 4]. Under these conditions, 1-hydroxylation of the vitamin D molecule does not occur. Only those vitamin D derivatives are effective which already contain a 1α-hydroxyl group (e.g. 1α, 25-$(OH)_2$-D_3) or a geometrically equivalent hydroxyl group (e. g., 3-OH in 5, 6-*trans*-vitamin D_3: for review see [9]). Competitive binding of *S.m.* factor to an intestinal cytoplasmic receptor with high specificity for 1,25-$(OH)_2$-D_3 was also observed [10]. From this, it is assumed that at least a part of the molecule displays a seco-steroid structure common to all vitamin D derivatives and, in addition, contains a hydroxyl group in the 1α- or in a biologically equivalent 3-position. This is substantiated by the present findings since the biological activity of highly purified *S.m.* extracts is associated with the presence of a vitamin D-like, 3-OH-$\Delta^{5,7}$-unsaturated steroid (table1). Furthermore, a u.v. absorption maximum at 274 nm would be indicative of a 5, 6-*trans*-triene system (for u.v. spectra of vitamin D derivatives see [11]), assuming no contribution to the absorption maximum from other parts of the molecule. The presence of carbohydrate groups on the molecule could explain the unusual hydrophilic properties and

the relatively high molecular weight. The differences in mobility of the active fractions on Silica Gel G might be due to different numbers of sugar residues attached to the steroidal skeleton or to more subtle structural or stereochemical differences. Since biological activity might require prior cleavage of a glycosidic linkage, this might explain the delayed response of some biological systems, e.g., the chick embryonic duodenum in organ culture to *S.m.* extracts [3].

The isolation procedure described above allows the preparation of biologically active substances of high purity and hopefully in sufficient amounts for the complete determination of their molecular structure.

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References

[1] Wasserman, R. H. (1974) Science 183, 1092–1094.
[2] Wasserman, R. H., Bar, A., Corradino, R. A., Taylor, A. N. and Peterlik, M. in: Proc. 5th Parathyroid Conf., Oxford 1974, Excerpta Medica, Amsterdam, in press.
[3] Corradino, R. A. and Wasserman, R. H. (1974) Nature 252, 716–718.
[4] Walling, M. W. and Kimberg, D. V., Gastroenterology, in press.
[5] Humphreys, D. J. (1973) Nature (N.B.) 246, 155–167.
[6] Taylor, A. N. (1974) Arch. Biochem. Biophys. 161, 100–108.
[7] Krebs, K. G., Heusser, D. and Wimmer, H. (1969) in: Thin-layer Chromatography (Stahl, E., ed.) pp. 859–909, Springer-Verlag, New York.
[8] Cassels, B. K., Rossi, F. M., Dallorso, M. E., Daskal, H. and Leiva, A. (1971) Contribuciones científicas y technológicas, Universidad técnica de estado, Santiago 4, 7–12.
[9] Okamura, W. H., Norman, A. W. and Wing, R. M. (1974) Proc. Natl. Acad. Sci. 71, 4194–4197.
[10] Procsal, D. and Norman, A. W. (1975) Fed. Proc. 34, 894.
[11] Holick, M. F. and DeLuca, H. F. (1974) in: Advances in Steroid Biochemistry and Pharmacology (Briggs, M. H., ed.) Vol. 4, pp. 111–155, Academic Press, New York.