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Metabolism of Cholecalciferol in Land Snails

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1. Radioactively labelled cholecalciferol was injected into the land snails *Levantina hiersolyma* and *Theba pisana*. Three metabolites (C, D and E), more polar than cholecalciferol, were found. 2. Metabolite C was found to be identical with 25-hydroxycholecalciferol. On injection of 25-hydroxy[26,27-^{3}H]cholecalciferol, metabolite E was predominantly formed. Metabolite D was predominantly formed from cholecalciferol. Metabolites D and E differ from any known cholecalciferol metabolites. 3. The intestine was found to be the tissue capable of carrying out the transformation of 25-hydroxycholecalciferol into metabolite E. 4. 25-Hydroxycholecalciferol and metabolite E were localized in the digestive gland of the snail, the tissue responsible for the absorption of Ca^{2+} and its storage. Metabolite D was not localized in any specific tissue.

Very little is known about the hormonal regulation of Ca^{2+} in molluscs. Wagge (1952) showed that cholecalciferol or ergocalciferol are essential nutrients for the terrestrial snail *Helix aspersa*, as their absence in the diet results in the death of the snails. On the other hand, increased concentrations of these sterols in the diet results in increased Ca^{2+} absorption from the food and increased ability to utilize Ca^{2+} reserves in the shell and digestive gland. *H. aspersa* fed on an increased cholecalciferol diet regenerated pieces of damaged shell with a richer organic matrix surrounding the inorganic crystals (Wagge, 1952).

The metabolism of cholecalciferol is at present known to occur only in vertebrates. More recent studies show a close association of cholecalciferol metabolites with Ca^{2+} absorption (Lawson & Emtage, 1974; Wasserman *et al.*, 1974) and skeletal formation (Ornoy *et al.*, 1978). The observations of Wagge (1952) together with the more recent findings raise the question as to whether molluscs, and in particular land snails, metabolize cholecalciferol. The present study reports that the two land snails analysed do metabolize cholecalciferol.

Experimental

Animals

Specimens of *Levantina* (*Levantina*) *hiersolyma* (Mousson, 1854) and *Theba pisana* (Muller, 1774) were collected while aestivating or immediately after the first rains in September. They were maintained in a humid environment in the laboratory and were fed lettuce, cucumbers and paper tissues. After an adjustment period of several days, they were injected intramuscularly in the foot region with a tracer dose (0.5μCi) of [1,2-^{3}H]cholecalciferol, 25-hydroxy[26,27-^{3}H]cholecalciferol or [1,2-^{3}H,4-^{14}C]-cholecalciferol. The radioactive vitamins were dissolved in propylene glycol containing 10% (v/v) ethanol, and each snail was injected with 0.05ml of this solution. After 10 days the snails were frozen, removed from the shells, and the entire body or specific tissues in the case of *L. hiersolyma* were subjected to lipid extraction and then analysed for cholecalciferol metabolites.

Lipid extraction and chromatography

The tissues were weighed, minced and lipids extracted with chloroform/methanol (2:1, v/v) (Bligh & Dyer, 1959). The lipid extracts were analysed for cholecalciferol metabolites by column chromatography with the aid of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala, Sweden). The lipids were dissolved in chloroform/light petroleum (b.p. 40–60°C) (13:7, v/v) and applied to a glass column (1cm×65cm) containing Sephadex LH-20 slurried and developed in the same solvent.

Some 80 fractions (3.5ml) were collected. Before chromatography each column was calibrated with authentic metabolites of cholecalciferol.

The region of each peak from the Sephadex LH-20 column was dried under a stream of N_2, dissolved in 10% propan-2-ol in n-hexane, and subjected to high-pressure liquid chromatography, by using a Waters model 6000A high-pressure-liquid-chromatographic system (Waters Associates, Milford, MA, U.S.A.) with a 10μm Micro-Porasil silicic acid column (0.4cm×30cm). The eluting solvent was

propan-2-ol/n-hexane (1:9, v/v). A flow rate of 1 ml/min was achieved at a pressure of 1.38 MPa.

Enzyme assays

For the assay of 25-hydroxycholecalciferol hydroxylase in the tissues of *L. hiersolyma*, tissue homogenates were incubated for 5 h at 27°C in a medium containing 25-hydroxy[26,27-^{3}H]cholecalciferol, essentially as described by Fraser & Kodicek (1970, 1973). The produced metabolites were analysed as described in the previous section.

Other laboratory procedures

Periodate cleavage was performed on *L. hiersolyma* cholecalciferol metabolites with aq. 5 % (w/v) $NaIO_4$ as previously described (Sheves *et al.*, 1978).

The competitive-protein-binding assay of cholecalciferol metabolites was carried out essentially by the method of Edelstein *et al.*, (1974), as modified by Weisman *et al.* (1977).

Radioactive materials and measurements of radioactivity

[1,2-^{3}H]Cholecalciferol (sp. radioactivity 12.6 Ci/mmol), [4-^{14}C]cholecalciferol (sp. radioactivity 9 Ci/mmol) and 25-hydroxy[26,27-^{3}H]cholecalciferol (sp. radioactivity 9 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, Bucks., U.K. [1,2-^{3}H,4-^{14}C]Cholecalciferol was obtained by mixing [1,2-^{3}H]cholecalciferol and [4-^{14}C]-cholecalciferol preparations so as to give a ^{3}H/^{14}C ratio of approx. 7.5. All radioactivity measurements were made in a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3390. Lipid samples were dried and counted for radioactivity in a solution of 100 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene and 4.0 g of 2,5-diphenyloxazole/litre of toluene. Quenching was corrected for by using the automatic external standardization and correlation curves for ^{3}H and ^{14}C, either individually or combined.

Results

Cholecalciferol metabolism in the land snails

Fig. 1 demonstrates a typical elution profile from Sephadex LH-20 of lipid extracts prepared from the soft part of *L. hiersolyma* and *T. pisana* 10 days after an injection of [1,2-^{3}H]cholecalciferol. Cholecalciferol is metabolized in these snails into at least four additional metabolites. Peaks B, C and E co-chromatographed on Sephadex LH-20 with cholecalciferol, 25-hydroxycholecalciferol and 24,25-dihydroxycholecaliferol respectively. An injection of 25-hydroxy[26,27-^{3}H]cholecalciferol into these terrestrial snails resulted in peak E being predominantly formed (Fig. 2).

Co-chromatography of peaks B, C and E with synthetic preparations of cholecalciferol, 25-hydroxycholecalciferol and 24*R*,25-dihydroxycholecalciferol on high-pressure liquid chromatography indicated that peaks B and C indeed co-eluted with cholecalciferol and 25-hydroxycholecalciferol respectively, whereas peak E was found to be more polar than 24*R*,25-dihydroxycholecalciferol (Fig. 3). Thus metabolite E is not 24,25-dihydroxycholecalciferol despite the fact that both possess similar chromatographic properties on Sephadex LH-20. This was confirmed by the periodate cleavage test in which the control 24,25-dihydroxy[26,27-^{3}H]cholecalciferol was

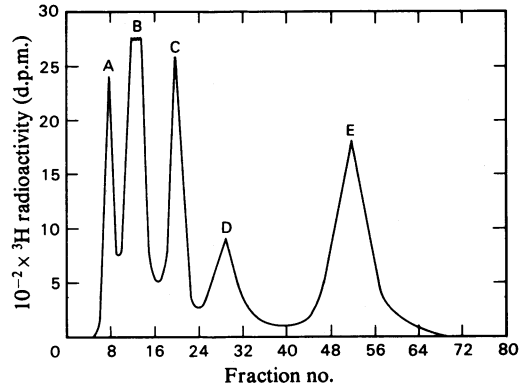

Fig. 1. *Sephadex LH-20 chromatographic profile of lipid extracts from L. hiersolyma 10 days after an injection of [1,2-^{3}H]cholecalciferol.*
A similar profile was obtained for *T. pisana*. For experimental details see the Experimental section. Fractions of 3.5 ml were collected.

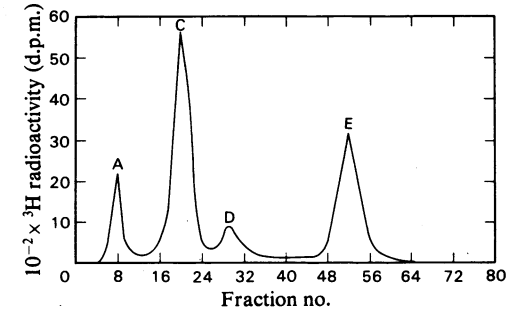

Fig. 2. *Sephadex LH-20 chromatographic profile of lipid extracts from L. hiersolyma 10 days after an injection of 25-hydroxy[26,27-^{3}H]cholecalciferol*
For experimental details see the Experimental section. Fractions of 3.5 ml were collected.

cleaved and lost its radioactive labelling, whereas metabolite E was unaffected.

None of the five peaks eluted from the Sephadex LH-20 column after injection with [1,2-^{3}H,4-^{14}C]-cholecalciferol showed any change in the ratio of ^{3}H/^{14}C radioactivity, thus excluding the possibility that hydroxylation occurred at C-1 or C-2 (Edelstein *et al.*, 1978).

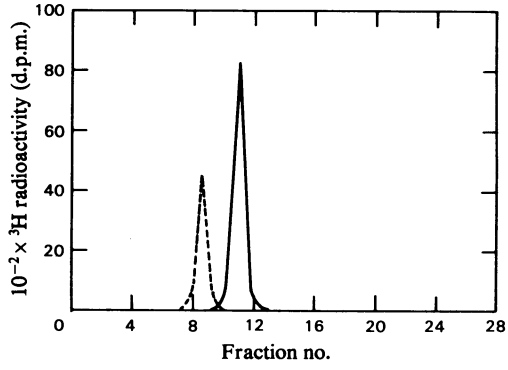

Fig. 3. *High-pressure liquid chromatogram of 24R,25-dihydroxycholecalciferol and peak E*

For experimental details see the Experimental section. ----, 24*R*,25-Dihydroxycholecalciferol; ——, peak E. Fractions of 1.0ml were collected.

Tissue distribution of cholecalciferol metabolites in L. hiersolyma

After injection of ^{3}H-labelled cholecalciferol or ^{3}H-labelled 25-hydroxycholecalciferol, the major portion of radioactivity was localized in the digestive gland (Tables 1 and 2). Sephadex LH-20 analysis of the lipid extracts from this tissue showed that this radioactivity is composed mainly of 25-hydroxycholecalciferol and metabolite E (Tables 1 and 2). The digestive gland in land snails is thus probably one of the sites at which cholecalciferol acts.

To investigate the distribution of the endogenous pools of cholecalciferol metabolites in *L. hiersolyma* we extracted the lipids from dissected tissues of specimens that were not treated and subjected them to the competitive protein binding assay for 25-hydroxycholecalciferol and for 24,25-dihydroxycholecalciferol. 25-Hydroxycholecalciferol and metabolite E were detected only in the digestive gland. The concentration of 25-hydroxycholecalciferol was 3.0ng/g of tissue. The concentration of metabolite E was 0.30ng/g of tissue. However, caution must be exercised with regard to the absolute concentrations of metabolite E

Table 1. *Distribution of cholecalciferol metabolites in the tissues of L. hiersolyma after an injection of* [1,2-^{3}*H*]*cholecalciferol*

For experimental details see the Experimental section.

Tissue	10^{-3} × Radioactivity in tissues (d.p.m./total tissue)	10^{-3} × Radioactivity in tissues (d.p.m./g of tissue)	Distribution of radioactivity (%)	Distribution of metabolites* A (%)	B (%)	C (%)	D (%)	E (%)
Digestive gland	2171	297	62.4	18.9	36.7	20.2	11.7	12.5
Intestine	327	126	9.4	20.8	51.8	10.3	10.4	6.7
Mantle	149	20	4.3	14.1	76.7	3.4	3.4	2.4
Muscle	602	83	17.3	16.4	67.3	6.1	6.1	4.1
Sexual organs	230	67	6.6	27.3	49.7	7.8	7.0	8.2

* Determined from the Sephadex LH-20 elution profiles.

Table 2. *Distribution of cholecalciferol metabolites in the tissues of L. hiersolyma after an injection of 25-hydroxy*[26,27-^{3}*H*]*cholecalciferol*

For experimental details see the Experimental section.

Tissue	10^{-3} × Radioactivity in tissues (d.p.m./total tissue)	10^{-3} × Radioactivity in tissues (d.p.m./g of tissue)	Distribution of radioactivity (%)	Distribution of metabolites* A (%)	C (%)	D (%)	E (%)
Digestive gland	8103	1000	87.3	8.1	39.4	8.1	44.4
Intestine	479	85	5.2	16.4	38.8	9.1	35.7
Mantle	255	28	2.7	26.6	34.5	6.9	32.0
Muscle	307	25	3.3	26.1	38.4	6.8	28.7
Sexual organs	140	28	1.5	34.1	31.3	10.0	24.6

* Determined from the Sephadex LH-20 elution profiles.

reported. Although metabolite E displaced ^{3}H-labelled 25-hydroxycholecalciferol, the binding affinity of this metabolite to the rat plasma binding protein is not known. The results do, however, show that the tracer study reflects the distribution of these two metabolites *in vivo*.

All attempts to observe preferential localization of metabolite D in a specific tissue of *L. hiersolyma* after injection of [1,2-^{3}H]cholecalciferol failed (Tables 1 and 2). Metabolite D was more or less evenly distributed among the various tissues studied, and compared with metabolite E, its percentage distribution was low.

Site of formation of metabolite E

Since our studies on the metabolism of cholecalciferol and of 25-hydroxycholecalciferol indicate that metabolite E is formed from 25-hydroxycholecalciferol, possibly by an additional hydroxylation, attempts were made to identify the tissue responsible for this metabolic transformation. When tissue homogenates of *L. hiersolyma* were incubated *in vitro* for 5h at 27°C with 25-hydroxy[26,27-^{3}H]cholecalciferol about 5 times more metabolite E was formed in the intestine than in the other tissues analysed (Table 3). Incubation at 37°C failed to promote this transformation.

Discussion

The present study shows that land snails do metabolize cholecalciferol. The metabolic pathway partially resembles that of vertebrates such as mammals and birds in which cholecalciferol is hydroxylated at C-25 to form 25-hydroxycholecalciferol. Whereas in mammals and birds, the latter compound is further metabolized into 1,25-dihydroxycholecalciferol, which is responsible for the intestinal absorption of Ca^{2+}, or to 24,25-hydroxycholecalciferol, which is related to skeletal ossification, in land snails 25-hydroxycholecalciferol is predominantly metabolized into the as yet unidentified polar metabolite E. Metabolite D on the other hand is predominantly formed from cholecalciferol itself.

The functions of cholecalciferol metabolites D and E are not yet known. The preferential concentration of metabolite E in the digestive gland of the snail *L. hiersolyma* suggests that its function is perhaps related to either Ca^{2+} absorption from the food, the predominant source of Ca^{2+} in these animals (Wagge, 1952), or the removal of Ca^{2+} from the particular cells of the digestive gland in which it is stored in the mineral form (Wagge, 1952; Burton, 1972). The location of the hydroxylating enzyme that forms metabolite E from 25-hydroxycholecalciferol in the intestine of *L. hiersolyma* is consistent with the notion that metabolite E is involved in Ca^{2+} absorption. The location of the hydroxylation activity in the intestine may have a regulatory function related to Ca^{2+} uptake. As the distribution of metabolite D is more or less uniform among the tissues studied, we have as yet no indication of what its function might be.

In almost all phylogenetic schemes of the animal kingdom, the molluscs and the chordates are at opposite extremes (Valentyne, 1977) and their common ancestor probably dates back to the very late Precambrian period soon after the metazoans evolved, but before the onset of biomineralization. The finding of cholecalciferol in molluscs is most easily understood by assuming that cholecalciferol or a close analogue was also required by the common ancestor. In this case, cholecalciferol is presumably present in marine molluscs, and perhaps too in many other invertebrates. Conceivably, the cholecalciferol requirement of land snails reflects their adaptation to the terrestrial environment, where their source of Ca^{2+} is almost entirely from food (Wagge,1952), compared with marine or freshwater molluscs, which derive their Ca^{2+} primarily from the salts dissolved in the water (Bevelander & Benzer, 1948; Van der Borght & Van Puymbroek, 1966). In this case, cholecalciferol should be confined to terrestrial gastropods.

The metabolism of cholecalciferol in marine and fresh water molluscs still requires investigation, as well as the determination of the chemical structures of metabolites D and E.

Table 3. *25-Hydroxycholecalciferol hydroxylase in L. hiersolyma tissues*

Tissues were homogenized and were incubated for 5h at 27°C with 25-hydroxy[26,27-^{3}H]cholecalciferol as described in the Experimental section.

Tissue	Formation of peak E (%)
Digestive gland	2.0
Intestine	11.2
Mantle	2.0
Muscle	2.0
Sexual organs	None

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