BIOLOGICAL DEACTIVATION OF THE ACTIVE ANALOGUE OF CHOLECALCIFEROL

M. SHEVES, Y. MAZUR, D. NOFF+ and S. EDELSTEIN+
Departments of Organic Chemistry and +*Biochemistry, The Weizmann Institute of Science, Rehovot, Israel*

Received 14 September 1978

1. Introduction

The hormonal form of vitamin D $1\alpha,25(OH)_2D_3$, is produced from cholecalciferol (vitamin D_3) by C-25 hydroxylation in the liver, followed by C-1 hydroxylation in the kidney. The important finding that this hormone is synthesized only in the kidney [1], and the demonstration that patients with advanced renal disease are unable to produce $1\alpha,25(OH)_2D_3$, prompted the synthesis of its analogues. The most widely-used analogue in the treatment of vitamin D resistant metabolic bone diseases is $1\alpha(OH)D_3$ [2–4]. The beneficial therapeutic effect of this analogue was shown to be due to its rapid conversion into $1\alpha,25(OH)_2D_3$ [5–7]. Thus, patients treated with $1\alpha(OH)D_3$ are in fact being treated with a potent calcium mobilizing agent, and the question has arisen therefore whether biological protective mechanisms are operating in order to eliminate toxic responses to such treatments.

Previous studies from our laboratories have shown that treatment with $1\alpha(OH)D_3$ in chicks results in high accumulation of a non-polar metabolite(s) in the liver [7]. This study describes the characterization of these non-polar metabolites, and the possible role that these metabolites play in the deactivation of $1\alpha(OH)D_3$ is discussed.

Abbreviations: $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxycholecalciferol; $1\alpha(OH)D_3$, 1α-hydroxycholecalciferol; $1\alpha,24,25(OH)_3D_3$, 1α,24,25-trihydroxycholecalciferol; $1\alpha[OH\text{-}7\text{-}^3H]D_3$, 1α-[*hydroxy*-7-^{3}H]cholecalciferol

Address correspondence to: S. Edelstein

2. Experimental

2.1. *Animals*

Male chicks (light cross; 1 day old) were fed for 3 weeks on a semi-synthetic vitamin D-free diet [8] based on soya-bean meal and glucose. At the end of week 3, a group of 5 vitamin D-deficient chicks received each a daily subcutanous injection of 0.15 μg $1\alpha[OH\text{-}7\text{-}^3H]D_3$ for 16 days. At the end of this period the animals were killed, their livers were removed and extracted for lipids.

2.2. *Lipid extraction and chromatography*

The livers were weighed, minced and extracted for lipids with chloroform/methanol (2:1, v/v) [9]. The non-polar peak was separated by column chromatography with the aid of Sephadex LH-20 (Pharmacia Fine Chemicals, Uppsala). The lipids were dissolved in chloroform/light petroleum (b.p. 40–60°C)/methanol (75:23:2, by vol.) and applied to a glass column (1 × 65 cm) containing Sephadex LH-20 slurried and developed in the same solvent. Some 50 fractions (3.5 ml) were collected. Aliquots from each fraction were counted for radioactivity. Before each run the columns were calibrated with authentic metabolites.

The isolated non-polar peak was further fractionated into two fractions, I and II (scheme 1) by column chromatography with the aid of silica-gel. Fraction I was eluted with 40 ml 5% (v/v) diethyl-ether in pentane, and fraction II was eluted with additional 40 ml 30% (v/v) diethyl-ether in pentane (1 × 10 cm columns). Repeated chromatographies were performed using either one of the two procedures described above.

High pressure liquid chromatography was per-

formed on a Model 6000A solvent delivery system (Waters Associates, Milford, MA) fitted with Lichrosorb Si100 column and using a 2-propanol: hexane (1:9, v/v) solvent system at a pressure of 400 p.s.i.

2.3. *Hydrolysis*

Fractions to be hydrolysed were dissolved in 15% (w/v) KOH in methanol, and were left to stand at room temperature for 3 days. At the end of this period, the mixture was diluted with water, and extracted with ethyl acetate and the organic fraction was washed several times with water and dried over $MgSO_4$. The solvent was evaporated, and the residue was subjected to column chromatography on Sephadex LH-20 as above.

2.4. *Periodate cleavage*

The material was dissolved in 0.2 ml methanol, and treated with 0.1 ml 5% (w/v) aqueous $NaIO_4$ for 16 h at room temperature. The mixture was then extracted with ethyl acetate and water, washed twice with water and dried over $MgSO_4$. The residue left after the solvent was evaporated was subjected to column chromatography on Sephadex LH-20 as above.

2.5. *Materials*

1α(OH)D_3 and 1α[*OH*-7-^{3}H]D_3 with spec. radioact. 2 Ci/mmol were synthesized in our laboratories as in [4,7].

2.6. *Measurement of radioactivity*

All radioactivity measurements were carried out in a Packard Tri-Carb automatic liquid-scintillation spectrometer no. 3390. Lipid samples were counted for radioactivity in a solution of 100 mg 1,4-bis-(5-phenyloxazole-2-yl) benzene and 4 mg 2,5-diphenyl-oxazole/l toluene. Quenching was corrected for by using the automatic external standardization and correlation curve for ^{3}H.

3. Results

Figure 1 demonstrates the elution profile from Sephadex LH-20 of lipid extract prepared from chick livers after long-term injections of 1α[*OH*-7-^{3}H]D_3. The elution profile of radioactivity is composed of

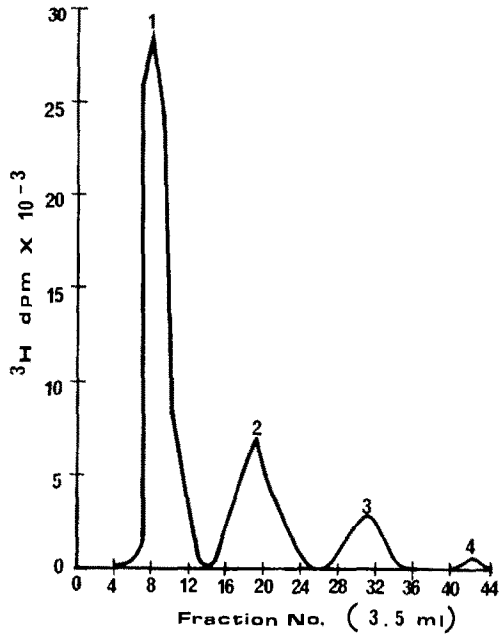
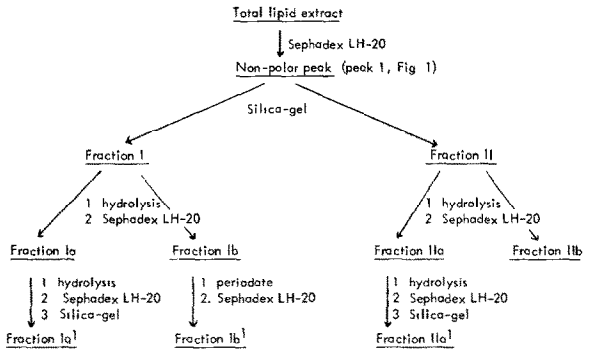


Fig.1. Sephadex LH-20 chromatographic profile of lipid extract from chick livers after injections with 1α[*OH*-7-^{3}H]D_3. For experimental details see section 2.2. Peaks: 1, non-polar, 2, 1α(OH)D_3, 3, 1α,25$(OH)_2D_3$, 4, 1α,24,25$(OH)_3D_3$; V_O 28.0 ml.

4 peaks, out of which peak 1, the non-polar peak, is the major one (63%). Peaks 2, 3 and 4 co-chromatographed with 1α(OH)D_3, 1α,25$(OH)_2D_3$ and 1α,24,25$(OH)_3D_3$, respectively.

When peak 1 was subjected to silica-gel column chromatography, two fractions were separated (I and II, scheme 1). Hydrolysis of fraction I followed by column chromatography on Sephadex LH-20 gave 2 fractions (Ia, Ib, scheme 1). Fraction Ia was eluted with void volume. When chromatographed on silica-gel following additional hydrolysis and separation on Sephadex LH-20 it was found to be more polar than the original fraction I (Ia^1). Fraction Ib co-chromatographed with an authentic sample of 1α,24(R),25$(OH)_3D_3$ on Sephadex LH-20. On treatment with $NaIO_4$ this material was cleaved, and resulted in the formation of a less polar material (Ib^1). This indicates that fraction Ib consist of 1α,24,25$(OH)_3D_3$ which upon treatment with $NaIO_4$ is cleaved to give a non-polar product which lost vicinal hydroxyl groups.

Scheme I
Fractionation procedure of the non-polar material extracted from livers of chicks that were dosed with 1α[*OH*-7-^{3}H]D_3



Hydrolysis of fraction II, followed by column chromatography on Sephadex LH-20 gave 2 fractions as well (IIa and IIb, scheme 1). Fraction IIa was eluted with the void volume and fraction IIb upon continuous elution. The later fraction was identified as 1α(OH)D_3 since it co-chromatographed with an authentic sample of 1α(OH)D_3 on Sephadex LH-20 and on high-pressure liquid chromatography. Additional hydrolysis of fraction IIa did not alter its chromatographic properties on either Sephadex LH-20 or silica-gel (IIa^1).

The distribution of the non-polar metabolites of 1α(OH)D_3 in chick liver is summarized in table 1.

4. Discussion

From the present data it is apparent that long-term administration of 1α(OH)D_3 results in the accumulation of non-polar metabolites in the liver. Since hydrolysis of these non-polar substances resulted in more polar metabolites, it is reasonable to assume that these are fatty acids esters, as it is known that liver contains high concentrations of fatty acids esters of vitamin D [10,11]. We suggest that the less polar ester fraction consists of fully esterified metabolites and the more polar fraction of partially esterified metabolites. We have identified part of the less polar ester fraction as esters of 1α,24,25$(OH)_3D_3$. This finding explains why only a negligible amount of free 1α,24,25$(OH)_3D_3$ was detected in tissues of chicks that were treated continuously with 1α(OH)D_3, despite the demonstration that kidney homogenates derived from vitamin D-fed chicks convert 1α,25$(OH)_2D_3$ into 1α,24,25$(OH)_3D_3$ [12]. It seems that excess 1α,25$(OH)_2D_3$ which is present in normocalcemic animals is being 24-hydroxylated and immediately thereafter, esterified. Part of the more polar ester fraction was identified as esters of 1α(OH)D_3. It appears that only one of the two hydroxyl groups in 1α(OH)D_3 is esterified, since this material has similar polarity on silica-gel as cholecalciferol which possess only one hydroxyl group. It is possible that excess 1α(OH)D_3 which is not being converted into 1α,25$(OH)_2D_3$ is mono-esterified so that its conversion to the active 1α,25$(OH)_2D_3$ by the liver 25-hydroxylase is prevented. The two unidentified metabolites in the non-polar fraction extracted from liver deserves further study. Possibly these are new compounds, or perhaps substances which were formed during fractionation.

Table 1
The distribution of the non-polar metabolites of 1α(OH)D_3 in chick liver

Fraction	Metabolite	Conc. (pmol/g)
I	Unidentified	0.30
	1α,24,25$(OH)_3D_3$-ester	0.34
II	Unidentified	0.25
	1α(OH)D_3-ester	0.18

In summary, the present study demonstrates the existence of two esterification mechanisms for the biological deactivation of 1α(OH)D_3. The first one is operating at the pre-activation stage by the liver 25-hydroxylase, and the second mechanism operates when normocalcemia is reached due to the action of 1α,25(OH) in its target cells. Excess 1α,25$(OH)_2D_3$ is deactivated by 24-hydroxylation followed by esterification of the resulting 1α,24,25$(OH)_3D_3$.

References

[1] Fraser, D. R. and Kodicek, E. (1970) Nature 288, 764–766.
[2] Barton, D. H. R., Hesse, R. H., Pecht, M. M. and Rizzardo, E. J. (1973) J. Am. Chem. Soc. 95, 2748–2749.
[3] Holick, M. F., Semmler, E. J., Schnoes, H. K. and DeLuca, H. F. (1973) Science 180, 190–191.

[4] Freeman, D., Acher, A. and Mazur, Y. (1975) Tetrahedron Lett. 261–264.
[5] Holick, M. F., Tavela, T. E., Holick, S. A., Schnoes, H. K., DeLuca, H. F. and Gallagher, B. M. (1976) J. Biol. Chem. 251, 1020–1024.
[6] Fukushima, M., Suzuki, Y., Tohira, Y., Matsunaga, I., Ochi, K., Nagano, H., Mishii, Y. and Suda, T. (1976) FEBS Lett. 65, 211–214.
[7] Edelstein, S., Noff, D., Freeman, D., Sheves, M. and Mazur, Y. (1978) Biochem. J. in press.
[8] Hurwitz, S. and Bar, A. (1972) Am. J. Physiol. 222, 761–767.
[9] Bligh, E. G. and Dyer, W. J. (1959) Can. J. Biochem. Physiol. 37, 911–916.
[10] Fraser, D. R. and Kodicek, E. (1968) Biochem. J. 106, 491–496.
[11] Fraser, D. R. and Kodicek, E. (1968) Biochem. J. 109, 457–467.
[12] Kleiner-Bosaller, A. and DeLuca, H. F. (1974) Biochim. Biophys. Acta 388, 488–495.