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Identification of 1,25-Dihydroxycholecalciferol, a Form of Vitamin D_3 Metabolically Active in the Intestine

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ABSTRACT **A biologically active vitamin D_3 metabolite ("peak V metabolite") more polar than 25-hydroxycholecalciferol has been isolated from chicken intestines in pure form as a mono(trimethylsilyl)ether derivative. The molecule has been identified as 1,25-dihydroxycholecalciferol by means of mass spectrometry, ultraviolet absorption spectrophotometry, and specific chemical reactions.**

It has been demonstrated (1) with tritiated vitamin D_3 of high specific activity that vitamin D_3 is converted to metabolites more potent and rapidly acting than the parent vitamin. The most abundant of these, 25-hydroxycholecalciferol (25-HCC) (2), acts more rapidly than vitamin D_3 in enhancing both intestinal calcium transport and bone mineral mobilization (3). The existence in the intestine of additional polar metabolite(s) active in promoting calcium transport is evident from work in a number of laboratories (4–8). In agreement with Haussler *et al.* (8) and Myrtle *et al.* (9), we have observed that one of these metabolite(s), the peak V metabolite, induces intestinal calcium transport in deficient chickens more rapidly than vitamin D_3 or 25-HCC, although it is less effective than 25-HCC in curing rickets or inducing bone mineral mobilization.

By blocking the conversion of 25-HCC to peak V metabolite by prior administration of actinomycin D, we have shown (10) that peak V, or a further metabolite of peak V, is the metabolically active form of vitamin D in the intestine. In the presence of actinomycin D, the peak V compound stimulates intestinal calcium transport, whereas 25-HCC does not. This polar metabolite has now been isolated in pure form and identified as 1,25-dihydroxycholecalciferol (1,25-DHCC).

ISOLATION

1450 vitamin D-deficient chicks were each given 2.5 μg of [1,2-^{3}H]vitamin D_3 (specific activity, 3.2 $\times$ 10^5 dpm/μg), orally, in vegetable oil (Wesson). 24 hr later the chicks were killed. Methanol–chloroform extracts of the small intestines were prepared, and the chloroform extract was subjected to a sequence of chromatographic separations on Sephadex LH-20 and BioRad (beads) S-X8 (BioRad Labs., Richmond, Calif.). 11 μg of highly purified peak V metabolite was obtained. 8 μg of the metabolite was then reacted with 5 μl of TBT (a combination of TMS-imidazole, bis-TMS-acetamide, and trimethylchlorosilane, Pierce Chemical Co., Rockford, Ill.) and 10 μl of pyridine for 10 min. The resulting tri(trimethylsilyl)ether derivative was chromatographed on a 1 $\times$ 60 cm column of Sephadex LH-20 in 50% chloroform in petroleum ether (bp 67–69°C). The tri(trimethylsilyl) derivative was partially desilylated in 100 μl of 3.6 $\times$ 10^{-4} M HCl in MeOH + 50 μl pyridine at 60°C for 4 hr. This gave a mixture of di(trimethylsilyl), mono(trimethylsilyl), and unsilylated products, which were separated by chromatography on Sephadex LH-20. The metabolite and the mono(trimethylsilylated) metabolite were separately chromatographed in methanol on Sephadex LH-20 (11) before being used for structural identification.

IDENTIFICATION OF THE METABOLITE AS 1,25-DHCC

Several lines of evidence established the structure of the active metabolite. The mass spectrum of the metabolite exhibited a molecular ion at m/e 416; this requires the incorporation of two additional oxygen atoms into the basic carbon skeleton of vitamin D_3 (cholecalciferol). The presence of three hydroxyl

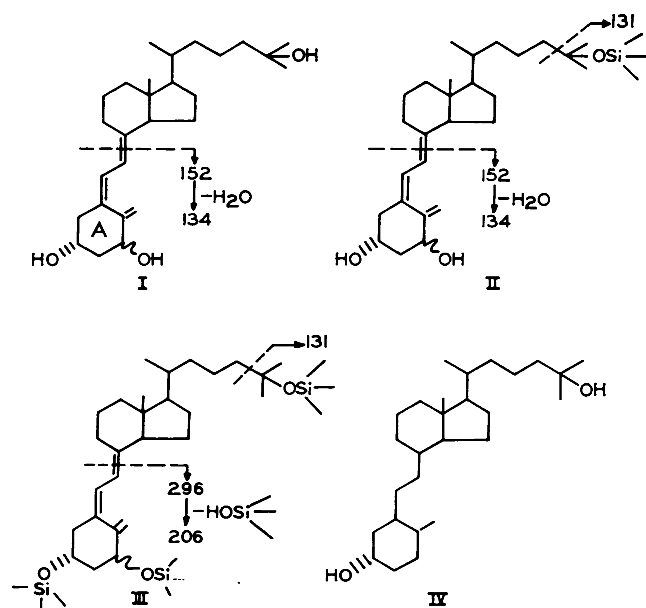

FIG. 1. Structures of 1,25-DHCC and its derivatives. (The numbers indicate schematically the origin of prominent peaks in the mass spectra of these compounds.) I = 1,25-DHCC; for discussion of II–IV, see text.

Abbreviations: 1,25-DHCC, 1,25-dihydroxycholecalciferol; 25-HCC, 25-hydroxycholecalciferol; TMS, trimethylsilyl.

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functions in the metabolite was demonstrated by the formation of a tri(trimethylsilyl)ether derivative (structure III, MW = 632, Fig. 1) and a mono(trimethylsilyl)diacetate derivative (MW = 572).

The mass spectrum of the tri(trimethylsilyl)derivative (III) and the mono(trimethylsilyl) derivative [MW = 488; structure II, produced from the tri(trimethylsilylated) compound by partial desilylation] and of the trimethylsilylether-diacetate exhibited an intense peak at m/e 131, which established the presence of a hydroxyl function at C-25 (2, 11, 12). In addition, the mass spectrum of the mono(trimethylsilyl) compound (II, Fig. 1) showed peaks at m/e 152 and 134 (152 − 18), which correspond to the characteristic ions at m/e 136 and 118 in the spectra of cholecalciferol, 25-HCC, and other cholecalciferol metabolites (2, 11, 12).

Since the ions at m/e 136 and 118 (136 − 18) are known to derive from ring A of the basic skeleton of vitamin D, the shift of these ions by 16 mass units (i.e., 136 + 16 = 152; 118 + 16 = 134) in the mass spectrum of the metabolite mono(trimethylsilyl)ether established the presence of an additional hydroxyl function in ring A. The same peaks (152, 134) are also observed in the mass spectrum of the metabolite itself, whereas in the mass spectrum of the tri(trimethylsilyl)ether derivative (structure III) these peaks are shifted to m/e 296 (152 + 2 silyl groups) and 206 [296 − $HOSi(CH_3)_3$]. In addition to providing conclusive proof for the presence of two hydroxyl functions in ring A, the peaks at m/e 152 and 134 (and 296 and 206 in the case of structure III) also establish the existence of the triene double-bond system in the metabolite, since these mass spectral peaks are very characteristic for the vitamin D-type triene structure (2, 12, 13). Ultraviolet absorption at 265 nm provided further strong evidence for a vitamin D-triene system. For the position of the additional hydroxyl function in ring A, carbons 1, 2, or 4 are thus the only possibilities. The presence of a C-1 hydroxy group was established by the following experiment: catalytic reduction (PtO_2/H_2) of the metabolite yielded a product that was shown to be identical (by cochromatography) with 25-hydroxyhexahydrocholecalciferol (structure IV). Since formation of compound IV from the metabolite requires hydrogenolysis of a hydroxyl group, the hydroxyl function lost in this process must have been located at either C-1 or C-4 (i.e., allylic to the double-bond system). The failure of the metabolite to react with periodate eliminates a C-4 (as well as a C-2) hydroxyl group; this observation requires that the additional hydroxyl group be a carbon 1. The observation that the biosynthesis of this metabolite from [1-^{3}H]cholecalciferol results in the loss of the tritium provides corroborative evidence for a C-1 hydroxy compound (6). The data above thus establish structure I, 1,25-DHCC, for this new metabolite.

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