IN VITRO PRODUCTION OF 25-HYDROXYCHOLECALCIFEROL*

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SUMMARY

The metabolism of vitamin D_3 to its biologically active form, 25-hydroxycholecalciferol, has been investigated in perfused rat livers and in rat liver homogenates. In both systems, significant conversion of vitamin D_3 to 25-HCC was observed, supporting earlier data which indicate that the liver is the major, if not the only, site of conversion of vitamin D_3 to 25-HCC.

INTRODUCTION

In 1966, Lund and DeLuca (1) discovered a polar vitamin D_3 metabolite which can cure rickets, increase intestinal calcium transport and stimulate bone mobilization as does the parent vitamin D_3. Blunt et al. (2) isolated and identified this active metabolite as 25-hydroxycholecalciferol (25-HCC). They showed that a dose of 25-HCC increases intestinal calcium transport and stimulates bone mobilization more rapidly than does a similar dose of vitamin D_3. These results suggest that vitamin D_3 must be converted to 25-HCC before the well-known functions of the vitamin can be manifested.

Olson and DeLuca (3) have shown that 2.5 µg of 25-HCC stimulates calcium transport in an isolated, perfused rat intestine, while 250 µg of vitamin D_3 brings about no

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stimulation. Trummel et al. (4) have demonstrated that 0.9 I.U./ml of the active metabolite induces mobilization of calcium from bone organ cultures while as much as 320 I.U./ml of crystalline vitamin D_3 has no effect. These data provide strong evidence that 25-HCC represents the metabolically active form of the vitamin in both intestinal transport and bone mobilization.

Ponchon and DeLuca (5) found that during two to four hours after D-deficient rats receive a 10 I.U. dose of ^{3}H-vitamin D_3, the radioactivity in the liver shows a decrease at a time corresponding to a rebound in the blood radioactivity. During this same time period, 25-HCC appears in the blood. Its appearance accounts for the decrease in liver radioactivity and increase in blood radioactivity, suggesting that the liver at least in part is responsible for the conversion of vitamin D_3 to 25-HCC. In continued investigations, Ponchon and DeLuca (6) demonstrated that hepatectomized rats are unable to produce significant amounts of 25-HCC while control rats show normal production of 25-HCC from ^{3}H-vitamin D_3. From this it appears clear that the liver is the major, if not the only, site of conversion of vitamin D_3 to 25-HCC. Recently it has been possible to demonstrate that perfused liver and cell-free liver homogenates from rats can readily convert vitamin D_3 to 25-HCC.

Data presentation

Livers from 150-200 g male, Holtzman rats were perfused with heparinized stock rat blood diluted 1:1 with Krebs-Ringer bicarbonate buffer, pH 7.4. The perfusion apparatus was that described by Northrop and Parks (7). The bile duct was cannulated in the first run and bile flowed continuously

during the course of the experiment. When the perfusate flow through the liver was seen to be 1.3 ml/min/g liver, 6 I.U. of ^{3}H-vitamin D_3 was injected into the perfusate at a point just before it entered the liver.

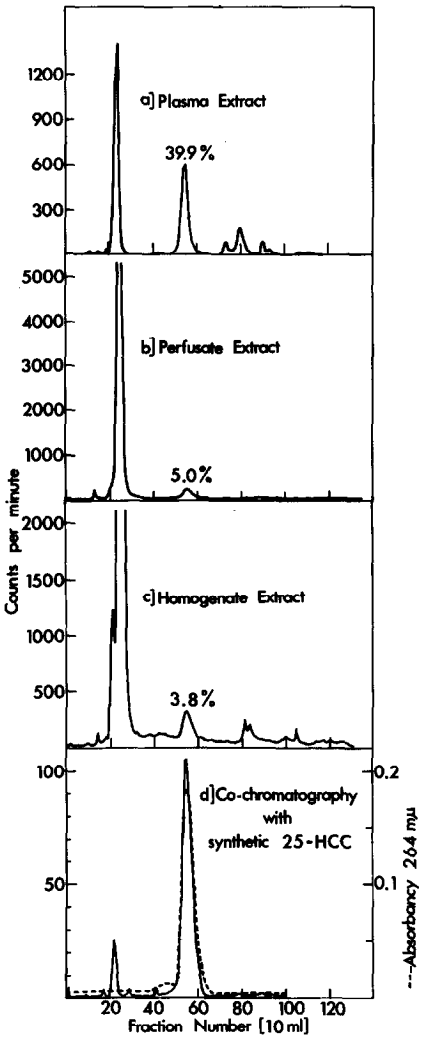

Figure 1. Silicic acid column chromatography of *in vivo* and *in vitro* metabolites of vitamin D_3. a) Extract of plasma from D-deficient rats given 0.25 μg ^{3}H-vitamin D_3 intrajugularly 24 hours before sacrifice. b) Extract of perfusate containing 0.15 μg ^{3}H-vitamin D_3 after circulation through stock rat liver for 4 hours. c) Extract of stock rat liver homogenate after incubation for 4 hours with 0.15 μg ^{3}H-vitamin D_3. d) Co-chromatography of homogenate metabolite with synthetic 25-HCC. (--- O.D. (264 mμ) ___ CPM/fraction)

Four hours after the injection, the perfusate and liver were extracted separately by the method of Bligh and Dyer (8). The chloroform layers of the extracts were evaporated to dryness and chromatographed on silicic acid columns. The solvent system used to analyze for metabolites of vitamin D_3 was that described by Ponchon and DeLuca (6). A profile of a perfusate extract is shown in Figure 1. As shown in Table 1, approximately 5% of the radioactivity in both the liver and the perfusate chromatographed in a position corresponding to that of 25-HCC. The original dose of D_3 contained no radioactivity in this region. When perfusate containing labeled ^{3}H-vitamin D_3 was circulated in the perfusion chamber for 4 hours with no liver present, no radioactivity appeared in the 25-HCC region. Thus the metabolite was not produced by non-enzymatic oxidation during circulation of vitamin D_3 in the perfusate equilibrated with 95% O_2, 5% CO_2. Clearly, the liver had to be present to mediate the conversion.

When a liver was perfused for 4 hours with 5,000 I.U. of

Table 1. Metabolism of vitamin D_3 by perfused rat livers.

Experiment no.	Substance analyzed	Total DPM present after perfusion	% 25-HCC
1	Perfusate	125,000	4.9
1	Liver	14,000	5.4
1	Bile	4,800	---
2	Perfusate	---	5.0
2	Liver	---	5.6

Experiments were performed as described in the text.

^{3}H-vitamin D_3, enough labeled compound appeared in the 25-HCC region of the perfusate chromatogram to test for its anti-rachitic activity by the line test method (9). All four rats dosed with 0.1 µg of the metabolite showed a response equal to or greater than that of 0.1 µg of vitamin D_3. The average score for the metabolite was 1.1 times that of vitamin D_3. Thus the isolated, perfused rat liver was able to convert vitamin D_3 to a compound with the chromatographic properties and anti-rachitic activity of 25-HCC, the in vivo metabolite of vitamin D_3.

Further studies were carried out with rat liver homogenates. After 4 hours of incubation under the conditions described in Figure 2, ^{3}H-vitamin D_3 was converted to a compound which chromatographed like 25-HCC. Figure 1 shows

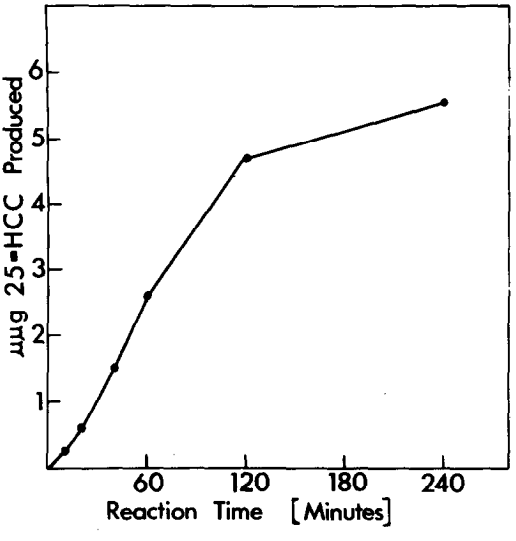

Figure 2. Time-course study of 25-HCC production in rat liver homogenates. Ten ml volumes of 25% stock rat liver homogenate containing 0.075 g ^{3}H-vitamin D_3, 6 mM glucose, 5 mMATP, 10^{-3} mM TPN, 40 mM nicotinamide, 25 mM potassium phosphate buffer, pH 7.4, 1.25 mM $MgCl_2$, and 25 mM KCl were incubated at 37°C for various time periods. The incubation mixtures were extracted and chromatographed on silicic acid columns as in Figure 1.

a chromatogram of the homogenate extract and one of the homogenate metabolite co-chromatographed with synthetic 25-HCC. As shown in Figure 2, the reaction proceeds almost linearly for the first 2 hours of incubation. Reactions carried out with and without added TPN, glucose, and others run in the presence of 1 μg of N,N'-diphenyl-p-phenylenediamine (DPPD) indicate that the reaction is stimulated by the addition of TPNH and is not inhibited by DPPD, an inhibitor of lipid peroxidation described by Ernster et al. (10).

In conclusion, then, the liver perfusion data and liver homogenate data presented in this paper demonstrate that the rat liver can convert vitamin D_3 to a compound which has the chromatographic properties and anti-rachitic activity of 25-HCC, the in vivo biologically active metabolite of vitamin D_3. Work is now in progress to study the nature of the enzyme system and its distribution among tissues and cell fractions. It appears likely that this system may well play a key regulatory role in calcium and phosphate metabolism as well as in the metabolism of vitamin D.

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