STUDIES ON CALCIFEROL METABOLISM I. PRODUCTION OF VITAMIN D METABOLITE 4B FROM 25-OH-CHOLECALCIFEROL BY KIDNEY HOMOGENATES*

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Summary: Incubations of 26, 27-^{3}H-25-hydroxycholecalciferol (25-OH-vitamin D_3) with kidney homogenates produced significant amounts of a compound which was chromatographically identical with and had biological activity equivalent to intestinal Metabolite 4B. Thus, the kidney appears to be a major site of synthesis of Metabolite 4B, the proposed biologically active form of cholecalciferol in the intestine.

Introduction

We have previously reported evidence for the existence of a polar metabolite of cholecalciferol (vitamin D_3), designated Metabolite 4B, which stereospecifically associated with the nucleus and chromatin fraction of the intestinal mucosa cell (1, 2) and which was highly active in promoting intestinal calcium absorption in the rachitic chick (3). More recently Myrtle and Norman (4) have conclusively shown that Metabolite 4B isolated from chick intestines is over 2 times as effective as 25-hydroxycholecalciferol (25-OH-CC), a metabolite found primarily in the blood (3), and almost 5 times as active as the parent cholecalciferol (CC), in stimulating intestinal calcium transport 24 hours after administration. More importantly, Metabolite 4B is capable of considerably shortening the lag in the transport response to cholecalciferol. At 9 hours, 4B was found to be over 13 times as active as the parent cholecalciferol at 24 hours. Thus, Metabolite 4B probably represents the biologically active form of cholecalciferol in the intestine. Similar results have been reported by Kodicek and coworkers (5, 6).

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Metabolite 4B, which is the predominant metabolite found in the intestinal mucosa 8 to 48 hours after a physiological dose# of cholecalciferol, has been shown to be produced *in vivo* from 25-OH-CC (3). Current efforts to chemically characterize Metabolite 4B are rendered exceedingly difficult due to an apparent control of its production. A maximum of about 1.6 ng of 4B are found per gram of intestinal mucosa cells (1, 3, 5, 6), despite administration of massive amounts of CC or 25-OH-CC. Accordingly, we have initiated a search for tissues which might produce Metabolite 4B *in vitro*. It is the purpose of this paper to report that the kidney is a major site of production of Metabolite 4B.

Methods

White Leghorn cockerels (H and N of California, Inc.) were fed a calciferol deficient diet (7) and used in the fourth week when they became rachitic. Tissues of interest were removed immediately after sacrifice and placed in ice cold 0.25 M sucrose in 0.1 M KH_2PO_4-K_2HPO_4, pH 7.4. Ten percent homogenates were made and 3.25 ml added to an incubation system containing 12 µmoles $MgCl_2$, 600 µmoles potassium phosphate, pH 7.4, 60 µmoles malate, 41.4 µmoles glucose-6-phosphate, 2.6 µmoles NADP, 5 µg of glucose-6-phosphate dehydrogenase (5 Kornberg units), and 80 to 850 pmoles (1.2 to 13 IU) of 26, 27-^{3}H-25-OH-cholecalciferol (Amersham/Searle, Inc.), in a final volume of 6 ml. The 26, 27-^{3}H-25-OH-CC, (specific activity 7.5 Ci/mmole or 196 mCi/mmole) was added to the incubation system in 100 µl ethanol.

After incubation at 37°C for 1.5 to 4 hours, 15 ml of methanol-chloroform (2:1) were added to obtain total lipids as described earlier (3). The chloroform soluble lipids were dissolved in hexane for subsequent silicic acid chromatography. In each instance, homogenates heated at 100°C for 5 minutes were employed in control incubations.

^{3}H-Metabolites produced via incubation *in vitro* were mixed with ^{14}C-intestinal metabolites obtained from rachitic chicks which had received 4-^{14}C-CC,

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The minimum daily requirement for cholecalciferol in the chick (7) is 0.65 to 1.3 nmoles (10 to 20 IU). One IU of cholecalciferol is equivalent to 25 ng or 65 pmoles.

as described elsewhere (1, 3). The combined metabolites were chromatographed on a 30 g (1 x 80 cm) silicic acid column. Elution was carried out with exponential gradients of diethyl ether, 1,2-dichloroethane, and methanol generated as previously described (1, 3), with the following modifications; the 300 ml of 50% (v/v) 1,2-dichloroethane were extended to 400 ml, and 300 ml of acetone were employed in place of 300 ml of 100% 1,2-dichloroethane. Rechromatography of ^{3}H- and ^{14}C-Metabolite 4B, obtained from a silicic acid column as described in Figure 1, was carried out on a 20 g (1 x 80 cm) Celite liquid-liquid partition column employing a mobile phase of 20% (v/v) 1,2-dichloroethane saturated with a stationary phase of 90% (v/v) methanol - 10% water (1). In both chromatographic systems, fractions were prepared for liquid scintillation counting as described earlier (3).

Metabolite 4B produced *in vitro* by kidney homogenates was examined for biological activity in rachitic chicks by the method of Coates and Holdsworth (8), as modified by Hibberd and Norman (7). This assay measures the ability of the test compound to stimulate intestinal calcium transport *in vivo*.

Results and Discussion

The primary tissues known to be related to the action of calciferol include the skin (site of production of cholecalciferol), the liver (site of hydroxylation of CC to 25-OH-CC (9)), the intestine and bone (sites of biological response, and localization of Metabolite 4B (10)), and possibly the kidney (11). Since we have previously shown that in the intact chick, 25-OH-CC is a precursor to Metabolite 4B (3), we have employed 26,27-^{3}H-25-OH-CC as a substrate for the production of 4B *in vitro*. To date we have carried out incubations of 26,27-^{3}H-25-OH-CC with homogenates of liver, intestinal mucosa, bone, and kidney. Only the kidney homogenate produced a ^{3}H-metabolite which exactly migrates with authentic *in vivo*-produced Metabolite 4B from intestines. Typical results are shown in Figure 1. Here, when 77 pmole of 26, 27-^{3}H-25-OH-CC were incubated 1.5 hours with a kidney homogenate, 22% or 17 pmole were converted to a compound which migrates with intestinal Metabolite 4B.

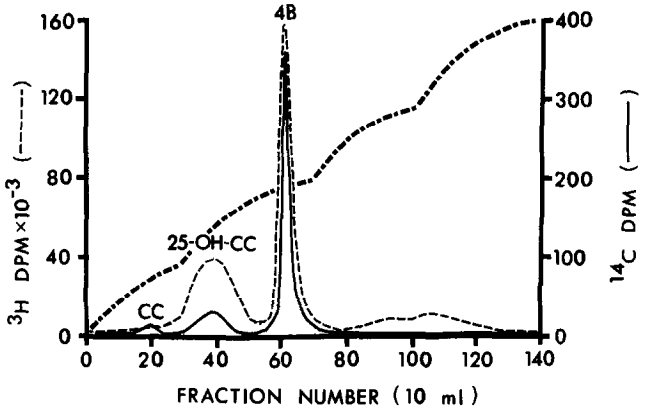

Fig. 1. Silicic acid chromatography of ^{3}H-metabolites produced *in vitro* from a kidney homogenate and ^{14}C-metabolites obtained from chick intestines *in vivo*. A kidney homogenate was incubated with 26, 27-^{3}H-25-OH-CC as described in the Methods section. The homogenate lipids were extracted and cochromatographed on a 30 g silicic acid column with intestinal lipids obtained from rachitic chicks which had received 0.65 nmole of 4-^{14}C-CC intracardially 17 hours prior to sacrificing. Metabolites were eluted with successive exponential gradients of diethyl ether, 50% (v/v) 1,2-dichloroethane, acetone, and methanol.
——— = ^{14}C; ---- = ^{3}H; — - — - — = gradients.

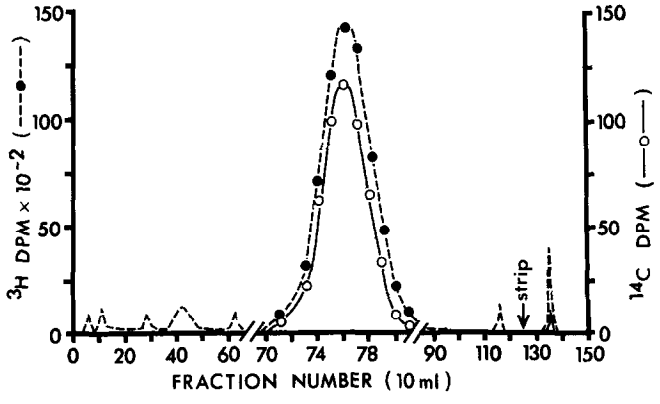

Fig. 2. Cochromatography of ^{3}H-Metabolite 4B (*in vitro*) and ^{14}C-Metabolite 4B (*in vivo*). The *in vitro*- and *in vivo*-produced metabolites were obtained from a silicic acid column exactly as described in Figure 1, and applied to a 20 g Celite liquid-liquid partition column, as described in the Methods section. Stripping of the column with 100% 1,2-dichloroethane was started with the collection of Fraction 125.

Inasmuch as this comigration occurred in a gradient elution system, the apparent comigration of the ^{3}H-metabolite obtained from kidney *in vitro*, and intestinal Metabolite 4B obtained *in vivo* was critically examined by rechromato-

graphy on a Celite liquid-liquid partition column, shown in Figure 2. Celite partition chromatography is capable of separating steroids as closely related as estriol and 16-epiestriol (12). Seventy-seven percent of the kidney ^{3}H-4B (*in vitro*) exactly comigrates with the intestinal ^{14}C-4B (*in vivo*)in this highly sensitive chromatography system, suggesting that Metabolite 4B from these two sources is identical.

The results of a biological assay of Metabolite 4B produced *in vitro* by kidney homogenates are reported in Table 1. The *in vitro* Metabolite 4B was found to be highly biologically active (over 4 times as active as the parent CC) in its ability to stimulate intestinal calcium absorption in the rachitic chick. In agreement with our earlier results with intestinal Metabolite 4B (4), the *in vitro* Metabolite 4B is also able to greatly shorten the lag which precedes the

Table 1

Biological Assay of *in vitro* Kidney Metabolite 4B

Compound Tested, nmoles (IU)	Hours[a]	Number of chicks	$^{45}Ca^{++}$ cpm/0.20 ml plasma
Rachitic control	25	4	207 ± 21 [b]
1.52 (23.4) CC	25	5	844 ± 34
0.76 (11.7) CC	25	5	451 ± 107
0.11 (1.6) Kidney 4B	25	4	386 ± 95 [c]
0.11 (1.6) Kidney 4B	10	4	406 ± 80 [c]

Kidney Metabolite 4B, obtained *in vitro* and isolated exactly as described in Figure 1, and standard amounts of CC, were tested for their ability to stimulate intestinal absorption of a dose of 4.0 mg of $^{40}Ca^{++}$ and $^{45}Ca^{++}$ (4 μCi) by the procedure of Hibberd and Norman (7). The appearance of $^{45}Ca^{++}$ in the blood is measured 30 minutes after placing the $^{45}Ca^{++}$ in the intact duodenum.

[a] Time in hours between administration of test compound and assay.
[b] Data are reported as the mean ± S.E.M.
[c] $P < 0.1$ above rachitic control.

transport response. Only 10 hours after administration, the kidney Metabolite 4B produced a significant stimulation of calcium transport.

To date, the kidney appears to be the sole tissue capable of converting significant quantities of 25-OH-CC to Metabolite 4B *in vitro*. This metabolite is chromatographically identical to, and possesses the high biological activity of, intestinal Metabolite 4B produced *in vivo*. While this work was in progress, Frazer and Kodicek (13) reported that kidney homogenates converted a mixture of 1-^{3}H- and 4-^{14}C-25-OH-CC to a polar compound. This compound, of unknown biological activity, was detected by thin layer chromatography and found to be deficient in tritium. This is a characteristic but not necessarily unique property of Metabolite 4B (3, 5, 10). The prospect now exists that Metabolite 4B, whose production is apparently limited *in vivo*, can now be produced in reasonable amounts via *in vitro* incubations, so that a serious attempt at its chemical identification may be attempted.

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