25-HYDROXYCHOLECALCIFEROL: SPECIFIC BINDING BY RACHITIC TISSUE EXTRACTS

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SUMMARY: In vitro incubation of ^{3}H-25-hydroxycholecalciferol with 105,000 x g supernatants from homogenates of rachitic rat kidney and skeletal muscle resulted in sedimentation of radioactivity at 5-6 S, in contrast to the 3-4 S sedimentation observed when the sterol was incubated with rachitic serum. The binding of ^{3}H-25-hydroxycholecalciferol by these tissue extracts was reduced by exposure to anti-rachitic sterols containing an open B ring with C_{10} methylene and C_9 hydrogenation, and by pre-incubation of the extracts with trypsin.

Several steroid hormones are thought from accumulated evidence to modify intracellular events at target tissues by first complexing with soluble cytoplasmic molecules (1,2) and subsequently associating with chromatin acidic proteins (3). Vitamin D is now known to undergo 25-hydroxylation in the liver (4) and subsequent 1-hydroxylation in the rat or chick kidney (5). The resulting dihydroxy metabolite is a potent stimulator of ^{45}calcium uptake by intestinal epithelium (6). Vitamin D is taken up by the liver (7), but the circulating reserve substance which also may be tissue-active, or undergo other transformations to permit specific tissue functions is thought to be 25-hydroxycholecalciferol (25-HCC) (8,9). 25-HCC has recently been implicated as an inducer of enzyme(s) responsible for its further transformation(s) (10). Since 25-HCC is apparently the major circulating form of the vitamin, we have examined the possibility of specific binding of 25-HCC to soluble tissue extracts.

MATERIALS AND METHODS

Weanling Sprague-Dawley rats were kept in the dark and fed a vitamin D-

free chow (Nutritional Biochemicals) for 4 weeks. The animals were bled from the aorta, and skeletal muscle and kidneys were dissected. Tissue minces were rinsed in cold 0.15M saline, homogenized in 5 mM EDTA, pH 7.4, and subjected to ultracentrifugation at 105,000 x g. The non-floating material in the supernatant fraction was diluted in 50 mM Tris-buffer, pH 7.4, to give a final protein concentration of 4 mg/ml (12). Absolute ethanol containing 2 ng of (^{3}H-26,27) 25-hydroxycholecalciferol (^{3}H-25-HCC) (11 Ci/m Mole, Amersham Searle) was pipetted into each of three tubes and the ethanol evaporated under a stream of nitrogen. One ml of diluted serum (4 mg protein) or 1 ml of the 105,000 x g tissue extract was added, incubated at 4° C for 30 minutes, and 200 µl of this solution was pipetted onto a 4.7 ml linear 5-20% sucrose gradient. Sixteen hour centrifugations at 40,000 rpm on a SW 50.1 rotor in a Beckman L2-65B ultracentrifuge were performed at 4° C and the gradients were collected through a bottom puncture into liquid scintillation vials.

The specificity of binding by the 105,000 x g supernatants was examined. A competitive binding assay was employed, in which the unbound sterol was removed by adsorption to charcoal coated with Dextran-20. Aliquots of tissue extracts were incubated with ^{3}H-25-HCC and various sterols for 1 hour at 25° C. Coated charcoal suspension (Norit, 2.5 gms and Dextran-20, 0.25 gms per 100 ml of 50 mM phosphate buffer, pH 7.4) was added (0.25 ml per tube) to precipitate the free sterol, and aliquots of supernatant containing the protein-bound sterol were assayed for radioactivity.

RESULTS

Figure I depicts the sedimentation patterns observed. The rachitic serum contained a single radioactive peak of 3-4 S, whereas the kidney and skeletal muscle extracts bound the labelled sterol on material sedimenting at 5-6 S.

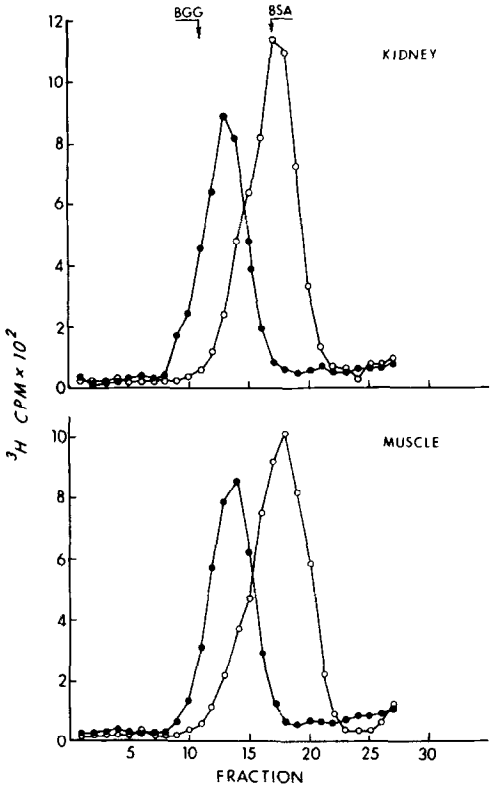

Figure 1. Sucrose gradient centrifugation of ^{3}H-25-hydroxycholecalciferol (11 Ci/m Mole) (^{3}H-25-HCC) bound to rachitic rat serum (o——o) and 105,000 x g supernatants from homogenates of rachitic rat kidney and skeletal muscle (●——●). Serum and tissue extracts (800 μg of protein) were pre-incubated at 4° C for 30 minutes with 400 picograms (8,000 cpm) of ^{3}H-25-HCC, and then layered onto 4.7 ml linear 5-20% sucrose gradients in 50 mM phosphate buffer, pH 7.4. Centrifugation was carried out at 40,000 rpm for 16 hours in a SW 50.1 rotor at 4° C. Bovine gamma globulin (BGG) and bovine serum albumin (BSA) were detected by their absorbance at 280 mμ.

The same sedimentation behavior occurred in sucrose gradients prepared in 1 or 100 mM sodium phosphate, pH 7.4. The serum sedimentation behavior was not changed by pre-homogenization and centrifugation at 105,000 x g prior to incubation with ^{3}H-25-HCC and sucrose gradient ultracentrifugation. Similar experiments in another group of rachitic rats have revealed essentially identical sedimentation patterns. We have observed that incubations of ^{3}H-25-HCC with equal protein mixtures of rachitic kidney or muscle extract and rachitic

Table I. Displacement of ^{3}H-25-hydroxycholesterol (^{3}H-25-HCC) (11 Ci/m Mole) from rachitic tissue extracts of kidney and muscle. Incubation mixtures consisted of 1 ml of 50 mM phosphate buffer, pH 7.4, containing 125 µg of 105,000 x g supernatant protein from rachitic rat kidney or skeletal muscle, 25 µl of ethanol containing the competitor at final concentration indicated, and 25 µl of ethanol containing ^{3}H-25-HCC (4000 cpm) at a final concentration of 0.0005 µM. Tubes were incubated at 25° C for 60 minutes. Unbound sterol was precipitated on Dextran 20-coated charcoal, and protein-bound (supernatant) radioactivity measured.

Competitor	Concentration (µM)	Supernatant Radioactivity (cpm)*	
		Kidney	Muscle
None	—	2136 ± 56	1859 ± 43
25-hydroxycholecalciferol	0.0025	1506 ± 22	1381 ± 28
"	0.025	679 ± 18	744 ± 22
Cholecalciferol	0.25	1220 ± 43	1125 ± 21
Ergocalciferol	0.25	1341 ± 35	1208 ± 28
7-dehydrocholesterol	0.25	2004 ± 54	1720 ± 26
25-hydroxy-7-dehydrocholesterol	0.25	2189 ± 53	1842 ± 33
Cholesterol	2.5	2087 ± 62	1878 ± 43
25-hydroxycholesterol	0.25	2068 ± 45	1920 ± 54
Dihydrotachysterol	2.5	2033 ± 43	1796 ± 27
Cortisol	2.5	2004 ± 54	1792 ± 50

* Mean ± S.E.M. of quadruplicate analyses

serum exhibit single radioactive peaks at 5-6 S. Pre-incubation of kidney and muscle extract with trypsin markedly reduced their ability to bind ^{3}H-25-HCC.

Table I shows the specificity of the binding of ^{3}H-25-HCC by soluble extracts of kidney and muscle. Of the potential competitors examined, only vitamin D_3 and vitamin D_2 at 100 fold higher concentrations than 25-HCC were able to displace ^{3}H-25-HCC from the binding material in these extracts. It appears that the open B ring is an important characteristic in this system. The failure of dihydrotachysterol to be competitive implies that the C_{10} methylene bond and C_9 hydrogenation are necessary determinants as well. Similar specificity has been observed with rachitic sera as well if the infranatant from 105,000 x g centrifugation was used. Native sera contains lipid moieties

which may compete with the charcoal for the free sterol. It has been possible to employ rachitic kidney extracts in the development of a radiocompetitive assay for 25-HCC in chromatographed extracts of human plasma (11).

Our findings suggest the presence of specific, soluble binders of 25-HCC in rachitic rat kidney and skeletal muscle. The unique role of the kidney in the controlled 1-hydroxylation of 25-HCC (5), and the inhibition of this step by metabolic inhibitors (10) is consistent with our finding of a specific binding substance in this organ. Our observation of similar binding in muscle was unexpected, but myopathic syndromes are commonly described in clinical states resulting from lack or "resistance" to the vitamin, and muscle has been shown to harbor a large amount of radioactivity following oral administration of ^{3}H-cholecalciferol to rachitic rats (13). It has been recently reported that human muscle is a significant source of anti-rachitic material (14). It is tempting to surmise that a variety of target tissues contain binding substances which might have an important preliminary role in different transformations and/or biological expressions of 25-HCC. The recent proposals of target organ "receptors" of various steroid hormones is supportive in this regard (2,3). In addition, previous tissue extract utilization in radio-competitive assay of steroids (15) suggests the reasonable goal of a relatively simple quantitative assessment of anti-rachitic sterols.

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