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25-Hydroxyvitamin D_3-1-Hydroxylase. Inhibition *in Vitro* by Rat and Pig Tissues†

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ABSTRACT: Although 25-hydroxyvitamin D_3-1-hydroxylase can easily be demonstrated *in vitro* with chick kidney preparations, attempts to demonstrate this activity *in vitro* with rat kidney homogenates have been unsuccessful, despite the fact that nephrectomized rats cannot produce 1,25-dihydroxyvitamin D_3. The failure of the rat preparation to carry out this hydroxylation has now been shown to be due to the presence of a heat-labile, highly potent inhibitor of the reaction. This inhibitor was found in all kidney cell fractions, but the most potent appeared to be the microsomes which released the inhibitor during incubation. A similar inhibitor was found in intestine and blood serum, both being potent sources. Pig kidney tissue also contained such an inhibitor.

The metabolism of vitamin D_3 to its more biologically active hydroxylated derivatives has been reviewed extensively (Olson and DeLuca, 1973; Omdahl and DeLuca, 1973; Wasserman and Taylor, 1972). 25-Hydroxyvitamin D_3 (25-OH-D_3)[1] is known to be synthesized mainly in the liver (Horsting and DeLuca, 1969) and represents the major circulating metabolite of the vitamin (Ponchon and DeLuca, 1969; DeLuca, 1969; Mawer *et al.*, 1969).

The second hydroxylation step, which has been shown by experiments with nephrectomized rats to be carried out only in the kidney (Fraser and Kodicek, 1970; Gray *et al.*, 1971; Omdahl *et al.*, 1972), occurs either at the 1 or the 24 position of the 25-OH-D_3 molecule (Holick *et al.*, 1971a,b, 1972) depending on the vitamin D, calcium, and phosphorus status of the animal. 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the most active form of vitamin D_3 known in both induction of intestinal calcium transport and bone mobilization (Holick *et al.*, 1971a; Haussler *et al.*, 1971; Omdahl *et al.*, 1971), is produced by hyposphosphotemic and hypocalcemic animals, whereas normal calcemia, hypercalcemia, or hyperphosphatemia leads to the production of 24,25-dihydroxyvitamin D_3 (24,25-$(OH)_2D_3$) (Boyle *et al.*, 1971; Tanaka and DeLuca, 1973).

In vitro hydroxylation of 25-OH-D_3 in the 1 position (Fraser and Kodicek, 1970; Gray *et al.*, 1971, 1972; Norman *et al.*, 1971) and in the 24 position (Omdahl *et al.*, 1972; Knutson and DeLuca, 1974) has been demonstrated using both homogenates and isolated mitochondria from kidneys of chicks fed the appropriate diet. Midgett *et al.* (1973) have reported the presence of a compound which co-chromatographs with 1,25-$(OH)_2D_3$ after incubation of 25-OH-D_3 with human, rat, and dog kidney homogenates; however, all attempts made in this laboratory to establish a rat kidney system which will synthesize either 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ *in vitro* have been unsuccessful.

This paper reports the presence of a factor in rat kidney homogenates which inhibits the formation of 1,25-$(OH)_2D_3$ from 25-OH-D_3 by homogenates and isolated mitochondria prepared from kidneys of vitamin D deficient chicks. As it seems possible that this factor may represent a regulator of 1,25-$(OH)_2D_3$ synthesis, some of its properties are reported.

Materials and Methods

Animals. One-day-old white Leghorn cockerels (Northern Hatcheries, Beaver Dam, Wis.) were maintained on a vitamin D deficient purified diet (Omdahl *et al.*, 1971) for 4–6 weeks before use.

Male albino rats (Holtzman Co., Madison, Wis.) were housed in hanging wire cages and fed a low vitamin D stock diet (Steenbock, 1923) or a vitamin D deficient diet containing 0.47% calcium and 0.3% phosphorus (Suda *et al.*, 1970). Variation in the calcium content of the diet was balanced by appropriate changes in the sugar content. When required, vitamin D_3 (1 or 2 IU daily) was given orally in 0.1–0.2 ml of cottonseed–soybean oil (Wesson Co., Fullerton, Calif.). Rats were allowed food and water *ad libitum* for 5–8 weeks before being used in experiments.

Pig kidneys were taken from two animals of mixed breed weighing 250–300 lb which had been fed stock rations, supplemented with enough vitamin D_2 to supply each pig with 500,000 IU daily for 28 days (Suda *et al.*, 1969).

For *in vivo* experiments rats were injected intrajugularly (under ether anethesia) with 650 pmol of 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) in 0.05 mol of 95% ethanol 24 hr be-

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[1] Abbreviations used are: 25-OH-D_3, 25-hydroxyvitamin D_3; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3.

fore death. The kidneys were then extracted and chromatographed as described below.

Rat Kidney Perfusion. Rats were injected with 500 units of sodium heparin intraperitoneally approximately 5 min before death. Perfusion was carried out *in situ* by forcing physiological saline solution into the kidney blood vessels, *via* the dorsal aorta and renal arteries, until the tissue was completely blanched. Kidneys were then removed and treated as described below.

Preparation of Tissue Homogenates. Animals were killed by decapitation and blood was collected when required. Kidneys were removed immediately after death and placed in ice cold buffered sucrose (0.32 M sucrose–0.05 M Tris-acetate, pH 7.4). The mesentary tissue was carefully dissected away, and the remaining kidney tissue was minced and weighed. A 10% w/v homogenate was prepared using 5 strokes with a motor driven glass Teflon Potter-Elvehjem homogenizer (A. H. Thomas Co., Philadelphia, Pa.). Homogenates (10% w/v) of pig kidney and rat tissues other than kidney were prepared in the same way.

Chick mitochondria were prepared as described by Knutson and DeLuca (1974). The final mitochondrial pellet was resuspended in the buffered sucrose solution to give a concentration of approximately 16 mg of protein/ml.

Rat and pig kidney homogenates were fractionated by differential centrifugation, essentially by the method of Hogeboom (1955). The crude 10% w/v homogenate was centrifuged at 700*g* for 10 min at 4° to remove cell debris and nuclei. This pellet is referred to as the 700*g* fraction. The resulting supernatant was centrifuged at 5000*g* for 10 min at 4°; the pellet obtained was expected to contain most of the mitochondria (5000*g* fraction). The supernatant was centrifuged at 54,000*g* for 60 min at 4°, producing a pellet expected to contain microsomal material (54,000*g* fraction), and the final supernatant. All the pellets were washed once and finally resuspended in ice cold buffer solution (0.32 M sucrose–0.05 M Tris-acetate, pH 7.4) to give a suspension corresponding to a 10% w/v homogenate.

The protein content of homogenates, subcellular fractions, and blood sera was determined by the method of Lowry *et al.* (1951).

Incubation Conditions. Incubations were carried out for 1 hr in 25-ml erlenmeyer flasks at 37° in the presence of 100% oxygen with continuous agitation of 80 oscillations/min with a 1-in. stroke length. Each flask contained 1 ml of chick kidney homogenate (10% w/v) or 0.5 ml of chick kidney mitochondrial preparation (approximately 8 mg of protein), 1 ml of rat or pig tissue preparation, 1 ml of cofactor solution, and 10 ng of 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) in 10 μl of 95% ethanol. In control experiments 1 ml of buffer replaced the rat or pig preparations. In experiments when rat tissue was tested for 25-OH-D_3-1-hydroxylase activity the chick kidney preparation was replaced by buffer. When the level of rat tissue preparation was varied, the homogenate was diluted to the appropriate strength before addition to the incubation flask. In early experiments, the cofactor solution used was the NADPH-generating system of Fraser and Kodicek (1970); later the reaction was supported with succinate (5 mM) and magnesium (1.87 mM $MgCl_2$) (Omdahl *et al.*, 1972). Experiments using NADPH as the reducing equivalents for the hydroxylation in calcium swollen mitochondria were as described by Ghazarian and DeLuca (1974).

Reactions were terminated by the addition of 10 ml of 2:1 methanol–chloroform.

Preincubations. Preincubations were carried out under identical conditions to incubations except that the 25-OH-[26,27-^{3}H]D_3 was not present. Rat kidney 54,000*g* fraction (1 ml) was incubated with the appropriate cofactor solution (1 ml) for 10–60 min. After this time the mixture was centrifuged at 96,000*g* for 60 min at 4° to remove the particulate material. The supernatant was tested for inhibition under the usual incubation conditions.

Extraction. The contents of the incubation flask were poured into a separatory funnel. The flasks were rinsed with 5 ml of 2:1 methanol–chloroform. The resulting one-phase system was allowed to stand at room temperature for 1 hr after which time a two-phase system was produced by addition of 5 ml of chloroform and 4 ml of water. After separation, the aqueous phase was reextracted with 5 ml of chloroform. The combined chloroform extracts were evaporated to dryness on a rotary evaporator in the presence of a small amount of absolute ethanol, added to remove any residual water. The residue was dissolved in 2 ml of 65:35 chloroform–Skellysolve B (petroleum ether, bp 67–69°). A small aliquot (20 μl) was taken for radioactivity determination. Ninety to one-hundred per cent recovery of the tritium added to the incubation flask was obtained.

Chromatography. The lipid extract from each incubation was chromatographed on a 10- or 16-g Sephadex LH-20 column (2 × 30 cm) equilibrated with 65:35 chloroform–Skellysolve B. The relative positions of 25-OH-D_3 and 1,25-$(OH)_2D_3$ have been determined previously by chromatography of pure metabolites under identical conditions (Holick and DeLuca, 1971). The column was developed with the same solvent, 50 5-ml fractions being collected into 1-dram vials at a flow rate of 1 ml/min. The solvent was removed under a stream of air and the residue was dissolved in 4 ml of toluene counting solution (Bhattacharrya and DeLuca, 1973). Samples were assayed for radioactivity in a Nuclear-Chicago Model No. 6868 Isocap liquid scintillation counter. Efficiency of counting was 48–50%. Recovery of radioactivity from the column represented 80–100% of that applied.

Radioactive 25-OH-D_3. 25-OH-[26,27-^{3}H]D_3 (1.2 Ci/mmol) was synthesized in this laboratory by the method of Suda *et al.* (1970). The radioactive material was purified periodically by chromatography on a Sephadex LH-20 column (1 × 60 cm) eluted with 50:50 chloroform–Skellysolve B (Holick and DeLuca, 1971).

Results

In agreement with previous work (Gray *et al.*, 1971, 1972) incubation of 25-OH-D_3 with kidney homogenates from chicks fed a vitamin D deficient diet produced 1,25-$(OH)_2D_3$ (Figure 1a); however, kidney homogenates from rats fed a diet marginal in vitamin D_3 failed to carry out the conversion (Figure 1b), all the radioactivity being recovered as 25-OH-D_3. Addition of the rat kidney homogenate to the chick kidney preparation before incubation completely blocked the hydroxylation reaction (Figure 1c) and the radioactivity was recovered as unchanged substrate. Heating the rat tissue to 100° for 5 min prior to addition to the incubation mixture destroyed the inhibitory effect (Figure 1d). The ratio of rat protein to chick protein present in these experiments was approximately 1:2. Inhibition was observed at ratios up to 1:20. The rats used in the experiments described above were shown to synthesize 24,25-$(OH)_2D_3$ *in vivo* and not 1,25-$(OH)_2D_3$.

As it is possible that the observed inhibition by rat tissue

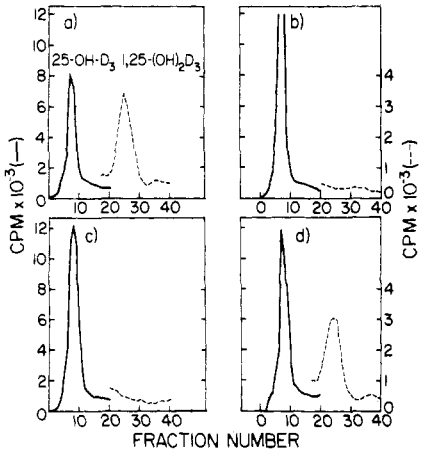

FIGURE 1: Sephadex LH-20 (10 g, packed and eluted as described in the text) chromatographic profiles of lipid extracts from incubations: (a) chick kidney homogenate (approximately 20 mg of protein) plus buffer; (b) chick kidney homogenate plus rat kidney homogenate (approximately 12 mg of protein); (c) rat kidney homogenate plus buffer; (d) chick kidney homogenate plus previously heated rat kidney homogenate. Rats were fed a diet low in vitamin D_3. Incubations were carried out as described in the text, using the NADPH-generating system described by Fraser and Kodicek (1970). Metabolites were identified from their relative position in the chromatographic profile (Holick and DeLuca, 1971): (—) substrate radioactivity; (- - -) 1,25-$(OH)_2D_3$ radioactivity.

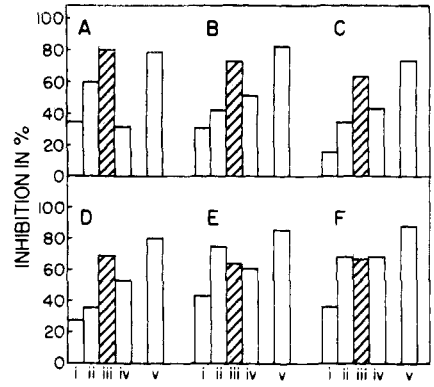

FIGURE 2: Comparison of the effect of subcellular fractions from kidneys of rats fed diets varying in calcium and vitamin D_3 content on 25-OH-D_3-1-hydroxylation by chick kidney homogenates: (A) low calcium (0.02%) vitamin D deficient diet; (B) normal calcium (0.47%) vitamin D deficient diet; (C–E) normal calcium diet supplemented with vitamin D_3 (1 IU per day) for 3, 7, and 14 days prior to death, respectively; (F) high calcium diet (2.0%) supplemented with vitamin D_3 (2 IU per day) for 21 days prior to death. Chick kidney homogenate (1 ml = approximately 18 mg of protein) was incubated with 1 ml of rat kidney 700*g* (i), 5000*g* (ii), 54,000*g* (iii), and supernatant (iv) fractions (approximately 5–8 mg of protein/ml) and also with rat kidney whole homogenates (v) which contained approximately 18 mg of protein/ml. Incubation conditions were as described under Materials and Methods. Malate and NADPH were used as sources of reducing equivalents (Fraser and Kodicek, 1970).

in vitro is important in the regulation of 25-OH-D_3 metabolism to 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ *in vivo*, the effects of varying the vitamin D supplementation of the rats on the inhibitory factor and its subcellular location were examined. The *in vivo* product of hydroxylation of 25-OH-D_3 in the kidney of each group of rats was also determined (Table I). The results are shown in Figure 2. Kidney homogenate from rats making exclusively 1,25-$(OH)_2D_3$ as well as those making exclusively 24,25-$(OH)_2D_3$ *in vivo* inhibited the chick 25-OH-D_3-1-hydroxylase *in vitro*. Neither 1-hydroxylase nor 24-hydroxylase activity was detected in the rat whole homogenate or 5000*g* preparations. Kidney tissue from rats fed all diets studied contains the inhibitor (Figure 2). In the case of the vitamin D deficient, low calcium, and normal calcium diets, and those diets supplemented with vitamin D for 3 days and 1 week, the inhibition effect is most marked in the 54,000*g* fraction, while in the rats given vitamin D_3 supplemented diets for 2 and 3 weeks, the inhibitor was found more equally distributed between the 5000*g*, 54,000*g*, and supernatant fractions. The *in vivo* study showed that the animals changed from the synthesis of 1,25-$(OH)_2D_3$ to 24,25-$(OH)_2D_3$ after supplementation with vitamin D_3 for more than 3 days (Table I).

TABLE I: *In Vivo* Product of 25-OH-D_3 Hydroxylation in the Kidneys of Rats Fed Diets Varying in Calcium and Vitamin D_3 Content.

Diet	1,25-$(OH)_2D_3$	24,25-$(OH)_2D_3$[a]
0.02% calcium, vitamin D deficient	+	ND[b]
0.47% calcium, vitamin D deficient	+	ND
0.47% calcium, vitamin D_3 (1 IU per day) for 3 days	+	Trace
0.47% calcium, vitamin D_3 (1 IU per day) for 1 week	Trace	+
0.47% calcium, vitamin D_3 (1 IU per day) for 2 weeks	ND	+
2.0% calcium, vitamin D_3 (2 IU per day) for 3 weeks	ND	+

[a]Identified from its relative position on Sephadex LH-20 column chromatography (Knutson and DeLuca, 1974). [b]Not detected.

It is clear from the data shown in Figure 2 that the inhibition effect is, at least in part, associated with particulate cell fractions especially in the vitamin D deficient preparations. However, the chick kidney 25-OH-D_3-1-hydroxylase is known to operate within the mitochondrion; thus, it seems likely that a soluble form of the "inhibitor" is produced by the rat tissue under the incubation conditions, and the soluble form is then taken up by the chick mitochondria. In order to test this hypothesis the most potent inhibitory fraction from the kidneys of normal calcium, vitamin D deficient rats, that is the 54,000*g* fraction, was preincubated and the particulate matter removed as described in the Materials and Methods section. The preincubation medium was then tested for inhibition in the isolated chick kidney mitochondrial system. Table II shows that the inhibitor is found in the preincubation medium. Heating the preincubation medium before addition to the chick system prevents inhibition. It was found that the inhibitory potency of the medium increases with time of preincubation up to 30 min, and then begins to fall.

It has been shown that 25-OH-D_3-1-hydroxylase has a specific requirement for NADPH (Ghazarian and DeLuca, 1974). As the mitochondrial membrane is impermeable to reduced pyridine nucleotides (Lehninger, 1951) it is possible that the "inhibitor" exerts its effect by blocking the formation of NADPH within the mitochondria. If this were so, then supplying externally generated NADPH in mitochondria rendered permeable by swelling with 10 mM calcium

TABLE II: Effect of Medium from Preincubation of Rat Kidney 54,000*g* Fraction on Chick Kidney Mitochondrial 25-OH-D_3-1-Hydroxylation.[a]

Incubation[b]	% Conversion to 1,25-$(OH)_2D_3$	% Inhibition of Hydroxylation
Buffer	21.63	0
Preincubation medium of rat kidney 54,000*g* fraction[c]	8.46	61.9
Preincubation pellet of rat kidney 54,000*g* fraction[d]	12.18	43.7
Rat kidney 54,000*g* fraction (diluted 1:1 with buffer)	8.54	61.5
Previously heated preincubation medium from rat kidney 54,000*g* fraction	19.68	9.0

[a] Rats were fed on a normal calcium, vitamin D deficient diet for 4 weeks. [b] The preparation listed (2 ml) was added to approximately 8 mg of chick kidney mitochondrial protein suspended in 1.5 ml of buffer (0.32 M sucrose–0.05 M Tris-acetate, pH 7.4) containing the NADPH-generating system described by Fraser and Kodicek (1970). Total volume in each flask was therefore 3.5 ml. [c] Rat kidney 54,000*g* fraction (1 ml) was preincubated under the conditions described in the text for 10 min before centrifugation. [d] After centrifugation of the preincubated rat kidney 54,000*g* fraction the pellet was resuspended in 2 ml of buffer.

TABLE III: Comparison of the Effect of Rat Kidney Homogenate and Subcellular Fractions on Calcium Swollen and Nonswollen Chick Kidney Mitochondria.[a]

Incubation	% Conversion to 1,25-$(OH)_2D_3$	% Inhibition of Hydroxylation
(A) Calcium swollen mitochondria[b]		
Buffer	49.08	0
Rat kidney whole homogenate	0	100
Rat kidney 54,000*g* fraction	0	100
Preincubation medium from rat kidney 54,000*g* fraction[c]	31.9	35.01
(B) Nonswollen mitochondria[d]		
Buffer	28.48	0
Rat kidney whole homogenate	0	100
Rat kidney 54,000*g* fraction	6.44	77.42
Preincubation medium from rat kidney 54,000*g* fraction	12.72	54.40

[a] Rats were fed a normal calcium, vitamin D deficient diet for 6 weeks. All incubations were carried out for a period of 15 min. [b] Chick kidney mitochondria were suspended in the calcium swelling system described by Ghazarian and DeLuca (1974). The rat kidney whole homogenate and 54,000*g* fractions were diluted 1:1 with buffer, and 2 ml of the preparations listed was added to each incubation mixture. [c] The rat kidney 54,000*g* fraction was preincubated under the conditions described in the text for 30 min before centrifugation. [d] Chick kidney mitochondria were suspended in a medium containing the NADPH-generating system decribed by Fraser and Kodicek (1970).

(Guerra *et al.*, 1966; Ghazarian and Deluca, 1974) should circumvent the inhibition. Table III shows the results of experiments with calcium swollen mitochondria compared to the system usually employed. The inhibition is clearly observed in the calcium swollen mitochondria as well. In addition, results not shown in Table III indicate that calcium swollen kidney mitochondria (5000*g* fraction) from vitamin D deficient rats do not carry out the 1-hydroxylation of 25-OH-D_3.

Homogenates of kidneys from pigs that had been fed a diet high in vitamin D_2 produce small amounts of a compound chromatographing with 24,25-$(OH)_2D_3$ and no detectable 1,25-$(OH)_2D_3$ as might be expected. The effect of addition of the pig kidney preparations to the chick kidney mitochondrial system is shown in Table IV. An inhibition effect was observed with the 5000*g* and 54,000*g* fractions; however, the 700*g* fraction and the supernatant showed no inhibition.

TABLE IV: Effect of Pig Kidney Whole Homogenate and Subcellular Fractions on Chick Kidney Mitochondria 25-OH-D_3-1-Hydroxylation.

Incubation[a]	% Inhibition
Buffer[b]	0
Whole homogenate	43.24
700*g* fraction	0
5000*g* fraction	56.71
54,000*g* fraction	48.29
Supernatant	0

[a] Preparations (6–10 mg of protein) were added to approximately 8 mg of chick kidney mitochondrial protein in the presence of succinate and magnesium (Omdahl *et al.*, 1972). The total volume in each flask was 3.0 ml. [b] During the incubation period, 20.8% of the substrate was converted to 1,25-$(OH)_2D_3$ by the control flask containing no pig tissue.

A comparison of the effects of other rat tissues to that of rat kidney on the chick 25-OH-D_3-1-hydroxylase is shown in Figure 3A. The tissues used were taken from vitamin D deficient rats, and were chosen because of their connection with vitamin D_3 circulation (blood serum), action (intestine), or metabolism (liver). Muscle was considered as a tissue with no established function of vitamin D. Intestinal homogenates and blood serum have a greater inhibitory effect than the kidney per milligram of protein added, whereas liver homogenate is somewhat less effective and although only one protein level was tested for muscle, it was found to be much less inhibitory than the other tissues examined (Figure 3A). Figure 3B shows a comparison between the inhibitory effect of rat and chick blood serum. Surprisingly the chick serum shows some inhibition of the kidney hydroxylation reaction; however, rat serum is much more effective.

It seems possible from these data (Figure 3) that the inhibition seen in vascular organs such as the kidney or liver

may be due to the presence of blood trapped in the organ on the death of the animal. However, homogenates and cell fractions from kidneys which had been perfused *in situ* were unable to produce detectable amounts of 1,25-$(OH)_2D_3$ from 25-OH-D_3 and, furthermore, the subcellular fractions from these preparations inhibited the chick mitochondrial 25-OH-D_3-1-hydroxylation.

Discussion

The results presented clearly demonstrate the presence in rat kidney tissue of a heat-labile factor which inhibits the conversion of 25-OH-D_3 to 1,25-$(OH)_2D_3$ by isolated chick kidney mitochondria. The factor appears to be associated at least partly with the microsomal cell fraction, and this location is more evident in tissue from vitamin D deficient animals. It is very likely that it is this factor which is responsible for the failure to demonstrate 25-OH-D_3-1-hydroxlase activity in homogenates and cell fractions from rat kidney.

Ghazarian and DeLuca (1974) have shown that when chick kidney mitochondria are swollen with 10 mM calcium, extra-mitochondrial NADPH is capable of supporting the 1-hydroxylase reaction in the absence of Krebs cycle intermediates, and in the presence of respiratory chain inhibitors or uncouplers of oxidative phosphorylation. This, taken together with the facts that rat kidney tissue strongly inhibits the reaction under conditions when respiratory chain inhibitors are ineffective and that rat kidney mitochondria isolated from similar homogenates carry out normal oxidative phosphorylation (DeLuca *et al.*, 1957), indicates that it is unlikely that the rat kidney factor is an inhibitor of the respiratory chain or oxidative phosphorylation.

The possibility that the inhibition effect of rat kidney tissue is due to strong binding of the substrate by a protein other than the 1-hydroxylase enzyme cannot be completely ruled out. A 25-OH-D_3 binding protein has been demonstrated to be present in rachitic rat kidney cytosol (Knutson, 1973) and used as the basis for a competitive binding assay for 25-OH-D_3 (Haddad and Chyu, 1971); however, the data presented here show clearly that in vitamin D deficiency the inhibitory factor is associated with a particulate cell fraction, probably microsomal in character. As the chick kidney cytosol also contains a 25-OH-D_3 binding protein (Colston *et al.*, 1973) it seems unlikely that this is important in the inhibition reported here.

Boyle *et al.* (1971) have shown that under conditions of normal calcemia, vitamin D deficient rats produce 1,25-$(OH)_2D_3$, whereas animals supplied with the vitamin synthesize 24,25-$(OH)_2D_3$. If the "inhibitor" in rat kidney tissue has physiological significance, then presumably it must be present at the site of the enzyme reaction within the kidney cell when the 1-hydroxylase is not operating *in vivo*. The results show that in normal calcemic, vitamin D deficient rats the "inhibitor" is most markedly associated with a "microsomal" cell fraction, while animals fed low levels of vitamin D_3 showed an increase in the "inhibitor" in the supernatant and "mitochondrial" cell fractions.

These data, therefore, are consistent with the possibility that the "inhibitor" is transferred from the microsomes to the mitochondria *via* the cytosol when 1,25-$(OH)_2D_3$ production is turned off. This hypothesis is supported by the finding that the "microsomal" fraction from the rat kidney is able to release a soluble factor which also inhibits the chick kidney 1-hydroxylation reaction. Assuming that the subcellular site of the rat kidney 1-hydroxylase is similar to

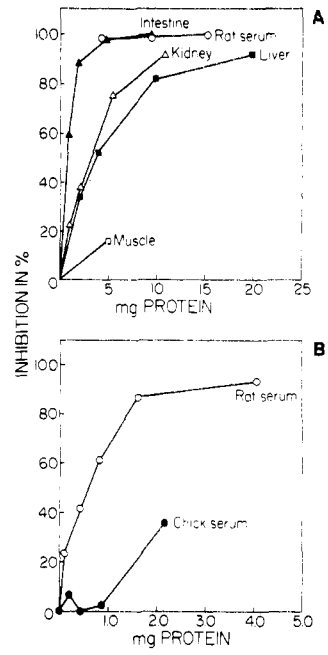

FIGURE 3: Inhibition of chick kidney mitochondrial 25-OH-D_3-1-hydroxylation by: (A) various rat tissue homogenates and (B) rat and chick blood serum. Rats were fed a vitamin D deficient diet for 5 weeks prior to the experiment. Succinate and magnesium were used to generate NADPH in intact mitochondria (Omdahl *et al.*, 1972), and incubations were carried out as described under Materials and Methods. Heat-denatured serum did not inhibit the hydroxylation reaction.

that of the chick enzyme, then the evidence suggests that the rat kidney "inhibitor" could be present at the subcellular site of action of the 1-hydroxylase under conditions when 24,25-$(OH)_2D_3$ is the major product formed from 25-OH-D_3.

The relative residual blood volumes of the different rat tissues examined are: kidney > liver > intestine > skeletal muscle (Sharpe *et al.*, 1950); thus, with the exception of intestine, the pattern is similar to that observed for the inhibition of chick kidney 25-OH-D_3-1-hydroxylation (Figure 3A). The strong inhibition effect observed with rat blood serum therefore suggested that the kidney, liver, and skeletal muscle inhibition may be caused by residual blood in the organs. The experiment with perfused tissue showed that this was not the case for kidney, indicating two possibilities: either (1) the observed inhibitions by rat kidney and blood serum are independent, unrelated phenomena, the kidney "inhibitor" being synthesized by kidney cells, or (2) the blood and kidney inhibitory factors are the same and the kidney cells absorb it from the blood. Perfusion of the other rat tissues was not attempted; however, the strong inhibition effect of intestinal homogenates cannot be explained by residual blood content.

Pig kidney tissue is capable of inhibiting chick kidney 25-OH-D_3-1-hydroxylation and the subcellular location of the inhibitor was similar to that found with rat kidney. It can be postulated that the inhibitory factor is in higher concentrations in mammalian tissue; however, more species would need to be tested to show this convincingly.

Midgett *et al.* (1973) reported production of 1,25-$(OH)_2D_3$ from 25-OH-D_3 by human, dog, and rat kidney tissue *in vitro* which might appear to conflict with the results reported here. However, the levels of substrate and radioactivity used by Midgett *et al.* (1973) were very much higher than those employed in experiments reported here,

the 1,25-$(OH)_2D_3$ produced was not positively identified in each case, and the reported level of 1-hydroxylase in mammals was very small. Thus, it is difficult at this stage of investigation to determine the reasons for the apparent disparity of the results.

The physiological significance of the inhibitory factor present in rat and pig tissue homogenates is unknown; however, the data accumulated so far suggest that further investigation into its nature and properties is warranted, and its presence should be taken into account by anyone seeking to measure the 25-OH-D_3-hydroxylase activity of the kidney, at least in mammalian species.

Acknowledgment

We are grateful to Dr. E. Burt Olson for his assistance with kidney perfusion techniques.

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