The production of 1,24,25-trihydroxyvitamin D₃ in vivo in vitamin D-deficient rats has been demonstrated from either 25-hydroxyvitamin D₃ or 1,25-dihydroxyvitamin D₃. The stereochemical configuration of the hydroxyl on the 24 position of 1,24,25-trihydroxyvitamin D₃ has also been unambiguously established to be R. Nephrectomy failed to eliminate the conversion of 1,25-dihydroxyvitamin D₃ to the 1,24,25-trihydroxyvitamin D₃, establishing that the 24-hydroxylase can be demonstrated in at least one organ in addition to kidney in contrast to the 1-hydroxylase. 1,24,25-Trihydroxyvitamin D₃ can also be produced in vivo from 1α-hydroxyvitamin D₃ or from 24-hydroxyvitamin D₃, analogs of vitamin D₃ which are not naturally occurring. Using chick kidney mitochondrial preparations it has been demonstrated that the 24-hydroxylase is able to utilize 1,25-dihydroxyvitamin D₃ as a substrate whereas it is unable to utilize 1α-hydroxyvitamin D₃. In addition, the chick kidney 1-hydroxylase is known to convert 24,25-dihydroxyvitamin D₃ to the 1,24,25-trihydroxyvitamin D₃, but this hydroxylase is unable to act on the 24-hydroxyvitamin D₃. These results demonstrate that the renal vitamin D hydroxylases require that a hydroxyl be on the 25 carbon of the vitamin D molecule before it can be 1- or 24-hydroxylated.

The abbreviations used are: 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; 24,25-(OH)₂D₃, 24,25-dihydroxyvitamin D₃; 25-OH-D₃, 25-hydroxyvitamin D₃; 1α-OH-D₃, 1α-hydroxyvitamin D₃; 1α-OH-D₃, 1α-hydroxyvitamin D₃; 1,24,25-(OH)₃D₃, 1,24,25-trihydroxyvitamin D₃; 24 OH D₃; 24 hydroxyvitamin D₃; 1,24-(OH)₂D₃; 1,24-dihydroxyvitamin D₃; 1α-OH-D₃, 1α-hydroxyvitamin D₃.
satisfactorily from 25 OH (26,27-3H)D3 with chick kidney preparations (3, 16). The 1α-OH-6-3HID3 was chemically synthesized as described by Holick et al. (17). The 24(R)-OH-24-3HID3 was chemically synthesized by the method of Tanaka et al. (18).

Animals—Male weanling rats, purchased from Holtzman Co. (Madison, Wis.), were fed either a low calcium, adequate phosphorus, vitamin D-deficient diet or a high calcium, low phosphorus, vitamin D-deficient diet for 3 weeks (19, 20). Where indicated the animals were subjected to sham operation or bilateral nephrectomy prior to the administration of the radioactive compounds.

One-day old white Leghorn chickens were obtained from Northern Hatcheries (Beaver Dam, Wis.) and were housed in an incubator at 34°C devoid of ultraviolet light. For 24-hydroxylase the chicks were fed a high calcium vitamin D-deficient diet for 9 days and then given 325 pmol 1α-OH-D3 on the 9th day to stimulate the 24-hydroxylase activity (21). They were used for the isolation of kidney mitochondria 24 h later as described earlier (16). For the 1-hydroxylase the chicks were fed the rachitogenic diet (22) for 4 weeks and then used to prepare kidney mitochondria (16).

Administration of Compounds—A quantity of one of the radioactive compounds (325 pmol) was dissolved in 0.05 ml of 95% ethanol and administered to rats intrajugularly. They were killed 24 h later, and their blood was collected. Nonradioactive 1,25-(OH)2D3 (325 pmol/chick) was dissolved in 0.05 ml of 95% ethanol and administered subcutaneously.

In Vitro Incubation of Kidney Mitochondria—The measurement of the vitamin D hydroxylase activities was performed as described by Gray et al. (16) as modified by Tanaka et al. (21). A 20% (w/v) homogenate of chick kidney was prepared in ice-cold buffer (pH 7.4) containing 0.19 M sucrose, 15 mM Tris/acetate, and 1.9 mM magnesium acetate. The homogenate was centrifuged at 300 × g for 10 min, and the resulting supernatant was centrifuged at 3000 × g for 10 min. The mitochondria were suspended in 1.5 ml of the same buffer containing 25 mM sucinate in a 20-ml flask (4 mg of protein/flask), and then the flask was flushed for 30 s with 100% oxygen. The reaction mixture was incubated at 37°C for 10 min with gentle shaking, and the reaction was stopped by adding a 2/1 (v/v) methanol/chloroform mixture.

Extraction and Chromatography—Serum from rats administered radioactive compounds was extracted with 2/1 (v/v) methanol/chloroform by the method of Tanaka et al. (21). The lipid extract from either rat serum or in vitro incubation medium was chromatographed on a 16-g Sephadex LH-20 column (2 x 18 cm) developed with a solvent system of chloroform/hexane (75/25). An aliquot of each fraction was counted with a Packard scintillation counter (model 3375) with the use of a toluene counting solution containing 0.4% 2,5-diphenyloxazole and 0.03% 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene.

Identification of 1,24R,25-Trihydroxyvitamin D3—Suspected 1,24,25-(OH)D3 obtained from Sephadex LH-20 column was co-chromatographed with chemically synthesized 1,24(R),25-(OH)D3 and 1,24(S)-25-(OH)D3 on a Du Pont Zorbax SIL column (0.21 mm x 25 cm) eluted with 3.5% MeOH in CH2Cl2 with a pressure of 92 kg/cm2 and a DuPont 830 or 840 high pressure liquid chromatography apparatus. The stereochemical isomers of 1,24,25-(OH)D3 are separated on this system. The radioactivity of each fraction was determined. Another portion of 1,24,25-(OH)2D3 was dissolved in 0.02 ml of 95% ethanol and added to an incubation vessel. The reaction mixture was incubated at 37°C for 10 min with gentle shaking, and the reaction was stopped by adding a 2/1 (v/v) methanol/chloroform mixture.

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Results
The administration of radioactive 25-OH-D3, 1,25-(OH)2D3, 1α-OH-D3, and 24-OH-D3 to vitamin D-deficient rats fed a high calcium, low phosphorus diet results in the production of significant amounts of 1,24,25-(OH)3D3 as shown in Fig. 1. Similar results were obtained with a low calcium, normal phosphorus diet. It is, therefore, reasonable to assume that the normal sequence of conversion of vitamin D is 25-OH-D3, to 1,25(OH)2D3, which induces the appearance of the 24-hydroxylase (23), resulting ultimately in the appearance of 1,24,25-(OH)3D3. There is, therefore, little doubt that 1,24,25-(OH)3D3 represents a physiological metabolite of vitamin D3. It is of some interest that 1α-OH-D3 is also converted to the 1,24,25-(OH)3D3 as is the analog 24(R)-OH-D3. It is unknown whether the 24-OH-D3 is first 1-hydroxylated and subsequently 25-hydroxylated or whether it is first 25-hydroxylated and then 1-hydroxylated. It will, however, be shown subsequently that 24-OH-D3 is first probably hydroxylated at C-25 and then at C-1 to form 1,24,25-(OH)3D3.

By means of co-chromatography of the in vivo generated 1,24,25-(OH)3D3 from either 25-OH-D3 or 1,25(OH)2D3 with a mixture of crystalline 1,24(R),25-(OH)3D3 and 1,24(S)-25-(OH)3D3 by means of high pressure liquid chromatography on a microparticulate silica column, it was possible to demonstrate unambiguously that the natural product has the 24-hydroxyl in the R configuration as shown in Fig. 2. The results not only demonstrate that the natural configuration is R but also provide strong evidence that a compound considered to be 1,24,25-(OH)3D3 is in fact that compound.

Because the 24-hydroxylase has only been demonstrated in kidney, it seems possible that nephrectomy could prevent the
conversion of 1,25-(OH)₂D₃ to the 1,24,25-(OH)₃D₃. If this were the case, it could provide an important tool for elucidating the importance of 24-hydroxylation to the function of 1,25-(OH)₂D₃. However, as shown in Table I, nephrectomy surprisingly did not eliminate the production of 1,24,25-(OH)₃D₃ and in fact did not significantly diminish this conversion. It is, therefore, obvious that the major 24-hydroxylation occurs in extrarenal tissue, at least when 1,25-(OH)₂D₃ serves as the substrate.

An examination of the conversion of 1,25-(OH)₂D₃ to the trihydroxy metabolite by in vitro chicken preparations reveals that the 24-hydroxylase, which has been studied with respect to conversion of 25-OH-D₃ to the 24,25-(OH)₂D₃, can convert 1,25-(OH)₂D₃ to the 1,24,25-(OH)₃D₃ (Fig. 3A). It has already been demonstrated previously and again in the present study that incubation of rachitic chick kidney mitochondria with radioactive vitamin D₃ compounds. Mitochondria from chicks fed the high calcium diet for 10 days and given a 325-pmol dose of nonradioactive 1,25-(OH)₂D₃ 24 h prior to being killed were used for A and B. For C and D chicks were fed the vitamin D-deficient diet for 1 month. The kidney mitochondria prepared from the former chicks were incubated with either 1,25-(OH)₂[26,27-3H]D₃ (A) or 1α-OH-[6-3H]D₃ (B), while the kidney mitochondria prepared from the latter chicks were incubated with either 24(R),25-(OH)₂[26,27-3H]D₃ (C) or 24(R)-OH-124-3H]D₃ (D) as described in the text. The incubation medium was extracted and chromatographed on Sephadex LH-20 columns as described in the text. The elution position where the synthetic analog 1,24-(OH)₂D₃ would be expected is shown by an arrow in B and D. 2,25-(OH)₂D₃ 1,24,25-(OH)₃D₃ 1α, 1α-OH-D₃ 24, 24(R)-OH-D₃ 24, 24, 24,25-(OH)₃D₃.

Of some interest is the fact that 1α-OH-D₃ cannot be converted to 1α,24-(OH)₂D₃ by the 24-hydroxylase. Similarly, 24-OH-D₃ cannot be converted to the 1α,24-(OH)₂D₃ by the 1-hydroxylase, illustrating the specificity of these hydroxylases for a hydroxyl on C-25. These results agree with in vivo results in which little or no 1,24-(OH)₂D₃ could be found either from 1α-OH-D₃ or from 24-OH-D₃.

**DISCUSSION**

It can now be concluded that 1,24,25-(OH)₃D₃ can be produced in vivo and in vitro from 1,25-(OH)₂D₃. Furthermore, one can be confident that the produced 1,24,25-(OH)₃D₃ has its 24-hydroxyl in the R configuration. Of even greater importance is the demonstration that 1,24,25-(OH)₃D₃ can arise from normal and expected physiologic processes illustrating that 1,24,25-(OH)₃D₃ is a naturally occurring metabolite of the vitamin. It is clear from previous work (21, 23) that the administration of 1,25-(OH)₂D₃ to a vitamin D-deficient animal results in the appearance of 25-OH-D₃-24-hydroxylation. It is, therefore, logical to assume, and it can now be demonstrated, that the administration of radioactive 25-OH-D₃ is first converted to the 1,25-(OH)₂D₃ and subsequently to 1,24,25-(OH)₃D₃. However, exactly what the role of the trihydroxyvitamin D₃ compound is in vivo remains unknown and is the subject of current investigation.

Although it is surprising that nephrectomy does not prevent the conversion of 1,25-(OH)₂D₃ to the 1,24,25-(OH)₃D₃, it is not totally unexpected because of the previous results of Garabedian et al. (24) demonstrating 24-hydroxylation of 25-OH-D₃ in nephrectomized animals. It was, however, quite surprising that nephrectomy did not significantly diminish the appear-

![Fig. 2. Co-chromatography of Synthetically prepared 1,24(R),25-(OH)₂D₃ and 1,24(S),25-(OH)₂D₃ and in vivo generated 1,24,25-(OH)₃D₃ from the Sephadex LH-20 columns (Fig. 1, A and B) was mixed with chemically synthesized stereochemical isomers of 1,24,25-(OH)₃D₃ and subjected to high pressure liquid chromatography using a Du Pont 830 instrument equipped with a column (25 x 0.21 cm) of Zorbax SIL at a pressure of 92 kg/cm². The solvent used was 3.5% methanol in CH₂Cl₂, and the flow rate was 0.44 ml/min. The 254 nm ultraviolet monitor was used to detect chemically synthesized isomers. The radioactivity of each 30-S fraction was determined by a Packard liquid scintillation counter model 3320 or 3375.

--- optical density at 254 nm; -- radioactivity of each fraction.

![Fig. 3. Metabolites produced by the in vitro incubation of chick kidney mitochondria with radioactive vitamin D₃ compounds. Mitochondria from chicks fed the high calcium diet for 10 days and given a 325-pmol dose of nonradioactive 1,25-(OH)₂D₃ 24 h prior to being killed were used for A and B. For C and D chicks were fed the vitamin D-deficient diet for 1 month. The kidney mitochondria prepared from the former chicks were incubated with either 1,25-(OH)₂[26,27-3H]D₃ (A) or 1α-OH-[6-3H]D₃ (B), while the kidney mitochondria prepared from the latter chicks were incubated with either 24(R),25-(OH)₂[26,27-3H]D₃ (C) or 24(R)-OH-124-3H]D₃ (D) as described in the text. The incubation medium was extracted and chromatographed on Sephadex LH-20 columns as described in the text. The elution position where the synthetic analog 1,24-(OH)₂D₃ would be expected is shown by an arrow in B and D. 1α, 1α-OH-D₃ 24, 24(R)-OH-D₃ 24, 24, 24,25-(OH)₃D₃ 1α, 1α-OH-D₃ 24, 24(R)-OH-D₃ 24, 24, 24,25-(OH)₃D₃.

**TABLE I**

<table>
<thead>
<tr>
<th>Operation</th>
<th>1,24,25-(OH)₂[26,27-3H]D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.33 ± 0.15</td>
</tr>
<tr>
<td>Nephrectomy</td>
<td>0.31 ± 0.08</td>
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It is unknown why Friedlander and Norman (11) were unable to detect 1,24,25-(OH)3D3 in the chromatographic profiles obtained from chicks given radioactive 25 OH-D3 and radioactive vitamin D3. It is possible that the low specific activity and the number of animals used in each of the experiments precluded detection of small amounts of 1,24,25-(OH)3D3 which could be expected. It is also possible that chronic administration of radioactive precursors would result in larger amounts of 1,24,25-(OH)3D3 appearing in the chromatographic profiles; in fact, work by Ribovich in this laboratory with the use of such an approach has also demonstrated the existence of 1,24,25-(OH)3D3 in significant amounts in rats on a variety of diets. These results coupled with those of Gray et al. (4) in man demonstrate that 1,24,25-(OH)3D3 is a physiologically significant metabolite, at least in mammals, and its possible role in metabolism and function of vitamin D must be considered.

Acknowledgment—We are indebted to Mrs. Helen Frank for her valuable technical assistance.

REFERENCES


TABLE II

<table>
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<th>Source of suspected 1,24,25-(OH)3D3</th>
<th>% H lost after periodate treatment</th>
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</thead>
<tbody>
<tr>
<td>A. Serum of rat given 1,25-(OH)2D3</td>
<td>88 ± 0</td>
</tr>
<tr>
<td>B. Serum of rat given 24,25-(OH)3D3</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>C. Serum of nephrectomized rat given 1,25-(OH)2D3</td>
<td>70 ± 4</td>
</tr>
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