A method based on affinity and hydrophobic chromatography has been developed for the partial purification of renal mitochondrial cytochrome P-450 (P-450). 2,4-Dichloro-6-phenylphenoxyethylamine coupled to Sepharose 4B provides the chromatographic medium which in the presence of two detergents, emulgen 911 (0.06%) and cholate (0.16%), retained P-450 but not most other mitochondrial proteins. P-450 was eluted from the column by increasing the detergent concentrations to 0.2% emulgen 911 and 0.5% cholate. Because of removal of 80% of the mitochondrial proteins and most of the chromophores, the method allows for the spectral quantitation of the mitochondrial P-450 and provides starting material for further purification. The specific content of the P-450 eluted from the 2,4-dichloro-6-phenylphenoxyethylamine columns varied between 0.5 and 2 nmol/mg of protein. The concentration of P-450 in the renal mitochondria of control rats was 0.26 ± 0.2 nmol/g of kidney or 0.016 nmol/mg of mitochondrial protein; renal mitochondria from vitamin D-deficient rats contained similar amounts of P-450.

The solubilized partially purified P-450 fraction catalyzed both the 1α- and 24-hydroxylations of 25-hydroxyvitamin D3. In vitamin D-replete animals, the turnover numbers of the 1α- and 24-hydroxylations reactions were 0.38 and 0.18 pmol/pmol of P-450/30 min. In vitamin D deficiency, there was an increase in the turnover number of both the 1α- and 24-hydroxylations to 4.00 and 1.75, respectively. The apparent K_m values of the 1α- and 24-hydroxylases were 50 and 25 nm, respectively, and were not different for control or vitamin-D-deficient animals. SKF 525A (10^{-4} M) inhibited these reactions by 70%. Metyrapone (10^{-3} M) enhanced the 24-hydroxylase from vitamin D-replete animals by 100% but had no effect on the other three activities. Calcium (10^{-4} M) caused a 2-fold stimulation of both hydroxylations while phosphate ions (2.5 x 10^{-2} M) inhibited both reactions.

Renal mitochondria and placental homogenates catalyze the conversion of 25(OH)D_3 to two metabolites, 1α,25(OH)_2D_3 and 24,25(OH)_2D_3 (1-3). 1α,25(OH)_2D_3 is involved in calcium absorption from the gut (4, 5) and mobilization of calcium from bone (6). The role of 24,25(OH)_2D_3 is involved in renal mitochondrial cytochrome P-450, is less clear, but there is evidence that it is involved in bone ossification (7).

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The two hydroxylation reactions are closely regulated by serum levels of calcium, phosphate, 25-hydroxyvitamin D, and parathyroid hormone. In vitamin D deficiency, in hypocalcemia, and in hypophosphatemia, there is an increase in the rate of 1α,25(OH)_2D_3 formation by the kidney, while in vitamin D-replete animals with a normal phosphate and calcium dietary intake, 24,25(OH)_2D_3 is the predominant metabolite (8-10). Both the 1α- and 24-hydroxylases are cytochrome P-450 mixed function oxidases (11, 12) but the biochemical mechanisms involved in the regulation of these enzymes are unknown. The quantitative and qualitative changes which occur in renal mitochondrial P-450s under conditions known to affect the 1α- and 24-hydroxylases have been difficult to assess for several reasons. First, the concentration of the enzyme in the membrane is low (estimated to be between 0.01-0.04 nmol/mg of mitochondrial protein (13, 14); second, the presence of other chromophores prevents the spectral quantitation of P-450; third, the enzyme recovery is low and the catalytic rates of the enzyme are slow after solubilization from the membrane (15, 16).

The first objective of this present work was to develop a method for solubilization and purification of renal mitochondrial P-450. In this report, such a method is described; it permits estimation of the P-450 content of individual rat kidneys and characterization of the enzymes by their catalytic activities.

The second objective was to compare renal mitochondrial P-450s from vitamin D-deficient and vitamin D-replete rats in order to determine whether the increase in the 1α-hydroxylase which occurs in vitamin D deficiency results from an increased enzyme concentration, an activation of existing enzymes, or the synthesis of a new form of P-450.

MATERIALS AND METHODS

The following generous gifts are acknowledged: 24,25(OH)_2D_3 from Dr. M. Gascon Barré (Hôpital St. Luc, Montreal); 2,4-dichloro-6-phenylphenoxyethylamine from Dr. A. Y. H. Lu (Merck Sharp and Dohme Research Laboratories); 25(OH)D_3 from the Upjohn Company (Kalamazoo); SKF 525A from Smith, Kline, and French (Montreal).

Emulgen 911 was purchased from Kao Atlas (Japan); CH-Sepharose 4B was from Pharmacia (Montreal); metyrapone was from Aldrich (Milwaukee, WI); glucose 6-phosphate, glucose 6-phosphate dehydrogenase, cholic acid, and cytochrome c were from Sigma (St. Louis); Bio-Beads SM-2 were from Bio-Rad (Richmond, CA); [3H]25(OH)D_3 (160 Ci/mmol) and [3H]1α,25(OH)_2D_3 (160 Ci/mmol) were from New England Nuclear.

Animals—125-g male Sprague-Dawley rats were maintained either on a normal diet of Purina Rat Chow or on a vitamin D-deficient diet containing 1% calcium and 1% phosphorus (ICN, Cleveland). After 8 weeks on the D-deficient diet, plasma levels of 25(OH)D_3, measured by high pressure liquid chromatography, were undetectable. Animals were killed by decapitation, and kidneys were removed, weighed, and minced with scissors in ice-cold 30 mM sodium phosphate buffer (pH 7.4) containing 1.15% KCl. The tissue was homogenized in centrifuge tubes with a Brinkmann Polytron PT10-35 fitted
with a PT20 probe, for 5 s at speed setting No. 5. Mitochondrial fractions were prepared by differential centrifugation. The nuclear fraction was removed by centrifugation at 10,000 × g for 10 min. The mitochondrial fraction was obtained by centrifuging the postnuclear fraction at 9000 × g for 10 min.

**Preparation of DPEA Columns**—Aliquots (5 g) of CH-Sepharose were swollen, washed, and coupled to DPEA as directed by the manufacturers. This involved incubating a 3-fold molar excess of DPEA with the swollen gel at room temperature for 2 h in 100 mM sodium bicarbonate buffer (pH 8.0) containing 1 M NaCl. After coupling, the gel was washed with three cycles of 100 mM Tris buffer, pH 8.0, 100 mM sodium acetate buffer, pH 4.5, both containing 1 M NaCl. The gel was then washed with 1 liter of deionized water and equilibrated in 30 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.06% emulgen 911, 0.16% sodium cholate (starting buffer). Equilibrated gel was packed in columns (0.5 × 10 cm) in starting buffer.

**DPEA Chromatography**—Mitochondrial fractions were resuspended with the use of a glass hand homogenizer in 30 mM sodium phosphate buffer, pH 7.4, containing 20% glycerol, 0.2% emulgen 911, 0.5% sodium cholate, 0.1 mM EDTA (solubilization buffer). The final suspension contained the equivalent of 1 g of tissue, wet weight/ml, and was allowed to stand at room temperature for at least 1 h prior to chromatography. Such suspensions can be left at room temperature for 4 h or at 4°C for 24 h with no loss of P-450. Before application to DPEA columns, the suspensions were diluted 3-fold with 30 mM sodium phosphate buffer containing 20% glycerol. This step brings the concentrations of the detergents to those in the starting buffer. These concentrations were found to be optimal for binding of P-450 to the columns. Samples were applied to DPEA columns at a flow rate of 0.5 ml/min. Following application of the sample, columns were washed with starting buffer containing 100 mM NaCl. The P-450 was eluted with solubilization buffer.

**Spectral Measurements**—The concentration of P-450 was determined from the CO-reduced difference spectrum after reduction with dithionite according to the method of Omura and Sato (17). An extinction coefficient of 91 mM⁻¹ cm⁻¹ was used. Protein was measured by the method of Lowry et al. (18) with bovine serum albumin as standard. Cytochrome c reductase was measured according to Phillips and Langdon (19) with 1 unit of activity defined as the reduction of 1 nmol of cytochrome c/min at 27°C.

**SDS Electrophoresis**—Electrophoresis was carried out in slab gels of 7.5% acrylamide with the buffer system of Laemmli (20). Gels were stained overnight with 0.25% Coomassie brilliant blue in acetic acid/methanol/water (1:1:10) and destained in acetic acid/methanol/water (1:1:10). Gels were calibrated with the following molecular weight markers: phosphorylase b, 98,000; bovine serum albumin, 68,000; ovalbumin, 45,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; and lysozyme, 14,400.

**Removal of Detergents**—Prior to measurement of catalytic activity, cholate was removed by dialysis for 2 days against 2 liters of 30 mM Tris-acetate buffer, pH 7.4, containing 20% glycerol. Emulgen 911 was removed with Bio-Beads as described previously (21). P-450 fractions were stirred with Bio-Beads at 4°C until emulgen 911 concentrations, as assayed by the method of Garewal (22), was less than 0.002%. This usually required at least 2 h of exposure to Bio-Beads.

**Measurement of Catalytic Activity**—Because of the increased instability of P-450 after removal of emulgen 911, catalytic activity was assessed within 4 h of removal of the detergent. The assay mixture consisted of Tris-acetate, 30 mM, pH 7.4, glucose-6-phosphate, 1 mM, glucose-6-phosphate dehydrogenase, 1 unit, NADPH, 100 µM, P-450 fraction, 0.1–1 ml, 30 µl of [1H]25(OH)D₃, (248,000 cpm), and unlabeled 25(OH)D₃ between 10 and 500 nM as indicated in individual experiments. The incubation volume was 5 ml. The substrate dissolved in ethanol was added to a 20-ml Erlenmeyer flask. The ethanol was evaporated under a stream of N₂. All other components of the system except NADPH were then added to the flask. The reaction was started with addition of NADPH. Incubations were done at 37°C. The reactions were stopped by decanting the contents of the Erlenmeyer flasks into 50-ml tubes containing 10 ml of chloroform/methanol (2:1). The tubes were vortexed and centrifuged, and the lower phase was removed. The extraction procedure was repeated. Both organic phases were pooled and evaporated under a stream of N₂. This procedure resulted in extraction of 95% of the radioactivity from the incubation medium. The residue was resuspended in 20 µl of isopropyl alcohol/hexane (1:9) and an aliquot of this was assayed for vitamin D metabolites on a Beckman Model 110 A liquid chromatograph.

**Assay of Metabolites**—Vitamin D metabolites were separated on a Zorbax-Sil column (4.6 mm × 25 cm) (DuPont) equilibrated with isopropyl alcohol/hexane (1:9) according to Jones and Deluca (23). The flow rate was maintained at 1 ml/min and 1.5-ml fractions were collected. Radioactivity in each fraction was measured in 10 ml of Aquasol by liquid scintillation counting.
RESULTS

DPEA Chromatography of Renal Mitochondrial P-450—Fig. 1 illustrates a typical elution profile from a DPEA column in which a bed volume of 10 ml was used to isolate mitochondrial P-450 from the kidneys of five control rats. Smaller columns of 1-ml bed volume have been used to isolate P-450 from individual rat kidneys. The yield of mitochondrial protein was 16.4 ± 1.2 mg/g of kidney. Upon DPEA chromatography of solubilized renal mitochondria, 85% of the protein was removed by washing the column with starting buffer containing 100 mM NaCl. Elution of P-450 was achieved with solubilization buffer. The specific content of P-450 eluted from DPEA columns varied between 0.5 and 2.0 nmol/mg of protein. The calculated mitochondrial P-450 content was 0.26 ± 0.02 nmol/g of kidney or 0.016 nmol/mg of mitochondrial protein. Rechromatography of the unbound protein fraction from the DPEA column wash did not result in further binding of P-450 to the column. A typical CO-reduced difference spectrum of the P-450 eluted from DPEA columns is illustrated in Fig. 2. The absorption maximum is at 451 nm and there was very little conversion to P-420. In vitamin D deficiency, there was no change in the renal mitochondrial content of P-450 and no difference in the CO-reduced difference spectral maximum (Table I). The P-450 was assessed in three separate groups of animals which were maintained on vitamin D-deficient diets on three separate occasions. The P-450 content was found to be the same as in control animals whether individual kidneys were examined or kidneys from groups of up to 20 animals were pooled and chromatographed together.

SDS Electrophoresis—A comparison of the SDS electrophoretic profiles of P-450 fractions from vitamin D-replete and D-deficient animals provided no evidence that the forms of P-450 present in these two groups of animals are different (Fig. 3). All known P-450s have Mr = 60,000–45,000 (24). In this molecular weight range of the gel, bands of 60,000 and 55,000 are present. Although none of these bands has been identified as P-450, the intensity of staining of the proteins reflects the concentration of P-450 protein applied to the gels.

Catalytic Activity—Upon removal of the detergents, the P-450 fraction catalyzed the hydroxylation of 25(OH)D₃ at both the 1α- and 24-positions. Fig. 4 is an example of a high pressure liquid chromatography elution profile of the chloroform/methanol extract after incubation. The system exhibited no requirement for added adrenodoxin or adrenodoxin reductase. Since the cytochrome c reductase activity of the P-450 fraction was 0.1 unit of cytochrome c reduced/min/pmol of P-450, it can be concluded that renal ferredoxin and renal ferredoxin reductase (14) are eluted from the DPEA column along with P-450. Both hydroxylations were inhibited by emulgen 911 concentrations above 0.002%. The hydroxylation rates were linear with time (Fig. 5) and P-450 concentration (Fig. 6) for 30 min. At a substrate concentration of 500 nm, P-450 fractions from vitamin D-replete animals catalyzed the 1α- and 24-hydroxylations of 25(OH)D₃ at rates of 0.38 and 0.18 pmol/pmol of P-450/30 min, respectively. With P-450 from vitamin D-deficient rats, the turnover numbers increased to 4.00 for the 1α-hydroxylase and 1.75 for the 24-hydroxylase (Table I).

Comparison of P-450s from Vitamin D-deficient and Vitamin D-replete Animals by Km and Sensitivity to Inhibitors—The increased catalytic rate in vitamin D deficiency was
Renal Mitochondrial 25(OH)D₃ Hydroxylase

Fig. 5. Time course of production of 1,25(OH)₂D₃ (■) and 24,25(OH)₂D₃ (○). Each incubation mixture contained 35 pmol of P-450 from vitamin D-replete animals and a substrate concentration of 500 nM. Each point represents ± one experimental value.

Fig. 6. Effect of P-450 concentration on the production of 1α,25(OH)₂D₃ (■) and 24,25(OH)₂D₃ (○). Aliquots of P-450 fractions from vitamin D-replete animals with P-450 contents varying between 7 and 35 pmol of P-450 were used in these incubations.

not due to an increased affinity for the substrate since the K_m values for the 24-hydroxylase (25 nM) and the 1α-hydroxylase (50 nM) were unaffected by the vitamin D status of animals (Fig. 7).

The standard modulators of P-450, SKF 525A and metyrapone, were examined for their effects on solubilized renal mitochondrial P-450 (Fig. 8). No differences were observed between P-450 fractions from vitamin D-deficient or vitamin D-replete animals in their response to SKF 525A (10⁻⁶ M), which inhibited both the 1α- and 24-hydroxylase by 70%. Metyrapone (10⁻³ M) caused a 2-fold stimulation of the 24-hydroxylase in the P-450 fraction from vitamin D-replete animals but had no effect on the 24-hydroxylase from vitamin D-deficient animals or on the 1α-hydroxylase from either animal group.

Effects of Calcium and Phosphate Ions on Catalytic Activity—The effects of calcium and phosphate ions were tested in order to determine whether these ions regulate the solubilized vitamin D hydroxylases in a fashion similar to that observed in renal mitochondria (26, 27). The results are shown in Table II. Both the 1α- and 24-hydroxylases were stimulated by calcium. There was a maximal 2-fold stimulation at a calcium concentration of 10⁻³ M. At concentrations above 10⁻² M, calcium had no effect. On the other hand, phosphate at concentrations above 2.5 × 10⁻³ M inhibited both hydroxylations. The 24-hydroxylase was completely inhibited and the 1α-hydroxylase inhibited by 86% at a phosphate concentration of 10⁻³ M. Similar results were obtained whether P-450 was derived from renal mitochondria of vitamin D-deficient or vitamin D-replete rats.

Fig. 7. Effect of substrate concentration on the metabolism of 25(OH)D₃ by solubilized renal mitochondrial P-450 from vitamin D-replete (A) and vitamin D-deficient (B) rats. Each point represents the mean and standard error of four experimental values. Insets show Lineweaver-Burk plots of the data. ■, 1α,25(OH)₂D₃; ○, 24,25(OH)₂D₃.
Fig. 8. Effect of SKF 525A (10⁻⁴ M) and metyrapone (10⁻³ M) on the metabolism of 25(OH)D₃ by solubilized P-450 from vitamin D-replete (A) and vitamin D-deficient rats (B). The P-450 concentrations in the incubation mixtures was 7 × 10⁻⁶ M from vitamin D-replete animals and 2 × 10⁻⁶ M from vitamin D-deficient animals. The substrate concentration was 50 nM. The results represent the means of duplicate experiments.

Table II

Effects of calcium and phosphate ions on catalytic activity

| Ion    | 24-Hydroxylase | 1a-Hydroxylase |
|--------|----------------|----------------|
|        | K₅₀ (nM) | % control activity | K₅₀ (nM) | % control activity |
| Ca²⁺   |           |                  |           |                  |
| 10⁻⁵   | 100       | 100              | 100       | 100              |
| 10⁻⁴   | 225       | 200              | 100       | 100              |
| 10⁻³   | 175       | 140              | 100       | 100              |
| 2 × 10⁻²| 100       | 100              | 100       | 100              |
| Phosphate |         |                  |           |                  |
| 10⁻²   | 100       | 100              | 100       | 100              |
| 2.5 × 10⁻²| 51       | 72               | 51        | 72               |
| 10⁻³   | 0         | 0                | 0         | 0                |

Discussion

Both the 1a- and 24-hydroxylations of 25(OH)D₃ are catalyzed by mitochondrial P-450s found in the kidney and placenta (1-3, 11, 12). Although it is well established that the 1a-hydroxylation predominates in vitamin D deficiency and the 24-predominates in control animals, very little is known about the biochemical mechanism of this regulation. Nor is it known whether two distinct forms of P-450 catalyze the two hydroxylations. As a first step toward a study of the regulation of these hydroxylases, a method has been developed for the partial purification and characterization of renal mitochondrial P-450s.

Cytochrome P-450 has been solubilized from kidney mitochondria and partially purified to a specific content of between 0.5 and 2.0 nmol/mg of protein. The mitochondrial content of P-450 from control rats is 0.016 nmol/mg of protein. Since the presence of cytochrome oxidase and other chromophores prevented the accurate measurement of P-450 in the intact mitochondria, the percentage of recovery has not been estimated. However, the value reported here of 0.016 nmol/mg of mitochondrial protein is close to the 10 nmol/g of mitochondrial protein reported by Pedersen et al. (14) and the 0.04 nmol/mg of mitochondrial protein reported by Ghanziyan et al. (13). A comparison of the P-450 fractions from control and vitamin D-deficient animals revealed no differences in the P-450 content of mitochondria, the CO-reduced difference spectral maxima, or protein profile on SDS electrophoresis. The solubilized P-450 catalyzed both the 1a- and 24-hydroxylations of 25(OH)D₃, at rates of 0.38 and 0.18 pmol/pmol of P-450/30 min with the enzyme from control animals, and 4.00 and 1.75 with enzyme from vitamin D-deficient animals. On the basis of the P-450 content of renal mitochondria reported herein, these values can be expressed as picomoles of product/mg of mitochondrial protein/min for comparison with values reported when rat kidney mitochondrial fractions were used (25). These calculated values are 0.18 and 0.09 for the 1a- and 24-hydroxylases in control animals and 2.1 and 0.93 in vitamin D-deficient animals. The corresponding values reported (25) with rat mitochondrial fractions are 2.9 for the 1a-hydroxylase in vitamin D-deficient rats and 0.29 for the 24-hydroxylase in control rats. Thus, the catalytic rates of the solubilized P-450 are close to those in intact mitochondria for these two reactions.

However, there was a major difference between the catalytic activity in the intact mitochondria and in the solubilized system, namely, the presence of both hydroxylases in approximately the same ratio in the solubilized P-450 from both vitamin D-deficient and vitamin D-replete animals. Previous reports have clearly demonstrated a reciprocal relationship between the two hydroxylases, with the 1a-predominating in vitamin D deficiency and the 24-in vitamin D-replete chickens. The significance of this difference between the solubilized system and intact mitochondria is not yet clear. Differences may be due to the removal of the enzyme from the mitochondrial membrane with loss of certain regulatory mechanisms, or may reflect a difference between the rat and chicken in the regulation of vitamin D hydroxylases.

Another unexpected but interesting observation of the present experiments was the demonstration that, during vitamin D deficiency, the quantity of P-450 in renal mitochondria is unaltered but the turnover number of the enzyme increases. The increase in the turnover number of P-450 may represent an activation of P-450, or the presence of new forms of the enzyme in vitamin D deficiency. Although in the present study no difference in the SDS electrophoretic profile of P-450 fractions from control and vitamin D-deficient animals could be detected, the presence of distinct forms of P-450 in vitamin D deficiency cannot yet be ruled out and must await further characterization of the enzymes.

Attempts were made to characterize the solubilized P-450s from vitamin D-deficient and vitamin D-replete animals by a study of their K₅₀ values and the effects of the known modulators of P-450: SKF 525A and metyrapone. Vitamin D deficiency did not result in a change in the affinity of the enzymes for the substrate 25(OH)D₃; the K₅₀ values for the 1a- and 24-hydroxylases were 50 and 25 nm, respectively. These values are an order of magnitude lower than those reported when kidney slices or mitochondrial fractions are used (25). At least two factors may contribute to the lower K₅₀ values reported herein: first, the removal of the mitochondrial membrane in which much of the substrate is sequestered when intact mitochondria are present; and, second, the possible removal of "inhibitory activity" in the form of a substrate binding protein which has been reported to be present in mammalian tissue (25).

No difference was seen between the P-450s from vitamin D-deficient or control animals in their response to SKF 525A which inhibited all the reactions by 70%. Metyrapone, on the other hand, had an effect only on the 24-hydroxylase from vitamin D-replete animals, where it caused a 2-fold stimulation.

Direct effects of calcium and phosphate ions on the 1a-hydroxylase have been postulated on the basis of the effects which these ions elicit in chick kidney mitochondria. Calcium has been reported both to inhibit (27) and stimulate (26) the...
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1α-hydroxylase while phosphate was reported to inhibit the enzyme (27). In the present study, addition of phosphate ions to the solubilized P-450 system caused an inhibition of both the 1α- and 24-hydroxylases while calcium ions stimulated both reactions. Although no physiological significance can yet be attached to these results, it is interesting that the relative proportions of 1α,25(OH)₂D₃ and 24,25(OH)₂D₃ are not influenced by the presence of these ions.

It is not yet known whether the 1α- and 24-hydroxylations of 25(OH)D₃ are catalyzed by two distinct forms of P-450. The difference in the $K_m$ values and response to metyrapone reported herein may be taken as evidence in support of this view. However, a definitive answer to this question awaits further purification and characterization of the enzymes.

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