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Assay and properties of 25-hydroxyvitamin D_3 23-hydroxylase

Evidence that 23,25-dihydroxyvitamin D_3 is a major metabolite in 1,25-dihydroxyvitamin D_3-treated or fasted guinea pigs

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Incubation of 25-hydroxyvitamin D_3 with kidney cortex mitochondria from 1,25-dihydroxyvitamin D_3-treated guinea pigs resulted in the formation of 23,25-dihydroxyvitamin D_3 as the major product. The identity of the product was verified by g.c.–m.s. and quantification was performed by h.p.l.c. The rates of the reaction were in the range 1.0–1.8 pmol/min per mg of mitochondrial protein (at 37 °C), which were 5–10 times the rates of formation of 24,25-dihydroxyvitamin D_3. In mitochondrial preparations from untreated guinea pigs, the rate of 23-hydroxylation was below detection limit (0.02 pmol/min per mg of mitochondrial protein). Fasting the animals for 24 h induced the 23-hydroxylase almost as efficiently as treatment with 1,25-dihydroxyvitamin D_3, with a concomitant depression of the 1α-hydroxylase. The 23-hydroxylase reaction required oxidizable substrate, was decreased by low O_2 partial pressures and inhibited by CO or the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. It was stimulated by the respiratory-chain inhibitors rotenone, antimycin A and KCN. These results indicate that the guinea-pig renal mitochondrial 23-hydroxylase is a cytochrome *P*-450 and that the reducing equivalents are primarily supplied by NADPH via the energy-dependent transhydrogenase.

INTRODUCTION

The metabolism of vitamin D_3 involves an initial sequence of hydroxylation reactions attacking both the ring structure and the side chain of the molecule (for a review, see [1]). After 25-hydroxylation in the liver [2,3], the product, 25-hydroxyvitamin D_3 [25(OH)D_3; 25-hydroxycholecalciferol] is further converted into several dihydroxylated metabolites in the kidney [4–7]. Of these, 1α,25-dihydroxyvitamin D_3 [1,25(OH)$_2D_3$; 1α,25-dihydroxycholecalciferol], the most active hormonal form of the vitamin [8], and 24,25-dihydroxyvitamin D_3 [24,25(OH)$_2D_3$; 24,25-dihydroxycholecalciferol], with unknown function, have attracted most attention.

The 1α-hydroxylase and the 24-hydroxylase appear to be under strict hormonal control and are regulated in a reciprocal manner by the calcium and phosphate status of the organism [10]. Studies on the mechanisms involved in this regulation have been hampered to a large extent by lack of suitable '*in vitro*' assay systems with reasonably high rates of hydroxylation activities.

It was recently suggested that 23-hydroxylation may be of importance for metabolism of 25(OH)D_3. Thus 23-hydroxylation of 25(OH)D_3 was demonstrated both *in vivo* and *in vitro* (using kidney homogenate) in vitamin D-supplemented chicken [11]. 23-Hydroxylation was also found to be important for metabolism of 25-hydroxy-24-oxovitamin D_3 in kidney homogenates from 1,25(OH)$_2D_3$-treated rats [12] and from vitamin D-supplemented chickens and rats [13]. In a fairly recent report, 25(OH)$_2D_3$ 23-hydroxylase activity was demonstrated in kidney homogenates from several species after treatment with 1,25(OH)$_2D_3$ [14]. The physiological and metabolic significance of 23,25-dihydroxyvitamin D_3 [23,25(OH)$_2D_3$; 23,25-dihydroxycholecalciferol] is, however, largely unknown. Also, the subcellular location and the general properties of the 25(OH)D_3 23-hydroxylase have not been clarified.

In the present work we report a very high activity of the 25(OH)D_3 23-hydroxylase in guinea pigs treated with 1,25(OH)$_2D_3$ or fasted, and show that 23,25(OH)$_2D_3$ is the major metabolite produced from 25(OH)D_3 by kidney mitochondria *in vitro*. In addition, some of the general properties of the 25(OH)D_3 23-hydroxylase have been defined.

EXPERIMENTAL PROCEDURES

Materials

[^{3}H]24,25(OH)$_2D_3$ was from Amersham International, Amersham, Bucks., U.K., and 25(OH)D_3 from Philips Duphar, Veenendal, The Netherlands. 1,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$ were gifts from Hoffmann–La Roche and Co., Basel, Switzerland. Other chemicals, biochemicals and solvents were standard high-purity materials.

Enzyme preparations

Male guinea pigs of the Dunkin–Hartley strain, weighing 250–740 g, were used. They were given a commercial pellet diet (Ewos AB, Södertälje, Sweden) containing 0.9% Ca and 0.5% phosphorus and 1000 i.u.

Abbreviations used: 25(OH)D_3, 25-hydroxyvitamin D_3; 1,25(OH)$_2D_3$, 1α,25-dihydroxyvitamin D_3; 23,25(OH)$_2D_3$, 23,25-dihydroxyvitamin D_3; 24,25(OH)$_2D_3$, 24,25-dihydroxyvitamin D_3; 25(OH)D_3-26,23-lactone, 25-hydroxyvitamin D_3-26,23-lactone; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

of vitamin D_3/kg. They received 1 μg of 1,25(OH)$_2D_3$/kg body wt. by intraperitoneal injection on three consecutive days before they were killed. This treatment raised serum calcium from 1.85±0.42 (n = 5) to 2.67±0.31 mmol/l (n = 13). In some experiments, female animals, untreated or treated with 1,25(OH)$_2D_3$, were fasted for 24 h before being killed. [The effect of fasting was investigated in view of a previous observation that fasting may stimulate 25(OH)$_2D_3$ 24-hydroxylase in rats (M. Warner, unpublished work)]. The animals were killed by decapitation. After removal of the medulla, mitochondria from the kidney cortex were prepared as described in [15]. The mitochondrial pellet was resuspended in an incubation medium consisting of 50 mM-Tris/acetate buffer, pH 7.4, 10 mM-$MgCl_2$, 12 mM-isocitrate and 2.5 μg of diphenyl-p-phenylenediamine to a protein concentration of about 20 mg/ml.

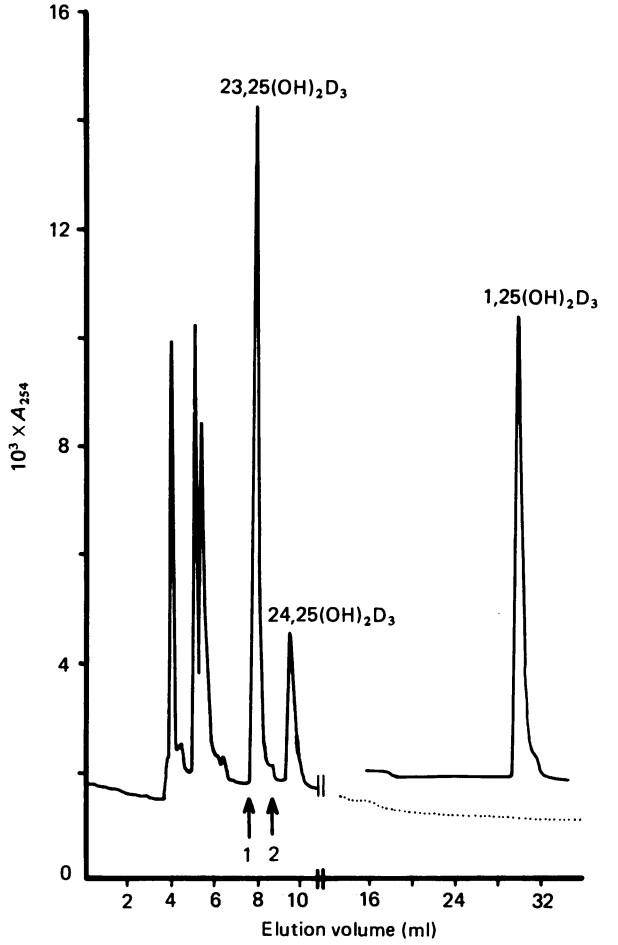

Fig. 1. Straight-phase h.p.l.c. profile of products [23,25(OH)$_2D_3$ and 24,25(OH)$_2D_3$] obtained after incubating 25(OH)D_3 with kidney mitochondria from a guinea pig treated with 1,25(OH)$_2D_3$

Incubation was performed as described in the Experimental procedures section, with 3.8 mg of mitochondrial protein and 20 μg of 25(OH)D_3/ml. After solid-phase extraction on Sep-Pak C_{18}, the extract was purified on reversed-phase h.p.l.c. The fraction containing the dihydroxylated metabolites was rechromatographed on a Zorbax-Sil (3 μm particle size) column (0.46 cm × 25 cm) as shown. The eluent was 8% propan-2-ol in hexane, and the flow rate was 1 ml/min. After 11 min the flow was increased to 2 ml/min. 1,25(OH)$_2D_3$ (250 ng) had been added as internal standard. The dotted line represents the corresponding h.p.l.c. profile from an incubation where no 1,25(OH)$_2D_3$ had been added as internal standard.

Assay of enzyme activities

After dilution to the desired protein concentration the incubation was started by addition of 20 μg of 25(OH)D_3 in 10 μl of acetone to 1 ml of the incubation medium. Unless otherwise specified, the reaction mixtures were incubated under air on a shaking water bath at 37 °C for 30 min. The reaction was terminated by the addition of 1 ml of acetonitrile. At this stage 250 ng of 1.25(OH)$_2D_3$ was added as internal standard.

Precipitated protein was separated by centrifugation (600 g, 10 min) and the supernatant applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, U.S.A.) that had been washed with 3 ml of methanol followed by 3 ml of water. After washing with 3 ml of 30% (v/v) water in methanol the material was eluted with 3 ml of acetonitrile. The solvent was removed under N_2 (temperature < 30 °C and light-shielded) and the sample redissolved in 100 μl of methanol. The concentration extract was subjected to reverse-phase h.p.l.c. (Nucleosil C_{18}; 5 μm particle size; column dimensions 4.6 mm × 250 mm) using 10% water in methanol at 1 ml/min as eluting solvent. The u.v.-absorbance peak at 254 nm, corresponding to 24,25(OH)$_2D_3$, appeared at 7 min 8 s, that of 23,25(OH)$_2D_3$ at 7 min 30 s, and that of 1,25(OH)$_2D_3$ at 7 min 40 s. The entire fraction corresponding to these three dihydroxylated metabolites was collected and the solvent evaporated under N_2. The material was further subjected to straight-phase h.p.l.c. (Zorbax-Sil; 3 μm particle size; column dimensions 4.6 mm × 250 mm) using 8% (v/v) propan-2-ol in hexane as eluent at a flow rate of 1 ml/min. The heights of the peaks corresponding to 24,25(OH)$_2D_3$ or 23,25(OH)$_2D_3$ formed were compared with standard curves constructed by plotting the ratio between the 23,25(OH)$_2D_3$-peak or the 24,25(OH)$_2D_3$-peak and the peak corresponding to the internal standard, 1,25(OH)$_2D_3$.

In some cases recovery of added [^{3}H]24,25(OH)$_2D_3$ was used for the calculation of the amount of 24,25(OH)$_2D_3$ formed, and it was shown that the two methods gave almost identical results. The use of 1,25(OH)$_2D_3$ as internal standard was justified by the finding that treatment of the animals with 1,25(OH)$_2D_3$ suppressed the formation *in vitro* of 1,25(OH)$_2D_3$ to below the detection limit (cf. the Results section).

25-Hydroxyvitamin D_3 1α-hydroxylase activity was measured by a modification [16] of a method based on isotope dilution–m.s. [17].

G.c.–m.s.

In order to identify the product, 23,25(OH)$_2D_3$ was isolated by the straight-phase h.p.l.c. Material from several incubations was pooled. After formation of the trimethylsilyl ether [18], a portion was used to record a full mass spectrum. Other portions were analysed by means of a multiple ion detector in order to record selected characteristic ions (see the Results section). In both cases an LKB 2091 instrument was used. The g.c. step was performed on a 1.5% SE-30 column (on Chromosorb W) operating at 280 °C. The carrier gas was helium, and the flow rate was about 30 ml/min.

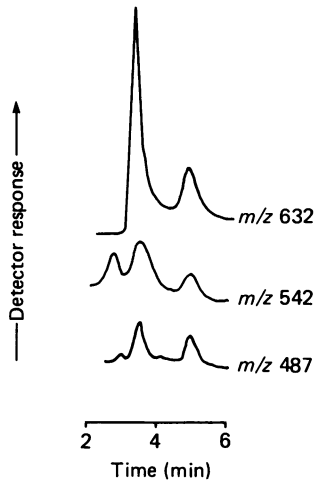

Fig. 2. Multiple-ion-detector recording of trimethylsilyl derivative of enzymically produced $23,25(OH)_2D_3$

The incubation extract was purified by two consecutive h.p.l.c. steps before analysis by g.c.–m.s. as described in Experimental procedures section. The ions shown had the same retention time as the peak at *m/z* 131.

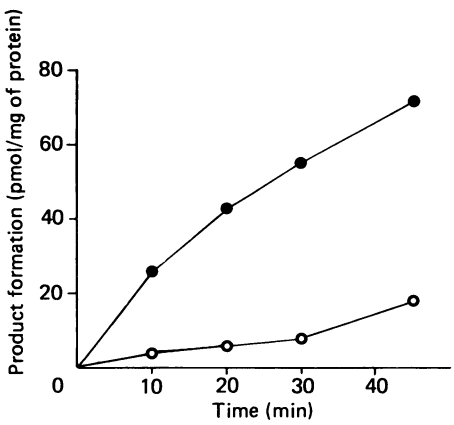

Fig. 3. Effect of incubation time on the production of $23,25(OH)_2D_3$ *in vitro* by kidney mitochondria of $1,25(OH)_2D_3$-treated guinea pigs (●)

The $25(OH)D_3$ 23-hydroxylase activity was assayed under standard incubation conditions as described in the Experimental procedures section. Each incubation contained 4.4 mg of mitochondrial protein/ml. For comparison, formation of $24,25(OH)_2D_3$ in the same preparation is also presented (○).

RESULTS

Incubation of $25(OH)D_3$ with kidney-cortex mitochondria from $1,25(OH)_2D_3$-treated guinea pigs resulted in formation of two products clearly separated by straight-phase h.p.l.c. (Fig. 1). The peak with an elution volume of 9.5 ml corresponds to $24,25(OH)_2D_3$ and was identified previously [15]. The fraction corresponding to the peak with an elution volume of 7.9 ml was collected and its u.v. spectrum recorded. This was a typical vitamin D spectrum, identical with that of the substrate, with a peak at 265 nm and a minimum aroung 230 nm. A mass spectrum of the trimethylsilyl ether derivative was dominated by a peak at *m/z* 131 characteristic for a trimethylsilyl oxo function in a C_{27}-steroid side chain [19]. Because of the great dominance of the *m/z* 131 peak, the other peaks were too small to allow a safe final identification (cf. ref. [11]). By multiple-ion-detector recordings, however, expected peaks with a retention time identical with that of *m/z* 131 were obtained at *m/z* 487, *m/z* 542 and *m/z* 632 (Fig. 2). These correspond to the characteristic peaks in a published mass spectrum of the trimethylsilyl ether derivative of $23,25(OH)_2D_3$ [11].

The rate of conversion of $25(OH)D_3$ into $23,25(OH)_2D_3$ was nearly linear with time of incubation up to 30 min (Fig. 3) and with the amount of mitochondrial protein up to at least 6 mg of protein (results not shown). Formation of $23,25(OH)_2D_3$ by kidney-cortex mitochondria required the presence of an oxidizable substrate such as isocitrate, malate or succinate, which were all about equally effective (Table 1). Inhibitors of the respiratory chain, such as rotenone, antimycin A or KCN, stimulated the activity, whereas the uncoupler FCCP was strongly inhibitory (Table 1). The reaction was decreased by low O_2 partial pressures and inhibited by CO (Table 2).

The rate of formation of $23,25(OH)_2D_3$ was about 5–10 times higher than the rate of formation of $24,25(OH)_2D_3$. In some experiments the rate of formation of $24,25(OH)_2D_3$ was even too low to be measured with sufficient precision. The relative rate of formation of $23,25(OH)_2D_3$ and $24,25(OH)_2D_3$ was also measured with kidney cortex mitochondria from $1,25(OH)_2D_3$-treated rat and untreated human. The ratio was highest in the guinea pig, lowest in the rat, with that for man in between (Table 3).

Table 1. Effect of oxidizable substrates, mitochondrial inhibitors and the uncoupler FCCP on the production of $23,25(OH)_2D_3$ *in vitro* by kidney mitochondria from $1,25(OH)_2D_3$-treated guinea pig

The incubations were performed in duplicate as described in the Experimental procedures section, except for the alterations shown. The concentration of mitochondrial protein used was 6 mg/ml.

Conditions	Production of $23,25(OH)_2D_3$ (pmol/30 min per mg)
No oxidable substrate	7
Succinate (12 mM)	44
Malate (12 mM)	42
Isocitrate (12 mM)	43
+ Rotenone (4 mM)	71
+ Antimycin A (4 μg)	79
+ KCN (4 mM)	81
+ FCCP (20 μM)	12

Substrate saturation was obtained at a $25(OH)D_3$ concentration of about 10 μg/ml (25×10^{-6} M) and the apparent K_m was of the order of 5×10^{-6} M (Fig. 4). The maximum rate of formation of $23,25(OH)_2D_3$ was in the range 1.0–1.8 pmol/min per mg.

In the h.p.l.c. profile of incubation extracts with

Table 2. Effect of CO on the production of $23,25(OH)_2D_3$ *in vitro* by kidney mitochondria from $1,25(OH)_2D_3$-treated guinea pigs

The incubations were performed as described in the Experimental procedures section, except that the temperature was 30 °C and the concentration of protein was 4 mg/ml. All the components of the incubation mixture were pipetted into the incubation flask and sealed with a screw-capped rubber stopper. The flasks were attached to a vacuum line by needles inserted through the stopper, evacuated, and flushed with the appropriate gas mixture alternatively. Thereafter, the flasks were removed from the vacuum line and incubated. The results are means ± S.D. for triplicate determinations.

Gas mixture	Production of $23,25(OH)_2D_3$ (pmol/30 min per mg)
Air	48 ± 2
O_2/N_2 (1:19)	21 ± 2
$O_2/CO/N_2$ (1:8:11)	5 ± 3

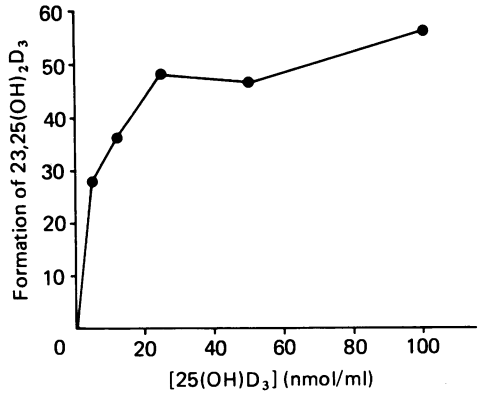

Fig. 4. Effect of substrate concentration on the rate of 23-hydroxylation by kidney mitochondria of $1,25(OH)_2D_3$-treated guinea pigs

The incubations were carried out as described in the Experimental procedures section, except that the temperature was 30 °C. The amount of $25(OH)D_3$ was varied as shown, and the mitochondrial protein concentration was 4 mg/ml. Incubations were carried out in triplicate and the mean results are presented.

mitochondria from $1,25(OH)_2D_3$-treated guinea pigs (Fig. 1), no measurable peak corresponding to $1,25(OH)_2D_3$ formed was observed. Without treatment of the animals with $1,25(OH)_2D_3$, however, a peak corresponding to $1,25(OH)_2D_3$ was clearly detectable (Fig. 5), whereas the peaks corresponding to $24,25(OH)_2D_3$ and $23,25(OH)_2D_3$ were barely discernible. As shown in Fig. 6, a decrease in the 1α-hydroxylase activity occurred rapidly after administration of $1,25(OH)_2D_3$. As soon as 18 h after the first injection this activity was barely detectable. Stimulation of the $25(OH)D_3$ 23-hydroxylase activity was considerably slower than was the decrease in the 1α-hydroxylase activity. The 23-hydroxylase activity increased about 10-fold as a result of 3 days of treatment with $1,25(OH)_2D_3$ (Fig. 6). Treatment for more than 3 days did not further stimulate the activity (results not shown).

Fasting female guinea pigs for 24 h had a marked stimulatory effect on the 23-hydroxylase activity (Table 4) and to some extent also on the 24-hydroxylase activity. Concomitantly, the 1α-hydroxylase activity was decreased. The effects of fasting and $1,25(OH)_2D_3$ treatment on the $25(OH)D_3$ hydroxylases were to some extent additive (Table 4). Serum calcium concentrations were 2.66 ± 0.09 mmol/l and 2.46 ± 0.25 mmol/l in fasted and control animals respectively.

DISCUSSION

The present study demonstrates a considerable increase in activity of a kidney mitochondrial $25(OH)D_3$ 23-hydroxylase in guinea pigs given $1,25(OH)_2D_3$ or fasted. Whether pre-existing 23-hydroxylase has been activated or whether there has been synthesis of new enzyme under these conditions has not been established, but the time-dependence (Fig. 6) indicates induction of enzyme. Under stimulating conditions the activity of the 23-hydroxylase exceeds by severalfold that of the $25(OH)D_3$ 24-hydroxylase. It is apparent that in this particular species, $23,25(OH)_2D_3$, rather than $24,25(OH)_2D_3$, is the major metabolite derived from $25(OH)D_3$ under conditions where the 1α-hydroxylase is suppressed. This is different from the situation in rats and chickens, where $24,25(OH)_2D_3$ is the major metabolite under similar conditions [20,21]. It should be noted also that, in these and other species, significant quantities of $23,25(OH)_2D_3$ are formed [11,14,22] (see also Table 3). In human kidney, $23,25(OH)_2D_3$ appears to be formed *in vitro* in quantities comparable with those of $24,25(OH)_2D_3$ (Table 1).

The physiological significance of $23,25(OH)_2D_3$ remains uncertain. According to preliminary studies this metabolite seems to have little biological activity in

Table 3. Rates of formation of $23,25(OH)_2D_3$ in kidney mitochondria from $1,25(OH)_2D_3$-treated guinea pig and rat and from untreated human

Source of kidney mitochondria	Formation (pmol/30 min per mg) of: $23,25(OH)_2D_3$ (*a*)	$24,25(OH)_2D_3$ (*b*)	(*a*)/(*b*)
Guinea pig (treated)	28	4.4	6.4
Rat (treated)	2.3	64	0.04
Man* (untreated)	2.2	7.5	0.3

* Non-tumour material obtained from cortex of a kidney removed from a 62-year-old male with a renal tumour.

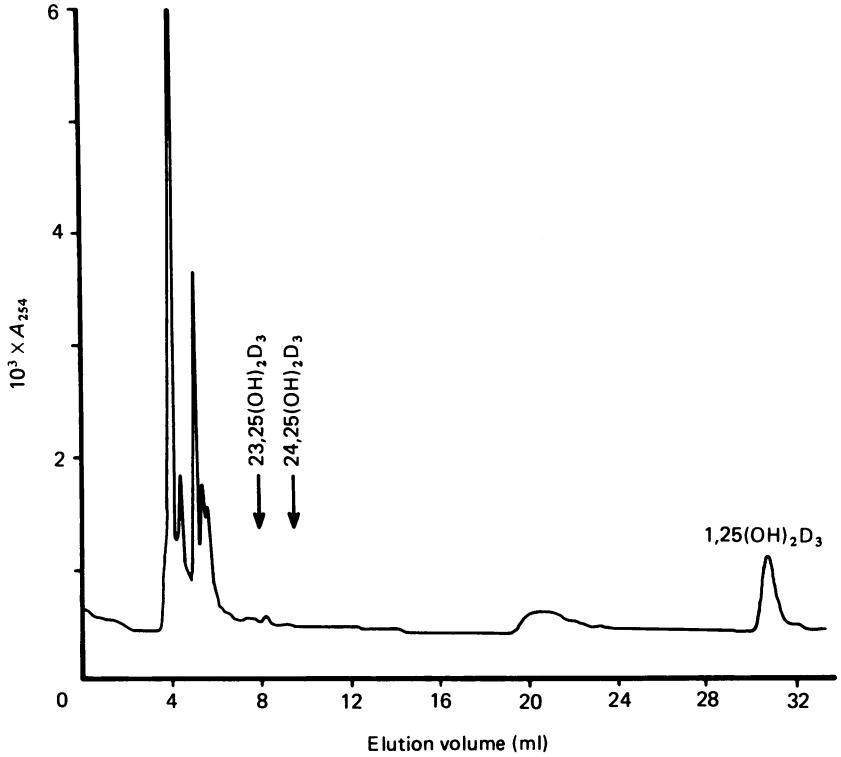

Fig. 5. Straight-phase h.p.l.c. of product [1,25(OH)$_2$D$_3$] obtained after incubating 25(OH)D$_3$ with kidney mitochondria from a guinea pig not treated with 1,25(OH)$_2$D$_3$

Incubation was performed as described in the Experimental procedures section, with 4.7 mg of mitochondrial protein and 20 μg of 25(OH)D$_3$/ml. After solid-phase extraction on a Sep-Pak C_{18} cartridge the extract was purified by reversed-phase h.p.l.c. as described in the Experimental procedures section. The fraction containing the dihydroxylated metabolites was rechromatographed on a Zorbax-Sil (3 μm particle size) column (0.46 mm x 25 cm) as shown. The eluent was 8% propan-2-ol in hexane, and the flow rate was 1 ml/min.

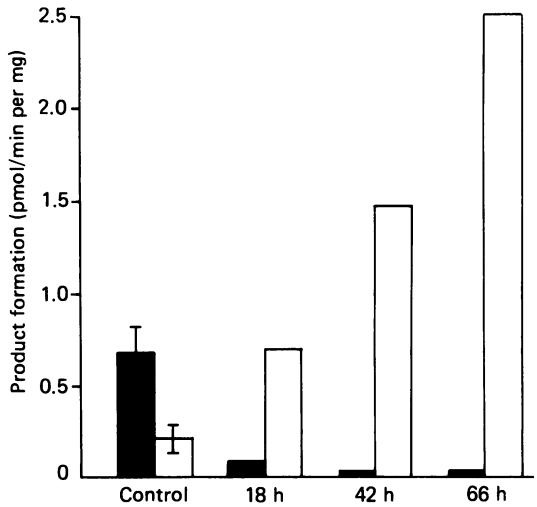

Fig. 6. Effect of administration of 1,25(OH)$_2$D$_3$ *in vitro* on renal mitochondrial 1α-hydroxylation (■) and 23-hydroxylation (□) of 25(OH)D$_3$

The results for control animals are the means ± S.D. obtained from five different animals. For further details, see the Experimental procedures section.

intestine and bone [11]. Most likely renal 23-hydroxylation represents the first step in a catabolic pathway for 25(OH)D$_3$. It may be mentioned that 23,25(OH)$_2$D$_3$ has been isolated from plasma of vitamin D-toxic pig [22], but no information is available on the presence of 23,25(OH)$_2$D$_3$ in plasma of untreated animals or man. A major metabolite of vitamin D_3 identified as 25(OH)D$_3$-26,23-lactone has, however, been detected in plasma of both man and animals [23–27], and it has been demonstrated that 23,25(OH)$_2$D$_3$ is an intermediate in the formation of this metabolite [22,27,28].

During the course of these studies we observed that 23,25(OH)$_2$D$_3$ was very unstable. The samples had to be treated very carefully to avoid loss and unacceptable variations in the results. This lack of stability may be part of the explanation as to why 23,25(OH)$_2$D$_3$ has remained unnoticed until recently.

Preliminary attempts were made here to characterize the 25(OH)D$_3$ 23-hydroxylase. The requirement of oxidizable substrate and the inhibition by CO (Table 2) suggest that it is a mitochondrial cytochrome *P*-450 enzyme similar to the 1α-hydroxylase [4,29] and the 24-hydroxylase [15]. The stimulatory effect of mitochondrial respiratory-chain inhibitors and inhibition by the uncoupler FCCP (Table 1) indicate that the reducing equivalents are mainly provided by NADPH generated through the energy-dependent transhydrogenase reaction.

Engstrom *et al.* [14], who used calf kidney homogenate, found that malate was the most efficient substrate to support the 23-hydroxylase. Under their conditions, cyanide and antimycin inhibited the activity, and the uncoupler dinitrophenol was without effect. Their findings support the contention that, under their

Table 4. Effect of fasting on 25(OH)D_3 hydroxylases in guinea-pig kidney mitochondria

The animals, all females, were fasted with free access to water for 24 h before being killed. The hydroxylase activities were measured as described in the Experimental procedures section. Results are means ± S.D. for the numbers (n) of animals shown.

Animals	Formation (pmol/min per mg) of:		
	1,25$(OH)_2D_3$	23,25$(OH)_2D_3$	24,25$(OH)_2D_3$
Controls ($n = 6$)	0.55±0.27	0.10±0.07	< 0.02
Fasted ($n = 6$)	0.17±0.13	0.94±0.36	0.16±0.08
Fasted and treated with 1,25$(OH)_2D_3$* ($n = 2$)	n.d.†	2.09, 2.98	0.54, 0.57

* Treated with 1,25$(OH)_2D_3$ for 3 days as described in the Experimental procedures section and fasted for 24 h.
† Abbreviation: n.d., not detectable.

conditions, 'malic' enzyme is a main source of reducing equivalents to the enzyme system. Presumably, NADPH may be provided by different mechanisms, the relative importance depending on the conditions used *in vitro* and the source of the enzyme preparation.

The mechanism by which fasting stimulates 25(OH)D_3 23-hydroxylase and 24-hydroxylase and suppresses the 1α-hydroxylase remains speculative for the moment. We noticed that serum calcium was slightly, although not significantly, elevated after 24 h of fasting, but 1,25$(OH)_2D_3$ and parathyroid hormone were not measured. If the elevated serum calcium is related to elevated 1,25$(OH)_2D$, this may be the factor directly responsible for induction of the 23- and 24-hydroxylases during long-term fasting.

Progress in the study of the 25(OH)D_3 hydroxylases at the molecular level and of their regulation has hitherto been slow, primarily because of the low enzyme activities in the '*in vitro*' systems used. In a recent study it was shown that kidney mitochondria from weanling guinea pigs on a normal diet containing physiological amounts of vitamin D exhibited high 25(OH)D_3 1α-hydroxylation activities [30] (cf. Fig. 5). The high rates of conversion of 25(OH)D_3 into 23,25$(OH)_2D_3$ that we have observed in the present work add to the potential usefulness of this particular animal model in more detailed studies of the kidney mitochondrial 25(OH)D_3 hydroxylases.

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