Synthesis of 1,25-dihydroxycholecalciferol and 24,25-dihydroxycholecalciferol by calvarial cells

Characterization of the enzyme systems

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The synthesis of 1,25-dihydroxycholecalciferol [$1,25(\text{OH})_2\text{D}_3$] and 24,25-dihydroxycholecalciferol [$24,25(\text{OH})_2\text{D}_3$] from 25-hydroxycholecalciferol [$25(\text{OH})\text{D}_3$] has previously been shown to occur in cells isolated from bone. The main findings of the present study are that the enzyme systems which catalyse these syntheses are: (1) active at 'in vitro' substrate concentrations over the range of 2–50 nM; (2) regulatable in a complex way by $1,25(\text{OH})_2\text{D}_3$, $24,25(\text{OH})_2\text{D}_3$, 25,26-dihydroxycholecalciferol and $25(\text{OH})\text{D}_3$, but not by cholecalciferol ('vitamin D_3 '); and (3) have relatively short half-lives (approx. 5 h).

INTRODUCTION

Extrarenal production of $1,25(\text{OH})_2\text{D}_3$ has been suspected to occur in a number of tissues. Included in these tissues are bone [1–4], intestine [5,6], liver [7], placenta [8–11], chorioallantoic membrane [12] and cells of the monocyte/macrophage lineage (including sarcoid tissue; [13,14]). The representative species include humans, birds and rodents. Many of these tissues have also been shown to synthesize $24,25(\text{OH})_2\text{D}_3$ [1,3,6,7,10,11]. For the most part, identification of $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ has been by chromatographic means. That is, the metabolites have been shown to be co-eluted with standard markers on open columns and on h.p.l.c. Inasmuch as h.p.l.c. results in an extremely high degree of resolution, it has been concluded that the extra-renally produced metabolites are indeed authentic. However, recent evidence indicates that there are some forms of vitamin D metabolites which are indistinguishable from true $1,25(\text{OH})_2\text{D}_3$ on h.p.l.c. systems and immunoassay [15].

At present there are only three extra-renal sites at which the production of $1,25(\text{OH})_2\text{D}_3$ has been unequivocally documented by m.s. analysis. They are rat placenta [8], pulmonary alveolar macrophages [14] and cells isolated from chick calvaria [16,17]. It is now generally accepted that at least these three cell sources possess an extra-renal 1-hydroxylase. That is not to say that the other tissues do not have the ability to convert $25(\text{OH})\text{D}_3$ into $1,25(\text{OH})_2\text{D}_3$, but rather that the products have not been analysed by m.s.

The issue of whether $1,25(\text{OH})_2\text{D}_3$ produced extra-renally is physiologically important raises a more important question. Two major drawbacks to assigning such an importance are: (1) most of the studies on the synthesis of $1,25(\text{OH})_2\text{D}_3$ have been performed in experiments *in vitro*; and (2) the rate of conversion of $25(\text{OH})\text{D}_3$ into $1,25(\text{OH})_2\text{D}_3$ by these tissues is less than

that of the kidney. However, the arguments which counter these observations centre around the supposition that, if $1,25(\text{OH})_2\text{D}_3$ is produced for local use, then the microenvironmental concentrations would be more than sufficient to evoke a biological response. Moreover, with the advent of sensitive methods for the measurement of $1,25(\text{OH})_2\text{D}_3$, it has now been shown, *in vivo*, that there are detectable levels of circulating $1,25(\text{OH})_2\text{D}_3$ in serum after total bilateral nephrectomy [18–21]. These studies have been performed in both humans and experimental animals.

If the extra-renal 1-hydroxylase enzyme system is biologically important, then it might be expected that this system would be responsive to regulatory factors and be able to utilize substrate at concentrations in the physiological range. The present paper provides evidence that chick calvarial cells contain a regulatable 1-hydroxylase enzyme system and that the apparent K_m for the system is in the low-nanomolar range. The characteristics of a chick cell 24-hydroxylase are also presented.

MATERIALS AND METHODS

Cell isolation and culture

Cells were isolated from embryonic chick calvaria of 17 days *in ova* by modification of previously reported methods [3,22]. Briefly, the calvaria were aseptically dissected and incubated, with shaking, in BGJ medium (Gibco; three calvaria/ml) containing penicillin (100 units/ml), streptomycin (100 µg/ml) and collagenase (Worthington; 2.0 mg/ml) for 20 min. At this time the enzyme-containing medium was decanted from the calvaria, centrifuged at 500 g for 3 min to remove red blood cells and loose connective-tissue cells and returned to the calvarial fragments for a further 100 min of incubation. The cells released during the 20–120 min

Abbreviations used: $1,25(\text{OH})_2\text{D}_3$, 1,25-dihydroxycholecalciferol; $24,25(\text{OH})_2\text{D}_3$, 24,25-dihydroxycholecalciferol; $25(\text{OH})\text{D}_3$, 25-hydroxycholecalciferol; $25,26(\text{OH})_2\text{D}_3$, 25,26-dihydroxycholecalciferol; 1-hydroxylase, 25-hydroxycholecalciferol 1-hydroxylase; 24-hydroxylase, 25-hydroxycholecalciferol 24-hydroxylase.

interval were collected by filtration through a 35 μ m-pore-size nylon mesh filter, washed three times in enzyme-free BGJ medium, counted, and plated in 60-mm-diameter culture dishes in serum-free BGJ medium at 1200 cells/cm² in 4.0 ml. The medium was changed after the first 24 h and every 48 h thereafter.

Metabolism and chromatography of 25(OH)D₃

All cultures were changed to 2.0 ml of fresh BGJ medium immediately before the addition of 25-hydroxy-[methyl-26,27-³H]cholecalciferol {[³H]25(OH)D₃; Amersham International; 9.6 Ci/mmol}. The radiolabel was added at a final concentration of 15 nM in 5.0 μ l of 95% (v/v) ethanol (except where noted in the Figure legends). Ethanol added as a solvent for the [³H]25(OH)D₃ never exceeded 0.25% (v/v). At various times after addition of the [³H]25(OH)D₃ the incubation was terminated by removal of the medium and addition of 100% ethanol to the cell layer, with scraping. The radioactivity recovered from the medium after three extractions with dichloromethane [23] was dried under N₂ gas, reconstituted in hexane/chloroform/methanol

(9:1:1, by vol.) and pooled with the radioactivity recovered from the dried ethanol extraction of the cell layer. Usually the metabolites from three dishes were pooled for further chromatographic analysis.

The metabolites were sequentially separated on a Sephadex LH-20 column (Pharmacia; 20 g of Sephadex LH-20; column size 75 cm $\times$ 1.5 cm) equilibrated with the '9:1:1' solvent and a Zorbax-Sil h.p.l.c. column (du Pont) equilibrated with hexane/propan-2-ol (9:1) as previously described [3]. Production of metabolites was quantified by peak-area analysis in either system.

In some experiments exogenous 1,25(OH)₂D₃, 24,25(OH)₂D₃, 25,26(OH)₂D₃ (donated by Dr M. Uskokovic, Hoffmann-La Roche) or cholecalciferol ('vitamin D₃') (Sigma) was added to the cultures (see the appropriate Figures for concentrations and duration of incubations).

Protein synthesis

The protein-synthetic activity of the chick calvarial cells in culture was estimated by determining the incorporation of ³H-labelled amino acids into trichloroacetic acid-precipitable material. The effect of various concentrations of puromycin on amino acid incorporation was studied after a 20 h incubation.

Data analysis

All values are expressed as means $\pm$ 1 S.E.M. unless otherwise noted. Comparisons are made with Student's *t* test.

RESULTS

Metabolism of 25(OH)D₃

Of the [³H]25(OH)D₃ added to the cultures for any one experiment, 85 $\pm$ 6% was recovered after extraction of the medium and cell layer. Of the radioactivity introduced into the columns, 99 $\pm$ 3% was recovered from chromatography.

Fig. 1 is an example of the profile from Sephadex LH-20 chromatography and h.p.l.c. When fractions 80–92 and 112–122 of the Sephadex LH-20 chromatography (Fig. 1a) were pooled and chromatographed by h.p.l.c. (Fig. 1b), more than 98% of the metabolites migrated as 1,25(OH)₂D₃ or 24,25(OH)₂D₃. The major contaminating substance appeared to be unmetabolized 25(OH)D₃. These data indicate that it is possible to quantify production of these metabolites after chromatography in either system.

Substrate saturation of 1- and 24-hydroxylase activity

As the concentration of substrate [25(OH)D₃] was increased from 2 to 50 nM, the velocity of the reactions for both hydroxylase systems approached a maximum. When results were subjected to an Eadie-Hofstee plot (*v* versus *v*/[S]) the apparent *K_m* and *V_{max}* for the systems could be calculated (Fig. 2). It should be pointed out that these kinetic constants are a characterization of the cell systems rather than of the enzymes themselves. This is because we are working with whole cells, because the enzymes are not purified and because we cannot be sure that substrate availability to the enzymes is maximal. Nevertheless, these constants define the 'in vitro' system and indicate that the cells can utilize the substrate at or below physiological concentrations. For the 1- and

Fig. 1. Representative profiles from Sephadex LH-20 chromatography and h.p.l.c.

(a) Shows the profile of radiolabelled metabolites produced by incubation of 25(OH)D₃ (15 nM) with approx. 12 $\times$ 10⁶ chick bone cells in culture. The reaction time was 2.0 h. Peak III is 25(OH)D₃ and peaks VI and VII are 24,25(OH)₂D₃ and 1,25(OH)₂D₃ respectively. (b) Is an h.p.l.c. chromatograph of pooled fraction nos. 80–92 and 112–122 from a Sephadex LH-20 separation. Three identifiable peaks are observed with retention volumes corresponding to 25(OH)D₃ (peak I), 24,25(OH)₂D₃ (peak II), and 1,25(OH)₂D₃ (peak III).

Fig. 2. Eadie-Hofstee plot (v versus $v/[S]$) for 1-hydroxylase and 24-hydroxylase

The values for K_m and V_{max} for the 1-hydroxylase (■) are 5.2 nM and 1.3 fmol/min per 10^6 cells with a correlation coefficient (r) of -0.92 . The values of K_m and V_{max} for the 24-hydroxylase (●) are 14.5 nM and 4.6 fmol/min per 10^6 cells with $r = -0.99$. The reaction time for both hydroxylases was 2.0 h. The rate of $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ production was linear for 2.0 h at all substrate concentrations.

24-hydroxylases the values of K_m are 5.1 nM and 14.5 nM and the values for V_{max} are 1.3 and 4.6 fmol/min per 10^6 cells respectively. The mean concentration of $25(\text{OH})\text{D}_3$ in chick and human serum is approx. 75 nM (20–40 ng/ml) [24,25]. As is discussed below, a direct comparison between the apparent concentration of metabolites in serum-free medium and in serum should not be made.

Regulation of hydroxylase activity

Exposure of these cells to exogenously added $1,25(\text{OH})_2\text{D}_3$ at a concentration of 10^{-8} M has previously been shown to stimulate $24,25(\text{OH})_2\text{D}_3$ production and inhibit $1,25(\text{OH})_2\text{D}_3$ production [3]. This result is again observed after a 20 h exposure at the same concentration (Fig. 3). However, the response to a wider range of $1,25(\text{OH})_2\text{D}_3$ concentrations reveals a complex pattern of regulation. At high concentrations, 10^{-7} M and above, both the 1- and the 24-hydroxylase activities are inhibited. As the concentration of $1,25(\text{OH})_2\text{D}_3$ is decreased the 24-hydroxylase activity is markedly stimulated and the 1-hydroxylase activity is gradually stimulated, both reaching a peak at 10^{-10} M. Both activities return to control levels at a $1,25(\text{OH})_2\text{D}_3$ concentration of 10^{-13} M.

The effects of $25(\text{OH})\text{D}_3$, $24,25(\text{OH})_2\text{D}_3$, $25,26(\text{OH})_2\text{D}_3$ and cholecalciferol on the 1- and 24-hydroxylase activities at selected concentrations is presented in Fig. 4. As can be seen, $25(\text{OH})\text{D}_3$,

Fig. 3. Regulation of $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ production by bone cells after treatment with exogenous $1,25(\text{OH})_2\text{D}_3$ for 20 h

The data (velocities) for the 1-hydroxylase (■) and the 24-hydroxylase (●) are expressed as a ratio (treated cells/control cells) for ease of comparison. The actual control values were 1.1 fmol/min per 10^6 cells for the 1-hydroxylase and 4.0 fmol/min per 10^6 cells for the 24-hydroxylase. The medium containing endogenous $1,25(\text{OH})_2\text{D}_3$ was removed and the cell layer rinsed before addition of the radiolabelled substrate, $[^3\text{H}]25(\text{OH})\text{D}_3$. These experiments were performed at substrate concentrations of 15 nM and 50 nM. The results were the same for both concentrations. The data with the substrate concentration of 15 nM are reported here. Values are means $\pm$ 1 S.E.M. ($n = 3$). Statistical significance: * $P < 0.05$; ** $P < 0.01$.

$24,25(\text{OH})_2\text{D}_3$ and $25,26(\text{OH})_2\text{D}_3$ have qualitatively similar effects to $1,25(\text{OH})_2\text{D}_3$, though displaced by approximately a 100-fold greater concentration. All three of the 25-hydroxylated metabolites maximally stimulate both hydroxylase activities at concentrations near 10^{-8} M and inhibit the activities at 10^{-5} M. The values return to control levels at 10^{-10} M for both activities and all three metabolites. Cholecalciferol is not statistically effective in regulating either of the hydroxylase activities.

Enzyme half-life

As evidenced in Figs. 3 and 4, the 1- and 24-hydroxylase activities appear to be under complex control. Consistent with this is the observation that the apparent half-life for the production of the metabolites is rather short (approx. 5 h). These data were obtained by inhibiting protein synthesis with a titrated non-lethal dose of puromycin and observing the decrease in activity. Protein synthesis was estimated by determining the incorporation of radiolabelled amino acids into trichloroacetic acid-precipitable material, and viability was assessed by microscopic examination. Table 1 indicates that a dose of 1.0 μg of puromycin/ml for 18 h inhibited protein synthesis by 97% without a visible effect on cell morphology. This concentration of puromycin was used in a time study of 1- and 24-hydroxylase activities. Fig. 5 indicates that, in the presence of puromycin, both hydroxylase activities decrease with a

Fig. 4. Effect of other vitamin D metabolites on the production of $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ by bone cells

Velocity values are, as in Fig. 3, expressed as a ratio (treated cells/control cells). The actual control values ranged from 0.85 to 1.35 fmol/min per 10^6 cells for the 1-hydroxylase (■) and from 3.80 to 4.70 fmol/min per 10^6 cells for the 24-hydroxylase (●). The cells were exposed to the metabolites for 20 h. Similar results were obtained at substrate concentrations of 15 nM and 50 nM (see the legend to Fig. 3). Values are means $\pm$ 1 S.E.M. ($n = 3$). Statistical significance is indicated as in Fig. 3.

Table 1. Effect of puromycin on protein synthesis and viability of cultured bone cells

Values given are means $\pm$ 1 S.E.M. ($n = 8$). Cells were exposed to puromycin for 18 h.

[Puromycin] ($\mu\text{g/ml}$)	Protein synthesis (fmol/h per 10^6 cells)	Morphology in culture
0 (control)	4.39 ± 0.19	Stellate, flattened, no cellular debris
0.5	2.91 ± 0.17	Stellate, flattened, no cellular debris
1.0	0.14 ± 0.02	Stellate, flattened, no cellular debris
5.0	0.17 ± 0.08	Some cells rounded, some cellular debris
25.0	0.09 ± 0.01	Most cells rounded, considerable cellular debris
100	0.09 ± 0.02	All cellular debris

half-life of approx. 5.0 h. The interpretation of these data is difficult, because the inhibition of protein synthesis influences both the production and the degradation of all of the components of the hydroxylase systems. These values for half-life, then, may not actually be true estimates of decay times of the enzymes themselves.

DISCUSSION

The hydroxylase activities characterized in the present study provide further evidence that cells from tissues other than the kidney can synthesize the principal dihydroxy metabolites of vitamin D, namely $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$. These data also demonstrate that the enzyme systems which catalyse these syntheses are under regulatory control, at least *in vitro*.

The identity of the metabolites synthesized by bone cells has been confirmed by chromatography in four different systems [3], chemical reaction with periodate [3] and m.s. analysis [16,17].

The fact that 99% of the radioactivity is recovered after chromatography is indicative of the resolving power of the chromatographic systems and of the lack of binding of interfering substances in the separations. Because there is no serum protein present and because there are relatively few (approx. 12×10^6) cells per extraction, the chromatographic profiles demonstrate little background and are relatively complete. It is under these conditions, we believe, that the demonstration of extrarenal synthesis of $1,25(\text{OH})_2\text{D}_3$ in chick calvarial cells is possible.

Fig. 2 demonstrates that the hydroxylases are active at or below substrate concentrations in the physiological range. Normal serum levels of $25(\text{OH})\text{D}_3$ in chicks and humans are 50–100 nM [24,25] and the range used in these experiments is 2–50 nM. The K_m values for the 1- and 24-hydroxylases were determined to be approx. 5 nM and

Fig. 5. Half-lives of the bone cell 1- and 24-hydroxylases

These data demonstrate the activity of the hydroxylases after exposure of the cells to a non-lethal dose of puromycin. Protein synthesis is inhibited by 97%, but there is no morphological change in the cells. The approximate half-life of both of the enzymes is 5 h. When plotted in the form of log activity against time, both decay curves demonstrate significant linearity (1-hydroxylase: $r = -0.99$, $P < 0.001$, half-life = 5.2 h; 24-hydroxylase: $r = -0.93$; $P < 0.01$, half-life = 5.0 h. Values are means $\pm$ 1 S.E.M. ($n = 3$).

15 nM respectively. The implications of these data are important, since they indicate that the 1- and 24-hydroxylase enzyme systems can utilize low concentrations of $25(\text{OH})_2\text{D}_3$ to produce $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ and are not simply non-specific hydroxylases active against a pharmacological dose of $25(\text{OH})_2\text{D}_3$.

One of the major determinants of 1- and 24-hydroxylase activity *in vivo* is $1,25(\text{OH})_2\text{D}_3$ itself [26,27]. This molecule has also been shown to regulate hydroxylase activity *in vitro* in both renal [27] and extra-renal [1,3] cells. The experiments previously performed in cultures of bone cells [3] indicate that exogenously added $1,25(\text{OH})_2\text{D}_3$ at 10 nM significantly inhibits 1-hydroxylase activity and significantly stimulates 24-hydroxylase activity. Studies using a wide range of added $1,25(\text{OH})_2\text{D}_3$ concentrations, however, reveal a more complex mechanism of regulation for both hydroxylases (Fig. 3). At $1,25(\text{OH})_2\text{D}_3$ concentrations between 10^{-12} and 10^{-10} M, both hydroxylases are stimulated. The activities return to control levels at 10^{-13} M $1,25(\text{OH})_2\text{D}_3$. The effects of exogenously added $24,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$ and $25,26(\text{OH})_2\text{D}_3$, but not cholecalciferol, on these enzymes is qualitatively the same (Fig. 4). In these '*in vitro*' experiments it appears as though the 25-hydroxy group is an important determinant of stimulation and inhibition of hydroxylase

activity and that hydroxy groups in other positions simply modify the dose response and magnitude of the effect. A result similar to this has been obtained in a perfused kidney system, where it was found that only metabolites containing a 25-hydroxylated carbon atom would stimulate 24-hydroxylase activity and inhibit 1-hydroxylase activity [28].

At this point in the discussion it is important to emphasize that a direct comparison between the concentrations of the vitamin D metabolites in serum-free and serum-containing systems cannot be made. This is because the two systems do not have the same compartments in which the metabolites can establish an equilibrium. We have previously shown [29] that the plastic culture dish has a very large binding capacity for vitamin D metabolites. Only small amounts of the metabolites enter the cells and still less remain in solution. Moreover, the distribution between the compartments is different for different metabolites. In the case of serum- or protein-containing medium the soluble compartment becomes a more dominant factor for binding of the metabolites and must certainly affect delivery of the metabolites to the cells. Whether binding to a binding protein enhances or inhibits specific delivery to the cells is still a topic of discussion. Therefore, to state that concentrations of vitamin D metabolites *in vitro* in serum-free or serum-containing systems compare directly with those *in vivo* is an over-interpretation of the data. What can be said, though, is that in the present system we have been able to measure cellular effects which compare with effects in other experiments *in vitro*, namely those utilizing renal cells in culture [27]. The fact that the renal-cell experiments have been shown to have a physiological relevance supports such a relevance for our experiments.

Many of the observations made in Figs. 3 and 4 regarding the stimulation of hydroxylase activity can be resolved with the hypothesis that $24,25(\text{OH})_2\text{D}_3$, $25(\text{OH})\text{D}_3$ and $25,26(\text{OH})_2\text{D}_3$ interact with a $1,25(\text{OH})_2\text{D}_3$ receptor and elicit $1,25(\text{OH})_2\text{D}_3$ effects. On the other hand, inhibition of hydroxylase activities by high concentrations of these molecules may be due to a competition with $[^3\text{H}]25(\text{OH})\text{D}_3$ as substrate or to a direct product inhibition. A result which is not easily interpreted, however, is the stimulation of 1-hydroxylase activity by $1,25(\text{OH})_2\text{D}_3$ and the other metabolites [if they are evoking a response by binding to the $1,25(\text{OH})_2\text{D}_3$ receptor]. This appears to be a type of positive feedback control, and, if it is a direct effect of the metabolites on the synthesis of the enzyme, it is an unusual mechanism. An alternative possibility is that 1-hydroxylase stimulation may be the result of an indirect action. For example, such indirect effects could occur if there were a general stimulation in mitochondrial or microsomal protein synthesis and may not necessarily be specific for the stimulation of the 1-hydroxylase as such.

The half-life for the 1- and 24-hydroxylase activity appears to be rather short. We have calculated that the time for decay to half-maximal activity is approx. 5 h for both systems. As frequently is the case in studies such as these, it cannot be stated that the half-life of the enzymes themselves is 5 h. This is because a number of factors enter into the interpretation of the data. For example, it is entirely possible that inhibition of protein synthesis in these cells causes a rapid decay in an obligatory

component of the enzyme system other than the actual hydroxylase. In this case we would be measuring the decay of the component and not necessarily that of the enzyme. Also, the inhibition of protein synthesis may also inhibit the synthesis of the enzymes which degrade the hydroxylases. In this case, the half-lives could actually be over-estimates of their true values. Summarizing, then, from our data we can only conclude that the half-life for the decay in overall production of $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ is of the order of 5 h. This value, however, agrees well with the 4–5 h reported for kidney mitochondria [30].

Regulation of any enzyme system can be exerted through changes in the activity of the enzyme (alteration in K_m) or changes in the number of enzyme molecules (alteration in $V_{\max}$). We have attempted to perform these experiments at a substrate concentration which is in the physiological range and yet is on the plateau for substrate saturation. However, we recognize that these experiments are performed in an 'in vitro' culture situation, and results so obtained may not actually represent the regulation that normally occurs *in vivo*. Nevertheless, the fact that these extra-renal cells have this complex mechanism indicates that $1,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3$ production *in vivo* can, if necessary, undergo a wide latitude of control.

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