Uptake and 25-Hydroxylation of Vitamin D₃ by Isolated Rat Liver Cells*

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The physiological roles played by hepatocytes and nonparenchymal cells of rat liver in the metabolism of vitamin D₃ have been investigated.

Tritium-labeled vitamin D₃ dissolved in ethanol was administered intravenously to two rats. Isolation of the liver cells 30 and 70 min after the injection showed that vitamin D₃ had been taken up both by the hepatocytes and by the nonparenchymal liver cells. The relative proportion of vitamin D₃ that accumulated in the nonparenchymal cells increased with time. Perfusion of the isolated rat liver with [²H] vitamin D₃ added to the perfusate confirmed the ability of both cell types to efficiently take up vitamin D₃ from the circulation.

By a method based on high pressure liquid chromatography and isotopic dilution-mass fragmentography it was found that isolated liver cells in suspension had a considerable capacity to take up vitamin D₃ from the medium. About 2.5 pmol of vitamin D₃ were found to be associated with each hepatocyte or nonparenchymal cell after 1 h of incubation. 25-Hydroxylation in vitro was found to be carried out only by the hepatocytes.

The rate of hydroxylation was about the same whether the cells were isolated from normal or rachitic rats (3.5 and 4 pmol of 25-hydroxyvitamin D₃ formed per h per 10⁶ cells, respectively). The possibility that the nonparenchymal cells might serve as a storage site for vitamin D₃ in the liver is discussed.

According to current concepts (1) vitamin D₃ must first undergo 25-hydroxylation in the liver to 25-hydroxyvitamin D₃, followed by 1α-hydroxylation in the kidneys to 1α,25-dihydroxyvitamin D₃, before it can exert its physiological functions. Since the liver is the only organ in which formation of 25-hydroxyvitamin D₃ takes place to any extent (2,3), this organ plays a central role in the metabolism of the vitamin. Recently it has become evident that both the hepatocytes and the nonparenchymal cells of the liver are involved in the metabolism of lipids and lipoproteins (4,5). In which of these types of liver cells the hydroxylation of vitamin D₃ takes place is not known. In the present work we demonstrate that both the hepatocytes and the nonparenchymal cells of the rat liver have a high capacity to take up vitamin D₃ both in vivo and in vitro. The further metabolism to 25-hydroxyvitamin D₃, however, takes place almost exclusively in the hepatocytes.

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The abbreviation used is: HPLC, high pressure liquid chromatography.
buffer, pH 7.5, 20 mM N-2-hydroxyethylpipеразине-N'-2-этиленаминная кислота, pH 7.5, and 10 mg/ml of bovine serum albumin. The osmolality was approximately 300 mosmol/liter. Cells from rachitic animals were incubated in the same medium except that it contained cytosolic fraction (4 mg of protein/ml) instead of bovine serum albumin. The cytosolic fraction had been prepared from a normal rat liver homogenate by centrifugation at 100,000 × g for 90 min. The procedure to determine the relative supernatant was 40 mg/ml, and 2 ml of the suspension of cells from rachitic rats the following additions were also made: 5.4 µmol of ATP, 6.4 µmol of malate, 1.3 µmol of NADP, 1.1 µmol of glucose-6-phosphate, 0.5 unit of glucose-6-phosphate dehydrogenase, 20 µmol of MgCl₂, 1.1 µmol of fructose, and 2.2 nmoles of N,N'-diphenyl-p-phenylenediamine. (The stimulation observed by these cofactors can be explained by activation of the 25-hydroxylase activity also in nonviable cells. No such stimulation was observed with cultured rat hepatocytes.) The reaction was started by the addition of 171 nmol of vitamin D₃ (cells from normal rats) or 520 nmol (cells from rachitic rats) in 20 µl or less of ethanol. A boiled cell suspension of identical composition was used as a blank incubation. The reaction was stopped after 2 h of incubation by the addition of 7 ml of methanol/chloroform (2:1). Deuterium-labeled 25-hydroxyvitamin D₃, 100 ng, was added at this point. After extraction (7) the chloroform phase was filtered through a 0.5-µm Millipore filter (Millipore Corp., Bedford, MA) and evaporated under nitrogen, and the residue was redissolved in the solvent used for HPLC. The formed 25-hydroxyvitamin D₃ was separated on two consecutive HPLC systems (7). The entire sample was first chromatographed on a Zorbax-ODS column (4.6 × 250 mm, particle size 5 µm) with 2.5% isopropanol in hexane as solvent (0.8 ml/min). The fraction that corresponded to eluted 25-hydroxyvitamin D₃ (retention time, 11 min) was collected and rechromatographed on a Zorbax-Sil column (2.1 × 250 mm, particle size 5 µm) with 2.5% isopropanol in hexane as solvent (0.8 ml/min). The eluted 25-hydroxyvitamin D₃ (retention time, 8.5 min) was converted into the trimethylsilyl/t-butyldimethylsilyl derivative, and the amount was determined by gas chromatography-mass spectrometry as described (13).

In some experiments the formed 25-hydroxyvitamin D₃ was quantitated by HPLC. In this case a Spherisorb-silica column (3 × 250 mm, particle size 5 µm) was used at the second chromatographic step with 2.5% isopropanol in hexane as eluting solvent (0.8 ml/min). The height of the UV-absorbing peak corresponding to 25-hydroxyvitamin D₃ (retention time, 9.5 min) was compared to that of a standard. Recovery was corrected for by the addition of tritium-labeled 25-hydroxyvitamin D₃ (10,000 cpm) prior to extraction. When the two methods of quantitation were compared identical results were obtained.

All tritium-containing samples were counted at about 50% efficiency in a Packard Tri-Carb liquid scintillation spectrometer. A standard amount of [³H]25-hydroxyvitamin D₃ was routinely used to assess constant counting efficiency.

A method based on isotope dilution-mass fragmentography (14) was used to quantify the amount of vitamin D₃ that had been taken up by the cells during the incubation. In these experiments the cells were separated from the medium by centrifugation immediately after the incubations. The cell fraction (after being washed 3 times) and the medium were extracted separately. [25,26,26,26,27,27,27-³H]vitamin D₃ (170 ng) was added to the cell fraction prior to extraction and purification by HPLC. The fraction corresponding to vitamin D₃ that eluted from the Zorbax-ODS column (retention time, 57 min) was collected and converted to the trimethylsilyl derivative and analyzed by gas chromatography-mass spectrometry essentially as described (14). One channel of the multiple ion detector was focused at the m/e 325 corresponding to the derivative of vitamin D₃ and the other channel at m/e 332 corresponding to the derivative of ³H-labeled vitamin D₃. The amount of unlabeled vitamin D₃ was calculated from the ratio between the height of the peak at m/e 325 and the height of the peak at m/e 332 with use of a standard curve. The standard curve was obtained by analysis of different standard mixtures of vitamin D₃ (0–50 ng) together with a fixed amount of ³H-labeled vitamin D₃.

Uptake of Tritium-labeled Vitamin D₃ into Liver Cells in Vivo-After Perfusion of the Liver—Tritium-labeled vitamin D₃ (approximately 0.75 × 10⁶ cpm, 50 pmol) dissolved in 100 µl of ethanol was given in one of the tail veins of rachitic rats. After varying periods of time the livers were perfused and the cells isolated and separated as described above. In one experiment, tritium-labeled vitamin D₃ was added to the perfusate (approximately 10⁶ cpm, 70 pmol), and the liver was perfused for 60 min. Prior to extraction of the cell preparations a known amount (310 ng) of vitamin D₃ was added to correct for recovery during extraction and chromatography. The samples were extracted with chloroform/methanol, 2:1, as above, and the upper layer was re-extracted once with chloroform. The extract was taken to dryness under a stream of N₂ and redissolved in the solvent used for HPLC. The samples were separated on two consecutive HPLC systems, but the order was reversed compared to the description above. The sample was first separated on a Spherisorb-silica column (3 × 250 mm, particle size 5 µm) with 2.5% isopropanol in hexane as solvent (1 ml/ml). From the distribution of radioactivity in the collected fractions (1/min) the relative amount of tritium-labeled vitamin D₃ (retention time 3.2 min) and 25-hydroxyvitamin D₃ (retention time 7.3 min) could be determined. Only one-tenth of the fraction that contained vitamin D₃ and vitamin D₃ was used for determination of radioactivity; the remainder was separated on a Zorbax-ODS column (4.6 × 250 mm) with 1.5% H₂O in methanol as eluting solvent (1.2 ml/min). This system separated vitamin D₃ and vitamin D₃ (retention times, 13.2 and 14.4 min, respectively). The radioactivity in the vitamin D₃ fraction was determined. After correcting for recovery of the added vitamin D₃ the amount of radioactive vitamin D₃ originally taken up by the cells was calculated. (The assumption was made that the losses during extraction and chromatography were identical for vitamins D₃ and D₃.)

RESULTS

**Uptake of [³H]Vitamin D₃ in Liver Cells in Vivo**

**Table 1**

<table>
<thead>
<tr>
<th>Time after injection (min)</th>
<th>Number of isolated cells</th>
<th>Recovered [³H]Vitamin D₃ (cpm/10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>48.5</td>
<td>460</td>
</tr>
<tr>
<td>70</td>
<td>49.5</td>
<td>230</td>
</tr>
</tbody>
</table>

**Fig. 1. Uptake of vitamin D₃ by isolated rat liver cells.** Hepatocytes (●, 10.6 × 10⁶ cells) and nonparenchymal cells (○, 5.1 × 10⁶ cells) from the liver of a normal rat were incubated in a volume of 2 ml as described under "Materials and Methods" in the presence of 171 nmol of vitamin D₃ for varying periods of time. The cells were separated from the medium, washed 3 times, and the amounts of vitamin D₃ associated with the cells were determined by isotope dilution-mass spectrometry as described under "Materials and Methods." The results are given as amount of vitamin D₃ per 10⁶ cells.
intravenous injection of [3H]vitamin D₃ into 2 rachitic rats approximately 58% of the radioactivity was recovered in the liver of one of the rats 30 min after the injection, and the same amount was also recovered from the liver of the other rat 70 min after the injection. The blood contained 6.9 and 4.8% of the radioactivity at these same times, respectively. In the blood, the fraction of the radioactivity that corresponded to 25-hydroxyvitamin D₃ increased from 1.5% at 30 min to 5.7% at 70 min.

After removal of the livers and separation of the hepatocytes from the nonparenchymal cells it was found that [3H] vitamin D₃ had been taken up by both cell types (Table I). The data showed that the relative proportion of vitamin D₃ that accumulated in the nonparenchymal cells increased with time.

It could be argued that the pronase treatment used to obtain pure nonparenchymal cells (11) would lead to liberation of vitamin D₃ from the hepatocytes and subsequent binding to the nonparenchymal cells. When the experiment reported in Table I was repeated and the cells were separated by the differential centrifugation method (10), essentially the same results as those reported were obtained.

Practically all the radioactivity recovered from the isolated cells cochromatographed with vitamin D₃ (96%) on HPLC. The amount that cochromatographed with 25-hydroxyvitamin D₃ was very small (at 70 min, 3% in the hepatocytes and 1.4% in the nonparenchymal cells).

TABLE I

<table>
<thead>
<tr>
<th>Number of isolated cells</th>
<th>Recovered radioactivity</th>
<th>Vitamin D₃</th>
<th>25-Hydroxyvitamin D₃</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>cpm</td>
<td>cpm/10⁶ cells</td>
</tr>
<tr>
<td>Perfusion medium</td>
<td>35,500</td>
<td>20,700 (58.5%)</td>
<td>6,200 (17.4%)</td>
</tr>
<tr>
<td>Hepatocytes</td>
<td>14,300</td>
<td>10,300 (72.6%)</td>
<td>1,600 (11.3%)</td>
</tr>
<tr>
<td>Nonparenchymal cells</td>
<td>4,700</td>
<td>4,000 (86.3%)</td>
<td>60 (1.3%)</td>
</tr>
</tbody>
</table>

Fig. 2. High pressure liquid chromatography on a Spherisorb silica column (3 x 250 mm) after prepurification on a Zorbax-ODS column of the methanol/chloroform extract of incubations of isolated liver cells and vitamin D₃. The experimental conditions including extraction and chromatography are given under "Materials and Methods." The cells were isolated from the liver of a rachitic rat. The incubation time was 2 h. A, authentic 25-hydroxyvitamin D₃ (43 ng); B, complete incubation system with 2 x 10⁷ parenchymal liver cells; C, blank incubation as in B, but the cell suspension had been boiled for 10 min prior to incubation; D, complete incubation system with 5 x 10⁷ nonparenchymal liver cells. The eluting solvent was 2.5% isopropanol in hexane, 0.8 ml/min.

Fig. 3. Effect of time on the formation of 25-hydroxyvitamin D₃ by liver cells isolated from normal (A) or rachitic (B) rats. The incubation conditions were as described under "Materials and Methods." 10.6 x 10⁶ hepatocytes (O) and 5.1 x 10⁶ nonparenchymal cells (O) were used per incubation in A, and 10.6 x 10⁶ and 7.6 x 10⁶, respectively, in B. The results are given as amount of 25-hydroxyvitamin D₃ formed per 10⁶ cells.
medium. After perfusion for 10-15 more min with the collagenase-containing buffer the cells were separated by use of the pronase method. About two-thirds of the radioactivity was recovered in the perfusion medium (Table II). Of this, as much as 17.4% cochromatographed with 25-hydroxyvitamin D$_3$ indicating that the added vitamin D$_3$ had been extensively metabolized by the liver cells during the perfusion. A considerable amount of the radioactivity was recovered both in the hepatocytes and in the nonparenchymal cells (Table II). When expressed per number of cells, this last cell type contained about 3 times as much radioactivity as the hepatocytes (Table II). The fraction of the radioactivity that cochromatographed with 25-hydroxyvitamin D$_3$ corresponded to about 11% in the hepatocytes but was rather insignificant in the nonparenchymal cells (Table II). This may indicate that the 25-hydroxylation of vitamin D$_3$ primarily takes place in the hepatocytes.

Uptake of Vitamin D$_3$ during Incubation of Isolated Liver Cells—Considerable amounts of vitamin D$_3$ were associated with both the hepatocytes and the nonparenchymal cells after incubation in a medium that contained 86 nmol/ml of vitamin D$_3$ (Fig. 1). Before extraction both cell preparations had been washed 3 times, and it is reasonable to assume that the vitamin D$_3$ had really been taken up by the cells and not only bound to the cell surface. The zero-time samples were left on ice during the entire incubation period (up to 4 h) before extraction. The very low content of vitamin D$_3$ in these cells (Fig. 1) is another argument in favor of a true uptake. The uptake was time dependent, and the nonparenchymal cells appeared to have a somewhat higher capacity than the hepatocytes (Fig. 1).

25-Hydroxylation of Vitamin D$_3$ in Isolated Liver Cells—When isolated hepatocytes were incubated in the presence of vitamin D$_3$ one more polar product could be detected by HPLC of the incubation extract. The retention time of this product was identical with authentic 25-hydroxyvitamin D$_3$ both by reversed-phase chromatography on the Zorbax-ODS column and by rechromatography on the silica column (Fig. 2B). Due to lack of sufficient amounts of material it was not possible to obtain a full mass spectrum of the product. The most prominent peaks in the mass spectrum of the trimethylsilie/t-butyldimethylsilie derivative of 25-hydroxyvitamin D$_3$ are those at m/e 131, m/e 439, and m/e 586 (13). After prepurification on HPLC, the mass fragmentogramic analysis showed that all these ions were present in the gas chromatographic peak corresponding to the derivative of the product isolated from the incubations with vitamin D$_3$. Furthermore, within the experimental errors, the ratios between the intensities of these different ions were identical with the corresponding ratios obtained in an analysis of derivative of authentic 25-hydroxyvitamin D$_3$. It is, therefore, reasonable to conclude that the identity of the product is indeed 25-hydroxyvitamin D$_3$. The quantitative determination by the mass fragmentogramic method (13) of the amount of product formed yielded results identical with that based on the peak height after the second HPLC step (cf. "Materials and Methods").

When nonparenchymal cells were incubated in the presence of vitamin D$_3$ alone or only an insignificant amount of the more polar product was formed (Fig. 2D). This formation could entirely be explained by the contamination of this cell fraction with parenchymal cells.

With cells isolated from normal rats the total formation of 25-hydroxyvitamin D$_3$ was linear with time at least up to 4 hr (Fig. 3A). In separate experiments it was found that about 50% of the formed 25-hydroxyvitamin D$_3$ was released to the medium. Linearity with time was also observed with cells isolated from rachitic rats (Fig. 3B). The rate of product formation by the hepatocytes was within the same range whether the cells were isolated from normal or from rachitic rats. Only after a prolonged time of incubation was formation of 25-hydroxyvitamin D$_3$ by the nonparenchymal cell preparations measurable (Fig. 3, A and B).

Linearity with the number of cells was found to be satisfactory up to about 10$^7$ cells (Fig. 4). In some experiments with a higher number of cells the reaction leveled off; this was observed in particular with hepatocytes isolated from the rachitic rats.

This lack of linearity at very high cell numbers was not
caused by hypoxia because continuous oxygenation of the
medium did not influence the results. Presumably, the expla-
nation is that the higher the cell concentration the higher the
tendency toward aggregation. Less substrate will thus be
available for uptake per cell. The very small formation of 25-
hydroxyvitamin D$_3$ observed with the nonparenchymal cell
preparation was only measurable at very high cell numbers
(Fig. 4).

With a fixed number of parenchymal cells (9.4 x 10$^6$) per
incubation the formation of 25-hydroxyvitamin D$_3$ was de-
pendent on the amount of substrate added up to about 130
nmol per incubation when a saturation level was reached (Fig.
5). Reciprocal plotting of the data indicated apparent
Km values of about 4 $\mu$M and 6 $\mu$M with hepatocytes from normal
and rachitic rats, respectively. The maximum rates of the
reaction were about 3.5 and 4 pmol x h$^{-1}$ x 10$^{-8}$ cells in the
two cases (Fig. 5, A and B).

**DISCUSSION**

Previous studies have established that vitamin D$_3$ given
intravenously to rats to a large extent is taken up by the liver
(3, 15-17). The present results have confirmed these findings.
Furthermore, the results have shown that both the hepa-
tocytes and the nonparenchymal liver cells of rachitic rats are
active in removing vitamin D$_3$ from the circulation. If one
assumes that about two-thirds of the liver cells are hepa-
tocytes and the remainder in the nonparenchymal cells
(18), from the data in Table I one can estimate that 30 min after the intravenous injection of labeled vitamin D$_3$ dissolved in ethanol about 90% of the radioactivity taken up by the liver is recovered in the
hepatocytes and the remainder in the nonparenchymal cells.

The importance of the nonparenchymal cells for the uptake
of vitamin D$_3$ was even more pronounced when the liver was
perfused in vitro and with vitamin D$_3$ added to the perfusion
medium (Table II).

The findings that vitamin D$_3$ is efficiently taken up both by
the hepatocytes and by the nonparenchymal cells suggests that
both cell types may be of importance in the metabolism of
vitamin D$_3$. It should be kept in mind, however that giving
D$_3$ dissolved in ethanol is rather unphysiological and
that the situation may be different under normal conditions.

25-Hydroxylation is the most important metabolic conver-
sion of vitamin D$_3$ that occurs in the liver (1-3). In a previous
study it was shown that isolated liver cells are able to carry
out this reaction (19). No attempt was made, however, to
separate the parenchymal from the nonparenchymal liver
cells. The incubation experiments reported here strongly
indicate that the 25-hydroxylation takes place only in the he-
patocytes and at comparable rates whether the cells are
derived from rachitic or from normal rats. For the moment
we can only speculate on what function the nonparenchymal
cells may play in the metabolism of vitamin D$_3$. One possibility
is that these cells may serve as a site of storage for vitamin
D$_3$. When the need for more 25-hydroxyvitamin D$_3$ arises the
stored vitamin D$_3$ might subsequently be transferred to the
hepatocytes for hydroxylation.

Two different vitamin D$_3$ 25-hydroxylases have been char-
acterized in rat liver. One is localized to the endoplasmic reticulum (20-23) and the other in the mitochondria (7, 13,
24) The first appears to have a high affinity but low capacity,
while the second has a low affinity and a high capacity. This
last one may be responsible for the continuous increase in
circulating 25-hydroxyvitamin D$_3$ after increasing dosage of
vitamin D$_3$ (25). Any feedback regulation of the 25-hydroxy-
ase activity does not appear to be very effective and is not
able to prevent toxic effects of large doses of vitamin D$_3$.
Uptake and storage of vitamin D$_3$ in the nonparenchymal cells
could be one way that the organism to some extent could
protect itself from the toxic effects of large doses of vitamin D$_3$.

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