Transport of Vitamin D₃ from Rat Intestine
EVIDENCE FOR TRANSFER OF VITAMIN D₃ FROM CHYLOMICRONS TO α-GLOBULINS

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The transport of vitamin D₃ from the intestine via the intestinal lymph to the plasma compartment in rats has been studied. Rats were cannulated in the intestinal lymph duct and given an intraduodenal bolus of [1α,25-(OH)₂] vitamin D₃ in soybean oil. The appearance of radioactivity was negligible during the initial 2 h and peaked 4-8 h after feeding. About 90% of the recovered radioactivity was associated with the chylomicron fraction (d < 1.006 g/ml), 0.5% with the combined low and high density lipoprotein fraction (1.006 < d < 1.21 g/ml), while 9.5% was found in the protein fraction of d > 1.21 g/ml. No asterisked vitamin D₃ was detected, but in the lymph fraction with d > 1.21 g/ml significant amounts of the radioactivity co-chromatographed with 25-hydroxyvitamin D₃. When [3H]vitamin D₃-containing intestinal chylomicrons were incubated in vitro with plasma of d > 1.006 g/ml, the amount of radioactivity transferred from the chylomicrons to this plasma fraction was zero at 4 °C while up to 80% was transferred at 37 °C during 3 h of incubation. The transfer was time-dependent, and dependent on the ratio between the amount of chylomicrons and plasma with d > 1.006 g/ml. The transfer of labeled vitamin D₃ from intestinal lymph to the plasma fraction with d > 1.006 g/ml was also found in vitro in functionally hepatectomized rats. Both in vitro and in vivo, 91-95% of the transferred vitamin D₃ was recovered in the plasma fraction with d > 1.21 g/ml. Agarose gel electrophoresis of this fraction (d > 1.21 g/ml) indicated that 95% of the radioactivity was associated with the α-globulins. The transfer of vitamin D₃ from chylomicrons to α-globulin in lymph and blood plasma might be of physiological importance for targeting of vitamin D₃ absorbed from the intestine.

A significant amount of information has accumulated over the recent years concerning the metabolism of vitamin D₃. It is accepted that the liver is of major importance in the 25-hydroxylation of the vitamin (1). Furthermore, 25-hydroxyvitamin D₃ undergoes 1α-hydroxylation in the kidneys to form 1α,25-dihydroxyvitamin D₃, the biologically active form of the vitamin (2). Previous studies on the absorption of radioactive vitamin D₃ in rats have revealed that about 72% of the radioactivity recovered in thoracic duct lymph was associated with chylomicrons (3). In mammals, the plasma transport of vitamin D₃ and its metabolites is mostly carried out by α-globulins called the binding protein for vitamin D and its metabolites (4-9), identical to group specific component (7). This protein is present in the plasma fraction with d > 1.21 g/ml and the molecular weight is approximately 53,000 (6,8).

All plasma lipoproteins float at d > 1.21 g/ml in the ultracentrifuge (9). 25-Hydroxyvitamin D₃ and 24,25-dihydroxyvitamin D₃ have significantly higher affinity for the vitamin D-binding protein than that of 1α,25-dihydroxyvitamin D₃ and vitamin D₃ (4, 10, 11).

In this paper, we present data on the uptake of [3H]vitamin D₃ in rat intestinal lymph. Thereby, we avoid possible errors due to hepatic and peripheral lymph which are also drained by the thoracic duct. We also demonstrate transfer of [3H]vitamin D₃ from chylomicrons to plasma proteins both in vitro and in vivo in hepatectomized rats.

**MATERIALS AND METHODS**

[1α,25-(OH)₂] Vitamin D₃ (cholecalciferol, specific activity 16 Ci/mmol) was obtained from the Radiological Centre, Amersham, England. The purity of the compound was verified before use by HPLC (see below). Male Wistar rats, 300-500 g, were fed on ordinary lab chow. The animals were given water and food ad libitum until the experiments started about 9 a.m.

**Lymph Collection**—The operation was performed under ether anesthesia through an anterior midline incision as described earlier (12). A cannula of clear vinyl tube (medical grade, 0.5 mm, inner diameter, 0.8 mm, outer diameter, Dural Plastics and England Pty. Ltd, Dural, Australia) was inserted in the major intestinal lymph duct, anchored with sutures, and brought to the outside in the lower left quadrant of the abdomen. The accessory duct distal to the superior mesenteric artery was ligated. A polyethylene tube (PP 90, Portex Ltd, Hythe Kent, England) was inserted through the upper right quadrant of the abdomen transgastrically into the lumen of the duodenum and anchored correspondingly. The abdominal incision was closed and the rats were kept restrained in cages with free access to water. When acceptable lymph flow was obtained (approximately 200 μl/h) and within 2 h after the operation, the rats were given [3H] vitamin D₃ (3.2 x 10⁷ cpm) in 500 μl of soybean oil through the duodenal tubing. This initial injection was followed by two 100-μl rinses with soybean oil. After 24 h the rats were killed by puncture of the aorta under anesthesia. The lymph flow during the collection period was approximately 600 μl/h.

**Functional Hepatectomy**—This was performed under ether anesthesia by evisceration. The gastrointestinal tract including the pancreas and spleen was removed from the distal esophagus to the rectum. The hepatic artery and the portal vein were ligated. Thus, all the blood flow into the liver hilus via the hepatic artery and portal vein was excluded (13). Rat intestinal lymph (0.5-0.8 ml) containing about 10⁵ cpm [3H]vitamin D₃ was then injected through the right femoral vein and the animals were killed by aortic puncture and exsanguination after 15 and 30 min, respectively.

**Separation of Lipoproteins**—Intestinal lymph and blood plasma were centrifuged at 4 °C in a Beckman L2-65 B ultracentrifuge using liquid chromatography.
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a 40.3 or Ti-60 rotor at 35,000 rpm. Chylomicrons were centrifuged at $d = 1.006$ g/ml for 20 h as reported by Havel et al. (9). The combined fraction of low and high density lipoproteins was isolated at $d = 1.21$ g/ml for 48 h at 35,000 rpm. The density was adjusted with potassium bromide.

**Incubation Experiments**—Intestinal lymph chylomicrons labeled with [3H]vitamin D$_3$, and isolated by preparative ultracentrifugation as stated above were incubated with plasma fractions with $d > 1.006$ g/ml for 48 h at 35,000 rpm. The density was adjusted with potassium bromide. Incubation Experiments—Intestinal lymph chylomicrons labeled with [3H]vitamin D$_3$, and isolated by preparative ultracentrifugation as stated above were incubated with plasma fractions with $d > 1.006$ g/ml from time 0 to 3 h. The incubations took place at 37 °C in a shaking water bath in the dark. After incubation, the samples were recentrifuged (+4 °C) at $d > 1.21$ g/ml. In several experiments the infranatants of the $d = 1.006$ g/ml were recentrifuged at $d = 1.21$ g/ml for 48 h. The top fraction of this centrifugation contained the intestinal chylomicrons, while the bottom fraction with $d > 1.006$ g/ml contained the nonlipoprotein plasma proteins, e.g. albumin and globulins. Both the top and bottom fractions from these two centrifugations were extracted, and the extracts were chromatographed by HPLC and assayed for radioactivity (see below). The samples were stored in the dark at -10 °C under N$_2$.

**Extraction, Chromatographic, and Analytical Procedures**—The samples were extracted with 20 volumes of chloroform/methanol (2:1, v/v) (14). The chloroform phase was evaporated under Nz at 40 °C, v/v, and the residue redissolved in a small volume of methanol. The entire sample was injected into an HPLC instrument (Spectra Physics, Santa Clara, CA) equipped with a Rheodyne loop (100 µl) injector and a Zorbax ODS column (4.6 × 250 mm). The eluting solvent was 1.5% H$_2$O in methanol at a flow rate of 1 ml/min. In this system, the retention time of 25-hydroxyvitamin D$_3$ was 6.3 min and that of vitamin D$_3$ was 19 min. The fraction corresponding to 25-hydroxyvitamin D$_3$ was rechromatographed on a Zorbax-Sil column (2.1 × 250 mm; particle size, 5 µm). The solvent was 2.5% isopropyl alcohol in hexane, and the flow rate was 0.8 ml/min. The retention time of authentic 25-hydroxyvitamin D$_3$ in the system was 10.7 min. In some experiments, the radioactive purity of the vitamin D$_3$ fraction that eluted from the ODS column was verified by rechromatography on a Spherisorb-Silica column (4.6 × 250 mm; particle size, 5 µm) with 1.75% isopropyl alcohol in hexane as solvent (1 ml/min). The retention time of vitamin D$_3$ in this system was 8.2 min. During the chromatography, one fraction was collected per min. After addition of 4 ml of counting solution the radioactivity was determined in a liquid scintillation counter. Quenching was corrected for by use of [1,2α-3H]cholesterol as internal standard. Recovery of radioactivity during chromatography was essentially complete. Protein was measured according to Lowry et al. (15) using bovine serum albumin as standard. Triacylglycerol was determined by a fluorometric method (16) adapted for the Auto-Analyzer. The bottom fractions of serum and lymph with $d > 1.21$ g/ml were separated on agarose gel electrophoresis as described by Johansson (17).

**RESULTS**

**Absorption of Vitamin D$_3$ via Intestinal Lymph**—A cumulative plot shows that 41 ± 7% (n = 4) of the [3H]vitamin D$_3$ given through the duodenal tube was recovered in the intestinal lymph collected during the first 24 h (Fig. 1). No significant amount of radioactivity was detected in the initial 2 h after duodenal tube feeding. The appearance of radioactivity in intestinal lymph peaked 4–8 h after feeding and very little appeared after 18 h. 90 ± 3% of the radioactivity recovered in the lymph was associated with the chylomicron fraction as determined by ultracentrifugation at $d = 1.006$ g/ml. 0.5% of the radioactivity was recovered in the combined low and high density lipoprotein fraction (1.006 < $d < 1.21$ g/ml) and 9.5% in the protein fraction of $d > 1.21$ g/ml.

**Appearance of Different Vitamin D$_3$ Metabolites in Intestinal Lymph**—When extracts of the lymph were subjected to reversed phase HPLC (ODS column) 100% of the radioactivity was recovered in the fractions of vitamin D$_3$ and more polar material (Table I, Fig. 2). This suggests that no esterification of vitamin D$_3$ occurs during absorption. In both the chylomicrons and the fraction with $d > 1.006$ g/ml, most of the radioactivity appeared as vitamin D$_3$ by reversed phase HPLC. In the lymph fraction with $d > 1.006$ g/ml, however, a significant amount of radioactivity co-chromatographed with 25-hydroxyvitamin D$_3$ (Table I, Fig. 2). By rechromatography of this fraction on the Zorbax-Sil column, the radioactivity had the same retention time as authentic 25-hydroxyvitamin D$_3$.

**Transfer of Labeled Vitamin D$_3$ from Lymph Chylomicrons to Plasma Proteins in Vitro**—Chylomicrons containing [3H]vitamin D$_3$ were obtained by intestinal lymph duct cannulation of the tube-fed male rats as stated under “Materials and Methods.” These chylomicrons were then incubated with rat plasma fraction with $d > 1.006$ g/ml. At 4 °C, no radioactive transfer was transferred from chylomicrons to plasma with $d > 1.006$ g/ml. At 37 °C, however, the transfer of vitamin D$_3$ from intestinal chylomicrons to plasma with $d > 1.006$ g/ml was significant and reached a plateau after 3 h of incubation (Fig. 3). At any time from 10 min to 3 h it was observed that 90 ± 7% (n = 10) of the radioactivity recovered in the plasma fraction with $d > 1.006$ g/ml was bound to the plasma protein fraction of $d > 1.21$ g/ml. This suggests a direct transfer of vitamin D$_3$ from the chylomicrons to the protein fraction with $d > 1.21$ g/ml.

**FIG. 1.** Total radioactivity recovered in rat intestinal lymph after 3.2 × 10$^6$ cpm (180 pmol) of [3H]vitamin D$_3$ dissolved in 0.5 ml of soybean oil was given through a duodenal tube. Data from one typical rat are presented.

**TABLE I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vitamin D$_3$</th>
<th>25-Hydroxyvitamin D$_3$</th>
<th>Recovery of radioactivity after intraduodenal feeding of [3H]vitamin D$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/ml</td>
<td>% of total radioactivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d &lt; 1.006</td>
<td>90.0 ± 13.1</td>
<td>0.8 ± 0.9</td>
<td>104.2 ± 13.0</td>
</tr>
<tr>
<td>d &gt; 1.006</td>
<td>79.0 ± 18.0</td>
<td>8.0 ± 4.0</td>
<td>98.3 ± 10.7</td>
</tr>
</tbody>
</table>
There was an increase in the transfer of vitamin D$_3$ from chylomicrons to the plasma with $d > 1.006$ g/ml when increasing amounts of the plasma fraction were incubated with a constant amount of chylomicrons (Fig. 4). A saturation level was reached when about 200 $\mu$l of chylomicrons containing 6.7 $\mu$mol of triglyceride were incubated with 115 mg of protein of the plasma fraction with $d > 1.006$ g/ml. Up to about 80% of the $[^3H]$vitamin D$_3$ was transferred to plasma with $d > 1.006$ g/ml after 3 h of incubation.

Of all the radioactivity recovered in the fraction with $d > 1.006$ g/ml after 3 h of incubation at 37 °C, 95 ± 2% ($n = 5$) was recovered in the plasma protein fraction with $d > 1.21$ g/ml. 78% of the recovered radioactivity was located in the $\alpha_2$-globulin fraction (see below). All the radioactivity transferred from lymph chylomicrons to plasma proteins in vitro was identified as unesterified vitamin D$_3$.

Transfer of $[^3H]$Vitamin D$_3$ from Chylomicrons to Plasma Proteins In Vivo—Intestinal lymph chylomicrons labeled with $[^3H]$vitamin D$_3$ were injected intravenously into functionally hepatectomized rats. 15 and 30 min after injection, 49.8 and 69.2% of the radioactivity, respectively, had been transferred to the plasma fraction with $d > 1.006$ g/ml (Table II). More than 91% of the radioactivity recovered in the $d > 1.006$ g/ml was localized to the plasma protein fraction with $d > 1.21$ g/ml. No radioactivity corresponding to 25-hydroxyvitamin D$_3$ was recovered in any fraction.

Agarose Gel Electrophoresis of Intestinal Lymph and Plasma Protein Fraction with $d > 1.21$ g/ml—When the intestinal lymph fraction with $d > 1.21$ g/ml from animals fed $[^3H]$vitamin D$_3$ through the duodenal tube was subjected to agarose gel electrophoresis, 78% of the recovered radioactivity co-migrated with $\alpha_2$-globulins and 17% was associated with the $\alpha_1$-globulins (Fig. 5A). When intestinal chylomicrons labeled with $[^3H]$vitamin D$_3$ were incubated with plasma ($d > 1.006$ g/ml) and the protein fraction with $d > 1.21$ g/ml of this incubation mixture was electrophoresed, 92% of the activity was detected in the $\alpha_2$-globulins and less than 4% in any of the other plasma protein fractions (Fig. 5B). Intestinal chylomicrons labeled with $[^3H]$vitamin D$_3$ were also injected intravenously to functionally hepatectomized rats. After 15 and 30 min, blood plasma was centrifuged at $d = 1.21$ g/ml and the bottom fraction was electrophoresed. 78% of the recovered radioactivity was located in the $\alpha_2$-globulin fraction and 9% was in the $\alpha_1$-globulin fraction (Fig. 5C).

![Distribution of radioactivity in extracts of intestinal lymph with $d < 1.006$ g/ml (chylomicrons, dotted line) and with $d > 1.006$ g/ml (solid line). The experiment was performed as described in the legend to Table I.](http://www.jbc.org/)

![Effect of time on the transfer in vitro of $[^3H]$vitamin D$_3$ from chylomicrons to the plasma fraction with $d > 1.006$ g/ml. Intestinal lymph chylomicrons labeled with 27,800 cpm of $[^3H]$vitamin D$_3$ and containing 5.00 $\mu$mol of triglycerides were incubated with 14.4 mg of a plasma fraction ($d > 1.006$ g/ml) at 37 °C for different periods of time. The incubation mixtures were ultracentrifuged and the percentage of radioactivity recovered in the plasma fraction of $d > 1.006$ g/ml was determined.](http://www.jbc.org/)

### Table II

<table>
<thead>
<tr>
<th>Time</th>
<th>Chylomicrons + VLDL ($d &lt; 1.006$ g/ml)</th>
<th>LDL + HDL ($1.006 &lt; d &lt; 1.21$ g/ml)</th>
<th>Plasma proteins ($d &gt; 1.21$ g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>% of total radioactivity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>50.2</td>
<td>2.9</td>
<td>46.9</td>
</tr>
<tr>
<td>30</td>
<td>30.8</td>
<td>5.8</td>
<td>63.6</td>
</tr>
</tbody>
</table>
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Fig. 5. Agarose gel electrophoresis of fractions with d > 1.21 g/ml. A, intestinal lymph collected from 2 to 18 h after intraduodenal instillation of 3.2 × 10⁶ cpm [³H]vitamin D₃ dissolved in 0.5 ml of soybean oil. After stepwise ultracentrifugation of the lymph at d = 1.006 and d = 1.21 g/ml the bottom fraction of the latter was electrophoresed and the distribution of radioactivity determined. B, intestinal lymph chylomicrons labeled with [³H]vitamin D₃ (25,200 cpm and 16.7 μmol of triglycerides) were incubated with a plasma fraction of d > 1.006 g/ml (21 mg of protein) for 3 h at 37 °C in a shaking water bath. The incubation mixture was ultracentrifuged at d = 1.006 g/ml (+4 °C) and then the bottom fraction (d > 1.006 g/ml) was adjusted to d = 1.21 g/ml and recentrifuged. The bottom fraction (d > 1.21 g/ml) of the latter was electrophoresed and the distribution of radioactivity determined. C, intestinal lymph chylomicrons labeled with [³H]vitamin D₃ (186,700 cpm and 160.8 μmol of triglycerides) were injected intravenously into two functionally hepatectomized rats. 15 and 30 min after the injection, plasma samples were taken and stepwise ultracentrifugation at d = 1.006 g/ml and d = 1.21 g/ml was performed at 4 °C. Samples from the bottom fraction (d > 1.21 g/ml) were electrophoresed and the distribution of radioactivity determined. Almost identical distribution was obtained at 15 and 30 min and the means of the data are shown.

DISCUSSION

In this report we have shown that radioactive vitamin D₃ is absorbed from rat intestine via the lymph and 90% is associated with chylomicrons (d < 1.006 g/ml). These findings confirm the previous results of Schachter et al. (3) who found that about 72% of the radioactivity was associated with the chylomicron fraction in thoracic duct lymph. The quantitative difference in the results may be explained by the different methods used for isolation of chylomicrons. In contrast to the high amounts of [³H]vitamin D₃ associated with chylomicrons, only trace amounts (0.5%) of the radioactivity were recovered in intestinal lymph associated with low and high density lipoproteins. The remainder of the lymph radioactivity was found in the fraction of d > 1.21 g/ml that electrophoretically corresponded to α-globulin.

While only unchanged [³H]vitamin D₃ was recovered in the chylomicron fraction of intestinal lymph, a significant amount of radioactive 25-hydroxyvitamin D₃ was found in the fraction of d > 1.21 g/ml (Fig. 2). One possible origin of the 25-hydroxyvitamin D₃ is the intestinal mucosal cells, although we cannot exclude the possibility that some vitamin D₃ is transported via small unligated lymph ducts to the liver where it could be hydroxylated in the 25-position. The liver might then release the radiolabeled 25-hydroxyvitamin D₃, which could be transferred from blood plasma to the intestinal lymph. A third possibility might be that some 25-hydroxyvitamin D₃ could be excreted with the bile (18) and reabsorbed by the intestinal mucosal cells (19, 20).

So far, very little information has been published on how vitamin D₃ is distributed when intestinal lymph chylomicrons mix with blood. We have demonstrated transfer of vitamin D₃ from chylomicrons to the plasma α-globulin fraction both in vitro (Figs. 3 and 4) and in vivo. To demonstrate transfer of [³H]vitamin D₃ in vivo we injected prelabelled intestinal lymph chylomicrons intravenously to hepatectomized rats, and about 50 and 70% of the radioactive in plasma was transferred to the plasma fraction with d > 1.006 g/ml after 15 and 30 min, respectively, with the predominant amount of radioactivity located in the plasma fraction with d > 1.21 g/ml. From our agarose gel electrophoresis data on both lymph and plasma fractions of d > 1.21 g/ml it is evident that most of the radioactive vitamin D₃ is bound to the α-globulins (Fig. 5A).

This was also true for the transferred vitamin D₃, both in vitro (Fig. 5B) and in vivo in functionally hepatectomized animals (Fig. 5C). The α-globulin fraction, responsible for carrying vitamin D₃, is presumably the well characterized binding protein for vitamin D and its metabolites (DBP) (4-6, 8).

The transport of vitamin D₃ has some features which are analogous to that of vitamin A but differs in several important respects. As fat-soluble substances, both vitamin D₃ and vitamin A are absorbed via the lymphatic route mainly in association with chylomicrons (21, 22) and they both have a carrier protein in plasma. During absorption, almost all vitamin A is esterified in the mucosal cells (21-23) and the apolar retinyl esters are probably incorporated in the chylomicron core. These esters are not removed from the chylomicron particle during in vitro incubations (24). In vivo, they seem to follow the chylomicron remnant to the liver (25). Release of vitamin A from the liver storage is a well regulated process with unesterified retinol attached to the retinol-binding protein.

In accordance with previous work (26) we have shown in this paper that all vitamin D₃ transported in lymph is unesterified. This makes it probable that the molecule is located
at the chylomicron surface and thus available for transfer to unfilled binding sites on the binding protein for vitamin D and its metabolites (4, 10, 11) as suggested by our in vitro and in vivo experiments.

When lymph chylomicrons are mixed with plasma in vitro, transfer of surface components like apoproteins, phospholipids, and unesterified cholesterol takes place between the different lipoproteins (27–30). Since vitamin D₃ has a specific binding protein, it was not surprising that in vitro transfer took place from chylomicrons to this protein. From experiments with functionally hepatectomized rats we could demonstrate that transfer also took place in vivo. When chylomicrons are subjected to lipolysis catalyzed by lipoprotein lipase (31), both the core material (triglycerides) and surface material (phospholipids, unesterified cholesterol, and most apoproteins) will decrease (32). It is likely that some vitamin D₃ also leaves the chylomicron particle during lipolysis. The resulting chylomicron remnants are taken up via receptor-mediated endocytosis in the liver (33–35) and vitamin D₃ remaining on the remnant particle is probably internalized in the hepatocytes at the same time. The vitamin D₃ transferred to the α-globulins might, however, have a different pathway ending up in other cell types or organs.

The formation of chylomicron remnants and their clearance by the liver takes place at a high rate (36). The physiological significance of vitamin D₃ transfer to α-globulins therefore depends on the transfer rate. Assuming that our in vitro data applies to the in vivo situation, we can estimate that there is capacity to transfer approximately 0.3 pmol of vitamin D₃ per min from chylomicrons to α-globulins in a rat with body weight of 390 g. During absorption of a physiological dose of vitamin D₃ (180 pmol) 0.3 pmol is absorbed/min assuming complete absorption during at least 10 h. From these estimates it is possible that a considerable amount of vitamin D₃ is transferred to plasma α-globulins when lymph chylomicrons mix with blood.

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