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LYMPHATIC ABSORPTION AND TRANSPORT OF RETINOL AND VITAMIN D-3 FROM RAT INTESTINE

EVIDENCE FOR DIFFERENT PATHWAYS

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The lymphatic absorption and transport of retinol and vitamin D-3 from rat intestine has been studied. When rats were cannulated in the intestinal lymph duct and given an intraduodenal bolus of [^{3}H]retinol and ^{14}C-labelled vitamin D-3, ^{14}C-labeled vitamin D-3 appeared later in the intestinal lymph than [^{3}H]retinol and the rate of absorption of vitamin D-3 was still maximal at a time when that of retinol had declined. Both vitamins were absorbed via the lymphatic route in association with chylomicrons. Almost all the retinol was esterified, while vitamin D-3 appeared in the chylomicrons as free vitamin D-3. In vitro incubations and in vivo studies using hepatectomized and normal rats showed that the retinyl ester was a relatively nonexchangeable component of the chylomicrons and their remnants. Hence, all the vitamin A followed the remnants in their clearance from plasma. In contrast, significant amounts of vitamin D-3 were transferred from the chylomicrons to other plasma fractions. Therefore, only a fraction of this vitamin may be removed in association with the chylomicron remnants.

Introduction

Vitamin A (retinol) and vitamin D-3 (cholecalciferol) are both absorbed in the small intestine and transported via the lymphatic route in chylomicrons [1–4]. Absorption studies have revealed that most of the vitamins recovered in lymph chylomicrons are in the form of retinyl ester [5–7] and free vitamin D-3 [4,8]. The esterification of retinol is catalyzed by a mucosal acyl-CoA : retinol acyltransferase [7]. Previous absorption studies [1–6] were done using thoracic duct lymph. In the present paper, we have studied the lymphatic uptake and transport of retinol and vitamin D-3 from the intestine in order to avoid possible errors due to hepatic and peripheral lymph which are also drained by the thoracic duct.

Zilversmit and collaborators [9–10] studied the exchange of retinyl ester between lipoproteins of rabbit plasma. They found that retinyl ester constitutes a relatively nonexchangeable marker for the chylomicrons in vitro. Recently we studied [8] the distribution of vitamin D-3 after mixing intestinal lymph with rat plasma. We demonstrated

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Abbreviations: HPLC, high-performance liquid chromatography; I.U., international units: LDL, low density lipoproteins; HDL, high density lipoproteins.

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transfer of vitamin D-3 from the chylomicrons to the plasma α-globulin fraction both in vitro [8] an in vivo [11].

The aim of the present study was to compare lymphatic absorption and transport of retinol and vitamin D-3 from rat intestine. The data presented suggest that the pathways differ to some extent in the mucosal cells, and diverge during chylomicron metabolism.

Materials and Methods

Chemicals. [15-^{3}H(N)]Retinol (all *trans*) (14.3 Ci/mmol) and (4-^{14}C)-labeled vitamin D-3 (cholecalciferol) (54.2 mCi/mmol) were obtained from New England Nuclear, Boston, MA and Amersham International, plc., Amersham, U.K., respectively.

Animals. Male Wistar rats (250–350 g) were fed an ordinary pellet diet (no. 3155, AREX, Møllesentralen, Norway) which contains about 9000 I.U. retinol (50% retinyl acetate and 50% retinyl palmitate) and 1500 I.U. vitamin D-3 per kg.

Lymph collection. The operation was performed under ether anaesthesia through an anterior midline incision as described earlier [8,12]. When acceptable lymph flow was obtained and within 2 h after the operation, rats were given [^{3}H]retinol and/or ^{14}C-labeled vitamin D-3 dissolved in soybean oil through a duodenal tubing [8]. The lymph was collected at room temperature and allowed to clot. Defibrination was therefore performed before analysis or experimental use. During the first 20 h, 155 ± 72 (S.D.) mg ($N = 6$) triacylglycerol was found in the lymph collected.

Functional hepatectomy. This was performed under ether anaesthesia 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) was then injected through the right femoral vein.

Separation of lipoproteins. Intestinal lymph and blood plasma were centrifuged at 4°C in a Beckman L2-65 B ultracentrifuge using a 40.3 or Ti-60 rotor at 35000 rpm. Chylomicrons were centrifuged at $d = 1.006$ g/ml for 20 h as reported by Havel et al. [14]. The combined fraction of LDL and HDL was isolated at $d = 1.21$ g/ml for 48 h. The density was adjusted by potassium bromide.

Extraction, chromatography and analytical procedures. The samples were extracted with 20 volumes of chloroform/methanol (2 : 1, v/v) [15]. The chloroform phase was evaporated under N_2 and the residue was redissolved in a small volume of methanol or hexane. 0.1–0.5-ml samples were mixed with 7 ml Instagel II (Packard Instruments Co.) for determination of radioactivity in a Packard Tri-Carb liquid scintillation counter. ^{3}H-containing samples and ^{14}C-containing samples were counted at about 50% and 65% efficiency, respectively. The data were corrected for ^{14}C activity counted in the tritium channel. Ratio of esterified and free [^{3}H]retinol was either determined by alumina column chromatography [2] or by high performance liquid chromatography (HPLC) [7]. Vitamin D-3 and its metabolites were separated by HPLC [8]. Triacylglycerol was determined enzymatically [16] and retinol was determined by the trichloroacetic acid method [17].

Results

Absorption of retinol and vitamin D-3 via intestinal lymph

The appearance of radioactivity in intestinal lymph after duodenal feeding of [^{3}H]retinol is shown in Fig. 1. The time-course of lymphatic absorption showed the same profile whether rats were fed 6000 I.U. or 600 I.U. retinol together with a constant lipid vehicle. The label started to appear in lymph 0.5 h after the radioactivity was given intraduodenally. There was a fairly constant absorption of retinol during the following 10 h, and it was completed within 12 h. Regardless of the amount fed, a considerable variation in the recovery of radioactivity was observed. The percentage of radioactivity recovered in the collected intestinal lymph after 18–24 h was 31.6 ± 10.2 (S.D.) % ($N = 5$) and 42.5 ± 24.8 (S.D.) % ($N = 6$) after feeding 6000 I.U. and 600 I.U. retinol, respectively.

When [^{3}H]retinol and ^{14}C-labeled vitamin D-3 were given simultaneously to rats through the duodenal tubing, the labeled retinol appeared in the intestinal lymph before the labeled vitamin

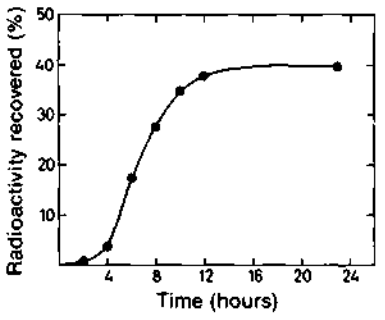

Fig. 1. Total radioactivity recovered in intestinal lymph after feeding [^{3}H]retinol. A rat was fed 600 I.U. [^{3}H]retinol (49 μCi) dissolved in 300 μl soybean oil, and the intestinal lymph was collected.

D-3 (Fig. 2A). After 10 h, the absorption of vitamin D-3 was still going on while the retinol absorption had almost stopped. The difference in absorption of the two vitamins are clearly shown by a decrease in the ratio of ^{3}H to ^{14}C in the lymph with time (Fig. 2B).

Retinol and vitamin D-3 in intestinal lymph

99.3 ± 0.2 (S.D.) % (N = 12) of the absorbed radioactivity from [^{3}H]retinol was associated with chylomicrons when a low dose of retinol (600 I.U.) was given to the animals. In rats fed 6000 I.U. of

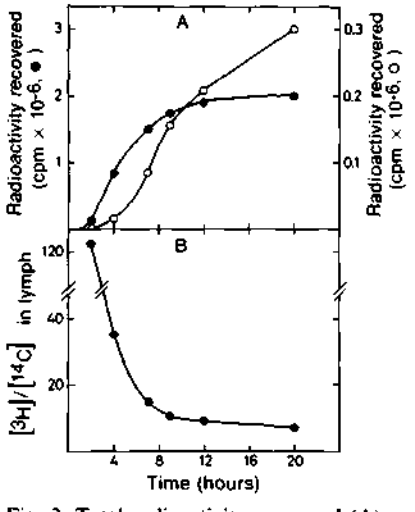

Fig. 2. Total radioactivity recovered (A) and ratio of [^{3}H] to [^{14}C] (B) in intestinal lymph after feeding [^{3}H]retinol and ^{14}C-labeled vitamin D-3 simultaneously. A rat was fed 64 μCi of [^{3}H]retinol (●) and 5 μCi of ^{14}C-labeled vitamin D-3 (○) dissolved in 100 μl soybean oil and the intestinal lymph was collected. The ratio of ^{3}H to ^{14}C is shown in B (■).

retinol, 96.9 ± 1.0 (S.D.) % (N = 4) of the radioactivity was recovered in the chylomicron fraction of the lymph. Thus, the chylomicron association of retinol was fairly independent of the amount of retinol fed.

Lipid extracts of intestinal lymph obtained after feeding [^{3}H]retinol were subjected to alumina column chromatography. Most of the radioactivity was found in the retinyl ester fraction. When rats were given the smallest (and most physiological) dose of retinol (600 I.U.), 99.0 ± 0.4 (S.D.) % (N = 6) of the retinol was esterified and no difference was observed throughout the absorption period. A slightly smaller percent (94.4 ± 1.0 (S.D.) % (N = 6)) was recovered in the retinyl ester fraction when 6000 I.U. retinol was fed.

Extracts of intestinal lymph obtained from rats after feeding 600 I.U. of [^{3}H]retinol were also applied to a HPLC column (Fig. 3). Very little radioactivity comigrated with the retinol standard, whereas 60–70% of the radioactivity had the same retention time as the retinyl palmitate standard. About 20% of the radioactivity had a longer retention time than the retinyl palmitate, suggesting that some of the retinol was esterified with fatty acids other than palmitic acid [5].

When double labeled intestinal lymph ([^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3) was ultracentrifuged, more than 98% of retinol and about 95% of vitamin D-3 was recovered in the chylomicron fraction. Only small amounts (0.8–0.9%) of retinol and vitamin D-3 were re-

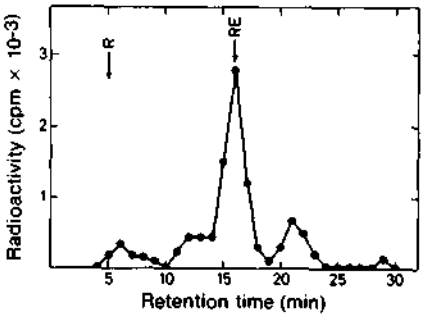

Fig. 3. Distribution of [^{3}H]retinol in extracts of intestinal lymph. A rat was fed 600 I.U. [^{3}H]retinol (49 μCi) dissolved in 300 μl soybean oil and the intestinal lymph was collected and extracted as described under Materials and Methods. Lipid residues were dissolved in methanol and analysed by HPLC with Spherisorb ODS column. Retinol (R) and retinyl palmitate (RE) were used as standards.

covered in the combined LDL and HDL fraction. 4–5% of vitamin D-3 and about 1% of retinol were recovered in the $d > 1.21$ g/ml fraction of the intestinal lymph.

Extracts of double labeled lymph were analysed by HPLC. No ^{3}H in the chylomicron fraction comigrated with the retinol standard. In contrast, almost all the ^{14}C was recovered as free vitamin D-3, suggesting that nearly all the retinol but no vitamin D-3 was esterified (Fig. 4A).

In lymph fractions with $d > 1.006$ g/ml, about 15% of the retinol radioactivity was identified as free alcohol. However, only about 2% of the total lymph retinol content is found in this fraction (Fig. 4B).

Again, in lymph fractions with $d > 1.006$ g/ml, 93% of the ^{14}C was recovered as free vitamin D-3, while about 7% of ^{14}C comigrated with the 25-hydroxyvitamin D-3 standard (Fig. 4B).

Plasma clearance of chylomicron [^{3}H]retinyl ester

Lymph containing [^{3}H]retinyl ester in the chylomicron fraction was injected intravenously into rats, and the radioactivity in plasma was followed. Rats were given lymph containing various doses of triacylglycerol and retinyl ester. Fig. 5 shows that removal from plasma was rapid, and relatively independent of both the triacylglycerol and retinyl ester concentrations. Increasing the triacylglycerol concentration from 0.5 mg to 50 mg, and the retinyl ester concentration from 3.3 I.U. to 720 I.U. did not change the half-time of the label in plasma, which in all experiments was about 10 min.

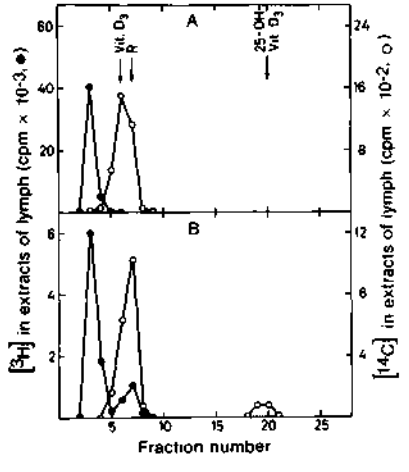

Fig. 4. Distribution of radioactivity in extracts of intestinal lymph with $d < 1.006$ g/ml (A) and $d > 1.006$ g/ml (B) after feeding [^{3}H]retinol and ^{14}C-labeled vitamin D-3 simultaneously. A rat was fed 64 μCi of [^{3}H]retinol and 5 μCi of ^{14}C-labeled vitamin D-3 dissolved in 100 μl soybean oil and the intestinal lymph was collected and ultracentrifuged at $d = 1.006$ g/ml. The top fraction (chylomicrons) and the bottom fraction ($d > 1.006$ g/ml) were extracted, dissolved in methanol and subjected to HPLC with a Zorbax Silica column [8].

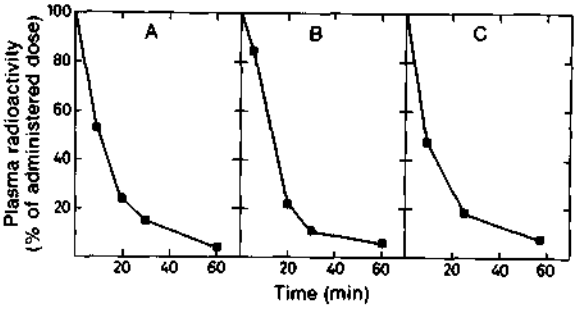

Fig. 5. Disappearance of chylomicron [^{3}H]retinyl ester from plasma. Lymph containing 0.5 mg triacylglycerol and 3.3 I.U. retinol (A), 4.8 mg triacylglycerol and 3.8 I.U. retinol (B), or 50 mg triacylglycerol and 720 I.U. retinol (C) was injected intravenously into rats, and radioactivity in plasma was determined after different periods of time. The total plasma volume was assumed to be 3.2% of body weight [2].

Plasma clearance of lymph labeled with [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3

In order to compare the plasma decay of chylomicron retinyl ester and chylomicron vitamin D-3, we injected double labeled lymph intravenously into rats. Fig. 6 shows that the removal from plasma was almost identical for the two vitamins, with $t_{1/2}$ about 6 min. The plateau of ^{3}H in plasma was about 20% of the administered dose. In most other experiments this plateau was only about 10% of the administered dose (see Fig. 5).

Aliquots of plasma were ultracentrifuged at $d = 1.006$ g/ml at different times after injection of the lymph. Fig. 7 shows the distribution of radioactivity recovered in the fraction with $d > 1.006$ g/ml. Of the radioactivity found in plasma, 10–20% of [^{3}H]retinol and 80–90% of ^{14}C-labeled vitamin D-3 was recovered in this fraction. Moreover, vitamin D-3 appeared in this fraction as soon as 2 min after the intravenous injection.

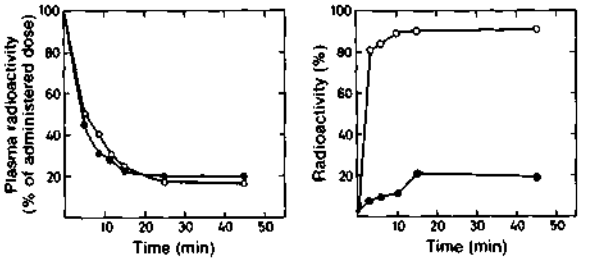

Fig. 6. Clearance of chylomicron [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3 from plasma. 0.5 ml of labeled lymph (0.29 μCi of [^{3}H]retinol (●) and 0.02 μCi of ^{14}C-labeled vitamin D-3 (○)) containing 9.2 mg triacylglycerol was injected intravenously into rats and radioactivity in plasma was determined after different periods of time. The total plasma volume was assumed to be 3.2% of body weight [2].

Fig. 7. Percent of radioactivity recovered in the plasma fraction with $d > 1.006$ g/ml after intravenous injection of lymph labeled with [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3. 0.4 ml of labeled lymph (0.23 μCi of [^{3}H]retinol (●) and 0.03 μCi of ^{14}C-labeled vitamin D_3 (○)) containing 7.4 mg triacylglycerol was injected intravenously into rats. Plasma samples were ultracentrifuged at $d = 1.006$ g/ml after different periods of time.

Exchange of [^{3}H]retinyl esters between lipoproteins in vivo

Lymph containing [^{3}H]retinyl ester in the chylomicrons were injected intravenously into functionally hepatectomized rats. The animals were killed after 30 or 60 min. 50–80% of the radioactivity was recovered in the plasma. Less than 2% of the radioactivity was recovered in the liver verifying that the liver was almost out of function. About 90% of the radioactivity recovered in the plasma was associated with the $d < 1.019$ g/ml fraction, indicating a small transfer of labeled vitamin A from chylomicrons and their remnants to other lipoproteins in vivo (Table I).

About 10% of plasma radioactivity was found in the combined LDL and HDL fraction . In the fractions with $d < 1.019$ g/ml and $d > 1.019$ g/ml, 96.1 ± 3.7 (S.D.) % ($N = 9$) and 93.1 ± 2.3 (S.D.) % ($N = 4$) of the radioactive retinol was esterified, respectively.

Transfer of [^{3}H]retinyl ester from lymph chylomicrons to plasma fractions in vitro.

Chylomicrons containing [^{3}H]retinyl ester were prepared from intestinal rat lymph after duodenal tube-feeding of 600 I.U. retinol. Transfer of radioactivity from these chylomicrons to normal rat plasma fraction with $d > 1.006$ g/ml were then tested during in vitro incubations at 37°C. As shown in Fig. 8, transfer of about 2% was found after 10–30 min. In control incubations with 0.9% NaCl instead of plasma, only 0.5% transfer of radioactivity was found. The amount of chylomicron triacylglycerol used in this experiment was 1.17 mmol/l. With prolonged incubation time up to 60 min or threefold increase in the plasma protein fraction, still only 4–7% of the radioactivity was transferred to the plasma fraction with $d > 1.006$ g/ml.

TABLE I

DENSITY DISTRIBUTION OF RADIOACTIVITY IN PLASMA AFTER INTRAVENOUS INJECTION OF CHYLOMICRON [^{3}H]RETINYL ESTER INTO FUNCTIONALLY HEPATECTOMIZED RATS

Lymph containing 3.8–34.0 I.U. retinol and 2.3–4.8 mg triacylglycerol were injected intravenously into functionally hepatectomized rats. The plasma from the rats were analysed after 30 min. Values represent mean ± S.D. of the given number of rats.

Fraction	Percent of total
$d < 1.019$ g/ml	87.8 ± 5.1 ($N = 9$)
1.019 g/ml < d < 1.21 g/ml	9.1 ± 2.3 ($N = 4$)
$d > 1.21$ g/ml	1.6 ± 1.4 ($N = 4$)

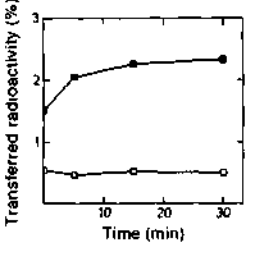

Fig. 8. Transfer of [^{3}H]retinol from chylomicrons to the plasma fraction with $d > 1.006$ g/ml in vitro. 0.3 ml labeled lymph chylomicrons (0.5 μCi of [^{3}H]retinol) containing 2.7 mg triacylglycerol were incubated with 2 ml of rat plasma (■) or 2 ml of 0.9% NaCl (□) at 37°C for different periods of time. The incubation mixtures were ultracentrifuged and the percentage of radioactivity recovered in the bottom fraction ($d > 1.006$ g/ml) was determined.

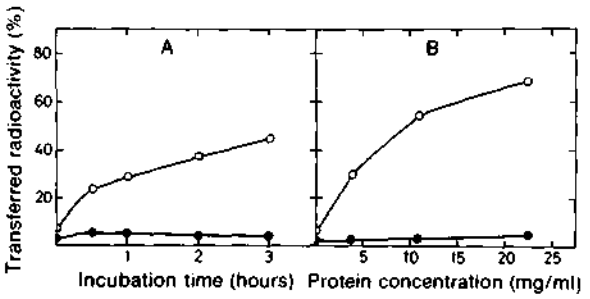

Fig. 9. In vitro transfer of [^{3}H]retinol and ^{14}C-labeled vitamin D-3 from chylomicrons to the plasma fraction with $d > 1.006$ g/ml as a function of incubation time (A) and protein concentration (B). Lymph chylomicrons containing 4.9 mg triacylglycerol and labeled with 0.2 μCi [^{3}H]retinol (●) and 0.01 μCi ^{14}C-labeled vitamin D-3 (○) were incubated at 37°C with the protein fraction with $d > 1.006$ g/ml in a final volume of 1.0 ml. At the end of the incubation, the mixtures were ultracentrifuged at $d = 1.006$ g/ml and the percent of radioactivity transferred to the fraction with $d > 1.006$ g/ml was determined. For the time course shown in A, protein concentrations of 8.0 mg/ml were used. For the data presented in B the incubation times were 3 h.

Transfer of [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3 from double labeled chylomicrons to plasma fractions in vitro

Intestinal lymph chylomicrons labeled with [^{3}H]retinyl ester and ^{14}C-labeled vitamin D-3 were incubated with a plasma fraction ($d > 1.006$ g/ml) at 37°C for different periods of time. The incubation mixture was ultracentrifuged and percentage of radioactivity of the two isotopes recovered in the fraction with $d > 1.006$ g/ml was determined. With increasing incubation time, we observed an increase in the amount of vitamin D-3 transferred. After 3 h, about 50% of the radioactivity from vitamin D-3 was recovered in the $d > 1.006$ g/ml fraction (Fig. 9A). Again, there was no increase in retinyl ester transfer with time, and less than 4% of the retinyl esters were transferred to the $d > 1.006$ g/ml fraction.

When incubation mixtures with increasing plasma protein concentrations were incubated at 37°C for 3 h, the amount of transferred vitamin D-3 increased up to 70% of the total vitamin D-3. However, no increase in the transfer of retinyl ester to the $d > 1.006$ g/ml fraction was observed with increasing plasma protein concentrations (Fig. 9B).

Discussion

The data presented here suggest that the absorption of the vitamins A and D-3 in gut differ to some extent. When the two labeled vitamins were given simultaneously, ^{14}C-labeled vitamin D-3 appeared later in the intestinal lymph than [^{3}H]retinol and the rate of absorption of vitamin D-3 was still maximal at a time when that of retinol had declined. Earlier reports indicate that retinol and vitamin D-3 are absorbed by facilitated diffusion and passive diffusion, respectively [18]. The different time course of absorption may also reflect differences in intraluminal events, rather than differences in absorption mechanism or intracellular processing.

Our analysis of the [^{3}H] retinol-labeled intestinal lymph confirms previous observations by others using thoracic duct lymph [2,5,9,10,19]. However, we found a higher degree of esterification and of chylomicron association than the previous reports. This may be due to our use of intestinal lymph. When using intestinal lymph, we avoid interference from hepatic and peripheral lymph which are also drained by the thoracic duct.

In a previous publication [8], it was pointed out that about 90% of the recovered radioactivity in lymph after feeding ^{3}H-labeled vitamin D-3, was in association with chylomicrons, and that about 10% was found in the protein fraction with $d > 1.21$ g/ml. No esterified vitamin D-3 was found in the lymph. Most of the radioactivity was recovered as free vitamin D-3, but a significant amount of ^{14}C radioactivity recovered in the protein fraction comigrated with authentic 25-hydroxyvitamin D-3. 25-Hydroxylase activity against vitamin D has been detected in the intestinal mucosa of chicken [20]. Therefore, radioactivity recovered in the 25-hydroxyvitamin D-3 fraction may by 25-hydroxyvitamin D-3 produced by the intestinal mucosal cells. On the other hand, it may originate from ^{14}C-labeled vitamin D-3 which has reached the liver through some unligated lymph vessels. Enterohepatic circulation of vitamin D-3 has been reported [21,22]. However, in this connection one should be aware that comigration of species in a single chromatographic system never fully proves identity with a standard compound.

Analysis of the plasma at different times after

injection of lymph, showed that less than 20% of the remaining [^{3}H]retinol was at any time recovered in the fraction with $d > 1.006$ g/ml. This is probably due to rapid receptor mediated endocytosis in the hepatocytes of the chylomicron remnants [23,24]. In contrast, more than 90% of the ^{14}C-labeled vitamin D-3 was recovered in the plasma fraction with $d > 1.006$ g/ml, and the transfer to this fraction was rapid. This result is in accordance with previous reports where we found that vitamin D-3 was transferred from chylomicrons to α-globulins (presumably the binding protein for vitamin D and its metabolites [23]) both in vitro [8] and in vivo [11].

Taken together, the results indicate that vitamin D-13, which is absorbed into lymph, is not exclusively transported in chylomicrons and their remnants. The plasma clearance of the vitamin D-3 was, however, as effective as the clearance of [^{3}H]retinyl ester which remained in the chylomicron remnants.

Using hepatectomized rats, we found that about 90% of the injected chylomicron associated [^{3}H]retinyl ester was recovered in the chylomicron remanant fraction after 30–60 min. The rest of the radioactivity was mostly in the combined LDL and HDL fraction. This radioactivity may represent chylomicron remnants which have a density higher that 1.019 g/ml. It is possible that chylomicrons which are metabolized in the periphery for extended periods are transferred to lipoproteins of $d = 1.019–1.063$ g/ml [19]. The halftime of remnants in plasma of normal animals is about 6 min [9,24,25]. Alternatively, a small fraction of the retinyl esters may be transferred to a type of HDL which is derived from the chylomicrons when they are attacked by lipoprotein lipase [26].

In an earlier study, we injected intravenously chylomicrons labeled with ^{3}H-labeled vitamin D-3 into hepatectomized rats [8]. After 30 min, more than 50% of the injected ^{3}H-labeled vitamin D-3 was recovered in the plasma fraction with $d > 1.21$ g/ml. These results indicate that the transport of retinol and vitamin D-3 differs.

When lymph chylomicrons were mixed with plasma in vitro, only a small transfer of [^{3}H]retinyl ester from the chylomicrons to other plasma fractions was observed. In contrast, Zilversmit et al. [10] found in rabbits that up to 29% of the retinyl ester from chylomicron remnants was transferred to higher density lipoproteins. They suggested that retinyl ester transfer was mediated by the cholesteryl ester transfer protein. The different results are probably due to the fact that rat plasma in contrast to rabbit plasma contains negligible amounts of this protein.

Our data indicate that the [^{3}H]retinyl ester is a relatively nonexchangeable marker for the chylomicron remnants in vivo in rats. Hence, [^{3}H]retinyl ester could be used as a marker to study the chylomicron catabolism, including formation and hepatic uptake.

When double labeled chylomicrons were incubated with plasma fraction in vitro, we demonstrated a remarkable difference in exchangeability of the two vitamins. The amount of transferred vitamin D-3 increased both with incubation time and increased plasma protein concentration. Only a small amount of retinyl ester was recovered in the non-chylomicron fraction.

In conclusion, the transport of retinol and vitamin D-3 from the intestine, differs in several ways. Both vitamins may be absorbed via the lymphatic route in association with chylomicrons.

Almost all the chylomicron retinol is esterified, while vitamin D-3 appears in the chylomicrons as free vitamin D-3. The retinyl ester is a relatively nonexchangeable component of the chylomicrons and their remnants. In contrast, significant amounts of vitamin D-3 are transferred from the chylomicrons to other plasma fractions. These results indicate that nearly all the retinol and only a fraction of the vitamin D-3 follow the chylomicron remnants in their uptake by the hepatocytes.

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