Effects of Triton WR 1339 and Orotic Acid on Biliary and Serum Dolichols in Rats

Kari Humaloja, Risto P. Roine, and Mikko Salaspuro

Two lysosomal storage diseases, aspartylglucosaminuria and mannosidosis, are associated with highly elevated serum dolichol concentrations. To eludicate possible mechanisms leading to elevated serum dolichols, we studied the effects of Triton WR 1339 (known to increase serum cholesterol) and orotic acid (known to decrease serum cholesterol) on blood and biliary dolichol and beta-hexosaminidase levels in rats. In Triton WR 1339–treated rats, serum dolichol was markedly increased compared with saline-treated controls 1 (400 ± 70 ng/mL, n = 7 *v* 85 ± 11 ng/mL, n = 8, $P < .001$), 4 (789 ± 70 ng/mL, n = 10 *v* 110 ± 10 ng/mL, n = 7, $P < .0001$), and 8 (549 ± 43 ng/mL, n = 8 *v* 87 ± 8 ng/mL, n = 7, $P < .001$) days after administration of the drug. By contrast, serum dolichol was decreased (64 ± 5 ng/mL, n = 8 *v* 119 ± 7 ng/mL, n = 8, $P < .0001$) after a 7-day orotic acid feeding compared with controls. Serum beta-hexosaminidase was unaffected by both treatments. Orotic acid also increased biliary dolichol (280 ± 47 ng/100 g body weight [BW]/h, n = 7 *v* 83 ± 15 ng/100 g BW/h, n = 7, $P < .01$) and beta-hexosaminidase (21 ± 3 mU/100 g BW/h, n = 7 *v* 8.3 ± 2 mU/100 g BW/h, n = 9, $P < .01$) excretion compared with controls. Thus, both Triton WR 1339 and orotic acid have an effect on dolichol metabolism, and it is conceivable—based on our results—that serum dolichol concentrations are regulated, at least in part, by a mechanism similar to that for serum cholesterol levels.

DOLICHOLS are long-chain polyisoprenoid alcohols generally restricted to chain lengths of 14 to 24 isoprene units.[1] The phosphorylated form of dolichol acts as an essential intermediate in the biosynthesis of asparagine-linked glycoproteins.[2,3] The biosynthesis of dolichol originates at acetyl-coenzyme A (CoA) and proceeds along the same pathway as cholesterol to all -*trans*-farnesyl pyrophosphate (FPP).[4] Dolichol synthesis in the liver has been suggested to be independent of cholesterol synthesis subsequent to the branch point at FPP.[4] Diet has been shown to have little effect on the liver dolichol pool.[5] Cholesterol feeding has been reported to have little effect[6,7] or to decrease[8] hepatic dolichol levels. Biliary excretion of dolichol has been suggested to be an important elimination route for dolichols from the body.[9]

The level of dolichol in the blood is rather constant under normal conditions,[10] and there is no diurnal variation.[11] Serum dolichol has been reported to be elevated in two rare lysosomal diseases, aspartylglucosaminuria and mannosidosis,[12] and to lesser extent in alcoholics.[13]

On the other hand, patients with neuronal ceroid lipofuscinosis have decreased serum dolichol compared with normal controls.[14] A disturbance in lysosomal function has been suggested to be the mechanism behind these changes in serum dolichol levels.[12,14] Human liver cirrhosis and hepatocarcinoma have been shown to be associated with a low dolichol content in the liver.[15,16]

The liver has been suggested to play a central role in the regulation of blood dolichols. It has been postulated that blood dolichol is synthesized in the liver and transported through the endoplasmic reticulum–Golgi system to the blood like other lipids that are associated with apoproteins.[1] In addition, bile has been suggested to be the only route for excretion of substantial amounts of dolichol from the body,[9] although phosphorylated dolichol has not been detected in rat bile.[17]

Triton WR 1339 is known to reduce the influx of plasma cholesterol into the liver in rats.[18,19] It also increases the biliary excretion of lysosomal enzymes and certain lipids (bile acids and phospholipids)[20] and markedly increases serum cholesterol in experimental animals.[20,21] Triton WR 1339 also accumulates in rat hepatocyte lysosomes, and this accumulation exhibits morphological characteristics that are in many respects similar to those found in lysosomal storage diseases.[22]

Furthermore, it has been shown that Triton WR 1339 increases hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase) activity in the liver,[23] and an inhibitor of HMG-CoA reductase, simvastatin, has been reported to decrease dolichol and ubiquinol levels in the liver of hypercholesterolemic rats.[24] On the other hand, it has been demonstrated that another HMG-CoA reductase inhibitor (mevinolin) had only limited effects on blood dolichol, although liver dolichol levels increased after this treatment.[25] In humans, HMG-CoA reductase inhibitors have been demonstrated to have no marked effect on blood dolichols.[26,27]

Orotic acid is known to inhibit the efflux of hepatic cholesterol to the serum.[28,29] It also increases hepatic cholesterol synthesis[30] and biliary cholesterol excretion.[21]

The aim of the present investigation was to study the effects of Triton WR 1339 and orotic acid on biliary and serum dolichols in rats to better understand the mechanisms underlying the altered serum dolichols found in rare lysosomal storage diseases.

MATERIALS AND METHODS

General Experimental Procedure

Male Wistar rats weighing 220 to 605 g were used for all experiments. Room temperature was maintained at 22° ± 2°C, and a 12-hour light/dark cycle was applied. All rats had free access to standard rat chow (Altromin 1324 pellets; Altromin, Lage, Germany) and water until the time of the experimental procedure. Triton WR 1339 (Sigma

From the Research and Treatment Unit of Alcohol Diseases, Helsinki University Central Hospital, Helsinki, Finland.

Submitted February 12, 1997; accepted December 2, 1997.

Supported by grants from the Emil Aaltonen Foundation and the Helsinki University Central Hospital Research Fund.

Address reprint requests to Mikko Salaspuro, MD, PhD, Research Unit of Alcohol Diseases, University of Helsinki, Tukholmankatu 8F, 00290 Helsinki, Finland.

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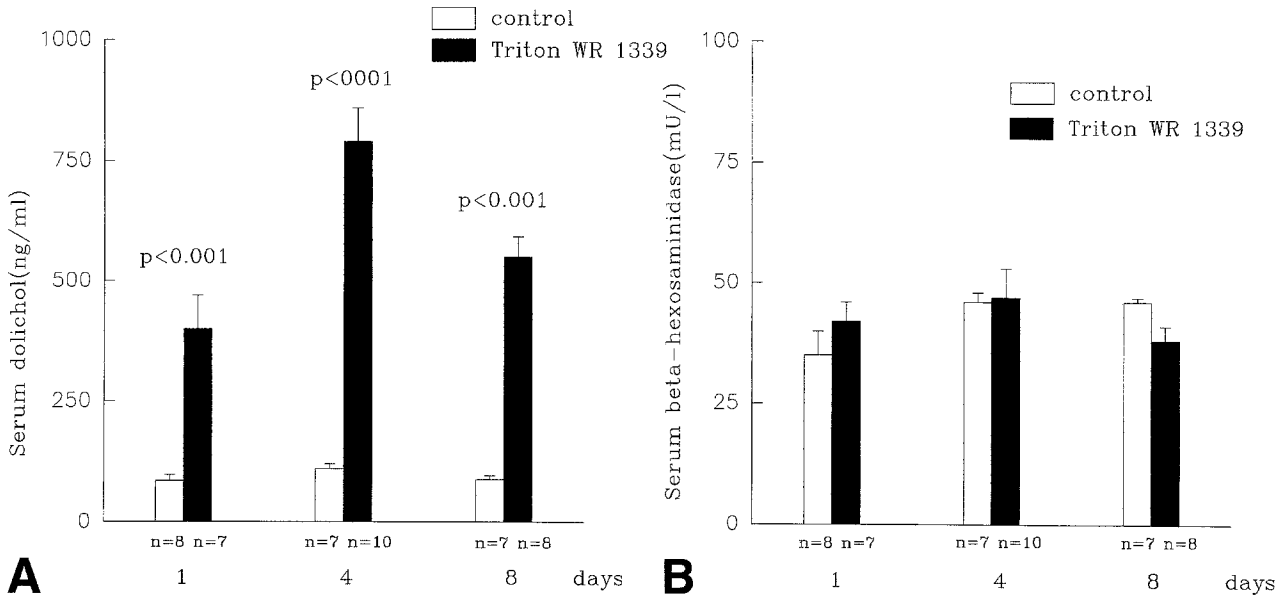

Fig 1. Effect of Triton WR 1339 (1 g/kg BW) on (A) serum dolichol and (B) serum beta-hexosaminidase levels in male Wistar rats 1, 4, and 8 days after IP administration of the drug compared with saline-treated controls.

Chemical, St Louis, MO) was dissolved in saline to a final concentration of 10% (vol/vol) and administered to the rats intraperitoneally (IP) 1, 4, or 8 days before the experiments (1 g/kg BW). Control rats were given an equal amount of saline (IP).

In another set of experiments, 15 g powdered regular diet (Altromin 1324 pellets) supplemented with 2% orotic acid (Sigma Chemical.) was fed to rats daily for 7 days. Control rats were given the same regular diet without orotic acid. All rats had free access to water until the time of the experimental procedure.

A bile fistula was created under sodium pentobarbital anesthesia (40 mg/kgIP). The common bile duct was cannulated with PE 10 polyethylene tubing just below the hepatic duct bifurcation, above the entrance of the pancreatic duct. Bile was then collected for 3 hours into plastic tubes, and the bile volume was determined by weighing, assuming a density for bile of 1.0 g/mL. Afterwards, blood was obtained from the heart by syringe. The rats were then killed and the livers removed and weighed.

The study was approved by the local animal welfare committee.

Analytical Methods

All blood samples were transferred into vacuum tubes and centrifuged at 4,000 rpm for 10 minutes. The sera, liver tissue, and collected bile were kept at −20°C until analysis. Dolichols were analyzed using a

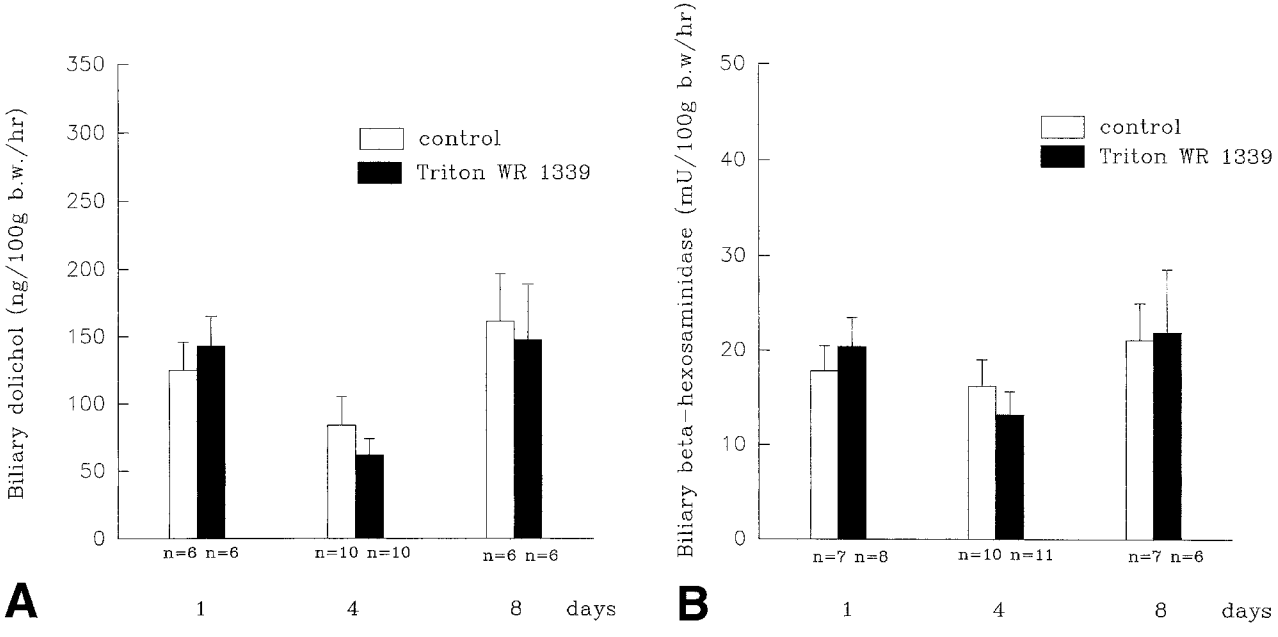

Fig 2. Effect of Triton WR 1339 (1 g/kg BW) on (A) biliary dolichol excretion and (B) biliary beta-hexosaminidase excretion in male Wistar rats 1, 4, and 8 days after IP administration of the drug compared with saline-treated controls.

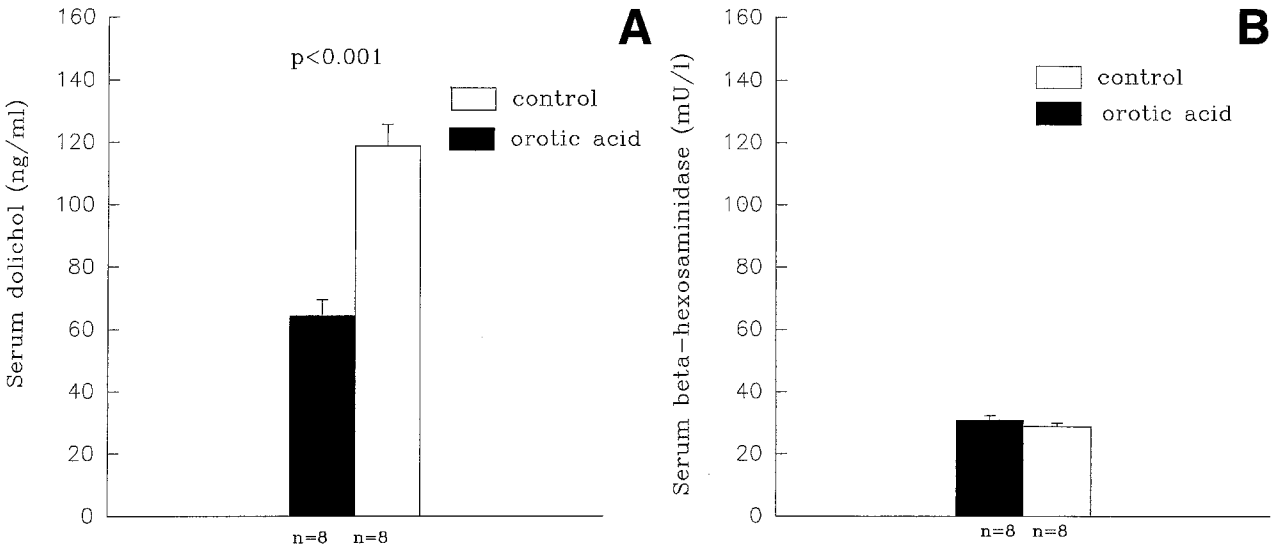

Fig 3. Effect of orotic acid feeding for 7 days on (A) serum dolichol and (B) serum beta-hexosaminidase levels in male Wistar rats.

high-performance Varian Vista 5500 liquid chromatograph as described previously.[11,14] Briefly, the saponification and extraction of dolichols from the serum (1 mL), bile (0.5 mL), and liver (0.5 g minced tissue) sample included the liberation of dolichols from their fatty acid esters or protein complexes by alkaline hydrolysis, the extraction of the free dolichols with *n*-pentane, and their purification on bonded silica C8 extraction columns. From the C8 extraction columns, dolichols were then eluted with 2 mL ethanol/methanol/2-propanol (90/5/5 by volume) and analyzed at 210 nm with a gradient elution program. Heneicosaprenol was used as an internal standard, and dolichol levels were expressed as the sum of the three homologs of 18, 19, and 20 isoprene units. When analyzing liver tissue samples, dolichol-23 was used as an internal standard.

Beta-hexosaminidase activity (EC 3.2.1.30) was determined by a spectrophotometric method using *p*-nitrophenyl-*N*-acetyl-b-glucosamine (Sigma Chemical) contained in 0.34 mol/L citrate buffer, pH 4.5, as a substrate.[31,32]

Statistical Calculations

All results are expressed as the mean ± SEM. Statistical differences between treatment groups and controls were calculated using parametric or nonparametric *t* tests according to the distribution of results. Spearman's correlation was used in the calculation of correlations. A P value less than .05 was considered significant.

RESULTS

Neither Triton WR 1339 nor orotic acid had any significant effect on the weight gain of rats in the different treatment groups compared with the controls. Liver weight was not statistically different between the treatment groups and controls, except in orotic acid–fed rats, in which the livers were heavier than in the controls (12.9 ± 0.2 g, n = 8 *v* 10.6 ± 0.5 g, n = 10, $P < .0001$).

Triton WR 1339 significantly increased serum dolichol in the

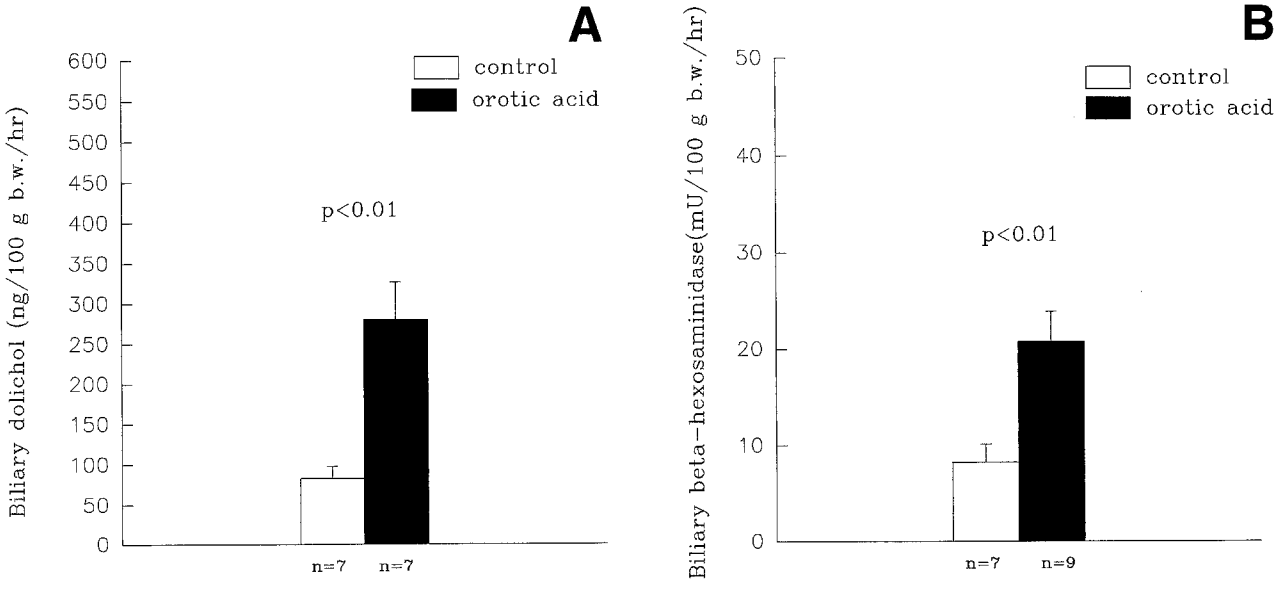

Fig 4. Effect of orotic acid feeding for 7 days on (A) biliary dolichol excretion and (B) biliary beta-hexosaminidase excretion in male Wistar rats.

Table 1. Effects of Orotic Acid (7 days) and Triton WR 1339 (4 days) Treatment on Liver Dolichol Content (μg/g liver) in Rats Compared With Untreated Controls

Group	D17	D18	D19	D20	D21	Total
Controls (n = 6)	3.0 ± 0.2	10.0 ± 0.6	12.4 ± 0.6	5.1 ± 0.4	0.55 ± 0.09	31 ± 2
Triton WR 1339 (n = 6)	3.2 ± 0.2	9.6 ± 0.6	12.0 ± 0.8	4.9 ± 0.3	0.49 ± 0.08	30 ± 2
Orotic acid (n = 6)	3.8 ± 0.3	10.9 ± 0.7	13.3 ± 0.6	5.3 ± 0.2	0.45 ± 0.05	34 ± 2

NOTE. Results are the mean ± SEM. None of the differences reached statistical significance.
Abbreviation: D, dolichol.

rats 1, 4, and 8 days after IP administration as compared with the saline-treated controls (day 1, 400 ± 70 ng/mL, n = 7 *v* 85 ± 11 ng/mL, n = 8, $P < .001$; day 4, 789 ± 70 ng/mL, n = 10 *v* 110 ± 10 ng/mL, n = 7, $P < .0001$; day 8, 549 ± 43 ng/mL, n = 8 *v* 87 ± 8 ng/mL, n = 7, $P < .001$; Fig 1A). By contrast, serum beta-hexosaminidase was unaffected by IP Triton WR 1339 administration as compared with the control level (Fig 1B).

Biliary excretion of dolichol (Fig 2A) and beta-hexosaminidase (Fig 2B) was unaffected by Triton WR 1339 compared with saline treatment. Bile flow (milliliters per hour) did not differ between Triton WR 1339–treated rats and controls.

On the other hand, orotic acid feeding clearly decreased serum dolichol concentrations as compared with the control levels (64 ± 5 ng/mL, n = 8 *v* 119 ± 7 ng/mL, n = 8, $P < .0001$; Fig 3A), but serum beta-hexosaminidase did not change in orotic acid–fed rats (31 ± 2 mU/L, n = 8 *v* 29 ± 1 mU/L, n = 8; Fig 3B). Furthermore, orotic acid increased biliary dolichol (280 ± 47 ng/100 g BW/h, n = 7 *v* 83 ± 15 ng/100 g BW/h, n = 7, $P < .01$; Fig 4A) and beta-hexosaminidase (21 ± 3 mU/100 g BW/h, n = 7 *v* 8.3 ± 2 mU/100 g BW/h, n = 9, $P < .01$; Fig 4B) excretion compared with the controls. This increase in excretion was higher than would be expected from the observed increase in bile flow (0.99 ± 0.06 *v* 0.64 ± 0.07 mL/h) alone.

The liver dolichol content was unaffected after 7 days of feeding orotic acid and also 4 days after administration of Triton WR 1339 (Table 1).

Combining all of the data for analysis showed that the absolute amount of biliary dolichol during the 3-hour collection period correlated highly significantly with the absolute amount of biliary beta-hexosaminidase ($r = .73$, $P < .0001$; Fig 5).

DISCUSSION

Triton WR 1339 significantly increased serum dolichol concentrations in rats in these experiments. The effect was maximal 4 days after administration of the substance. Triton WR 1339 has been shown to rapidly increase plasma cholesterol, and the mechanism underlying this phenomenon has been suggested to be a "trapping" of cholesterol in the blood compartment as a consequence of physical alteration of serum lipoproteins[33-35] and impaired function of lipoprotein lipase.[36,37] It has also been proposed that the liver is the main organ causing increased serum cholesterol, since hepatectomy followed by Triton injection did not increase serum cholesterol levels in rabbits.[35] It is conceivable that mechanisms described also explain, at least in part, the increase of serum dolichols found in our present experiments in rats.

Triton WR 1339 had no effect on hepatic dolichol levels in this study. This is in line with previous results concerning its effect on the hepatic cholesterol level.[21] It seems—based on our present results—that the hepatic dolichol content is not directly reflected in the serum dolichol level. Orotic acid feeding decreased serum dolichol in treated rats compared with controls in our setting. It has been shown that orotic acid feeding decreases serum high-density lipoprotein (HDL) cholesterol[21] in rats, and the mechanism for this phenomenon has been suggested to be a decreased release of very–low-density lipoprotein (VLDL) from the liver into the circulation,[28,29] which is proposed to reduce HDL formation from VLDL when serum VLDL is low and decrease the serum HDL cholesterol level.[21] Since dolichols are mainly associated with the HDL lipoprotein fraction in rat blood,[17] the mechanism for the decreased serum dolichols after orotic acid feeding could also be mediated by reduced HDL in the serum. In addition, reduced HDL in the serum have previously been suggested to explain the reduced serum dolichol concentrations found in abusers of anabolic androgenic steroids.[38]

On the other hand, intravenously injected dolichol has been shown to be associated first with VLDL, and after 4 days to be translocated extensively to HDL.[39] A transfer factor responsible for accelerating the translocation of dolichol from VLDL to HDL has also been demonstrated in human serum.[40] It may be that the direct effects of orotic acid on VLDL profoundly affect

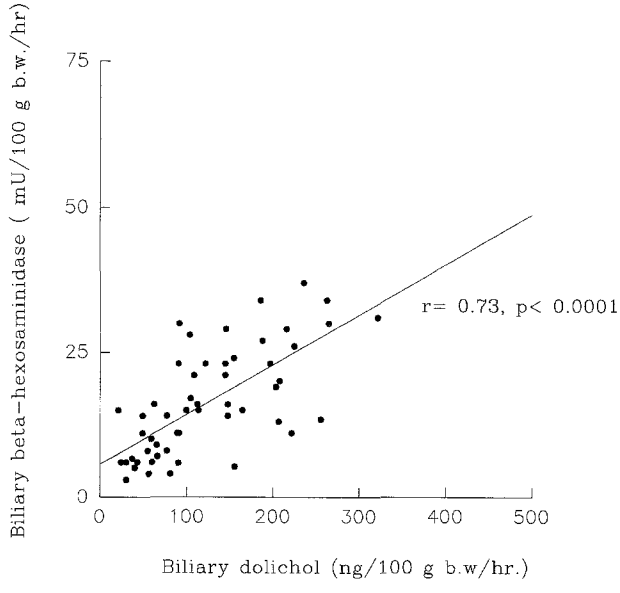

Fig 5. Correlation between absolute biliary dolichol and beta-hexosaminidase excretion during a 3-hour bile collection period.

serum dolichol levels in rats, but we do not have data to support this suggestion.

Orotic acid increased biliary dolichol and beta-hexosaminidase excretion in our setting after a 7-day treatment period. Orotic acid has been shown to induce fatty liver[28,29,41] and to increase hepatic cholesterol synthesis.[21,41] In our present experiments, the liver was heavier in orotic acid–fed rats than in controls, which could reflect an increased lipid content in the liver. Orotic acid has also been shown to increase biliary excretion of cholesterol in rats,[21,41] but no change in hepatic free cholesterol has been found.[41] It has been suggested that increased hepatic free cholesterol available for biliary secretion could be the cause for elevated cholesterol output to the bile in rats after orotic acid feeding.[41] In the present experiments, hepatic dolichol content was unchanged after 7 days of orotic acid feeding, but it may be that orotic acid increased the hepatic dolichol available for biliary excretion, and this excess of dolichol could explain the elevated biliary excretion of dolichol found in the present study.

Combining all of the data for analysis revealed that biliary dolichol excretion correlates highly significantly with biliary beta-hexosaminidase excretion, which suggests that lysosomes are, at least in part, involved in the biliary excretion of dolichols. This observation supports the previous suggestion that a disturbance in lysosomal function might be the cause of the altered serum dolichols found in lysosomal diseases.[12,14] However, we found no correlation between serum dolichol and biliary beta-hexosaminidase in the present study. Consequently, it seems that serum and biliary dolichols may be regulated independently of each other.

Both Triton WR 1339 and orotic acid profoundly affect the hepatic metabolism of lipids. Since these substances also significantly alter serum dolichol levels, the present results support the previous suggestion that the liver is an important regulatory site for the blood dolichol supply.[42] There may be several mechanisms that explain the alterations in serum dolichol levels in rats treated with Triton WR 1339 and orotic acid. However, their effect on lipoprotein metabolism may be the most important explanation for the observed changes in serum dolichol concentrations in this study.

ACKNOWLEDGMENT

The technical assistance of Tuula Heiskanen is gratefully acknowledged.

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