Effect of infusion of "tris-galactosyl-cholesterol" on plasma cholesterol, clearance of lipoprotein cholesteryl esters, and biliary secretion in the rat

Herman Jan Kempen,* Folkert Kuipers, Theo J. C. van Berkel,† and Roel J. Vonk****

Gaubius Institute TNO,* Herenstraat 5d, 2313 AD Leiden; Department of Pediatrics, University of Groningen,** Bloemsingel 10, 9712 KZ Groningen; and Department of Biochemistry I, Erasmus University,† P. O. Box 1738, 3000 DR Rotterdam, The Netherlands

Abstract As shown by us previously (van Berkel et al. 1985. *J. Biol. Chem.* **260:** 2694–2699 and van Berkel et al. 1985. *J. Biol. Chem.* **260:** 12203–12207) the clearance of both low density lipoproteins (LDL) and high density lipoproteins (HDL) from the blood can be greatly enhanced by pretreatment of these lipoproteins with a tris-galactosylated cholesterol derivative, which makes these particles recognizable by hepatic galactosyl-receptors. Here we report that intravenous infusion of the (water-soluble) tris-galactosyl-cholesterol in rats caused a dose-dependent decrease of the plasma cholesterol level. This fall was sustained long after termination of the infusion. It was not observed upon infusion of tris-glucosyl-cholesterol. The fall in plasma cholesterol was accompanied by an increase in hepatic cholesterol. Upon injection of rat HDL and LDL labeled in their cholesteryl ester moieties, plasma clearance of label in both lipoproteins was enhanced in rats infused with tris-galactosyl-cholesterol, the stimulation being more pronounced when the label was in HDL. The appearance of label in bile was also enhanced in the rats receiving the compound, again more markedly when the label was given as HDL. Ninety four percent or more of the radioactivity excreted in the bile was in the form of bile salts, with conjugated cholate being the major species in both control and treated rats; 6% or less of the radioactivity in the bile was as free cholesterol. Infusion of tris-galactosyl-cholesterol constitutes a new and defined method of lowering plasma lipoprotein levels by enhancing their uptake in the liver.—**Kempen, H. J., F. Kuipers, T. J. C. van Berkel, and R. J. Vonk.** Effect of infusion of "tris-galactosyl-cholesterol" on plasma cholesterol, clearance of lipoprotein cholesteryl esters, and biliary secretion in the rat. *J. Lipid Res.* 1987. **28:** 659–666.

Supplementary key words low density lipoproteins • high density lipoproteins • tris-glucosyl-cholesterol • hepatic galactosyl-receptors

Hyperlipoproteinemia Type II (familial hypercholesterolemia) is a disease caused by absence or malfunction of LDL-receptors, thus giving rise to increased serum levels of LDL (1). Patients heterozygous for this genetic defect have a tenfold higher risk of a myocardial infarction before age 60 (2). A number of treatments aimed at lowering the LDL level in such patients is presently available, ranging from diet modification and drugs such as cholestyramine and mevinolin, to surgical intervention (partial ileal bypass) and repeated plasma exchange (3). Some of these treatments are quite effective, but can have undesirable side effects; others are less effective. Rationally, a treatment for these patients should aim at restoring the impaired clearance of LDL from the blood by the liver. Indeed, drugs that primarily act as bile acid sequestrants or as blockers of cholesterol synthesis were actually shown to lower the serum LDL level by increasing the hepatic LDL-receptor activity (4, 5). We have looked for a new way to increase hepatic clearance of LDL that is independent of the LDL-receptor pathway.

The liver has an effective uptake system for asialoglycoproteins (6). These proteins are cleared from the circulation by high affinity binding to receptors on hepatocytes which specifically recognize their galactosyl-terminated oligosaccharide moieties. This binding is followed by endocytosis of the glycoprotein-receptor complex, dissociation of this complex (salvaging the receptor), and lysosomal breakdown of the glycoprotein. The asialoglycoprotein receptor also recognizes various "neoglycoproteins" like galactosylated albumin (7) and lactosylated LDL (8).

In previous reports we described the synthesis (9) and properties (9–11) of a water-soluble galactosyl-containing cholesterol derivative, denoted as tris-galactosyl-cholesterol, which was found to attach itself to the surface of lipoproteins when added to whole serum or plasma, or to isolated lipoproteins. Pretreatment of radiolabeled hu-

Abbreviations: tris-galactosyl-cholesterol, N-(tris(β-D-galactopyranosyl-oxymethyl)methyl)N"-(4 (5-cholesten-3β-yloxy)succinyl)glycinamide; LDL, low density lipoproteins; HDL, high density lipoproteins; PBS, phosphate-buffered saline.

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man LDL (10) or HDL (11) with tris-galactosyl-cholesterol caused a striking increase in the rates at which these particles disappear from the circulation, and are taken up by the liver. Surprisingly, tris-galactosyl-cholesterol-loaded human HDL was found nearly exclusively in the liver parenchymal cells (11), whereas tris-galactosyl-cholesterol-loaded human LDL was recovered mainly in the Kupffer cells (10).

We now describe the effect of intravenous administration of tris-galactosyl-cholesterol to rats. When given as infusion the substance causes a clear fall in plasma cholesterol, accompanied by a rise in liver cholesterol, clearance of cholesteryl ester from the plasma, and biliary excretion of cholesterol.

MATERIALS AND METHODS

Materials

Tris-galactosyl-cholesterol was synthesized as described (9). Tris-glucosyl-cholesterol was prepared in the same way, using glucosyl-beta-1-bromide instead of the galactosylbromide. These compounds were dissolved in phosphate-buffered saline (PBS) in concentrations of up to 12 mg/ml (the maximum solubility in water) for intravenous administration. [^{3}H]cholesterol was purchased from Amersham International.

Lipoprotein isolation and labeling

Rat LDL and HDL were obtained by density gradient ultracentrifugation (12). The rat lipoproteins were labeled with [^{3}H]cholesteryl ester by injection of [^{3}H]cholesterol as previously described (13).

Animal experiments

Male Wistar rats (300–350 g) were equipped with two catheters (one for infusion, the other for blood sampling) in the heart, and some also with one in the bile duct (14). The catheters were placed in such a way that the rats were conscious and unrestrained during the experiments and had a normal eating pattern. In rats with a bile catheter, bile was diverted for 8 days before the start of the experiment in order to establish a new steady-state in cholesterol and bile salt synthesis. On the day of the experiment, hourly bile collections were started at 9:00 AM. Infusion of PBS or of tris-galactosyl-cholesterol (4 mg/ml or 10 mg/ml) or tris-glucosyl-cholesterol (4 mg/ml) dissolved in PBS, was started at 10:00 AM via one of the two heart catheters. The infusion was initiated by giving a bolus injection of 0.4 ml, and then continued at a rate of 0.6 ml/hr for 4 or 24 hr. In this way, 11 or 28 mg (9 or 22 μmoles) was administered during the 4-hr period and 59 mg (47μmoles) during the 24-hr infusion period. At certain times (specified under Results) samples of 300 μl of blood were taken (via the second heart catheter) and placed in a centrifuge tube containing 5 μl of heparin solution. After mixing, the tubes were centrifuged for 15 min at 1500 *g*, and the plasma was collected and stored for further analysis. Some rats infused for 24 hr were killed at the end of the infusion by pentobarbital injection and bleeding from the heart catheter. The liver was removed, weighed, frozen in liquid nitrogen, and stored at −20°C for subsequent cholesterol determination. To other rats infused for 24 hr, lipoproteins with [^{3}H]cholesteryl esters were administered 3 hr after start of the infusion as a bolus injection of 1.0 ml via the same catheter used for blood sampling. These rats received 12,000 Becquerel in HDL, or 5,500 Becquerel in LDL. Blood and bile samples were collected from these rats for a subsequent 2-day period.

Analytical techniques

Total cholesterol in plasma was determined with an enzymatic method (Boehringer Monotest-R, cat. no. 236691). In separate tests, we found that the cholesterol esterase present in the Monotest did not hydrolyze tris-galactosyl-cholesterol when this compound was added to rat plasma in a final concentration of 0.5 mM. For cholesterol determination in liver tissue, the livers were macerated by extrusion through a steel screen and then homogenized in a Potter-Elvehjem tube with a Teflon pestle in four volumes of a buffer containing 200 mM sucrose, 25 mM tris-HCl, and 2 mM EDTA, pH 7.2. For total cholesterol, triplicate aliquots (200 μl) of each homogenate were mixed with 1 ml of a freshly prepared solution of 0.3 N NaOH in 97% ethanol; for free cholesterol, three other aliquots were mixed with 1 ml of 97% ethanol. These mixtures were incubated for 60 min at 37°C, and then supplemented with 1 ml of distilled water. In separate tests, long-chain acyl esters of cholesterol and tris-galactosyl-cholesterol were hydrolyzed completely in the alkaline ethanol, and not in the neutral ethanol. Lipids were then extracted with 4 ml of hexane. The hexane extracts were blown to dryness and free cholesterol was quantitated using an enzymatic test for free cholesterol (Boehringer, cat. no. 310328).

Bile flow, as well as biliary excretion of bile salts, phospholipids, and free cholesterol were assessed as described before (15). Tritium radioactivity was measured by liquid scintillation counting in aliquots of the bile samples, either directly after decolorization with an equal volume of hydrogen peroxide or after extraction of the neutral lipids in chloroform to separate cholesterol from the bile salts. In some bile fractions radioactivity was determined in individual bile salts after HPLC separation. The latter was carried out essentially as described (16), using a Varian 5000 apparatus equipped with a Waters C18 radial compression column. Bile salts were first extracted from total bile using Sep-Pak C18 cartridges (Waters

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Assoc.) After injection, the column effluent was collected in 15-sec fractions to be assayed for activity. For identification, retention times of peaks were compared with those of bile salt standards.

Statistical analysis

Significance of differences between groups of rats was assessed using Student's *t*-test (two-sided) for paired or unpaired observations.

RESULTS

Short-term infusions

In preliminary experiments with pentobarbital-anaesthetized rats, tris-galactosyl-cholesterol was given in the form of intravenous bolus injections in doses of up to 8 mg, dissolved in 1 ml. No consistent changes in the serum cholesterol level were observed in periods up to 3 hr after the injection, while plasma samples were clearly hemolytic. In order to avoid this problem, we decided to give the substance as a slow intravenous infusion, reasoning that in this way all tris-galactosyl-cholesterol might become fully incorporated in the circulating plasma lipoproteins and thus not reach a hemolytic concentration. **Fig. 1** shows that total plasma cholesterol was markedly decreased upon infusion of 11 or 28 mg of tris-galactosyl-cholesterol during a 4-hr period. The level was not appreciably affected by infusion of PBS or of 11 mg of tris-glucosyl-cholesterol. The effect was clearly dose-dependent, as seen by the more rapid onset and the higher extent of cholesterol-lowering obtained with the higher dose. Although infusion was stopped after 4 hr, the cholesterol levels at 24 hr in the tris-galactosyl-cholesterol-treated rats were still significantly below the pretreatment value, and below the values in rats receiving saline or tris-glucosyl-cholesterol. Infusion of 11 mg of tris-galactosyl-cholesterol for 4 hr in rats with permanent biliary drainage resulted in the same degree of lowering of the serum cholesterol level as in the rats with intact enterohepatic circulation (not shown). No hemolysis was observed in the plasma samples obtained in these experiments.

Long-term infusions

As shown in **Fig. 2,** rats receiving 59 mg of tris-galactosyl-cholesterol as an infusion over a 1-day period had 70% lower plasma cholesterol levels than rats given PBS, 24 hr after start of the infusion. The drug treated rats still had 60% or 40% lower levels at 24 hr or 48 hr, respectively, after terminating the infusion. None of the plasma samples obtained in these experiments gave evidence of hemolysis.

At the end of the 24-hr infusion, the hepatic cholesterol content was higher in the rats that received tris-galactosyl-cholesterol than in the control rats **(Table 1).** This increase was statistically significant for free and total cholesterol, but not for esterified cholesterol (calculated as total minus free cholesterol).

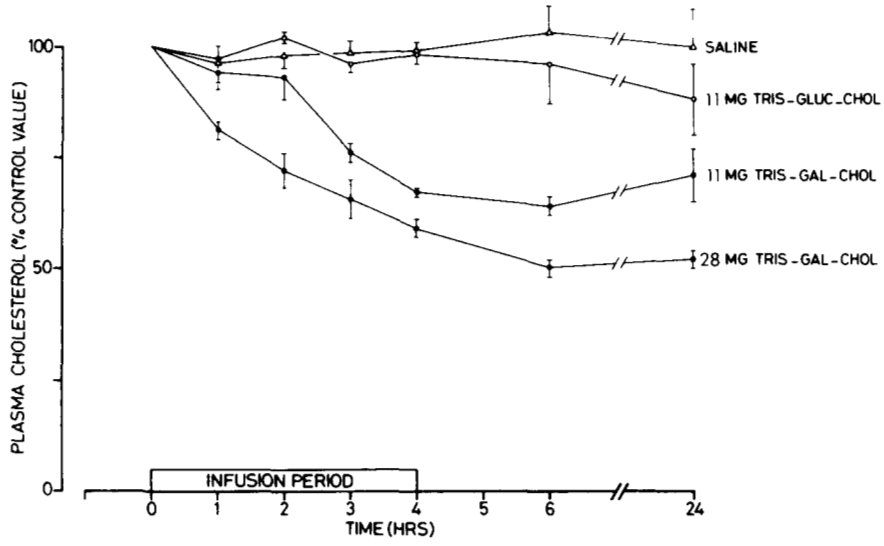

Fig. 1. Effect of intravenous infusion of 11 mg or 28 mg of tris-galactosyl-cholesterol, 11 mg of tris-glucosyl-cholesterol, or phosphate-buffered saline over a period of 4 hr in rats with intact enterohepatic circulation. Data are means ± SD of three animals for each treatment, and represent the percentage of the average of cholesterol concentrations at 60 and 10 min before start of infusion (control). Absolute control levels were: 1.72 ± 0.05, 2.05 ± 0.15, 1.73 ± 0.18 and 1.85 ± 0.20 mM, respectively.

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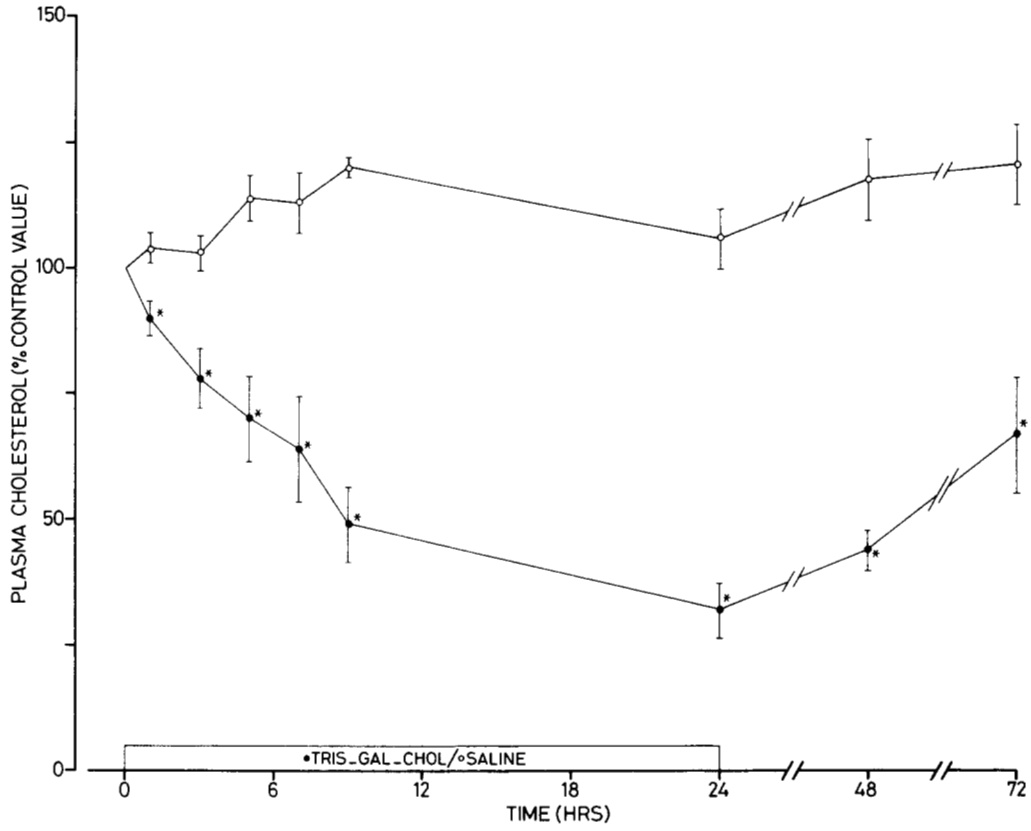

Fig. 2. Effect of intravenous infusion of 59 mg of tris-galactosyl-cholesterol (n = 4; —●—) or saline (n = 4; —○—) over a period of 24 hr on plasma cholesterol concentration in rats having a bile fistula for 8 days. Data are means ± SD and represent percentage of the average cholesterol concentrations 48, 24, and 1 hr before start of infusion (control). Absolute control levels were 1.59 ± 0.36 and 1.86 ± 0.28 mM, respectively.

Biliary excretion of fluid, bile salts, and phospholipids was not affected by tris-galactosyl-cholesterol administration, but excretion of cholesterol was significantly increased above the rate in rats receiving PBS, during the second, third, and fourth 12-hr period after start of the infusion **(Fig. 3).**

In these same experiments, a trace amount (20 μg of protein) of rat LDL or HDL, containing ^{3}H-labeled cholesteryl ester, was administered 3 hr after the start of the infusion. The rate at which labeled HDL was cleared from the circulation was considerably increased in the rats receiving tris-galactosyl-cholesterol **(Fig. 4A),** the effect being apparent immediately after injection of the tracer. In contrast, when labeled rat LDL was injected, the decay rate began to accelerate 6 hr after injection of tris-galactosyl-cholesterol, and the overall effect was less marked (Fig. 4B). The excretion of the tritium label in the bile during the first 21 hr after injection of these tracers is depicted in **Fig. 5.** In the rats receiving the tracer in the form of HDL, excretion of radioactivity was strongly and immediately stimulated by tris-galactosyl-cholesterol (Fig. 5A); the stimulatory effect was delayed and less impressive when the label was given as LDL (Fig. 5B). Total recovery of radioactivity in bile at 24 hr was about 28% of the injected dose in the rats infused with PBS (no difference between HDL and LDL as vehicle). This value was increased nearly 2-fold or 1.5-fold by infusion of tris-galactosyl-cholesterol in rats receiving labeled HDL or LDL, respectively. In all fractions of all experiments, label was present for more than 94% in the form of bile salts, the remainder occurring as free cholesterol. Distribution of ^{3}H radioactivity among the different bile salts

TABLE 1. Effect of infusion of tris-galactosyl-cholesterol (59 mg/rat per 20 hr) in rats on hepatic cholesterol contents

	Free Chol	Total Chol	Esterified Chol[a]
	μmol/g wet weight		
Control	4.30 ± 0.19	5.09 ± 0.08	0.80 ± 0.23[b]
Tris-gal-chol	5.83 ± 0.23[c]	7.08 ± 0.27[c]	1.25 ± 0.21[b]

[a] Calculated as total minus free cholesterol.
[b] Significantly greater than 0 ($P < 0.05$ in paired t-test).
[c] Significantly different from value in control rats ($P < 0.05$ in unpaired t-test).

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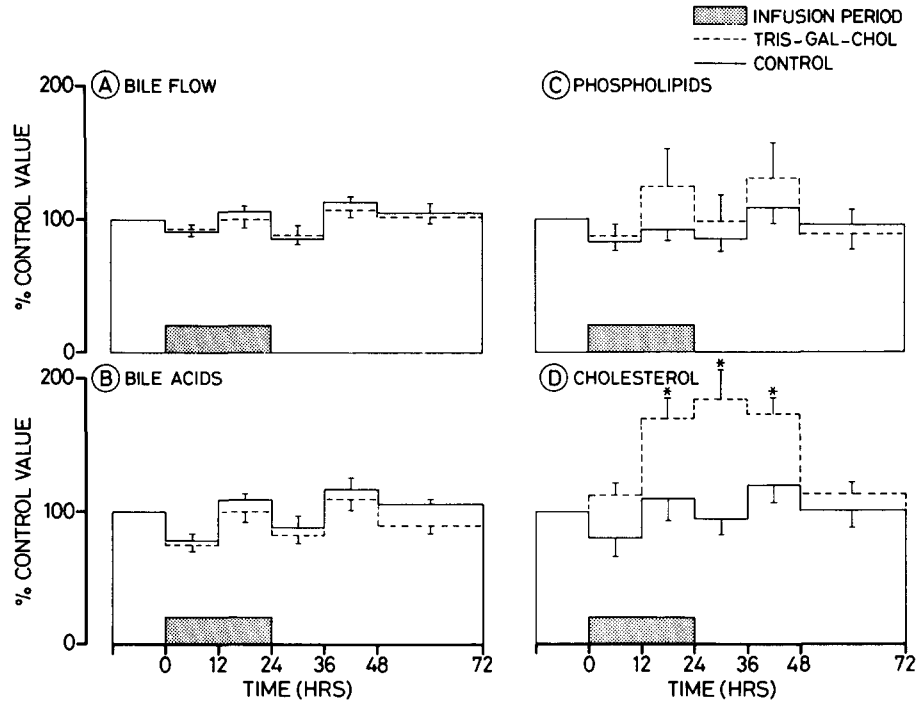

Fig. 3. Effect of intravenous infusion of 59 mg of tris-galactosyl-cholesterol (n = 4; ····) or saline (n = 5; ——) over a period of 24 hr on biliary fluid and lipid secretion by rats having a fistula for 8 days. Data are means ± SD given as percentage of secretion rates in the 24-hr period before start of infusion (control). The absolute control values were for saline: bile flow, 0.61 ± 0.05 ml/hr; bile acids, 16.4 ± 2.2 ml/hr; phospholipids, 1.70 ± 0.66 μmol/hr; cholesterol, 0.19 ± 0.06 μmol/hr; and for tris-galactosyl-cholesterol: bile flow 0.57 ± 0.11 ml/hr; bile acids, 13.4 ± 1.7 ml/hr; phospholipids, 2.16 ± 1.37 μmol/hr; cholesterol 0.25 ± 0.04 μmol/hr.

was investigated by HPLC only for the experiment with labeled HDL. As shown in **Fig. 6,** there was no obvious change in distribution pattern among the various bile salts in the rats infused with tris-galactosyl-cholesterol in comparison to control rats, the majority of label occurring in both cases in the form of conjugated cholate.

Infusion of tris-galactosyl-cholesterol for 24 hr did not affect the amount or the pattern of eating by these rats, nor did it have any apparent effect on the animals' behavior.

DISCUSSION

In this work we describe a new way of lowering the plasma cholesterol level by administration of a substance purposely designed to enhance lipoprotein uptake by the galactosyl-receptors in the liver. The cholesterol-lowering effect of tris-galactosyl-cholesterol was found to be dose-dependent, and specific for the galactosyl moiety in the molecule. Infusion of the substance resulted in an enhanced removal of labeled cholesteryl ester, incorporated in homologous HDL or LDL, from the circulation. In earlier studies we found that homologous radioiodinated HDL also was cleared more rapidly, and taken up to a greater extent in the liver, in rats infused with tris-galactosyl-cholesterol (17). Therefore, increased clearance of lipoproteins from the blood by the liver was probably the main reason for the fall of the serum cholesterol level. Presently, it cannot be excluded that inhibition of cholesterol synthesis (by the cholesterol set free from tris-galactosyl-cholesterol in the liver) additionally contributed to the lowering of the serum level. However, treatment of rats with compactin, a potent inhibitor of cholesterol synthesis, does not cause an appreciable decrease of plasma cholesterol (15, 18), so this possibility seems less likely.

The mass of cholesterol introduced by infusion of tris-galactosyl-cholesterol (47 μmoles) could not be completely accounted for by the increase in hepatic and biliary cholesterol. At the end of 24 hr of infusion, we measured an increase of 2 μmol/g wet weight in the liver which, with a liver weight of 11 g, results in an increment of 22 μmol. The increase in biliary cholesterol excretion in that period amounted to about 2 μmol above that in control rats. It is unclear how much of this total increment of 24 μmol was due to uptake of lipoprotein cholesterol, and how much originated from the infused compound itself. If all of the lipoprotein cholesterol removed from the plasma by infusion of the galactosyl compound were inside the liver, this should elevate the hepatic cholesterol

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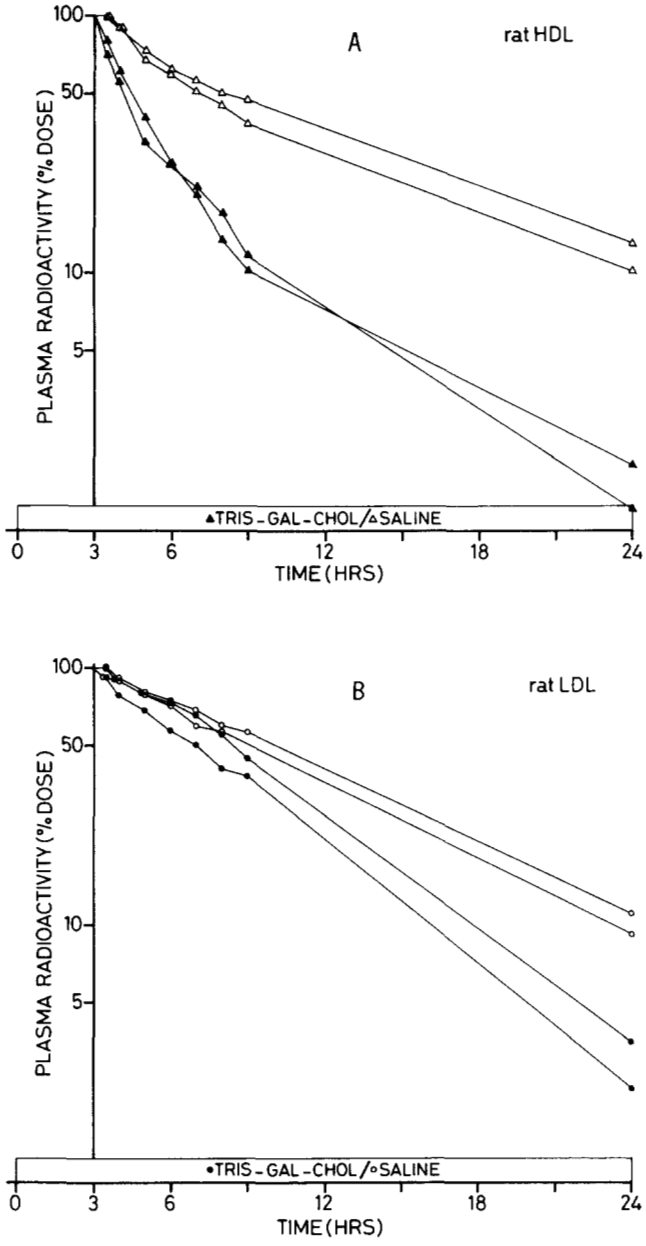

Fig. 4. Plasma decay of ^{3}H radioactivity after intravenous injection of [^{3}H]cholesteryl ester-containing rat HDL (A, upper panel) or rat LDL (B, lower panel) in rats having a bile fistula for 8 days, receiving an intravenous infusion of tris-galactosyl-cholesterol (2.4 mg/hr, ▲, ●) or saline (△, ○). Labeled lipoproteins were injected 3 hr after start of infusion. Data are percent of injected radioactivity, each curve representing a separate animal.

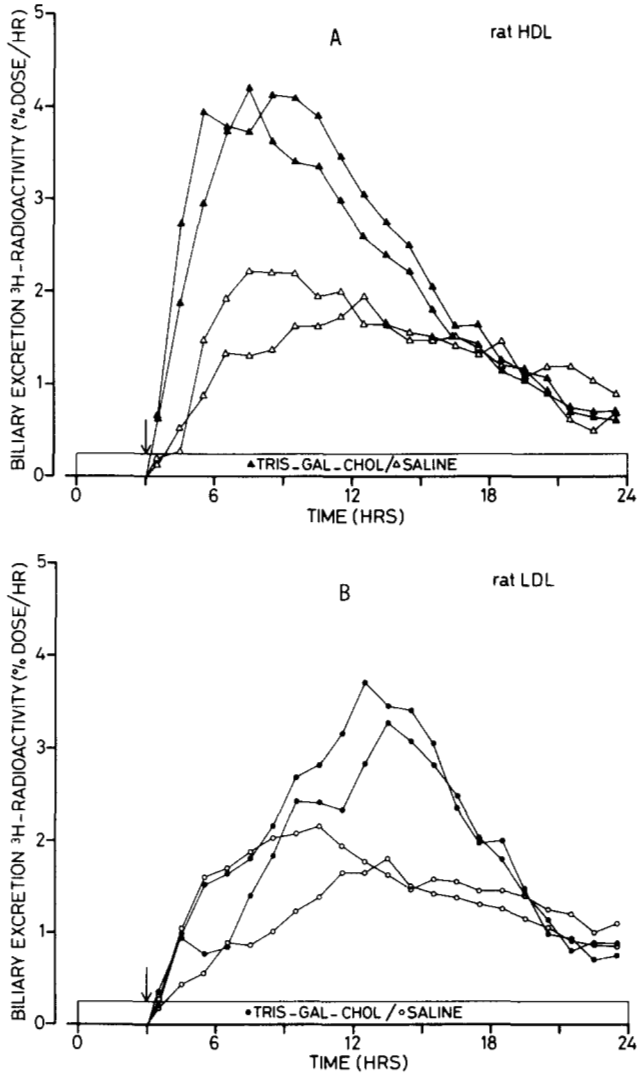

Fig. 5. Biliary secretion of ^{3}H radioactivity after intravenous injection of [^{3}H]cholesteryl ester-containing rat HDL (A, upper panel) or rat LDL (B, lower panel) in rats having a bile fistula for 8 days, and receiving intravenous infusion of tris-galactosyl-cholesterol (2.4 mg/hr, ▲, ●) or saline (△, ○). Labeled lipoproteins were injected 3 hr after start of infusion. Data are percent of injected radioactivity secreted per hr; each curve represents a separate animal. Total secretion (as % of injected dose) 21 hr after injection) was 44.0 and 49.4 after injection of labeled HDL and 41.6 and 36.6 after injection of labeled LDL, for the rats receiving tris-galactosyl-cholesterol, and 30.5 and 24.6 after labeled HDL and 23.2 and 31.6 after labeled LDL in rats receiving saline.

content by about 12 μmoles (assuming a plasma volume of 12.5 ml with an original cholesterol concentration of 1.8 mM, lowered by the compound to 0.8 mM). In any case, the observed increment in the liver is too small to account for the infused cholesterol esterified in tris-galactosyl-cholesterol. The location of the missing fraction is presently obscure, and will be the subject of further study.

There was a marked difference between HDL and LDL with respect to the effect of tris-galactosyl-cholesterol infusion on the plasma clearance of labeled cholesteryl ester carried in either of these particles. The stimulation was immediate and pronounced when HDL was used as the carrier, but delayed and less marked with LDL as the vehicle. Consequently, the rate of appearance of radiolabeled bile salts in bile increased promptly and sharply in the drug-treated rats when the label was conveyed in the form of HDL, but rose only after a lag period when LDL was

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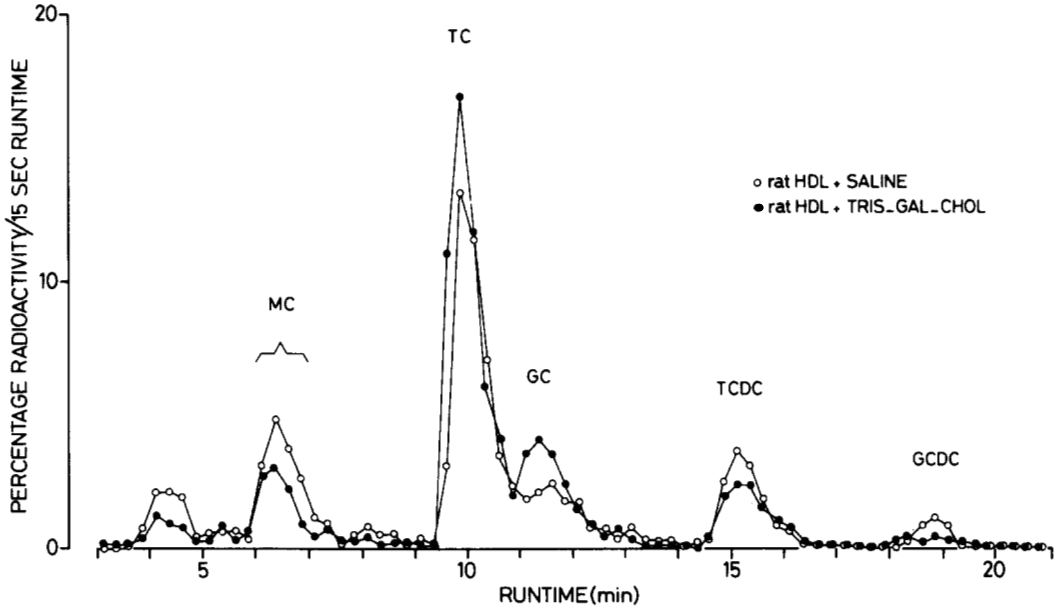

Fig. 6. Reverse-phase HPLC elution profile of ^{3}H-labeled bile acids in bile secreted by a rat having a bile fistula for 8 days, 4 hr after intravenous injection of ^{3}Hcholesteryl ester-labeled rat HDL. The rats received an intravenous infusion of tris-galactosyl-cholesterol (2.4 mg/hr, ●) or saline (○). The main peaks were identified as muricholic acid conjugates (MC), taurocholic acid (TC), glycocholic acid (GC), taurochenodeoxycholic acid (TCDC), and glycochenodeoxycholic acid (GCDC).

used as carrier. Possibly, rat LDL must be loaded with more galactosyl residues per particle than rat HDL for its recognition and sequestration by the hepatic receptors, but other explanations cannot be excluded.

According to current knowledge, bile salt formation from cholesteryl ester can only take place after hydrolysis of the ester and subsequent oxidation of the liberated free cholesterol. The enhanced biliary excretion of radioactivity, and the nearly complete appearance of the label in the form of bile salts also in the drug-treated animals, therefore indicate that infusion of tris-galactosyl-cholesterol leads to an increased uptake and hydrolysis of plasma cholesteryl esters in the liver.

We found that tris-galactosyl-cholesterol caused enhanced synthesis of labeled bile salts, but not of total bile salt mass. This would seem to indicate that plasma cholesteryl esters become a more important substrate for bile salt formation during infusion of tris-galactosyl-cholesterol, without an increase in the net flux through cholesterol 7α-hydroxylase. However, another explanation could be that the substrate pool for bile salt synthesis receives labeled cholesterol merely by exchange, and that the increased radioactivity in bile salts in the tris-galactosyl-cholesterol-treated rats is a reflection of increased intrahepatic availability of labeled cholesterol for exchange, without a real increase in mass flux into this substrate pool. The biliary excretion of free cholesterol, but that not of bile salts or phospholipids, was found to increase about 12 hr after start of tris-galactosyl-cholesterol infusion. As discussed above, the additional biliary cholesterol may be derived from the hydrolysis of tris-galactosyl-cholesterol molecules, as well as from plasma or hepatic cholesterol pools. Recently, other substances have been reported to cause an isolated increase of biliary cholesterol excretion, not parallelled by increases in bile salt and phospholipid excretion (19–22). These substances were found to decrease cholesterol esterification (19–20) or to increase cholesterol synthesis (21, 22), and were thought to channel cholesterol directly into bile. However, we think it improbable that cholesteryl ester formation would be depressed or cholesterol synthesis stimulated in the liver by tris-galactosyl-cholesterol treatment, since introduction of lipoproteins into various cells is invariably found to be accompanied by increased esterification and decreased cholesterol synthesis. For this reason we submit that the extra cholesterol, secreted in bile during and after tris-galactosyl-cholesterol infusion, is not of plasma or endogenous hepatic origin but is derived from the administered compound itself.

Surprisingly, the plasma cholesterol level remained depressed long after termination of tris-galactosyl-cholesterol infusion. As a possible explanation we suggest that the substance during its infusion becomes incorporated not only in the plasma lipoproteins but also in the plasma membranes of blood or tissue cells. These membranes then could serve as a storage compartment of the compound, slowly releasing it to lipoproteins which enter the circulation.

The present data show that tris-galactosyl-cholesterol has a potential as a new cholesterol-lowering drug. Since its HDL-lowering action reflects a higher cholesterol transport to the liver, this action may not be as unde-

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sirable as it would seem at first sight, provided that HDL production is not lowered by the substance. The drug probucol also lowers the plasma level of HDL, but is effective in treatment of xanthomatosis (23). It could be argued that the drug might enhance LDL uptake by tissue (arterial) macrophages, in view of its effect on LDL uptake by Kupffer cells. However, although LDL pretreated with tris-galactosyl-cholesterol was taken up much faster by freshly isolated rat Kupffer cells than untreated LDL, such a stimulation was not observed in cultured mouse peritoneal macrophages (T. van Berkel, unpublished observation). Clearly, further studies in other species, including man, are required to delineate in more detail the mode and safety aspects of this way of lipoprotein lowering. In the meantime, the substance can have considerable use as a tool in unravelling metabolic and cellular events concerned with hepatic cholesterol transport and catabolism.

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