Cholesterol-lowering effect of soyabean lecithin in normolipidaemic rats by stimulation of biliary lipid secretion

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The purpose of the present study was to assess the role of the liver in the plasma-cholesterol-lowering effect of soyabean lecithin. Normolipidaemic rats were fed on lecithin-enriched or control diets with the same amount of protein. The lecithin diets contained 200 g/kg high-fat commercial semi-purified soyabean lecithin (230 g/kg total lipids as soyabean phosphatidylcholine) or 200 g/kg high-fat purified soyabean lecithin (930 g/kg total lipids as soyabean phosphatidylcholine). The control diets were a low-fat diet (40 g fat/kg) and a high-fat triacylglycerol-rich diet (200 g fat/kg). The high-fat diets were isoenergetic. The cholesterol-lowering effect of the lecithin-enriched diets was associated with significantly lower levels of plasma total- and HDL-cholesterol and significantly higher levels of bile phosphatidylcholine (PC), bile salts and cholesterol. These findings suggest that the liver plays a major role in the reduction of plasma cholesterol, the increased biliary lipid being provided by both HDL and the hepatic microsomal pools of PC and cholesterol.

Soyabean lecithin: Cholesterol: Bile: Liver

Many studies have revealed that, in humans as well as in other species (i.e. rats), HDL are implicated in the process of bile lipid secretion (Halloran *et al.* 1978; Esnault-Dupuy *et al.* 1987; Rivabene *et al.* 1992) and that HDL, particularly HDL_2, are a major source of biliary steroids (Bravo & Cantafora, 1990). Furthermore, it has been demonstrated recently that an extrahepatic HDL–phosphatidylcholine (PC) pool is a major source of biliary PC (Chanussot *et al.* 1990; Portal *et al.* 1993). These observations have raised speculation concerning the metabolic effects of changes in HDL or HDL-like particles induced by administration of soyabean lecithin. Martins *et al.* (1989) and Williams *et al.* (1984) showed that a lecithin-rich diet could lead to a direct transfer of lecithin to plasma HDL or to the formation of HDL-like particles having a high potential of PC linkage.

The cholesterol-lowering effect of lecithin is not clearly established (Tompkins & Parkin, 1980; Williams *et al.* 1984; Knuiman *et al.* 1989). It has been suggested that polyunsaturated lecithin lowers plasma cholesterol, possibly by enhancing HDL formation (Jimenez *et al.* 1990). In addition, a soyabean-lecithin-enriched diet was shown to increase bile lipid secretion (Rioux *et al.* 1994). Thus, the present study was carried out in order to determine if the cholesterol-lowering effect can be correlated to a stimulation of bile production by HDL or HDL-like particles in normolipidaemic rats fed on diets enriched with soyabean lecithin.

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MATERIALS AND METHODS

Animals and diets

Male Wistar rats (Iffa Credo, L'Arbresle, France) were kept in pairs for 2 weeks in an animal room under controlled conditions (temperature: 22°; light cycle: 07.00–19.00 hours; humidity: 50%). A standardized diet UAR AO4 (UAR, Epinay sur Orge, France) and tap water were supplied *ad lib*. Mean weight at the end of this baseline period was 292 (SE 9) g. The rats were divided into four groups receiving the following diets: control (C): low-fat diet (40 g fat/kg diet; *n* 7); control-triacylglycerol (C-TG): high-fat diet containing sunflower-seed oil (200 g oil/kg diet; *n* 7); semi-purified lecithin (NPLE): high-fat diet (200 g semi-purified lecithin/kg diet; *n* 8); purified lecithin (PLE): high-fat diet (200 g lecithin/kg diet) containing pure soyabean lecithin (*n* 6) (Table 1). The commercial semi-purified lecithin contained 230 g pure soyabean PC/kg. The pure soyabean lecithin contained 930 g pure soyabean PC/kg.

The C, C-TG, NPLE and PLE diets in paste form were supplied *ad lib*. for 2 weeks. At the end of this period, mean weight gain was 38 (SE 4) g and there was no significant difference in weight gain between the four groups. Feed intake was similar in the four groups (18·5 (SE 2·0) g/d per rat). The three high-fat diets were chosen in order to be isoenergetic (16·2, 15·7 and 15·6 kJ/g for the C-TG, NPLE and PLE diets respectively). Thus, the rats in these three groups were given the same daily lipid energy intake, enabling the effects of the C-TG, NPLE and PLE diets to be compared in terms of triacylglycerol or phospholipid energy. Under such conditions the composition of fatty acids provided by the C-TG diet was different from that provided by the NPLE and PLE diets (Table 2). The C-TG diet did not contain linolenic acid whereas the NPLE and PLE diets were enriched in this fatty acid (7 g/100 g total fatty acids). Iwata *et al.* (1992) showed that such a difference (7%) in dietary linolenic acid did not induce changes in plasma cholesterol levels of rats given safflower-seed lecithin or soyabean lecithin. Thus, it is the amounts of fatty acid in the form of triacylglycerols or lecithin that are implicated in cholesterolaemic changes rather than the composition of these fatty acids. The results of the C-TG, NPLE and PLE groups can, therefore, be compared.

Experimental design

After diet administration, rats previously feed-deprived overnight were anaesthetized at 08.00 hours by an intraperitoneal injection of 48 mg pentobarbital sodium (Sanofi, Montpellier, France)/kg body weight and placed under an infrared heating lamp to maintain a constant body temperature of 37°. The main bile duct was cannulated with a polyethylene PE-10 catheter (Clays-Adams, Parsippany, NJ, USA) and bile was recovered in 15 min fractions for 2 h. After the first 15 min period of bile collection, 0·22 MBq in 1 ml of prepared HDL-[³H]PC containing 0·108 μmol PC was infused through a PE-50 catheter in the right jugular vein over a 15-min period. This was followed by a 15 min intravenous infusion of a solution containing 35 g/l bovine serum albumin (BSA, Sigma, Coger, Paris, France) in saline (9 g NaCl/l) at a rate of 4 ml/h. Bile flow was determined gravimetrically assuming a density of 1 g/ml. After 120 min, rats were killed by puncture of the abdominal aorta. Livers were removed and stored in a 1 mM-NaHCO₃ buffer solution, pH 7·5, at 4° and then homogenized for subcellular fractionation.

Preparation of [³H]phosphatidylcholine-HDL

[³H]PC-HDL was prepared as previously described (Portal *et al.* 1993). Plasma (23 ml) was collected from four feed-deprived (16 h) donor rats, and HDL were isolated by

Table 1. *Composition of the diets* (g/kg)

Diet...	C	C-TG	NPLE	PLE
Ingredients				
Casein*	240	240	240	240
Sunflower-seed oil†	40	200	—	—
Semi-purified soyabean lecithin‡	—	—	200	—
Purified soyabean lecithin‡	—	—	—	200
Starch*	240	140	140	140
Sucrose*	183	123	123	123
Cellulose*	40	40	40	40
Vitamin mixture*§	10	10	10	10
Minerals*‖	47	47	47	47
Distilled water	200	200	200	200
Energy content (kJ/g)	12·58	16·26	15·72	15·63

C, control diet (40 g lipid/kg); C-TG, control lipid-rich diet (200 g lipid/kg); NPLE, diet-enriched with 200 g semi-purified lecithin/kg; PLE, diet enriched with 200 g purified lecithin/kg.

* Provided by UAR, Villemoisson-sur-Orge, France.

† Lesieur, Paris, France.

‡ Lucas Meyer, Chelles, 77645 France.

§ The vitamin mixture (UAR 200) provided the following (mg/kg diet): retinol 12, cholecalciferol 0·125, thiamin 40, riboflavin 30, nicotinic acid 140, pyridoxine 20, pyridoxal 300, cyanocobalamin 0·1, ascorbic acid 1600, α-tocopherol 340, menadione 80, calcium pantothenate 200, choline 2720, pteroylmonoglutamic acid 10, *p*-aminobenzoic acid 100, biotin 0·6.

‖ The salt mixture (UAR B205) provided the following (g/kg diet): Ca 4·0, K 2·4, Na 1·6, Mg 0·4, Fe 0·12, Mn 0·032, Cu 0·005, Zn 0·018, Co 0·0004, I 0·002.

Table 2. *Lipid and fatty acid composition of the diets*

Diet...	C	C-TG	NPLE	PLE
Fatty acids (mol/100 mol fatty acids)				
16:0	5·6	5·6	14·0	14·0
18:0	2·2	2·2	4·0	4·0
18:1	25·1	25·1	11·0	11·0
18:2	66·2	66·2	64·0	64·0
18:3	—	—	7·0	7·0
Others	0·9	0·9	—	—
Phospholipid classes (mol/100 mol phospholipids)				
Lysophosphatidycholine	—	—	< 3	< 3
Phosphatidylcholine	—	—	22	93
Phosphatidylinositol	—	—	20	Tr
Phosphatidylserine	—	—	2	Tr
Phosphatidylethanolamine	—	—	23	Tr
Phosphatidic acid	—	—	5	Tr
Sphingomyelin	—	—	—	—
Soyabean sterols	—	—	13	< 0·5
Other phospholipids	—	—	9	—
Moisture and residual solvent	—	—	—	< 1·5

C, control diet (40 g lipid/kg); C-TG, control lipid-rich diet (200 g lipid/kg); NPLE, diet enriched with 200 g semi-purified lecithin/kg; PLE, diet enriched with 200 g purified lecithin/kg; Tr, trace.

ultracentrifugation and flotation at 120000 g for 22 h at 10° in a sucrose solution of density
1·21 g/ml according to the method of Hatch & Less (1968). Ultracentrifugation was carried
out in a Beckman L7 ultracentrifuge equipped with a fixed-angle rotor type 60Ti (Beckman,
Gagny, France). The resulting HDL fraction was dialysed against saline for 2 h to remove
sucrose.

HDL-PC were labelled by direct transfer of Lα-dipalmitoyl [choline-methyl-
^{3}H]phosphatidylcholine (specific radioactivity: 1850 GBq/mmol, NEN, Paris, France).
The purity (93%) of the commercial [^{3}H]PC solution was prechecked by TLC on silica gel
(F 1500, Schleicher and Schüll, Dassel, Germany) using a chloroform–methanol–water
(65:35:4, by vol.) mixture. Labelling of HDL was achieved by gently stirring HDL and
22·2 MBq of the [^{3}H]PC aqueous solution for 18 h at 37° (Martins *et al.* 1989; Tijburg *et
al.* 1991). A 1 ml portion of the prepared HDL-[^{3}H]PC contained 0·22 MBq [^{3}H] and
0·108 μmol PC.

Subcellular liver fractionation

Livers were fractionated by sequential centrifugation and sucrose-gradient ultracentrif-
ugation into two liver-cell plasma membrane fractions, one enriched in bile canalicular
complexes (BCM) and the other containing mainly lateral sinusoidal membranes (PM)
(Yousef *et al.* 1975). Microsomes were isolated by ultracentrifugation. The purity of the
cellular fractions was estimated after determination of rotenone-insensitive NADPH
ferrihaemoprotein reductase (*EC* 1.6.2.4), a microsomal marker (Sottocasa *et al.* 1967),
leucine aminopeptidase (*EC* 3.4.11.1), a canalicular marker (Yousef & Tuchweber, 1984)
and 5′-nucleotidase (*EC* 3.1.3.5), a sinusoid marker (Yousef & Tuchweber, 1984). The
purity of the fractions was similar to that obtained in previous metabolic studies (Portal *et
al.* 1993; Yousef *et al.* 1990). Enrichment of both leucine aminopeptidase and 5′-
nucleotidase was greater in BCM than in PM.

Assays in the plasma, bile and liver membrane subfractions

Total phospholipids in bile and membranes were analysed by measuring inorganic
phosphate as described by Amic *et al.* (1972) after digestion in perchloric acid. Cholesterol
in bile and membranes was measured by the method of Huang *et al.* (1961). Total and/or
unesterified plasma cholesterol was assayed using the enzymic method of Lie *et al.* (1976).
HDL-cholesterol was assayed by the method of Aufenanger *et al.* (1988). PC in plasma and
HDL was analysed by the enzymic method of Takayama *et al.* (1977). Protein in HDL
and liver membranes was determined by the method of Lowry *et al.* (1951). Bile acids were
enzymically determined using 3α-hydroxysteroid dehydrogenase (Sigma) (Domingo *et al.*
1972).

[^{3}H] radioactivity in bile and liver membranes was counted in 10 ml scintillation liquid
(Beckman Ready Solv MP) using a Packard liquid scintillation counter (Tri Carb 1600 TR,
Packard, Rungis, France) equipped with an external standard, and results were expressed
in disintegrations/min. The distribution of the radioactivity on PC, lyso-PC and free
choline in the bile was determined after separation by TLC using the conditions described
above. The specific radioactivity of PC in bile and cellular organelles was calculated as the
percentage of [^{3}H] injected per nmol PC.

Tissue processing assay

Pieces of liver, no more than 1 mm in size, were fixed for up to 6 h in 20 g/l glutaraldehyde
in phosphate-buffered saline (0·1 M, pH 7·4 at 4°). After several washes in the same buffer,
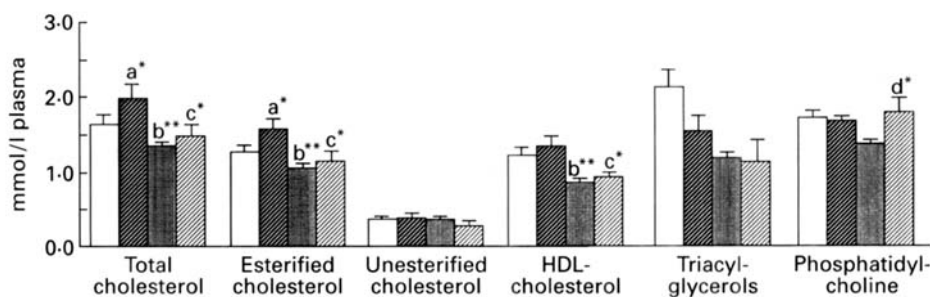samples were postfixed by 10 g/l OsO_4 in cacodylate buffer (0·1 M pH 7·4), then dehydrated

Fig. 1. Plasma cholesterol, triacyglycerol and phospholipid concentrations in rats fed on a low-fat control diet (C, □, n 7), a high-fat control diet (C-TG, ▨, n 7), a diet containing 200 g semi-purified soyabean lecithin/kg (NPLE, ▩, n 8) or a diet containing 200 g purified soyabean lecithin/kg (PLE, ▨, n 6). Values are means with their standard errors represented by vertical bars. Differences were assessed by ANOVA for factorial non-repeated values at a probability of 95 % by Fisher's post-hoc least significant difference test or the Scheffe F test. * Significant difference as assessed by Fisher's test; ** significant difference by both tests. [a] C v. C-TG; [b] C-TG v. NPLE; [c] C-TG v. PLE; [d] NPLE v. PLE.
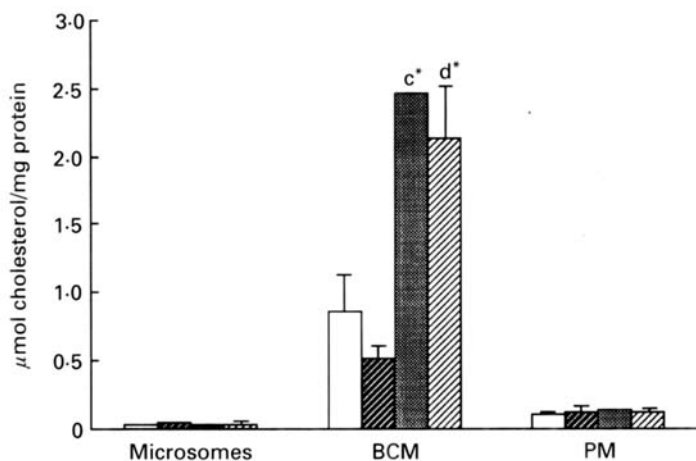

Fig. 2. Concentration of cholesterol in the microsomes, bile canalicular membranes (BCM) and total plasma membranes (PM) of rats fed on a low-fat control diet (C, □, n 7), a high-fat control diet (C-TG, ▨, n 7), a diet containing 200 g semi-purified soyabean lecithin/kg (NPLE, ▩, n 8) or a diet containing 200 g purified soyabean lecithin/kg (PLE, ▨, n 6). Values are means with their standard errors represented by vertical bars. Differences were assessed for ANOVA for factorial non-repeated values at a probability of 95 % by Fisher's post-hoc least significant difference test or the Scheffe F test. * Significant difference as assessed by Fisher's test. [c] C-TG v. PLE; [d] NPLE v. PLE.

through ethanol and embedded in Epon 812 (Luft, 1961). Semi-thin sections (2 μm in thickness) were stained with toluidine blue.

Statistical analysis

Results are expressed as the arithmetical means of each group with their standard errors. We previously verified the homogeneity of the data in all conditions. Thus differences between groups were compared by using ANOVA for factorial experiments (Figs 1–3) or for repeated values (Fig. 4). The significant differences were determined by Fisher's post-hoc least significant difference test and/or the Scheffe F test at a probability value of 95 %. However, we restricted the statistical test to comparisons with only one variable (C v. C-TG; C-TG v. NPLE; C-TG v. PLE; NPLE v. PLE).
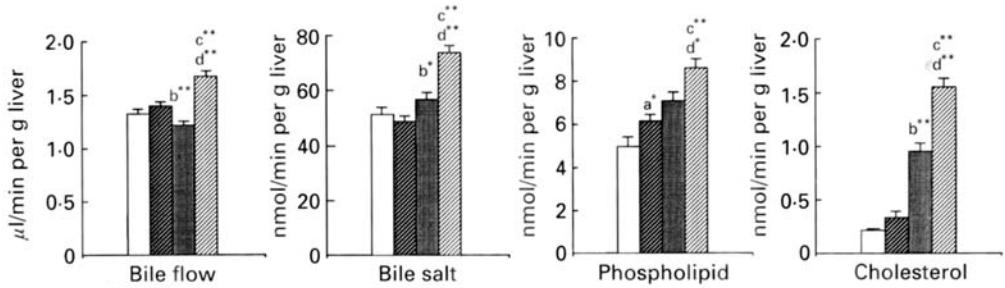
Fig. 3. Lipid composition of bile from rats fed on a low-fat control diet (C, □, n 7), a high-fat control diet (C-TG, ▨, n 7), a diet containing 200 g semi-purified soyabean lethicin/kg (NPLE, ▨, n 8) or a diet containing 200 g purified soyabean lecithin/kg (PLE, ▨, n 6). Values are means with their standard errors represented by vertical bars. Differences were assessed by ANOVA for factorial non-repeated values at a probability of 95% by Fisher's post-hoc least significant difference test or the Scheffe F test. * Significant difference as assessed by Fisher's test; ** significant difference by both tests. [a] C v. C-TG; [b] C-TG v. NPLE; [c] C-TG v. PLE; [d] NPLE v. PLE.

RESULTS

Plasma

As shown in Fig. 1, rats fed on the C-TG diet had significantly greater total cholesterol and esterified cholesterol compared with the control (low fat) group C. However, both NPLE and PLE lecithin diets led to a significant decrease of the total and esterified cholesterol compared with the C-TG group. This reduction was associated with a significant reduction in HDL-cholesterol. Lecithin-containing diets lowered plasma triacylglycerols non-significantly. Plasma PC was not significantly different between C and C-TG groups, or between C-TG and PLE groups. However, there was a significant increase in plasma PC in the PLE group, compared with the NPLE group (Fig. 1).

Liver

Although both the NPLE and PLE diets caused a significant increase in cholesterol in the BCM compared with the C-TG diet, there were no changes in the cholesterol content of the microsomes or the PM (Fig. 2). On the other hand, phospholipid contents of the BCM microsomes and PM were not significantly affected by the different diets (about 0·6 μmol phospholipid/mg protein in each subcellular fraction; results not shown).

Bile

Bile flow as well as bile salt, phospholipid and cholesterol secretion rates were significantly higher in the PLE group than in the C-TG group (Fig. 3). Bile salt and cholesterol outputs were significantly higher in the NPLE group than in the C-TG group. The level of radioactive PC increased in the C and C-TG groups with the duration of the experiment, and reached a maximum between 60 and 75 min; thereafter it remained constant until the end of the experiment (Fig. 4(a)). This plateau was at a significantly higher level in the C-TG group than in the C group. The PLE diet also significantly increased the secretion of radioactive PC as compared with C-TG, without reaching a maximum as in the C-TG group. The quantities of radioactive PC secreted in the bile were not significantly different between the NPLE and C-TG groups, from the beginning up to 75 min of experiment. Between 90 and 120 min the quantities of radiolabelled PC were significantly higher in rats consuming the NPLE diet than in those fed on the C-TG diet. However, there was no
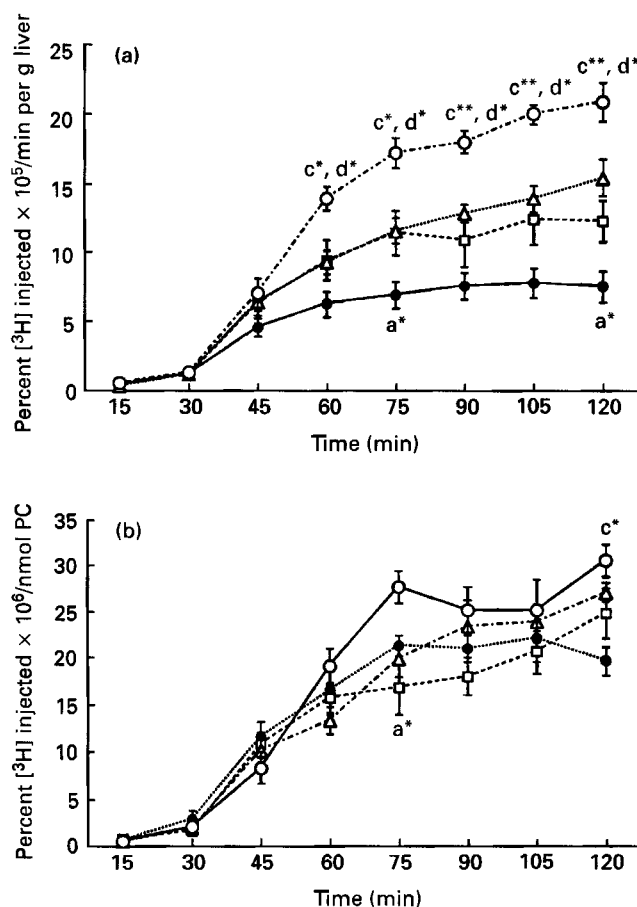
Fig. 4. (a) Quantities and (b) specific radioactivity of [³H]phosphatidylcholine ([³H]PC) secreted in the bile of rats fed on a low-fat control diet (C, —●—, n 7), a high-fat control diet (C-TG, --□--, n 7), a diet containing 200 g semi-purified soyabean lecithin/kg (NPLE, --△--, n 8) or a diet containing 200 g purified soyabean lecithin/kg (PLE, --○--, n 6). Values are means with their standard errors represented by vertical bars. Differences were assessed by ANOVA for repeated values at a probability of 95 % by Fisher's post-hoc least significant difference test or the Scheffe F test. * Significant difference as assessed by Fisher's test; ** significant difference by both tests. ᵃ C v. C-TG; ᵇ C-TG v. NPLE; ᶜ C-TG v. PLE; ᵈ NPLE v. PLE.

marked difference between the four groups in regard to the specific radioactivity of bile PC; the only significant differences occurred at 75 min between groups C and C-TG, and at 120 min between groups C-TG and PLE (Fig. 4(b)).

Histological study of the liver

The observations were carried out in order to determine eventual disturbances in the liver of the lecithin group. The histological structure was well preserved (Fig. 5). At the cellular level some observations can be made. In the lecithin group, at the periphery of the dilated bile canaliculi (↑), the pericanalicular areas appear deprived of mitochondria. Unstained small vacuoles (▲) are numerous and located in these areas; larger vacuoles (*) with a clear content are present facing the spaces of Disse.
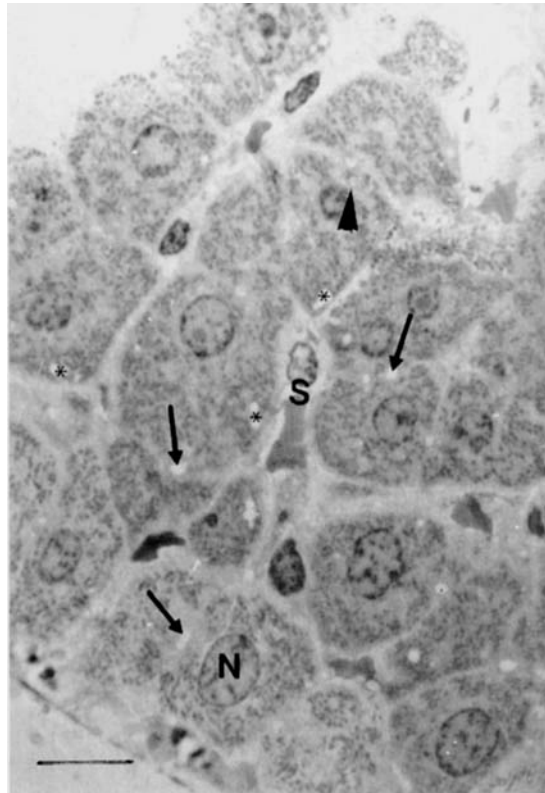
Fig. 5. Semi-thin sections, 2 μm (toluidine blue) through rat livers. N, nucleus; S, sinusoid; *, large vacuoles; ▲, small peribiliary vacuoles; ↑ bile canaliculi; bar, 100 μm.

DISCUSSION

Previous findings have shown that HDL is the main lipoprotein implicated in the physiological transport of lecithins (Martins *et al.* 1989) involving a plasma PC transfer protein (Pownall *et al.* 1991) and that the HDL fraction is largely implicated in the transport and/or exchange of cholesterol between tissues and plasma (Schwartz *et al.* 1993).

In the present experiment soyabean lecithin led to a significantly reduced level of esterified plasma cholesterol. HDL-cholesterol was significantly lower in both PLE and NPLE groups compared with the C-TG group. Our findings on plasma cholesterol partially corroborate those of Jimenez *et al.* (1990). These authors observed that polyunsaturated lecithin also induces a reduction in plasma cholesterol of hypercholesterolaemic rats by lowering VLDL-, intermediate-density-lipoprotein (IDL-) and LDL-cholesterol despite a rise in HDL-cholesterol. This enhancement of HDL-cholesterol by phospholipid-enriched diets in hypercholesterolaemic rats was confirmed by Iwata *et al.* (1993). The opposite effect of lecithin on HDL-cholesterol observed in these latter studies and in our work could be explained by differences in the experiments, which used hypercholesterolaemic and normolipidaemic rats respectively. In addition, the composition of soyabean lecithins is different according to their origin. Since our commercial semi-purified lecithin contained the same amounts of PC and phosphatidylethanolamine, part of the plasma-cholesterol-

lowering effect observed with the semi-purified NPLE lecithin could have been due to dietary phosphatidylethanolamine which shows this effect in hypercholesterolaemic rats (Imaizumi *et al.* 1991). A possible cholesterol-lowering effect of other main components of commercial NPLE lecithin, such as phospholipids (phosphatidylinositol, phosphatidic acid) and vegetable sterols (phytosterols), might be considered.

Podrez *et al.* (1993) suggested that VLDL and bile salt productions are inversely correlated. Moreover, Robins *et al.* (1990) demonstrated that bile and HDL secretions by the liver are regulated by the bile salts. In the present experiment bile salt production was stimulated by lecithin feeding and this may represent a protective effect against atherogenic VLDL and LDL. Lecithin raised cholesterol in the bile canalicular vesicles. The abundance of small vacuoles formed at the periphery of the bile canaliculi suggest an enhanced traffic in this part of the cell. Such observations could be related to a significant increase in bile flow as well as in all biliary lipids. These results are in agreement with those of Rioux *et al.* (1994), obtained in normolipidaemic rats fed on diets enriched with commercial semi-purified lecithin, and showing a higher level of all bile lipid components. In the present study the lecithin diets resulted in a significant rise of radiolabelled biliary PC originating from HDL-PC, compared with C-TG, while at each experimental time-point specific radioactivity of the bile PC did not show marked differences between the four groups. Thus, the higher amounts of total bile PC in the lecithin groups could result not only from a stimulation of [³H]HDL-PC uptake by the liver but also from enhanced release by hepatic microsomes of unlabelled neosynthesized or preformed PC. Our data show that the molar ratio of unesterified cholesterol to phospholipids in the bile was higher in the NPLE and PLE groups than in the C-TG group. This increase in both lecithin groups could not be correlated to the stable unesterified cholesterol:phospholipids ratio observed in the plasma of rats in all groups. Thus, lecithin could mobilize another source of bile cholesterol as plasma (HDL)-cholesterol, possibly from the preformed or newly synthesized hepatic microsomal cholesterol pool. This hypothesis is based on other results (Ide *et al.* 1994) showing that soyabean lecithin stimulates hepatic cholesterol biosynthesis.

Rioux *et al.* (1994) found no evidence of modification of the nature of PC fatty acids in the bile and liver plasma membranes, after lecithin administration, as could be explained by studies of Iwata *et al.* (1993). These authors proposed that the hypocholesterolaemic effect of lecithin was unrelated to the degree of polyunsaturation of lecithin fatty acids. Thus the cholesterol-lowering effect of lecithin could be linked to the hydrophilic nature of the lecithin head. However, such a hypothesis needs further investigation.

In conclusion, the specific cholesterol-lowering effect of dietary lecithin can be explained in part by a stimulation of uptake of a PC carrier-HDL by the liver. The stimulation results in higher amounts of bile lipids provided by both HDL and hepatic microsomal pools of PC and cholesterol.

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