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EFFECTS OF NIACIN ON BILIARY LIPID OUTPUT IN THE RAT

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Abstract—The mechanisms for the hypocholesterolaemic action of niacin (nicotinic acid) were examined in rats administered niacin at a dose of 400 mg/kg body wt/day for either 2 or 4 weeks. Another group of rats were administered diosgenin, an inhibitor of acyl-CoA:cholesterol acyltransferase, as a 1% (w/w) supplement in the diet for 7 days. Both agents produced small increases in bile flow rates (up to 40%) and mild hepatotoxicity evidenced by small increases in serum transaminase activities. Niacin treatment for 2 or 4 weeks lowered serum cholesterol concentrations by 13% or 29%, respectively, with the greatest decrease occurring in the low density lipoprotein fraction. This was accompanied by relatively large increases in biliary cholesterol output (114% and 130% after 2 and 4 weeks treatment, respectively) with smaller increases in the biliary output of phospholipid (18% and 45%) and bile acid (26% and 14%). Diosgenin treatment increased serum cholesterol by 29% and increased the biliary output of cholesterol, phospholipid and bile acid by 800%, 10% and 45%, respectively. Thus, both agents increased the cholesterol saturation of bile (100% by niacin, 500% by diosgenin). Cholesterol and phospholipid in fistula bile from control rats were present in lamellar and micellar forms. Niacin treatment did not alter the physical form of biliary lipids whilst diosgenin caused the appearance of vesicular lipid in fistula bile. Thus, increased biliary secretion of cholesterol explains, at least in part, the hypocholesterolaemic action of niacin. In addition, since aggregation of biliary vesicles is involved in cholesterol gallstone formation in humans, the non-appearance of vesicular material in fistula bile from niacin-treated rats may be of some importance.

Niacin (nicotinic acid) is a well-established agent for lowering circulating lipid concentrations and hence decreasing cardiovascular events [1]. It is administered either alone at doses of 3–6 g/day [2], or in combination with other hypolipidaemic agents, e.g. cholestyramine [3]. A variety of benign, but often unpleasant, side effects of niacin have been reported and include transient increases in serum transaminases [4]. An analogue of niacin, acipimox, has recently been introduced as a hypolipidaemic agent and is effective at much lower doses [5].

The hypolipidaemic effects of niacin are thought to be mediated primarily by decreased hepatic output of very low density lipoprotein (VLDL§) [6]. During niacin therapy in humans, total serum cholesterol concentrations are decreased by up to 40%; atherogenic low density lipoprotein (LDL) cholesterol being reduced whilst atherosclerosis-protecting high density lipoprotein (HDL) cholesterol is increased [7]. Niacin has also been reported to inhibit lipolysis, resulting in reduced circulating concentrations of both non-esterified fatty acids and triglycerides [6].

Bile represents the only route for excretion of cholesterol, either by direct secretion into bile or via conversion into bile acids. In bile, cholesterol was initially thought to be solubilized in mixed micelles with bile acids and phospholipids [8]. Subsequently, unilamellar vesicles were identified as lipid carriers in human bile [9], and stacked lamellar structures have recently been identified in human bile [10] and may also be of importance as biliary lipid carriers.

The present study was undertaken to investigate whether the hypocholesterolaemic action of niacin is mediated, at least in part, via increased biliary secretion of either cholesterol itself or bile acids. These experiments were undertaken in niacin-treated rats. Whilst the rat shows similar HDL metabolism to humans, it must be remembered that the conversion of VLDL to LDL differs markedly, manifesting itself in low concentrations of LDL in rat serum. As a comparison, biliary lipid output was also investigated in diosgenin-treated rats. Diosgenin is a potent inhibitor of acyl CoA:cholesterol acyltransferase (ACAT), and markedly increases biliary cholesterol output in rats [11]. Gel exclusion chromatography was employed to investigate possible alterations in the physical forms of biliary lipids after niacin or diosgenin treatment.

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§ Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein; ALT, alanine aminotransferase; AST, asparate aminotransferase; ACAT, acyl CoA: cholesterol acyltransferase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA.

MATERIALS AND METHODS

Materials. Hydroxysteroid dehyrogenase (grade II, from *Pseudomonas testosteroni*), NAD^+, 5α-cholestane and other fine chemicals were from the

Sigma Chemical Co. (Poole, U.K.). All other chemicals and solvents were from BDH (Speke, U.K.) and were the highest grade available. Sephacryl S-300HR was from Pharmacia (Milton Keynes, U.K.). [1α,2α-^{3}H]Cholesterol (1.69 TBq/mmol) was from Amersham International (Aylesbury, U.K.) and Ecoscint H scintillation fluid was from Mensura Technology (Wigan, U.K.). Cannulation tubing PP10 was made by Portex (Hythe, U.K.).

Animals. Male Wistar rats (230–330 g), fed a standard laboratory diet and maintained at 22° under a constant 12 hr light–dark cycle, were used throughout.

Niacin was dissolved in water to a final concentration of 100 mg/mL and administered by stomach tube at a dose of 400 mg/kg body wt/day for either 2 or 4 weeks. This is approximately four times the highest dosages employed in humans, but on the basis of body surface area, is equivalent to a human dose of 5 g/day [12]. Diosgenin was administered for 7 days as a 1% (w/w) supplement in the diet. Diosgenin was dissolved in chloroform and sprayed uniformly onto the powdered chow and mixed; the solvent was allowed to evaporate in a fume hood for 24 hr before feeding commenced.

Animals were starved for 24 hr before bile duct cannulations were performed with PP10 tubing while the rats were under pentobarbitone (Sagatal) anaesthesia. The cannula was inserted into the bile duct close to the liver to avoid possible contamination with pancreatic juice [13]. Animals were maintained in a thermostatically controlled cabinet at 37° throughout bile collection. Bile was collected on ice (for assays) or at room temperature (for gel exclusion chromatography) for 1 hr into pre-weighed tubes; bile volume was determined as the increase in weight of the collection tubes, assuming bile has a density of 1 g/mL. All bile samples were stored at −20° for up to 2 weeks before assay.

After bile collection, blood was collected by severing the jugular vessels. Blood was allowed to clot at room temperature and serum separated by centrifugation. Livers were also removed and weighed.

Gel exclusion chromatography. [1α,2α-^{3}H]Cholesterol (22.2 kBq, 13.2 pmol) was placed in a clean glass tube and the toluene solvent evaporated under nitrogen. Fresh rat bile (0.75 mL) was added to the dry [^{3}H]cholesterol and incubated at 37° for 1 hr with gentle shaking, during which time the [^{3}H]-cholesterol equilibrates with endogenous cholesterol in bile [14]. Radiolabelled bile (0.5 mL) was loaded onto a column (1.6 x 70 cm) of Sephacryl S-300HR. The column was eluted by downward flow of buffer at 1 mL/min and fractions of 1.3 mL were collected. The elution buffer was 150 mM NaCl, 50 mM Tris–HCl, 1.5 mM EDTA, pH 8.0, and contained 10 mM sodium cholate to preserve micellar integrity [14]. Aliquots (0.3 mL) of the eluate fractions were mixed with 10 mL of Ecoscint H scintillation fluid and radioactivity determined in a Canberra-Packard liquid scintillation counter model 1600TR.

Blue dextran was used to determine the void volume of the column and bovine serum albumin (M_r 66,700), ovalbumin (M_r 43,000), chymotrypsin (M_r 24,300) and cytochrome *c* (M_r 12,400) were used to calibrate the column.

Assays. Biliary cholesterol was measured by gas liquid chromatography of its trimethylsilyl ester using 5α-cholestane as internal standard [15]. The column (152 cm x 3 mm) was 1.5% (w/w) SE30 on Diatomite CQ (80–100 mesh) and the operating conditions were: carrier gas (N_2) flow rate, 50 mL/min; injection temperature, 250°; column temperature, 235°; detector temperature, 270°. Total bile acid concentrations in bile were assayed using 3α-hydroxysteroid dehydrogenase [16]. Phospholipids in aliquots of bile or column eluate were extracted into chloroform [17] and organic phosphorous determined by the method of Bartlett [18] except that, after evaporation of chloroform, samples were digested with 70% (v/v) perchloric acid [19].

Serum total cholesterol was determined by the CHOD-PAP method and triglyceride by the GPO-PAP method using kits supplied by Boehringer Mannheim (Germany). Serum HDL was measured as the cholesterol remaining in the supernatant after precipitation of all other serum lipoproteins with phosphotungstic acid and Mg^{2+} ions (HDL precipitant, Boehringer Mannheim). Serum VLDL cholesterol was calculated using the Friedewald equation [20] which has been validated for human serum.

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed at 20° by NAD^+-coupled reactions using kits supplied by Boehringer Mannheim.

RESULTS

Serum lipid profiles

Treatment of rats with niacin produced a hypocholesterolaemic effect similar to that seen in man. After 2 or 4 weeks niacin administration, serum total cholesterol concentrations were decreased by 13% or 29%, respectively (Table 1). This decrease was primarily in the LDL cholesterol fraction although a small, but significant, decrease in HDL cholesterol was seen after 4 weeks niacin treatment (Table 1). In contrast, a hypotriglyceridaemic effect was not observed with niacin; indeed, after 4 weeks treatment, serum triglycerides were significantly increased by 57% (Table 1). Relatively small increases (<50%) in serum AST and ALT occurred during niacin treatment (Table 1).

In contrast to niacin, diosgenin increased both HDL and LDL cholesterol by similar amounts, resulting in a 29% increase in total serum cholesterol concentrations. Serum triglyceride concentrations were unchanged and small increases in serum AST and ALT were observed (Table 1).

Biliary lipid profiles

Niacin produced a small choleresis, increasing the average bile flow rate for 1 hr after interruption of the enterohepatic circulation by between 20% and 35% (Table 2). Niacin treatment for 2 or 4 weeks increased the biliary cholesterol concentration by 73% or 91%, respectively; no effect was observed upon the biliary concentration of bile acids, whilst biliary phospholipid concentrations were significantly

Table 1. Serum lipids and transaminases in niacin- and diosgenin-treated rats

	Treatment			
	Control (13)	Niacin 2 week (11)	Niacin 4 week (14)	Diosgenin (12)
Total cholesterol (mM)	1.78 ± 0.097	1.54 ± 0.071	1.26 ± 0.052‡	2.29 ± 0.102†
HDL cholesterol (mM)	0.882 ± 0.044	0.785 ± 0.102	0.703 ± 0.034*	1.115 ± 0.102
LDL cholesterol (mM)	0.663 ± 0.084	0.466 ± 0.051	0.175 ± 0.032‡	0.903 ± 0.118
Triglyceride (mM)	0.504 ± 0.037	0.617 ± 0.069	0.791 ± 0.108*	0.538 ± 0.042
AST (IU/L)	114.5 ± 8.0	159.2 ± 21.6	134.2 ± 27.0	154.5 ± 13.3*
ALT (IU/L)	34.3 ± 2.4	50.0 ± 8.4	49.8 ± 5.6*	58.0 ± 3.4‡

Values are means ± SEM for the number of observations given in parentheses.
Significant differences from controls were assessed by Student's *t*-test and are indicated by: * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$.

Table 2. Biliary lipid profiles in niacin- and diosgenin-treated rats

	Treatment			
	Control (13)	Niacin 2 week (11)	Niacin 4 week (14)	Diosgenin (12)
Bile flow rate (μL/min)	18.0 ± 1.4	22.3 ± 1.2*	24.3 ± 1.4†	25.1 ± 1.7†
Bile flow rate (μL/min/g liver)	1.60 ± 0.09	1.95 ± 0.06†	1.93 ± 0.11*	2.04 ± 0.14*
Phospholipid (mM)	6.45 ± 0.33	6.28 ± 0.24	7.63 ± 0.38*	5.41 ± 0.69
Total bile acids (mM)	21.0 ± 1.0	21.9 ± 0.8	19.7 ± 0.8	24.7 ± 1.1*
Cholesterol (mM)	0.218 ± 0.025	0.378 ± 0.056*	0.416 ± 0.051†	1.51 ± 0.27‡
Phospholipid output (nmol/min/g liver)	10.3 ± 0.78	12.2 ± 0.58	14.9 ± 1.26†	10.8 ± 1.57
Bile acid output (nmol/min/g liver)	33.8 ± 2.7	42.6 ± 1.7*	38.4 ± 3.4	49.3 ± 2.8‡
Cholesterol output (nmol/min/g liver)	0.347 ± 0.040	0.744 ± 0.122†	0.803 ± 0.107‡	3.10 ± 0.56‡
Cholesterol: (bile acid + phospholipid) ratio	0.0079 ± 0.00068	0.0134 ± 0.0018*	0.0156 ± 0.0022†	0.0486 ± 0.0072‡

Values are means ± SEM for the number of observations given in parentheses.
Significant differences from controls were assessed by Student's *t*-test and are indicated by: * $P < 0.05$, † $P < 0.01$ and ‡ $P < 0.001$.

increased by 18% after 4 weeks treatment with niacin (Table 2). When expressed as rates of output (nmol/min/g of liver), the increases in biliary cholesterol and phospholipid showed a time–effect relationship with the greatest increases occuring after 4 weeks of niacin treatment (Table 2). In addition, a small increase in bile acid output was observed. However, because niacin increased the biliary output of cholesterol to a much greater extent than that of either phospholipid or bile acids, the cholesterol:(bile acid + phospholipid) ratio was increased by 70% and 97% after treatment for 2 and 4 weeks, respectively (Table 2).

Diosgenin feeding produced much greater increases in biliary cholesterol concentrations (600%) and output (800%); a small increase in biliary bile acid output was observed whilst phospholipid output was unaffected (Table 2). This resulted in a 500% increase in the cholesterol:(bile acid + phospholipid) ratio over the control value (Table 2).

Physical form of biliary cholesterol and phospholipid

Chromatography of bile from control rats revealed a major peak of [^{3}H]cholesterol of apparent M_r 43,000 together with a partially resolved smaller peak of apparent M_r 15,000 (Fig. 1a). Phospholipid in bile from control rats also eluted as two partially resolved peaks of similar apparent M_r (Fig 2a). By comparison to elution profiles of human bile obtained under similar conditions [21], it is likely that the major cholesterol and phospholipid peaks of M_r 43,000 represent stacked lamellae whilst the peaks of M_r 15,000 represent mixed micelles.

Chromatography of bile from rats treated with niacin for 2 and 4 weeks revealed similar elution profiles of both cholesterol (Fig. 1b, c) and phospholipid (Fig. 2b, c). However, chromatography of bile from diosgenin-fed rats always revealed additional peaks of cholesterol (Fig. 1d) and phospholipid (Fig. 2d) which eluted at the void

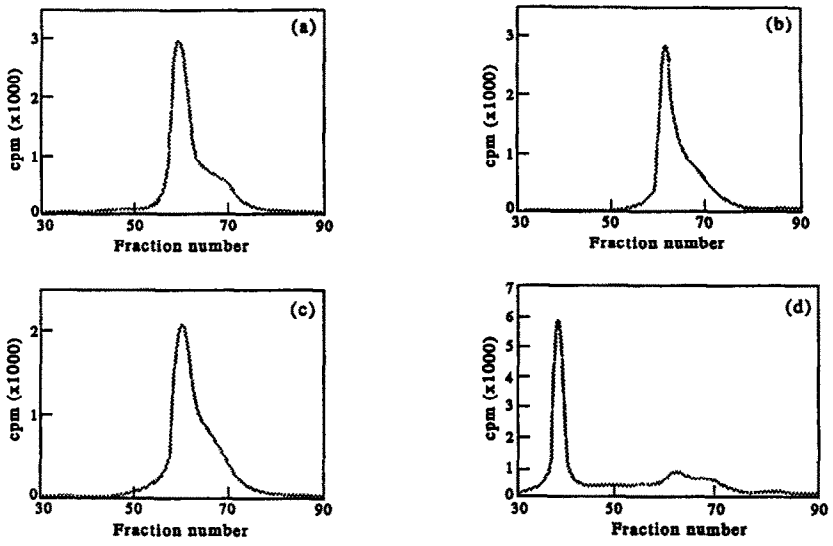

Fig. 1. Distribution of [^{3}H]cholesterol following gel exclusion chromatography of rat bile on Sephacryl S-300HR. (a) Control bile; (b) bile from a rat treated with niacin for 2 weeks; (c) bile from a rat treated with niacin for 4 weeks and (d) bile from a diosgenin-fed rat. Typical elution profiles are shown from at least five observations in each group. Full experimental details are given in Materials and Methods.

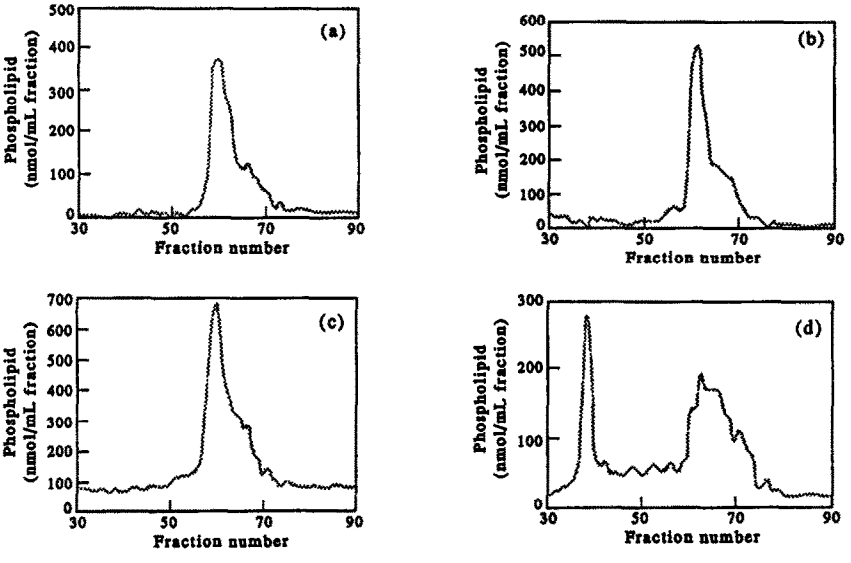

Fig. 2. Distribution of phospholipid following gel exclusion chromatography of rat bile on Sephacryl S-300HR. (a–d) are as described in the legend to Fig 1. Typical elution profiles are shown from at least five observations in each group.

volume of the column and, therefore, were of apparent M_r greater than the exclusion limit of Sephacryl S-300HR (i.e. >500,000). This form of cholesterol and phospholipid has been identified as vesicular by Sömjen and Gilat [14]. Lamellar (43,000) and micellar (15,000) peaks of cholesterol and phospholipid were also identified in bile from diosgenin-fed rats.

DISCUSSION

When administered to hyperlipidaemic patients, niacin decreases both serum total cholesterol and triglyceride concentrations by 10–40% (see, for example, Refs 2, 7, 22). Although serum cholesterol concentrations in laboratory rats are considerably lower than those in hyperlipidaemic patients, niacin

feeding for 2 or 4 weeks similarly reduced serum cholesterol by 13% or 29%, respectively (Table 1). By far the greatest decrease in serum cholesterol was seen in the LDL fraction although a small decrease in HDL cholesterol was also evident. Paradoxically, niacin feeding of rats appeared to increase serum triglyceride concentrations (Table 1). However, it must be remembered that serum triglyceride concentrations in rats are several-fold lower than those routinely seen in humans. Indeed, following sucrose feeding to rats, when serum triglyceride concentrations increased 4-fold and approached those seen in hyperlipidaemic patients, niacin dramatically lowered serum triglyceride concentrations (Crosbie and Rahman, unpublished observations). Thus, whilst niacin treatment of rats does not produce exactly the same alterations in serum lipid profiles as seen in humans, it does produce a similar and marked hypocholesterolaemia.

The hypolipidaemic action of niacin is thought to be due, at least in part, to its inhibition of lipolysis in adipose tissue. It is well known that lipolysis is stimulated by elevated intracellular cyclic AMP concentrations. Niacin has been shown to inhibit adenylate cyclase in both hamster [23] and human [24] adipocytes, with a concomitant decrease in intracellular cyclic AMP concentrations. Indeed, it has been suggested that niacin binds to a receptor on the adipocyte plasma membrane and inhibits adenylate cyclase via an inhibitory guanine nucleotide protein (G_i-protein), in an analogous fashion to adenosine and prostaglandin E_2 [25]. Decreased availability of free fatty acids is then believed to reduce the ability of the liver to assemble and secrete VLDL [6, 7, 25].

Whilst such a mechanism would explain how niacin decreases serum triglyceride concentrations, it does not explain the hypocholesterolaemic action of niacin. Table 2 shows that niacin markedly increases biliary cholesterol output whilst producing much smaller increases in the biliary output of bile acids and phospholipids. This results in an increased cholesterol saturation of bile, as evidenced by a doubling of the cholesterol:(bile acid + phospholipid) ratio after 4 weeks niacin feeding (Table 2). Angelin *et al.* [26] have similarly shown that niacin increases biliary cholesterol concentrations in humans with a resultant increase in the cholesterol saturation of bile. In addition, Grundy *et al.* [27] have reported a small increase in biliary cholesterol concentrations following niacin therapy, although this did not cause an increase in the faecal excretion of neutral steroids nor produce a significant increase in the cholesterol saturation of bile. It is interesting to note that acipimox, a closely related analogue of niacin, does not increase biliary cholesterol saturation; indeed, cholesterol saturation was decreased in eight out of nine patients [28].

Biliary cholesterol is derived from both *de novo* synthesis in the liver and a pre-formed hepatic cholesterol pool. The contribution from *de novo* synthesis is relatively small (approximately 20%) [29] and varies with the activity of HMG-CoA reductase, the rate-limiting enzyme of cholesterol synthesis [30]. Whilst it is possible that niacin increases biliary cholesterol output via increased HMG-CoA reductase activity, this is unlikely as such an effect would be expected to increase serum cholesterol concentrations as well. It is more likely that niacin increases the secretion of pre-formed cholesterol into bile. Hepatic cholesterol exists in a single metabolic pool, the whole of which is potentially available for biliary secretion [31]. The size of this pre-formed cholesterol pool (and hence biliary cholesterol output) is partly regulated by the rates of cholesterol esterification (by ACAT) and hydrolysis of its ester (by cholesterol ester hydrolase) [30]. Thus, diosgenin, an inhibitor of ACAT, expands the hepatic cholesterol pool, which results in increased biliary output [11, 32]. Similar results have been obtained in this study (Tables 1 and 2). However, it is unlikely that niacin inhibits ACAT since it lowers, rather than increases, serum cholesterol.

Some evidence is now available which suggests that biliary cholesterol and phospholipid output is controlled by intracellular second messengers [30]. Of particular interest is the observation that membrane-permeable cyclic AMP analogues and forskolin (a stimulator of adenylate cyclase) produce a sustained reduction in biliary lipid output without a sustained effect on biliary bile acid output [33]. Thus, by analogy to the known ability of niacin to reduce cyclic AMP concentrations in adipose tissue [23–25], it is tempting to speculate that niacin similarly decreases cyclic AMP concentrations in the liver. Such an effect would be expected to increase the biliary output of cholesterol from pre-formed sources.

Lipid is thought to be secreted initially into bile as a result of bile acid-induced outward vesiculation of fluid microdomains of the canalicular membrane [30]. These vesicles of cholesterol and phospholipid are further processed by bile acids to yield other physical forms such as stacked lamellae and mixed micelles. Gel exclusion chromatography of bile from control rats revealed two peaks of cholesterol (Fig. 1) and phospholipid (Fig. 2). The major peaks of cholesterol and phospholipid at apparent M_r 43,000 were identified as lamellar by comparison to the elution profiles of human bile obtained by Sömjen *et al.* [21]; indeed, these authors report an apparent M_r of 37,000 for lamellar cholesterol in human bile. Micellar cholesterol and phospholipid eluted after the lamellar material at an apparent M_r of 15,000. Despite niacin increasing the biliary output of cholesterol by considerably more than that of phospholipid or bile acids, no change was observed in the physical form of either cholesterol or phospholipid. In contrast, gel exclusion chromatography of bile from diosgenin-treated rats showed an extra peak of cholesterol and phospholipid at the void volume; such material has been identified by many workers as vesicular [8, 21, 34]. Diosgenin increases biliary cholesterol output to a much greater extent than niacin (Table 2). As a result, the cholesterol:(bile acid + phospholipid) ratio is higher in diosgenin-treated rats (0.049) than in niacin-treated rats (0.016) (Table 2). Therefore, it is likely that, although diosgenin also produces a small increase in biliary bile acid output (Table 2), insufficient bile acids are available to process all of

the vesicular lipid such that vesicles remain in fistula bile. On the other hand, sufficient bile acids are available in bile from niacin-treated rats and vesicles are not seen. These results may be of some significance since aggregation of vesicles is thought to be involved in the nucleation of cholesterol gallstones [34–36].

In conclusion, this study has demonstrated that increased biliary secretion of cholesterol contributes to the hypocholesterolaemic action of niacin in rats. Niacin did not cause any alteration in the physical form of cholesterol in rat bile although diosgenin, which increased biliary cholesterol secretion to a much greater extent, caused the appearance of vesicular lipid in fistula bile.

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