Hypocholesterolemic action of β-cyclodextrin and its effects on cholesterol metabolism in pigs fed a cholesterol-enriched diet

Jacqueline Férézou,[1,*] Michel Riottot,* Colette Sérougne,* Corinne Cohen-Solal,*
Isabelle Catala,‡ Christian Alquier,§ Michel Parquet,* Catherine Juste,† Huguette Lafont,§
Denis Mathé,* Tristan Corring,† and Claude Lutton*

Laboratoire de Physiologie de la Nutrition (INRA),* Université Paris-Sud, Orsay; UEPSD, INRA,†
Jouy en Josas; U130 INSERM,§ Marseille; and Service Nutrition et Santé,‡
Eridiana Beghin-Say, Neuilly sur Seine, France

Abstract To examine the effects of β-cyclodextrin (BCD), a non-absorbable carbohydrate, on lipid metabolism, growing pigs were fed a 0.3% cholesterol-enriched diet for 4 weeks or this diet containing 5% or 10% BCD. Pigs fed a basal diet without added cholesterol or BCD were used as controls. The cholesterol-rich diet induced hypercholesterolemia (1.75 vs. 0.84 g/l plasma) due to increased LDL concentration, delayed the plasma clearance of vitamin A, enhanced liver cholesterol storage, lowered the hepatic activities of LDL-receptors (by 47%) and HMG-CoA reductase (by 62%), stimulated cholesterol 7α-hydroxylase (×3), and accelerated the fecal output of neutral sterols (×4). Addition of BCD to the cholesterol-rich diet prevented the elevation of plasma cholesterol due to dietary cholesterol excess. Moreover, BCD produced a dose-dependent effect in reducing liver cholesterol storage, stimulating hepatic cholesterogenesis, increasing the proportion of primary bile acids in bile and in feces, and the fecal loss of neutral sterols and bile acids. Pigs receiving 10% BCD thus differed markedly from controls, especially for HMG-CoA reductase and cholesterol 7α-hydroxylase hepatic activities (×5), and fecal output of total bile acids (×3) and hyocholic acid (×20), and their overall cholesterol synthesis was higher (+50%), despite the abundant dietary cholesterol.■ Owing to the property of BCD to bind cholesterol and bile acids in vitro, these results suggest that this resistant carbohydrate accelerates body cholesterol turnover by reducing cholesterol absorption, increasing cholesterol and bile acid synthesis, and altering the action of the intestinal microflora.—**Férézou, J., M. Riottot, C. Sérougne, C. Cohen-Solal, I. Catala, C. Alquier, M. Parquet, C. Juste, H. Lafont, D. Mathé, T. Corring, and C. Lutton.** Hypocholesterolemic action of β-cyclodextrin and its effects on cholesterol metabolism in pigs fed a cholesterol-enriched diet. *J. Lipid Res.* 1997. **38:** 86–100.

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The hypocholesterolemic effect of β-cyclodextrin (BCD) has been recently reported in rats and hamsters (1–3). BCD is a circular carbohydrate containing 7 glucopyranose units linked in the α1–4 position (cycloheptaamylose) which is synthesized by the action of cycloglycosyl transferase (E.C. 2.4.1.19) on amylomaize starch (4). From X-ray diffraction studies, it appears as a water-soluble cone-shaped molecule fitted with a hydrophobic cavity (5). This structure confers the property of increased aqueous solubility of a variety of compounds in vitro (6). In particular, BCD forms inclusion complexes with hydrophobic molecules (or part of molecules) such as cholesterol (7), steroids (8), and vitamins (9), and can also bind bile salts (9–11). When orally administered, BCD is not toxic or genotoxic to rodents, even at high doses (up to 20% of the diet) (1, 12, 13). This carbohydrate, which is resistant to salivary and pancreatic α-amylase hydrolysis (12), is not absorbed by the small intestine in rat or human, but is partially or even totally metabolized by the intestinal microflora (14, 15). In the digestive tract, BCD is therefore able to interact with various nutrients and metabolites which probably compete for its binding sites (16). These actions result in perturbations into the intestinal transit and absorption processes and consequently affect lipid metabolism (1–3). Although the stimulation of bile acid synthesis was proposed to be the major cause of the hypocholesterolemic effect of BCD (1–3), its mode of action is an open question.

The aim of the present study was to examine the effects of BCD on lipid metabolism in domestic swine, an omnivorous animal which has many similarities with humans with regard to digestive physiology, cholesterol biodynamics, lipoprotein composition, and risk for coronary artery disease (17–19). Male growing pigs were fed a semi-purified diet enriched with 0.3% cholesterol for 4 weeks, or this diet containing BCD at the dose of 5 or 10%. Age-matched pigs fed the basal diet with neither cholesterol nor BCD added served as controls. In these four groups, fasting plasma lipid levels were measured and lipoprotein profiles were analyzed. Plasma levels of insulin, triglycerides, and retinol palmitate were measured over the 24 h following a test meal enriched in vitamin A, a liposoluble vitamin which binds to BCD in vitro (9). Possible toxic or unfavorable effect of BCD was evaluated on the basis of food intake, body weight gain, retinyl palmitate uptake, posprandial lipemia, and liver tissue histology. As the liver is the major target organ in the whole body cholesterol turnover (20), the effects of BCD on the main hepatic processes involved in cholesterol homeostasis were also studied. The activities of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and cholesterol 7α-hydroxylase, which are the key regulatory enzymes of cholesterol and bile acid synthesis, respectively, were determined. The expression of the hepatic LDL receptor (LDLr), which is the major route for plasma cholesterol entering the liver, was assessed by ligand-blotting, and hepatic cholesterol concentration was measured to estimate cholesterol storage. To study the effects of BCD on enterohepatic circulation of sterols (21), lipid and bile acid composition of the gallbladder bile and daily fecal output of neutral sterols and bile acids were also analyzed.

MATERIALS AND METHODS

Chemicals and isotopes

Solvents and chemicals of analytical grade were purchased from Prolabo (Paris, France). BCD (Kleptose[R]) was a gift from Société Roquette Frères (Lestrem, France) [1,2-^{3}H]cholesterol (sp. act. 40–60 Ci/mmol, Amersham, Les Ulis, France) and [^{14}C] taurocholate sodium salt (sp. act. 46.4 mCi/mmol, DuPont-NEN Products, Les Ulis, France) were used as internal markers for correction of procedural losses during saponification and extraction of sterols or bile acids from test meals or feces. No isotopes were given to pigs in vivo.

Animals and diets

Twenty-four Large White male castrated pigs, weighing 30 ± 1 (mean $\pm$ SEM) kg at the beginning of the study, were obtained from the Experimental Stock Farm (INRA, La Minière, France). They were studied in a facility approved by the French Department of Veterinary Management. The pigs were maintained in accordance with the Principles of Biomedical Research Involving Animals, developed by the Council for the International Organization of Medical Sciences. They were individually housed, maintained at about 25°C under normal lighting (7 AM–7 PM) and dark cycles. They were fed at 8 AM and 4 PM with free access to water at all times. Initially, all animals were stabilized on their normal stock diet for 3 days before being divided into four groups (6 pigs per group) according to the dietary conditions. **Table 1** shows the composition of the four semi-purified diets used in the study. Cholesterol (Pharmaceutical Codex) and BCD were included into the basal diet (N diet) at the expense of corn starch. Each animal received the same amount of experimental diet (1 kg mixed with 1 liter of water, twice a day) during the duration of the experiment. This was necessary to simplify sterol balance calculation. The control group (N group) was maintained on the basal diet (containing neither cholesterol nor BCD added) for 33 days. One group was maintained on the cholesterol-enriched diet without BCD added (CH group) and two other groups were fed the cholesterol-enriched diet for 7 days, then the same diet with BCD added at the dose of 5% (CH-

TABLE 1.   Composition of diets

Nutrient	Experimental Group			
	N	CH	CH-B5	CH-B10
Casein	17.8	17.8	17.8	17.8
Soybean oil	15.0	15.0	15.0	15.0
Corn starch	43.1	42.8	37.8	32.8
β-cyclodextrin	0	0	5.0	10.0
Sucrose	15.0	15.0	15.0	15.0
Cholesterol	0	0.3	0.3	0.3
Cellulose	5.0	5.0	5.0	5.0
Mineral mixture[a]	3.0	3.0	3.0	3.0
Vitamin mixture[b]	1.0	1.0	1.0	1.0
Antioxidant[c]	0.1	0.1	0.1	0.1

Composition (percent of weight) of the semi-synthetic diets, according to the experimental groups: N, control pigs fed the basal diet; CH, pigs fed the basal diet enriched with 0.3% cholesterol; CH-B5, pigs fed the cholesterol-enriched diet containing 5% β-cyclodextrin; CH-B10, pigs fed the cholesterol-rich diet containing 10% BCD.

[a] Mineral mixture (g per kg): CaHPO$_4$ · 2H$_2$O, 414.358; CaCO$_3$, 180; NaCl, 120; KCl, 160; MgSO$_4$, 80; FeSO$_4$ · 7H$_2$O, 20; ZnSO$_4$ · H$_2$O, 17.6; MnSO$_4$ · H$_2$O, 6.4; CuSO$_4$ · 5H$_2$O, 1.6; KI, 0.018; Na$_2$SeO$_3$, 0.024.

[b] Vitamin mixture prepared on cornstarch support (mg per 10 g); 50% choline chloride concentrate, 3000; α-tocopherol acetate (1000 IU/g), 22; vitamin A acetate (500,000 IU/g), 10; vitamin D$_3$ (100,000 IU/g), 10; nicotinic acid, 36; calcium D-pantothenate, 30; thiamine hydrochloride, 3; riboflavin, 6; pyridoxine hydrochloride, 3; p-aminobenzoic acid, 20; folic acid, 2; 1‰ vitamin B12 concentrate, 60; 2% D-biotin concentrate, 0.15; menadione, 4.4; myo-inositol, 200; cornstarch, 6593.45.

[c] Butylhydroxytoluene.

B5 group) or 10% (CH-B10 group) until the end of the study.

Experimental schedule

On day 26 of the experiment, a carotid catheter (surgical tygon microtube, internal/external diameter: 0.050/0.090 inch) was implanted under general anesthesia using halothane. A small amount of heparinized saline (50 IU heparin per ml, Choay, Gentilly, France) was infused in the catheter prior to blood collection (see below). On day 28, a fasting blood sample (20 ml containing Na_2EDTA as an anticoagulant) was drawn at 8 AM. The first experimental meal had 100,000 IU of vitamin A (retinyl palmitate, Avibon, Rhône-Poulenc Rorer, France) included. The second scheduled meal for 4 PM was omitted, in order to follow the postprandial changes in plasma lipid parameters. Blood samples (10 ml) were collected at 10 AM, 12 AM, 2 PM, 4 PM, 6 PM, 8 PM, and at 8 AM the following morning. After each blood collection, a minimal amount of slightly heparinized saline (10 IU/ml) was infused in the catheter in order to avoid eventual perturbation of lipolytic activities (22). For each blood sample, hematocrit was measured and the plasma was immediately separated from blood cells by centrifugation at 4°C and aliquoted for further analyses. Monoiodoacetamide (1 mg/ml) was added to fresh plasma samples used for lipid and lipoprotein analyses to inhibit cholesterol esterification. Aprotinin (40 µg/ml, Fluka, Buchs, Switzerland) was added to the remaining samples, and these were stored at −20°C. Stools excreted within the last 4 days of the experiment (from day 29 to day 33) were collected, weighed, and frozen until analyses. On the final day of the study (on day 33), a last blood sample was drawn (8 AM) after an overnight fast. The animals then underwent surgery to obtain a liver biopsy and a gallbladder bile sample under halothane anesthesia. The gallbladder bile was carefully aspirated through a 18-gauge needle into a 50-ml syringe. The needle hole on the apex of gallbladder was then sutured to minimize the risk of postoperative bile leakage. A liver sample (approximately 7 g) was obtained from the left lateral lobe and bleeding was controlled by electric hemostasia. Gallbladder aspirates (40–50 ml) were mixed thoroughly, then aliquoted for further analyses. Liver samples (1 g each) were immediately frozen for biochemical assays and slices were kept for histologic observations. Before returning to the Experimental Stock Farm, the animals were allowed to recover from surgery for 1 week under strict surveillance.

Analyses

Diets. After the addition of a known tracer amount of [³H]cholesterol, 10 g from three batches of the basal diet without cholesterol added (N diet) was extracted with ethanol for 48 h, by means of a Soxhlet apparatus. After lipid saponification in boiling ethanolic 2 M potassium hydroxide for 2 h, the unsaponifiable fraction was extracted with petroleum ether and radioactivity was measured by liquid scintillation (MR300 apparatus, Kontron, Montigny le Bretonneux, France). Sterols were analyzed and quantified by gas–liquid chromatography (GLC), as described below for fecal sterols.

Plasma and lipoproteins. Plasma levels of glucose and lipids were measured by commercially available test kits: glucose, phospholipids, and triglycerides (Wako, Osaka, Japan); total cholesterol (CHPOP-method, Boehringer, Meylan, France) by means of an automatic analyzer (Abbott-VP, Rungis, France). Free cholesterol was measured manually (CHOP-method without prior hydrolysis, Boehringer). Insulin was determined by radioimmunoassay (CIS-Bio International, Gif sur Yvette, France). Retinyl palmitate was measured under subdued lighting in plasma samples after the addition of retinyl acetate as an internal standard. After lipid extraction by hexane, retinyl esters were separated by high performance liquid chromatography (HPLC) on a Spherisorb C18 column (15 cm length × 4.6 mm i.d., Prolabo Rhone-Poulenc, Paris, France) using methanol as the mobile phase (flow: 1.5 ml/min) (23). The effluent absorbance was monitored at 330 nm and the retinyl ester peaks were integrated using a PE Nelson 1020 computer (Perkin-Elmer, Saint-Quentin en Yvelines, France). The efficiency of retinyl ester extraction was greater than 95%.

Plasma samples collected on day 28 at 8 AM (fasting state) and at 2 PM (postprandial state) were used to fractionate lipoproteins by ultracentrifugation in a density gradient (24), using a SW41 rotor (Beckman Instruments, Gagny, France) in a L8-70 apparatus (Beckman). The gradient was prepared by successively layering from the bottom of the tube (Nalgene, Nalge Company, Rochester, NY, USA): 1 ml distilled water, 1.2 ml NaBr solution of density (d, g/ml) 1.006, 1.5 ml of d 1.019, 2.5 ml of d 1.063, 3 ml of d 1.120 and 2 ml of plasma adjusted to d 1.210 g/ml with solid KBr (325 mg/ml). All solutions contained 0.02% (w/v) NaN_3. After a 24-h run at 100,000 g (40,000 rpm) at 15°C, 28 fractions (0.4 ml) were collected by syringe aspiration from the top of the tube. Each fraction was analyzed for lipids as described above and for proteins by the method of Lowry et al. (25) using dichloromethane for the delipidation of turbid samples. The density gradient was checked by measuring the refractive indices of saline fractions collected from a tube containing 2 ml of KBr solution, d 1.210 g/ml, instead of the plasma sample, using a calibration line to relate density to refractometry measurements. On the basis of the cholesterol pro-

file in the gradient, the level and the composition of the major lipoprotein classes, i.e., triglyceride-rich lipoproteins (TRL), low density lipoproteins (LDL), and high density lipoproteins (HDL), were calculated from the sum of appropriate fractions, according to their density. Pooled LDL and HDL fractions were checked for apolipoprotein composition by SDS-polyacrylamide-gel electrophoresis (26).

Liver. Histology. Small liver fragments were immediately fixed by immersion in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4) for 1 h at 4°C. After rinsing, the material was then fixed in 2% osmium tetroxyde in the same buffer as above, dehydrated in a graded series of ethanol solutions, and embedded in Epon. For each sample, 15 sections of 2 μm were stained by toluidine blue and examined by optical microscopy. Sections of 70 nm were performed in the most representative sites. After counterstaining with uranyl acetate and lead citrate, they were observed in a Philips electron microscope at 80 keV.

Chemical analyses. Frozen liver samples (0.5 g) were homogenized in 5 ml of isopropanol, using an Ultra-Turrax apparatus (Janke & Kunkel Gmb & Co., Staufen, Germany). After incubation at 60°C for 1 h and centrifugation for 5 min at 3000 g, the supernatant was collected and the pellet was re-extracted with 5 ml of isopropanol. Triglycerides and total cholesterol were measured enzymatically on pooled isopropanolic extracts, using appropriate kits, as described above. Free and esterified cholesterol were separated by thin-layer chromatography on silica gel plates eluted with hexane–ethyl acetate 70:30 (v/v), extracted with diethyl ether, taken to dryness, and dissolved in isopropanol prior to enzymatic cholesterol determination, as described above.

Enzyme activities. Frozen liver samples (1 g) were homogenized in 7 ml buffer (Tris/HCl 50 mM, sucrose 300 mM, DTT 10 mM, EDTA 10 mM, NaCl 50 mM; pH 7.4) at 4°C, by means of a Teflon pestle. Microsomal fractions were isolated according to the procedure described by Einarsson et al. (27). HMG-CoA reductase activity was determined in the presence of alkaline phosphatase in order to fully dephosphorylate the enzyme (active form) using the radioisotopic technique of Philipp and Shapiro (28). For the measurement of cholesterol 7α-hydroxylase activity, liver samples (1 g) were homogenized in the same buffer as above, except NaCl was replaced by NaF to protect the phosphorylation state of the enzyme (active form). The enzyme activity was evaluated by a radioisotopic method (29). Microsomal protein concentrations were measured using the Bradford method (30).

LDL receptor binding. Liver membrane preparation. Total membranes from frozen liver samples (1 g) were pre-

pared according to Kovanen, Brown, and Goldstein (31). The samples were finely minced, suspended in 7 ml of buffer A: 20 mM Tris-HCl, pH 8.0; 0.15 M NaCl; 1 mM CaCl$_2$ and 200 IU/ml aprotinin and homogenized at 4°C, using an Ultra-Turrax apparatus. The homogenate was cleared of debris by filtration through nylon mesh and centrifugation at 8000 g for 15 min at 4°C. The membrane fraction was prepared from the supernatant by ultracentrifugation at 100,000 g for 1 h at 4°C, in a Beckman 70 Ti rotor. The pellet was resuspended in 800 μl of buffer A by flushing 10 times through a 22-gauge needle. Solubilization of proteins was achieved by adding 800 μl buffer B (125 mM Tris-maleate, 2 mM CaCl$_2$, aprotinin 110 IU/ml, 2% Triton X-100, pH 6) and flushing 10 times through a 22-gauge needle (32). After gently mixing for 20 min at room temperature, the samples were centrifuged at 100,000 g for 1 h at 4°C. The supernatant was collected and Triton X-100 was removed from the samples by agitation for 1 h in the presence of Amberlite XAD2 (500 mg/ml, Sigma) which was previously washed 3 times with distilled water, once with ethanol 20%, and then extensively with distilled water. After syringe aspiration through a 26G × 1/2-gauge needle to discard Amberlite, the samples were centrifuged at 8000 g for 10 min at 4°C. The clear supernatant, which contained the solubilized proteins, was collected and assayed for protein content using the Bradford method (30).

Ligand. Porcine LDL was isolated from plasma (EDTA, 1 mM and aprotinin, 200 IU/ml) by sequential ultracentrifugation (33). Plasma was adjusted to d 1.020 g/ml with solid KBr. After centrifugation at 100,000 g for 24 h, lipoproteins of density inferior to d 1.020 g/ml were discarded. The subnatant was adjusted to d 1.060 g/ml with solid KBr and centrifuged at 100,000 g for 48 h. LDL was collected at the top of the tube and dialyzed against NaCl 0.154 M, EDTA 1 mM, pH 7.5, overnight. After protein determination using the Lowry method (25), LDL was adjusted to 1 mg/ml with deionized water. They were dialyzed overnight against EDTA 50 mM, pH 8.0, and protein was determined once again using the Lowry method. Labeling LDL with colloidal gold was achieved according to Roach, Zollinger, and Noel (34). Colloidal gold was prepared by the method of Frens (35), as described by Handley et al. (36). LDL samples (150 μg) were diluted to 500 μl with deionized water in 10-ml ultracentrifuge tubes and 5 ml of colloidal gold solution was added to the samples while mixing. After centrifugation at 20,000 g for 20 min at 4°C in a Ti70 rotor (Beckman), the supernatant was aspirated off and the pellet of colloidal gold-LDL conjugates was collected and stored at −20°C in the presence of sucrose (0.2 g/ml) until required.

Ligand blotting. The LDL-binding activity was deter-

mined by the method of Roach et al. (34). Samples of solubilized hepatic membrane proteins (4, 8, and 16 µg in 50 µl) were spotted onto nitrocellulose using a dot-blot apparatus (Bio-Rad, Richmond, CA). To determine total and nonspecific LDL-binding, the nitrocellulose was incubated with colloidal gold-LDL conjugates (20 µg/ml) in the absence or in the presence of excess un-labeled LDL (×12.5). After treatment with the gold en-hancement kit (Bio-Rad), the wet nitrocellulose was scanned with a laser densitometer (Ultroscan, LKB, Bromma, Sweden) that was interfaced with a micro computer. Peaks were integrated and the LDL-binding was expressed as peak height in millimeters. The spe-cific binding to the LDL receptor was calculated as the difference between total and nonspecific LDL-binding.

Bile. Duplicate aliquots (100 µl) of freshly collected bile were diluted 10-fold in isopropanol. The molar con-centration of total bile salts was measured on 5 and 10 µl of the isopropanolic samples, taken to dryness, using 3α-hydroxysteroid dehydrogenase (Sigma) (37). Com-mercial kits were used for cholesterol (CHOD-PAP method, Boehringer) and phospholipid (Enzymatic Color Test, Labo Express Service, Viry-Chatillon, France) assays on 10 or 20 µl of isopropanolic solutions, after decolorization under UV light. Individual molecu-lar species of conjugated bile acids were determined by HPLC, as previously described (38) and their molar proportions were expressed as percent of the total bile salt concentration. Hydrophobicity index of the bile acid pool was calculated for each bile sample on the basis of the hydrophobicity index previously deter-mined for common conjugated bile salts (39), setting that of the glycine conjugate of 3α-6-oxo-5β-cholanate to −0.35.

Fecal samples. Feces were homogenized in a minimal volume of distilled water and aliquots were lyophilized. After the addition of known amounts of radioactive markers ([1,2-^{3}H]cholesterol and [^{14}C]taurocholate so-dium salt), lipids from 2 g of dried fecal samples were extracted with ethanol for 48 h using a Soxhlet appara-tus and saponified in boiling ethanolic 2 M potassium hydroxide for 1 h (1). The sterol containing the unsa-ponifiable fraction was extracted with petroleum ether and ^{3}H radioactivity was measured. In the aqueous phase, bile acids were deconjugated by the method of Grundy, Ahrens, and Miettinen (40) and extracted with diethylether, and ^{14}C radioactivity was determined to ac-count for procedural losses. Free bile acids were methyl-ated with diazomethane. Before analysis, sterols were sylilated with Deriva-sil (Chrompack, les Ulis, France) in hexane, and methylated bile acids were silylated with both Deriva-sil and BSTFA (Pierce, Rockford, IL) in di-chloromethane. Trimethylsilyl (TMS) derivatives were assayed by GLC in the presence of cholestane as an in-ternal standard, using an HRGC 5160 apparatus (Carlo-Erba, Cachan, France) equipped with a standard fused silica WCOT capillary column (length: 25 m, film thick-ness; 0.2 mm) cross-linked with CPSil5CB (Chrompack) for sterols, or with OV1701 (Spiral, Dijon, France) for bile acids. The oven temperature was 240°C and the flow rate of hydrogen carrier gas was 2 ml/min. Molar proportions and total amounts of sterols and bile acids were assessed, using calibration lines obtained from the analysis of standard mixtures (1). Daily fecal outputs of cholesterol and bile acids were calculated after correc-tion for fecal flow, on the basis of a theoretical 90% recovery of dietary β-sitosterol, taking into account that this phytosterol is a reliable marker in swine (41).

Statistical analysis

Results are given as mean values and their SEM. Sta-tistical differences among the four groups were deter-mined by multivariant analysis and the Newman-Keuls test. A value of $P < 0.05$ was considered as the criterion of significance. In case of borderline difference be-tween two groups, in particular cholesterol-fed and con-trol pigs, Student's *t*-test for two groups was also used as an additional test to compare the results.

RESULTS

Growth

All the animals remained in good health throughout the entire experiment. They consumed essentially more than 95% of the daily meals, except two wasting pigs that were thus excluded from the study. As shown in **Table 2,** no intergroup difference appeared in the body weight gain which approximated 0.90–0.95 kg/day in all the groups.

Diets

Sterol analysis indicated that the basal diet (N diet) contained 48 ± 2 mg of cholesterol and 512 ± 23 mg of phytosterols (sterols from plant origin) per kg, which corresponds to 25% campesterol (C28 sterol), 21% stig-masterol (C29), and 54% β-sitosterol (C29). Assuming that the same phytosterol amount was contained in the 0.3% cholesterol-enriched diet (CH diet) and taking into account the daily food intake (2 kg), it was consid-ered that each pig consumed an average of 550 mg of β-sitosterol daily. This amount of poorly absorbable sterol was associated with approximately 100 mg of cho-lesterol in the control group, or with 6,100 mg of cholesterol in the three other groups fed the choles-terol-enriched diet.

TABLE 2. Biological and metabolic parameters of experimental groups

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Initial body weight	36.4 ± 1.0	37.4 ± 1.5	36.1 ± 1.5	34.4 ± 1.9
Final body weight	63.1 ± 1.0	62.6 ± 1.4	58.0 ± 2.5	59.0 ± 1.1
Hematocrit	34 ± 1	35 ± 1	31 ± 2	32 ± 1
Insulin	16.3 ± 3.0	11.8 ± 0.9	15.8 ± 2.6	12.5 ± 1.2
Glucose	1.13 ± 0.04[b]	0.88 ± 0.06[a]	0.81 ± 0.08[a]	0.99 ± 0.05[ab]
Total cholesterol	891 ± 49[a]	1846 ± 46[b]	1035 ± 87[a]	851 ± 21[a]
Free cholesterol	230 ± 23[a]	456 ± 38[b]	238 ± 23[a]	188 ± 99[a]
LDL-cholesterol	548 ± 33[a]	1373 ± 91[b]	616 ± 77[a]	464 ± 13[a]
HDL-cholesterol	305 ± 22	385 ± 33	356 ± 56	324 ± 23
Triglycerides	281 ± 56	294 ± 21	333 ± 79	282 ± 59
Phospholipids	1061 ± 114[a]	1622 ± 104[b]	1149 ± 100[a]	1002 ± 93[a]

Initial and final body weights (kg) for the overall observation period of 33 days, hematocrit (percent), plasma levels of insulin (mU/l), glucose (g/l) and lipids (mg/l), LDL- and HDL-cholesterol (mg/l), measured on day 28 of the experiment in fasting pigs fed the basal diet (N group), the basal diet containing 0.3% cholesterol without BCD (CH group) or with β-cyclodextrin at the dose of 5% (CH-B5 group) or 10% (CH-B10 group), according to the conditions detailed in Table 1. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.

Fasting blood parameters

The major blood parameters measured are shown in Table 2. Hematocrit was unaffected by the dietary conditions. The highest value of fasting glycemia was found in control animals fed the basal diet (N group), but no major difference was found between insulin values. Comparing N and CH groups shows that 4 weeks feeding of a cholesterol-enriched diet induces a 2-fold increase in the plasma cholesterol level (without alteration of the free/total cholesterol ratio), a 1.6-fold increase in phospholipid level, and no change in triglyceride level. Hypercholesterolemia of CH pigs was due to an increase in the LDL-cholesterol level, without any change in HDL-cholesterol concentration. In the animals fed the CH-B5 or the CH-B10 diet, fasting plasma levels of cholesterol and phospholipids were significantly lower than in the CH group, and similar to those of N pigs fed the diet without cholesterol added. In each group, lipid parameters were stable between days 28 and 33 (end of the study), except in the CH group in which plasma levels of cholesterol and phospholipids were significantly ($P < 0.05$) higher on day 33 than on day 28: the increment of plasma cholesterol (337 ± 64 mg/l) averaged 18% within the last 5 days of the experiment (final plasma cholesterol level at day 33 being 2194 ± 74 mg/l).

Cholesterol distribution among the lipoproteins, studied on day 28, is shown in **Fig. 1A** for the pigs fed the diets without BCD (N and CH groups) and in Fig. 1B for the pigs fed the BCD-supplemented cholesterol-rich diets (CH-B5 and CH-B10 groups). Apart from the first four fractions that correspond to triglyceride-rich lipoproteins (TRL, density inferior to 1.020 g/ml), cholesterol was distributed between two major peaks, LDL and HDL. In control pigs (group N), the LDL distribu-

tion was relatively wide (fractions 6–15, density range 1.026–1.082 g/ml) and peaked on fraction 10 (d 1.043 g/ml). In the CH group, the LDL distribution was slightly wider (fractions 4–15, density range: 1.019–1.082 g/ml), the LDL peak was more intense and shifted towards a lower density with a maximal value at fraction 8 (d 1.031 g/ml). It was verified that apoB was practically the sole apolipoprotein of the LDL fraction, whatever the cholesterol content of the diet (results not shown). In pigs from the CH-B5 and CH-B10 groups, cholesterol profiles were close to that of control animals. The LDL peak was slightly lower in the CH-B10 than in the CH-B5 group, but it did not reach statistical significance. The HDL peak (fractions 16–25, density range: 1.087–1.790 g/ml) was not influenced by the cholesterol content of the diet. As shown in **Table 3,** the overall composition of the LDL fraction was only slightly influenced by the dietary conditions. The lowest triglyceride proportion was found in CH pigs fed the cholesterol enriched-diet, whereas the lowest phospholipid proportion was found in CH-B10 pigs.

Postprandial changes in plasma lipid and insulin parameters

In the plasma samples collected on day 28, cholesterol and phospholipid concentrations did not change over the 12 h following the test meal. In each group, postprandial values remained similar to the fasting values, with the highest values found in the CH group, as in Table 2. Triglyceride, insulin, and vitamin A plasma levels displayed a postprandial wave, with increases observed between 4 and 8 h after the meal for triglycerides and vitamin A, and between 2 and 4 h for insulin. **Table 4** reports the plasma lipid levels measured 6 h after the meal (postprandial samples), the highest observed insu-
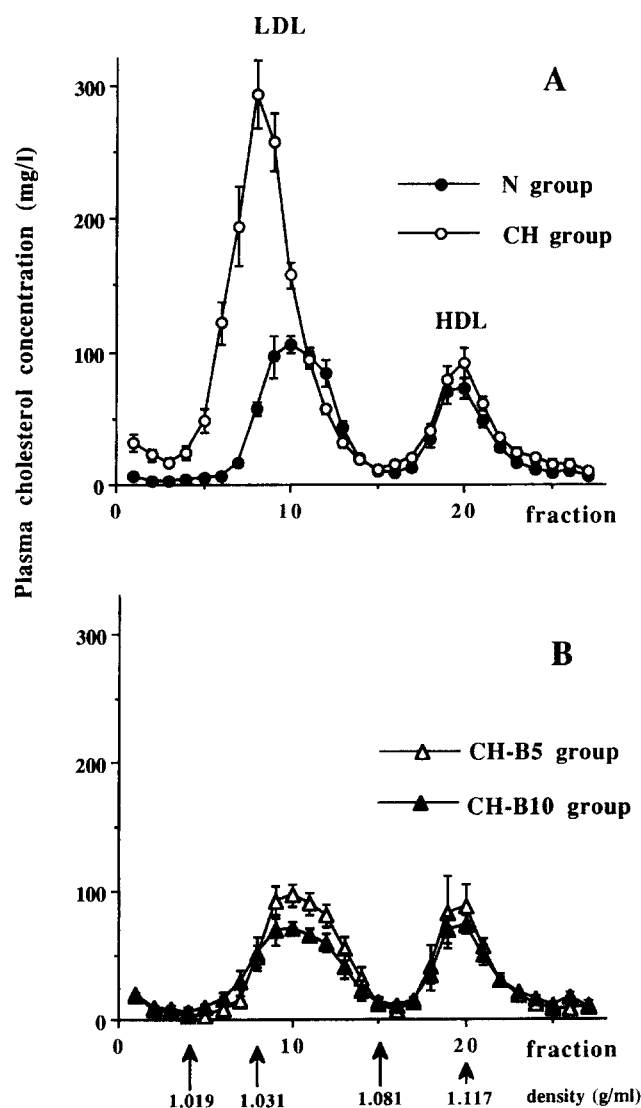
Fig. 1. Cholesterol distribution among the plasma lipoproteins separated in a density gradient as described in the Experimental section. A: In pigs fed the control diet (N group) or this diet with 0.3% cholesterol added (CH group). B: In pigs fed the cholesterol-enriched diet with BCD added at the dose of 5% (CH-B5 group) or 10% (CH-B10 group).

TABLE 3.   Composition of LDL fraction

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Cholesterol	42.6 ± 1.0	47.5 ± 2.0	42.0 ± 3.6	43.8 ± 2.4
Triglycerides	4.2 ± 0.7[a]	1.0 ± 0.1[b]	3.5 ± 0.5[a]	6.4 ± 1.8[a]
Phospholipids	23.2 ± 0.4[a]	24.5 ± 1.3[a]	23.0 ± 2.0[a]	18.3 ± 1.4[b]
Proteins	30.0 ± 1.1	27.0 ± 1.0	31.5 ± 2.0	31.5 ± 1.7

LDL composition (percent of total mass) measured on day 28 of the experiment in the four groups of fasting pigs, according to the dietary conditions detailed in Table 1. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.

lin levels (at 2 or 4 h after the meal), and the areas under the curves for plasma levels of triglycerides, insulin, and vitamin A, calculated over the 12 h following the test meal. For each separate group of pigs (e.g. N, CH, CH-B5, CH-B10), insulin levels were higher ($P < 0.05$, paired Student's t-test) than in fasting state (see Table 2). Although the mean postprandial levels of triglycerides were higher in each group, they did not reach statistical difference because of the large dispersion of the values. The areas under the curves for insulin and triglyceride levels were also not influenced by the different dietary conditions; however, the amplitude of the postprandial wave of vitamin A was significantly ($P < 0.05$) higher in the CH group than in the three other groups, as shown in **Fig. 2.** The peak height was 2.6-fold higher in the CH group (Fig. 2B) compared with the control group (Fig. 2A), whereas the curves obtained in the CH-B5 and CH-B10 groups (Fig. 2B) were similar to that obtained in the control group.

Cholesterol distribution among the lipoproteins, measured in the postprandial state (at 6 h after the meal), was similar for each group compared with that observed in the fasting state, although the total amount of TRL (calculated as the sum of the lipoprotein components) markedly increased, mainly due to higher triglyceride amounts (results not shown). The plasma concentration and the composition of postprandial TRL (summed fractions 1–2, d < 1.010 g/ml) are shown in **Table 5.** For CH compared with control animals (N), the amount and the proportion of cholesterol increased at the expense of triglycerides. In pigs fed the CH-B5 and the CH-B10 diets, TRL displayed a composition similar to that observed in pigs fed the basal diet (N); the concentration being slightly higher in the CH-B5 than in the CH-B10 group.

Liver

Histology and lipid contents. In pigs fed the cholesterol-enriched diet (CH group), the livers contained significant lipid infiltration that appeared as two different structures under microscopy. Lipids formed large droplets with an osmiophilic content, located essentially on the vascular part of lobules, near the sinusoids. These droplets were located intracellularly near the nuclei. The second type of lipid infiltration was represented by groups of small intracellular vesicles, sometimes osmiophilic, associated with numerous refringent bodies. Electron microscopy revealed that the large lipid droplets were in fat storing cells or Ito cells (42), while the microvesicles were located in the hepatocytes, frequently associated with secondary lysosomes and vesicles containing altered cell membranes. Furthermore, some modifications at the level of intracellular membranes, dilatations, and partial loss of villosities were ob-

TABLE 4. Plasma lipid levels

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Cholesterol	839 ± 49[a]	1725 ± 52[b]	1017 ± 92[a]	849 ± 34[a]
Triglycerides	354 ± 51	482 ± 103	453 ± 79	462 ± 116
	(34 ± 15)	(65 ± 23)	(126 ± 58)	(141 ± 38)
Phospholipids	1069 ± 114[a]	1697 ± 68[b]	1265 ± 111[a]	1141 ± 94[a]
Insulin	30.6 ± 4.6	33.0 ± 1.7	37.9 ± 6.9	35.8 ± 5.5
	(151 ± 55)	(179 ± 21)	(217 ± 69)	(161 ± 46)
Triglyceride area	3861 ± 548	5048 ± 1043	4242 ± 326	4665 ± 1179
Insulin area	218 ± 14	228 ± 29	268 ± 29	239 ± 26
Vitamin A area	1052 ± 138[a]	2327 ± 269[b]	1304 ± 73[a]	1316 ± 235[a]

Plasma lipid levels (mg/l) at 6 h after the vitamin A-loaded test meal, maximal insulin levels (mU/l) observed at 2 or 4 h after the meal, and the area under the curves of the plasma levels of triglycerides (mg/ l × h), insulin (mU/l × h) and vitamin A (μg/l × h) over the 12 h following the vitamin A-loaded test meal measured on day 28 of the experiment in the four groups, according to the dietary conditions detailed in Table 1. The mean percent increase in triglyceride and insulin levels, as compared with the corresponding fasting values (see Table 2), is indicated in parentheses. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.
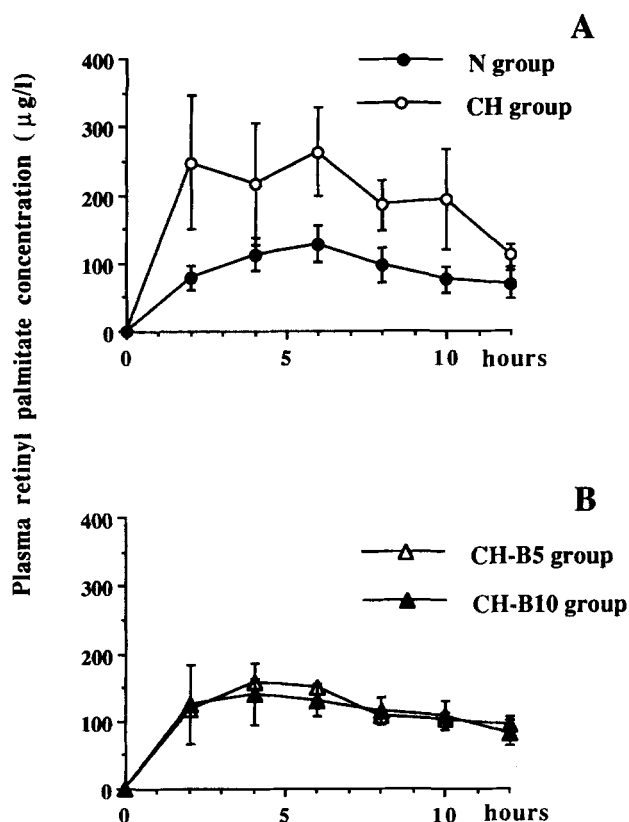

Fig. 2. Time-course of plasma concentration of retinylpalmitate (vitamin A) within the 12 h after the vitamin-A-loaded test meal. A: In pigs fed the control diet (N group) or this diet with 0.3% cholesterol added (CH group). B: In pigs fed the cholesterol-enriched diet with 5% (CH-B5 group) or 10% (CH-B10 group) BCD added.

served. In the CH-B5 group, the large lipid droplets occurred less frequently and microvesicles were less numerous and not associated with refringent bodies, as compared with the CH group. In the CH-B10 group, osmiophilic inclusions, droplets or microvesicles, were even less common and the livers were remarkably similar to those of control animals. Whatever the dietary conditions, biliary canaliculi showed no modification at the level of junctions and the ultrastructure appeared to be conserved. As shown in **Table 6,** chemical analysis of the liver samples indicated that the cholesterol concentration was markedly higher in the CH than in the N group (1.4-fold and 3.5-fold for free and esterified cholesterol, respectively). In the CH-B5 and CH-B10 groups, the mean liver cholesterol concentration was lower than in the CH group, but the difference was significant only in the CH-B10 group, and similar to that of the N group. There appeared to be no intergroup difference for hepatic triglyceride concentrations.

TABLE 5. Concentration and composition of postprandial TRL

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Cholesterol	13 ± 2[a]	63 ± 17[b]	32 ± 6[a]	20 ± 3[a]
	(6)	(14)	(7)	(5)
Triglycerides	167 ± 37[a]	247 ± 56[ab]	320 ± 72[b]	284 ± 83[ab]
	(70)	(54)	(74)	(75)
Phospholipids	34 ± 7	81 ± 26	45 ± 10	48 ± 21
	(14)	(18)	(10)	(13)
Proteins	25 ± 9	68 ± 25	33 ± 10	27 ± 9
	(10)	(14)	(8)	(7)

Cholesterol, triglyceride, phospholipid and protein concentrations (mg/l plasma) in the triglyceride-rich lipoproteins (TRL) isolated from the plasma collected at 6 h after the test meal (postprandial state) on day 28 of the experiment, in the four groups according to the dietary conditions detailed in Table 1. The mean proportion (in percent) of each lipoprotein component is shown in parentheses. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.

TABLE 6. Hepatic, lipids, enzyme activities, and LDL binding

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Free cholesterol	2.47 ± 0.08^a	3.36 ± 0.19^b	2.69 ± 0.22^a	2.61 ± 0.08^a
Esterified cholesterol	0.49 ± 0.04^a	1.71 ± 0.29^b	1.20 ± 0.32^{ab}	0.58 ± 0.07^a
Triglycerides	11.41 ± 1.63	9.41 ± 1.38	9.42 ± 0.67	7.10 ± 0.88
Cholesterol 7α-hydroxylase	4.3 ± 0.5^a	$14.4 \pm 3.7^{ab*}$	17.4 ± 3.8^{ab}	19.5 ± 4.3^b
HMG-CoA reductase	8.6 ± 0.9^{ab}	$3.2 \pm 0.3^{a**}$	11.1 ± 6.8^{ab}	43.7 ± 16.5^b
LDL-specific binding	115 ± 8^b	78 ± 9^a	69 ± 3^a	86 ± 7^a

Hepatic lipid concentrations (mg/g) and activities (pmol/min per mg protein) of cholesterol 7α-hydroxylase and HMG-CoA reductase, and the specific LDL-binding to liver membrane proteins (peak height in mm), determined at the end of the study (on day 33) in the four groups according to the dietary conditions defined in Table 1. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test. CH differs from N group at $*P < 0.004$, $**P < 0.001$ by Student's t test for two groups.

Enzymatic activities. As shown in Table 6, cholesterol feeding decreased the activity of HMG-CoA reductase, which was about 3-fold lower in the CH compared with the N group. In the CH-B5 group, this activity was similar to that measured in the N group, whereas it was 5-fold higher in the CH-B10 group. Conversely, cholesterol feeding led to a significant stimulation of the activity of the cholesterol 7α-hydroxylase, which was 3-fold higher in the CH compared with the N group. Inclusion of 5 or 10% BCD into the cholesterol-rich diet produced a trend to increase this enzymatic activity according to the dose, but no statistical difference was observed between CH, CH-B5, and CH-B10 groups.

LDL-binding to liver membranes. As compared with control N pigs (Table 6), the specific LDL-binding was significantly lower in CH pigs fed the cholesterol-enriched diet (-47%) and in pigs fed this diet with BCD added (-67% and -34% in CH-B5 and CH-B10 pigs, respectively). When the individual results from the pigs fed the diets without BCD added (N and CH groups) are considered together, a significant negative correlation links the specific LDL-binding and LDL-cholesterol concentrations ($y = -0.04x + 134.37$, $r = 0.665$, $P = 0.026$, $n = 11$).

Bile lipid composition

As shown in **Table 7,** cholesterol feeding significantly increased the molar proportion of cholesterol in the gallbladder bile at the expense of bile acids (compare CH with N), but did not alter the bile acid composition. By comparison, addition of BCD to the cholesterol-enriched diet did not alter the relative distribution of biliary lipids but produced a marked effect on the bile acid composition, which appeared to be dose-dependent. As compared with that observed in the CH group, the proportion of hyodeoxycholic acid decreased, whereas that of chenodeoxycholic and hyocholic acids increased. Therefore, a gradual increase in the relative proportion of primary bile acids (sum of chenodeoxycholic, cholic, and hyocholic acids) was observed when increasing

BCD in the cholesterol-enriched diet. Consequently, the mean primary/secondary bile acid ratio, which approximated 0.6 in N and CH groups, was 2.2 in the CH-B5 and 5.5 in the CH-B10 groups. Thus, the hydrophobicity index of the bile acid pool increased in parallel. No major intergroup difference appeared in the bile acid conjugation pattern.

Fecal neutral sterols and bile acids

Analysis of fecal samples indicated that the bulk of neutral sterols was composed of cholesterol and its two 5β-hydrogenated products, coprostanol and epicoprostanol (C27 sterols) in all the groups. However, β-sitosterol (and related 5β-reduced metabolites) represented up to $31.4 \pm 3.3\%$ of neutral sterols in control pigs fed the diet without cholesterol added. This proportion was decreased in cholesterol-fed pigs (to $12.0 \pm 2.0\%$) and further in pigs fed cholesterol-enriched diets with BCD ($8.8 \pm 1.6\%$ in the CH-B5 group, $6.8 \pm 0.8\%$ in the CH-B10 group). The fecal recovery of dietary β-sitosterol was used to obtain the daily output of neutral sterols and bile acids, after correction for the fecal flow. **Table 8** shows the proportions and the daily output of C27 neutral sterols and bile acids. In CH compared with N pigs, cholesterol feeding produced a 3.5-fold increase in the fecal elimination of C27 sterols. Addition of BCD to the cholesterol-enriched diet increased this output by a further 1.5- and 1.9-fold in CH-B5 and CH-B10 groups, respectively. In control pigs fed the basal diet, about 92% of cholesterol was eliminated in the form of 5β-hydrogenated derivatives, predominantly coprostanol. This proportion approximated 50% in pigs fed the cholesterol-enriched diet (CH group) or this diet containing 5% BCD (CH-B5 group) and 23% in pigs fed the diet containing 10% BCD (CH-B10 group). Neither the daily elimination nor the composition of bile acids was influenced by the cholesterol content of the diet. In both N and CH groups, the primary bile acids (chenodeoxycholic and hyocholic acids) represented about 20% of the total bile acids, whereas hyodeoxycholic acid

TABLE 7. Lipid and bile acid composition

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Cholesterol	2.0 ± 0.1^a	3.7 ± 0.4^b	3.2 ± 0.2^b	3.0 ± 0.3^b
Phospholipids	5.8 ± 0.4	7.0 ± 0.4	6.5 ± 0.3	7.4 ± 0.8
Bile acids	92.2 ± 0.3^a	89.3 ± 0.7^b	90.3 ± 0.3^{ab}	89.6 ± 1.0^b
Chenodeoxycholic	24.8 ± 4.8^a	24.8 ± 1.8^a	43.4 ± 2.5^c	57.6 ± 3.5^b
Cholic	—	0.8 ± 0.8	0.6 ± 0.6	2.8 ± 1.0
Hyocholic	12.4 ± 2.1	10.9 ± 1.7	25.0 ± 7.5	24.5 ± 3.0
Hyodeoxycholic	45.1 ± 4.9^a	51.0 ± 1.6^a	22.8 ± 6.3^c	6.7 ± 3.4^b
3αOH-6 oxo-5β cholanoic	17.7 ± 2.7	12.5 ± 1.1	8.2 ± 2.6	8.4 ± 2.9
Glyco/tauroconjugation	18.4 ± 4.1^a	12.7 ± 2.0^{ab}	7.7 ± 0.4^b	10.9 ± 1.6^{ab}
Hydrophobicity index	-0.12 ± 0.04^a	-0.12 ± 0.01^a	$+0.02 \pm 0.02^c$	$+0.14 \pm 0.06^b$

Lipid and bile acid composition (mol percent), glyco/tauroconjugated bile acid ratio, and hydrophobicity index of total bile acids in the gallbladder bile collected at the end of the experiment in the four groups according to the dietary conditions detailed in Table 1. Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.

was the most abundant secondary (or microbial) bile acid. Addition of 5 or 10% BCD to the cholesterol-enriched diet produced a dose-dependent increase in the fecal elimination of bile acids. The daily excretion of bile acids in the CH-B10 group was 3.5-fold greater than in the CH group. This change was associated with a progressive decrease in the proportion of hyodeoxycholic and lithocholic acids at the expense of hyocholic acid. Thus, the primary bile acids represented about 70% of the total bile acids in the CH-B10 group.

DISCUSSION

This study examines the effects of BCD, a nonabsorbable circular carbohydrate, on cholesterol and bile acid metabolism in developing pigs fed a cholesterol-enriched diet. Our results show that BCD does not affect body growth, hematocrit and plasma levels of glucose and insulin, even at a high dose of 10%. It exerts a marked hypocholesterolemic effect and modifies the action of the microflora in the intestinal lumen, confirming previous observations in rodents (1–3).

In control pigs fed the basal diet, plasma lipid parameters are in agreement with previous reports in swine (17–19, 43–45); plasma cholesterol (≈ 0.9 g/l) being equally shared between LDL and HDL. The bulk of LDL particles is found in the density range 1.040–1.080 g/ml. The broad characteristic of the LDL peak is consistent with the heterogeneity of the LDL fraction in pigs (43, 44), while LDL behave as a sharper peak centered around the density 1.030 g/ml in healthy humans (46). Consistent with previous reports (44, 45), the HDL fraction of control pigs forms a single peak without the distinction between HDL_2 and HDL_3 particles as observed in humans. Plasma triglyceride and TRL levels are relatively low compared with the values observed

TABLE 8. Distribution of fecal neutral sterols and bile acids

Group	N (n = 5)	CH (n = 6)	CH-B5 (n = 5)	CH-B10 (n = 6)
Neutral sterols				
Coprostanol	85.1 ± 4.9^b	45.2 ± 4.8^a	39.5 ± 9.9^a	23.2 ± 9.3^a
Epicoprostanol	6.9 ± 2.0^b	6.8 ± 2.6^b	8.9 ± 5.8^b	0.1 ± 0.1^a
Cholesterol	7.9 ± 3.4^a	47.9 ± 3.6^b	51.6 ± 12.9^b	76.7 ± 9.3^c
Elimination	1.08 ± 0.17^a	4.26 ± 1.04^b	6.47 ± 1.24^{bc}	8.21 ± 0.69^c
Bile acids				
Chenodeoxycholic	11.5 ± 1.0	12.6 ± 1.1	9.2 ± 2.7	12.3 ± 2.8
Hyodeoxycholic	50.0 ± 0.3^a	45.5 ± 3.6^{ac}	36.2 ± 13.3^c	12.4 ± 7.5^b
Hyocholic	10.8 ± 1.3^a	7.5 ± 2.5^{ac}	33.1 ± 12.0^{ac}	60.7 ± 8.4^b
Lithocholic	16.4 ± 0.7^{ac}	21.3 ± 1.9^a	9.7 ± 4.5^b	3.4 ± 1.6^b
Ketones	10.6 ± 1.1	13.0 ± 1.2	11.7 ± 4.0	11.0 ± 3.7
Elimination	1.63 ± 0.04^a	1.58 ± 0.28^a	4.33 ± 1.01^b	5.58 ± 0.99^b

Distribution (percent) of fecal neutral sterols (cholesterol and microbian metabolites) and bile acids, measured in pooled feces collected for the last 4 days of the experiment in the four groups according to the dietary conditions defined in Table 1. Daily eliminations (g/day) were corrected for fecal flow on the basis of the recovery of dietary β-sitosterol (unabsorbed C29 sterol). Groups with different letter superscripts are significantly different at $P < 0.05$ by the Newman-Keuls test.

in healthy human subjects (46). Such characteristics have been reported recently in swine (19).

After 4 weeks of feeding a cholesterol-enriched diet without BCD added, plasma cholesterol levels doubled, and the cholesterol profile in the density gradient approached that observed in healthy humans (46). The LDL peak increased and shifted towards a lower density region compared with control pigs, whereas the HDL peak was unchanged. There was no noticeable accumulation of apoE-rich HDL, an effect that is produced by much greater amounts of dietary cholesterol (19, 47). In the postprandial state, the altered composition of TRL reflects the accumulation of particles abnormally rich in cholesteryl esters, including chylomicron remnants and β-VLDL (19). As these cholesterol-enriched particles are cleared more slowly than normal TRL (48), the increased amplitude of the postprandial wave of vitamin A in cholesterol-fed pigs therefore reflects the delayed clearance of TRL bearing the newly absorbed vitamin A (49), rather than the improved absorption of vitamin A. Cholesterol feeding also induces an increase in fasting glycemia, an effect that is difficult to explain in so far as no difference appears in the insulin levels between hypercholesterolemic and control pigs.

Consistent with the view that the liver has a marked response to exogenous cholesterol input (50), dramatic effects on the liver status were induced by 0.3% dietary cholesterol feeding. Clearly, the excess dietary cholesterol was responsible for the morphological modifications observed in hepatocytes and the associated disorders of the intracellular membranes. The presence of numerous secondary lysosomes could indicate digestion of lipids by autophagy, resulting in a decrease in the intracellular lipid content (51). In cholesterol-fed pigs compared with controls, liver cholesterol concentration was markedly increased, the activity of HMG-CoA reductase was depressed, cholesterol 7α-hydroxylase activity was elevated, and LDLr expression was decreased. The inverse correlation between plasma LDL-cholesterol levels and specific LDL-binding to liver membranes, found in pigs fed the diet with and without added cholesterol, confirms the down-regulation of the liver LDLr by dietary cholesterol in swine (52), a regulatory mechanism that appears to be absent in rats (53). The reduced activity of both HMG-CoA reductase and LDLr in cholesterol-fed animals is probably due to a negative feedback mechanism to compensate for the increased absorption of exogenous cholesterol (20, 50). In a similar manner, the increase in cholesterol 7α-hydroxylase is an attempt to clear excess cholesterol from the liver. However, cholesterol feeding only induced an increase in the molar proportion of cholesterol in gallbladder bile, without any change in the bile acid composition. Despite the stimulation of the cholesterol 7α-hy-

droxylase activity, fecal daily output of bile acids was not changed, whereas that of neutral sterols (cholesterol and by-products) was enhanced markedly. These effects of dietary cholesterol differ from those observed in rats in which cholesterol feeding stimulated fecal bile acid excretion (54). However, they confirm the data of a previous study in minipigs (41). The poor microbial degradation of cholesterol into 5β-metabolites indicates that the microflora is probably encompassed by excess cholesterol in the luminal content, due to the secretion of cholesterol-enriched bile and increased amounts of unabsorbed (exogenous and endogenous) cholesterol transiting into the colonic content.

Addition of BCD to the cholesterol-rich diet clearly prevented the elevation of plasma cholesterol due to dietary cholesterol excess. Lipoprotein patterns in BCD-fed pigs were similar to those measured in control pigs fed the diet without added cholesterol. These observations support the notion that dietary cholesterol absorption is reduced markedly by BCD, although this effect was not demonstrated directly by an isotopic method. In contrast, the results of the retinyl-palmitate test (similar in BCD-fed and control pigs) indicate that BCD does not impair the intestinal absorption of vitamin A. On the basis of biochemical assays, morphology of liver tissue, similar body weight gain, food consumption, and physical fitness of pigs in all the groups, it can be concluded that BCD is not toxic.

Taking into account the high capacity of BCD to bind cholesterol (7, 8) or to mediate cellular cholesterol efflux in vitro (55), it may be assumed that BCD acts as a cholesterol trap in the lumen and thus inhibits cholesterol absorption in the small intestine. In terms of stoichiometry, all cholesterol molecules included in the 0.3% cholesterol-enriched diet can be bound to BCD; the BCD/cholesterol molar ratio being 5.6 or 11.3 for the 5% or 10% dose of BCD, respectively. However, endogenous cholesterol molecules entering the lumen from the bile (in mixed micelles) and from sloughed enterocytes can act as competitors for the binding process. Other hydrophobic molecules, such as monoglycerides, free fatty acids, liposoluble vitamins, coming from the digestive process, probably also interact with BCD. The spectacular stimulation of hepatic HMG-CoA reductase by BCD at the 10% dose (+500%, as compared to the control value) is probably in response to the enhanced loss of cholesterol due to reduced absorption of both dietary and endogenous (biliary) cholesterol. Such enhancement of cholesterol synthesis occurs in humans treated with high amounts of phytosterols that lower cholesterol absorption (56).

The inhibition of cholesterol absorption is not sufficient to explain the overall action of BCD, particularly with respect to bile acid metabolism. As BCD binds bile

acids with a relatively high affinity in vitro (9–12), it is likely that this carbohydrate also plays a role analogous to that described for bile acid chelators, such as cholestyramine (57). These compounds, which are not metabolized during the digestive transit, may act as hypocholesterolemic agents in several species. They interrupt the bile acid enterohepatic cycle and thus impede the cholesterol absorption that is normally facilitated by bile acids; consequently, cholesterol and bile acid synthesis are markedly enhanced (57, 58). In comparison, as BCD is a resistant (poorly digestible) carbohydrate fermented in the colon (2, 59), it probably produces a less efficient interruption of the bile acid circulation. Consistent with these ideas, the fecal excretion of bile acids is enhanced in BCD-fed pigs and the proportions of primary bile acids also increase in bile and feces according to the dose of BCD in the diet. This latter effect could be due to an altered intestinal transit time and/or decreased recycling of secondary bile acids due to their preferential binding by BCD. In these respects, no diarrhea was produced by BCD and the fecal excretion of non-reabsorbed secondary bile acids was not significantly affected by BCD. Thus, the most plausible explanation for the large change in bile acid composition is the action of BCD on the intestinal microflora. Increased proportions of primary bile acids suggest that the end-products of the fermentable BCD, such as lactic acid and short-chain fatty acids that lower the colonic pH, inhibit the microbial bile acid degradation. Similar effects have been observed with amylomaize starch in the hamster (60) and humans (61). In pigs, BCD prevents the transformation of chenodeoxycholic acid into lithocholic acid, a potential co-carcinogenic metabolite in the colon. Furthermore, the enhancement of the fecal output of hyocholic acid is partly explained by the poor passive absorption of trihydroxylated bile acids (62).

It is important to note that the enrichment of primary bile acids in bile by BCD increases the hydrophobicity index of bile. This change could be responsible for the lower expression of hepatic LDLr in BCD-fed pigs compared with controls, in spite of similar plasma LDL-cholesterol levels. Indeed, a recent report suggests that the nature of circulating bile acids influences the hepatic LDL clearance, as the LDL uptake by hepatocytes is stimulated in vitro by ursodeoxycholic acid, a very hydrophilic bile acid (63). Thus, it is logical that BCD could produce a decrease in hepatic LDLr with an increased hydrophobicity index of biliary bile acids. Another consequence of this large change in bile acid composition in BCD-fed pigs is a marked alteration of cholesterol nucleation in bile (64). In pigs of the present study, BCD favored the propensity of cholesterol to nucleate in native bile in vitro (65), despite a tendency

to decrease cholesterol concentration of bile. Thus, BCD may be considered as a potential lithogenic agent in swine, a species that is naturally protected against cholelithiasis by high proportions of hydrophilic bile acids in bile (38). Conversely, we have demonstrated that the bile enrichment of primary bile acids in BCD-fed hamsters produces a decrease in the hydrophobicity index of bile acids (1). In this species, in which the bile acid composition is similar to that in humans (66) and clearly different from that in pigs, BCD even prevents cholelithiasis induced by a sucrose-rich diet [unpublished data from the authors].

Further insight into the mechanism of action of BCD can be drawn from the results of fecal analysis, taking into account the negative sterol balance in rapidly growing animals (67). According to a previous study in minipigs (41), the whole body cholesterol concentration is not greatly influenced by the cholesterol content of the diet, despite large variations in liver cholesterol concentrations. From these data, the mean whole body cholesterol concentration in the present study may be assessed to 1 g per kg of body weight in the four groups of pigs. Thus, giving a similar body weight gain (1 kg per day for all the groups), the cholesterol amount deposited daily in tissues with growth approximates 1 g, regardless of the group. In terms of sterol balance, this value represents the difference between the daily cholesterol input (ingestion plus synthesis) and the daily fecal output of cholesterol, as neutral sterols (cholesterol and microbial metabolites) and bile acids (41, 67). Therefore, taking into account that the daily cholesterol ingestion was 0.1 g (control group) or 6.1 g (other groups), a mean rate of cholesterol synthesis may be evaluated in the four groups of pigs on the basis of the daily fecal excretion of cholesterol (neutral sterols and bile acids) measured at the end of the study. Although not strictly correct because of the necessary assumptions, the values obtained in this manner are very different among the four groups. Their comparison helps to appreciate the proper effect of excess dietary cholesterol and added BCD on cholesterol biodynamics in pigs. In the control group, the relatively high rate of cholesterol synthesis (3.7 g per day) is not surprising in such rapidly growing animals fed a cholesterol-poor diet (41). As expected, this rate is reduced markedly (to 0.7 g per day) in cholesterol-fed pigs, in agreement with decreased activity of HMG-CoA reductase. Between cholesterol-fed and control pigs, the difference in the fecal output of cholesterol as neutral sterols (4.26 g and 1.08 g per day, respectively) indicates that a large part of dietary cholesterol excess is eliminated without prior conversion into bile acids. This is consistent with the higher biliary cholesterol concentration in cholesterol-fed pigs than in controls. This also indicates the poor intestinal percent

absorption of large amounts of dietary cholesterol. At a dose of 5 or 10% in the cholesterol-rich diet, BCD increases cholesterol synthesis to 5.7 g and 8.7 g per day, respectively. These data strongly support the increased fecal output of neutral sterols in BCD-fed pigs resulting from both a reduced absorption of dietary cholesterol and an increased output of endogenous cholesterol originating predominantly from the liver and into bile. If BCD would impede only the intestinal absorption of dietary cholesterol, activity of cholesterol-7α hydroxylase in BCD-fed pigs would be returned to the baseline activity found in control pigs. In fact, this activity, like the fecal output of bile acids, was markedly increased by BCD, as is also the case after cholestyramine treatment (57).

This comprehensive study indicates that BCD, a resistant carbohydrate, accelerates the body cholesterol turnover in pigs when supplemented together with a cholesterol-enriched diet. It prevents hypercholesterolemia and stimulates both cholesterol and bile acid synthesis, in response to impaired cholesterol and bile acid absorption. BCD is not toxic in pigs, even at a very high dose of 10%, as it restores the liver status to the basal state of control pigs and does not alter intestinal absorption of vitamin A. As BCD appears to be a potent agent to reduce the microbial degradation of bile acids, further studies are necessary to elucidate its mode of action in the intestinal lumen and to compare it with established bile chelators, fibers, and resistant carbohydrates.

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