

## Chronically gorging *v.* nibbling fat and cholesterol increases postprandial lipaemia and atheroma deposition in the New Zealand White rabbit

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In the present study, we compared the effects of nibbling and gorging on postprandial lipaemia and lipoproteins, hepatic lipid uptake and atheroma deposition. New Zealand White rabbits were fed on a low-fat (LF) control diet or a peanut oil- (10 g/d) and cholesterol- (0.5 g/d) enriched (HF) diet with the fat and cholesterol components given either by nibbling (HF-N) or gorging (HF-G). After 4 and 8 weeks, rabbits were given a test meal, which was either nibbled or taken as a bolus. The LF diet did not noticeably alter postprandial lipid variables. Triacylglycerol levels, 0–35 h lipid responses and plasma accumulation of dietary lipids were significantly higher in the HF-G group than in the HF-N group, despite higher post-heparin plasma lipase activities. Furthermore, as studied on cultured isolated hepatocytes, the higher the rate of supply of triacylglycerol- and cholesterol-rich lipoproteins (TCRL), the lower the rate of lipid uptake and bile salt secretion. Atheroma deposition was significantly increased by gorging the HF diet and was correlated with levels of most postprandial lipid variables. We conclude that gorging *v.* nibbling a fat and cholesterol-enriched diet exacerbates postprandial lipaemia by reducing the rate of TCRL clearance and favours atheroma deposition.

### Dietary lipids: Hepatic lipid uptake: Lipoproteins

The importance of changes in plasma lipids and lipoproteins occurring in the postprandial state, as suggested by Zilversmit (1979), has gained increasing attention during the last decade (Tall *et al.* 1982; Cohen *et al.* 1988; Cohn *et al.* 1988; Karpe *et al.* 1993; Lairon, 1996; Bergeron & Havel, 1997). In fact, several human studies have already provided evidence that altered postprandial lipid and lipoprotein patterns are associated with atheroma plaque progression and CHD (Weintraub *et al.* 1987; Groot *et al.* 1991; Miesenböck & Patsch, 1992). Triacylglycerol-rich lipoprotein remnants, either of hepatic or intestinal origin, have been implicated in this process (Patsch *et al.* 1992; Karpe *et al.* 1993).

The detrimental effects of increasing the amounts of saturated fat and cholesterol in the diet on post-absorptive plasma- and LDL-cholesterol levels and the risk of CHD have been extensively documented (Gardner & Kraemer, 1996). More recently, the acute effects of the amount of ingested dietary triacylglycerols (Murphy *et al.* 1995; Dubois *et al.* 1998) or cholesterol (Dubois *et al.* 1994) on the postprandial responses have been reported in human subjects. A few studies regarding the influence of meal frequency on fasting lipid and lipoprotein levels (Jenkins *et al.* 1989, 1992; Arnold *et al.* 1993; McGrath & Gibney, 1994; Mann, 1997) have reported that increasing meal

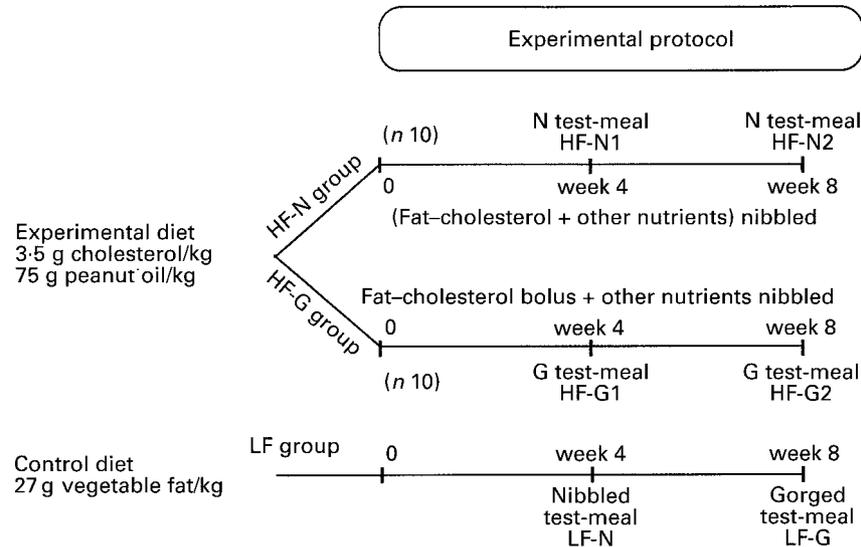
frequency attenuates the postprandial response to a daily prudent diet, improves post-absorptive lipid variables and especially lowers LDL-cholesterol, during studies performed in healthy or non-insulin-dependent diabetic subjects. Nevertheless, the long-term consequences of the acute metabolic changes induced by nibbling *v.* gorging have not been evaluated.

Taken together, these observations raised the important question of the link between the amount of dietary lipids ingested during the daytime, the resulting postprandial response and finally, the potential atherogenicity of the repetition of such metabolic events.

To investigate this point, we selected the rabbit model because of its acknowledged use in nutrition and lipid metabolism research and its ability to develop measurable atheroma deposition within a few months of exposure to a lipid-rich diet (Bocan *et al.* 1993; Daley *et al.* 1994*a,b*). It should be emphasized that the rabbit is a spontaneous nibbler. In a previous study (Juhel *et al.* 1997), we observed that rabbits fed on a regular low-fat diet showed a negligible postprandial response to a bolus test meal but exhibited an exacerbated response to a bolus meal when given a high-fat, high-cholesterol diet for a sufficient period. In fact, only a limited number of postprandial studies have been reported in the rabbit (Van Heek & Zilversmit, 1990; Juhel *et al.*

**Abbreviations:** AUC, area under the curve; G, gorged; HF, high-fat; LF, low-fat; N, nibbled; TCRL, triacylglycerol- and cholesterol-rich lipoproteins.

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**Fig. 1.** Experimental protocol. The LF group ( $n=8$ ) was fed on the low-fat control diet and the HF-N ( $n=10$ ) and HF-G ( $n=10$ ) groups were fed on the fat (10 g/d) and cholesterol (0.5 g/d) parts of the diet by nibbling exclusively or gorging fat and cholesterol and nibbling most other nutrients respectively. The nibbled test meals were ingested over 3–4 h. The gorged test meals were administered as a single bolus within 20 min.

1997) which indicate that this species is characterized by a long postprandial hypertriglycerolaemic response lasting 20–30 h after ingestion of a bolus fat meal. We recently documented that the well-known high ability to develop atheroma in this species could result from this protracted hyperlipidaemic postprandial state (Juhel *et al.* 1997).

In the present study, using the rabbit model, we aimed to modulate the postprandial lipid response by spreading the daily lipid and cholesterol intake or giving it as a bolus.

## Materials and methods

### Animals and diets

Twenty-seven male New Zealand White rabbits (Elevage Scientifique des Dombes, Romans, France), 14–15 weeks old and weighing 2.71 (SE 0.05) kg at the beginning of the experiment were used. They were housed individually at 20° and had free access to water and food. They were exposed to a 12 h light–dark cycle. The rabbits were randomly assigned to three groups (Fig. 1) and the overall protocol lasted 8 weeks. A group of seven rabbits was fed on 150 g/d of a commercial low-fat (LF) (27 g/kg), cholesterol-free diet in granular form (Usine d'Alimentation Rationnelle, Villemoisson-Sur-Orge, France) for 8 weeks. Two groups of ten rabbits were fed on a high-fat (HF), high-cholesterol diet providing 10 g peanut oil/d and 0.5 g cholesterol/d prepared by Usine d'Alimentation Rationnelle. The detailed nutrient composition of the diets is given in Table 1. As shown in Fig. 1, the HF-N group ( $n=10$ ) nibbled 135 g mixed HF diet in granular form over 24 h. The HF-G group ( $n=10$ ) ingested most of the diet during 24 h except the daily dose of cholesterol and peanut oil which was ingested once daily (within 20 min) in the form of an intrabuccal bolus given by using a 50 ml syringe in a restraining cage. Rabbits were trained to receive boluses for several days and were not

stressed by this treatment. There was no difference in the daily amounts of peanut oil (10 g) and cholesterol (0.5 g) ingested by HF-N and HF-G rabbits. LF and HF diets provided 220 and 240 kJ/kg respectively. Weight gains were comparable in the three groups, i.e. 10.5 (SE 1.7), 12.1 (SE 2.0) and 11.9 (SE 1.8) g/d for rabbits in groups LF, HF-G and HF-N, respectively. Seven rabbits from the HF-N or HF-G groups were given test meals while three rabbits from each group were used for collecting hepatocytes.

### Test meals

On the morning of the experiment day (weeks 4 and 8), after an overnight fast, the rabbits of the three experimental groups were given a test meal (55 g) of fixed composition as either an intrabuccal bolus given with a syringe within

**Table 1.** Nutrient content of experimental diets (g/d)\*

Nutrient (g/d)	LF diet (nibbling)	HF-N diet (nibbling)	HF-G diet (gorging)
Protein	19.50	15.60	3.90 (11.70)
Cellulose	24.00	19.20	6.25 (12.95)
Carbohydrate	74.20	59.30	14.75 (44.55)
Mineral–vitamin mixture†	12.00	9.60	2.40 (7.20)
Vegetable fat	4.50	3.60	(2.70)
Peanut oil	–	10.00	10.00
Cholesterol	–	0.50	0.50
Water	15.75	12.50	16.25 (9.45)

LF, low fat; HF, high-fat; N, nibbled; G, gorged.

\*The LF diet was a regular low-fat, cholesterol-free diet; HF-N and HF-G diets were comparable in terms of fat and cholesterol. Nibbling indicates that rabbits nibbled their diet during the whole day. Gorging indicates that rabbits received one part of the diet (including the daily amounts of peanut oil and cholesterol) as an intrabuccal bolus within 20 min and the remaining part in granular form (parenthesis values) during the rest of the day.

†UAR-205 (UAR, Villemoisson sur Orge, France).

20 min or a granular feed which was nibbled within 3–4 h. As described in Fig. 1, the LF rabbits received a nibbled test meal (LF-N,  $n$  7) on week 4 and another one as a bolus (LF-G) on week 8. Giving either the nibbled or the gorged test meal to LF rabbits allowed us to compare the acute effects of nibbling and gorging. On weeks 4 and 8, the HF-G group ( $n$  7) received test meals as bolus only (HF-G1 and HF-G2) whereas the HF-N ( $n$  7) rabbits had nibbled test meals only (HF-N1 and HF-N2). The test meals were prepared as follows: cholesterol (0.5 g), [ $^3\text{H}$ ]cholesterol (1480 kBq/g cholesterol) and [ $^{14}\text{C}$ ]triolein (74 kBq/g peanut oil) (CEA, Gif-sur-Yvette, France) were dissolved in chloroform-methanol (2:1, v/v). Phospholipids (0.5 g) and peanut oil (10.0 g) were added after the organic solvent was evaporated to dryness under  $\text{N}_2$ . Then, casein (4.0 g), cellulose (5.0 g), carbohydrates (15.5 g), mineral and vitamin mixture (2.8 g) and water (18.2 g) were added and blended. During the 35 h follow-up, rabbits had free access to water.

#### Analytical determinations

Blood was collected from the ear artery in tubes containing lithium-heparin. Blood samples were obtained either in the post-absorptive state (after an overnight fast) every 2 weeks or 10, 20, 25 and 35 h postprandially, after test-meal presentation in line with previous studies (Van Heek & Zilversmit, 1990; Juhel *et al.* 1997). In order adequately to store plasma samples, a cocktail of inhibitors was added as reported (Cardin *et al.* 1984). Blood was centrifuged for 15 min at 910 g at room temperature.

**Plasma lipid variables.** Triacylglycerols were determined by an enzymic procedure (Buccolo & David, 1973) using commercial kits (Kits BioMérieux, Marcy l'Etoile, France). Total and free cholesterol components were assayed by the cholesterol oxidase method (Siedel *et al.* 1983) with kits purchased from BioMérieux. Plasma phospholipids were assayed by an enzymic procedure (Takayama *et al.* 1977) with commercial kits (BioMérieux). Labelled dietary and plasma lipids were measured by dual scintillation counting with Packard 1600TR equipment (Packard, Meriden, CT, USA) with an external standard for quench correction.

**Lipoproteins.** Plasma (1 ml; overnight post-absorptive or 20 h postprandial peak samples) was used for separation of lipoprotein fractions, i.e. triacylglycerol and cholesterol-rich lipoproteins (TCRL) = chylomicrons + VLDL + IDL ( $d \leq 1.019$  kg/l), LDL ( $1.019 \leq d \leq 1.063$  kg/l) and HDL ( $1.063 \leq d \leq 1.21$  kg/l) by ultracentrifugation on a KBr discontinuous gradient (200 000 g for 24 h at 15° in a Beckman ultracentrifuge (SW 40Ti rotor; Beckman, Palo Alto, CA, USA) as previously described (Juhel *et al.* 1997). Lipoprotein lipids were adjusted for recovery, i.e. triacylglycerols (94 (SE 5) %), esterified cholesterol (88 (SE 5) %), free cholesterol (92 (SE 8) %), phospholipids (79 (SE 2) %).

**Lipoprotein lipase and hepatic lipase activities.** On weeks 4 and 8, at the end point (35 h postprandially) of the test-meal follow-up, post-heparin plasma lipoprotein lipase and hepatic lipase activities were measured as previously described (Van Heek & Zilversmit, 1990; Juhel *et al.* 1997). Briefly, blood was collected from the marginal ear artery 20 min after a single injection of 200 IU heparin/kg

(Heparine LEO, Paris, France). Lipoprotein lipase activity was calculated by subtracting hepatic lipase activity from total lipase activity and expressed as  $\mu\text{mol}$  free fatty acids/ml per min (Juhel *et al.* 1997).

**Liver lipids.** On week 8, just after killing of the rabbits, the liver was excised, rinsed with 0.15 M-NaCl, frozen and then kept at  $-20^\circ$  until analysis. Liver lipids were extracted and then quantified as previously described (Juhel *et al.* 1997).

#### Postprandial triacylglycerol- and cholesterol-rich lipoprotein uptake by cultured rabbit hepatocytes

**Isolation and culturing of liver cells.** On week 4, three rabbits from each experimental group (HF-N and HF-G) were killed. Rabbit hepatocytes were prepared by perfusion of a liver lobe with collagenase (0.005 g/l) as described previously (Whiting *et al.* 1989). After isolation and washing, the cells were suspended in William's medium E supplemented with fetal calf serum (100 ml/l), glutamine (0.1 mg/ml), kanamycin (0.1 mg/ml), penicillin (20 units/ml), streptomycin (0.1 mg/ml) and seeded on collagen-coated dishes ( $1 \times 10^5$  cells/cm<sup>2</sup>). The dishes were incubated at 37° in an atmosphere containing  $\text{CO}_2$  (50 ml/l) for 2–3 h. The medium was removed by aspiration and the cell monolayers washed with William's medium E supplemented as described earlier but without fetal calf serum. Experimental additions to the culture medium were made 24 h after this point and represented time zero.

**Characterization of postprandial triacylglycerol- and cholesterol-rich lipoprotein fractions.** TCRL is the  $d \leq 1.019$  kg/l fraction as described earlier. TCRL used for the cell culture experiments were isolated from HF-N and HF-G rabbit plasmas 20 h after test-meal consumption performed on week 4. The postprandial TCRL fractions isolated from HF-N and HF-G rabbits were called, for clarity, TCRL-N and TCRL-G respectively. The lipid compositions of TCRL-N and TCRL-G were not different. TCRL-N and TCRL-G contained (g/100 g): 11.3 (SE 1.7) v. 17.5 (SE 1.6) triacylglycerols, 22.5 (SE 0.9) v. 15.5 (SE 1.1) free cholesterol, 54.4 (SE 2.1) v. 53.5 (SE 4.0) esterified cholesterol and 12.8 (SE 0.4) v. 13.4 (SE 0.7) phospholipids. TCRL-N and TCRL-G total cholesterol concentrations were adjusted to 31 mmol/l with 0.15 M-NaCl. This value was chosen to get a final total cholesterol concentration in dishes comparable to the TCRL postprandial increment in rabbit plasma. TCRL were dialysed at 4° against culture medium and filtered through 0.22  $\mu\text{m}$  membranes, before addition to hepatocyte cultures.

**Incubation of cultured liver cells with TCRL.** To mimic *in vivo* situations, the total amount of TCRL was added to the liver cell medium either once at time zero (gorging) or stepwise, i.e. 0.25 of the TCRL dose at time zero and then 0.25 dose at 1, 2 and 3 h (nibbling). At 4, 8, 12 and 20 h after the beginning of the experiment, 0.8 ml culture cell medium was collected in order to measure remaining amounts of TCRL radiolabelled lipids, total cholesterol and triacylglycerols. The liver cell viability was controlled (by checking luminescence using an optical microscope) in every dish during the experiment and estimated at about 90%. At the end point (20 h), total bile acid content in the

culture medium was assayed by an established enzymic method (Domingo *et al.* 1972).

#### *Atheroma plaque quantification*

Immediately after killing (week 8), the arteries were carefully isolated from the arch of aorta to the iliac section. Lipids were removed on the external side of the aortas. Aortas were cut in the longitudinal axis, fixed in 100 ml/l formol overnight and stained for 20 min in red sudan IV as previously described (Holman *et al.* 1958). Total, arch, thoracic and abdominal aorta surfaces covered by stained atherosclerotic lesions were quantified by using video densitometry and a BioLab software package as before (Juhel *et al.* 1997).

#### *Statistical analysis*

The statistical significance ( $P < 0.05$ ) of the differences observed between the three groups of rabbits was assessed by using ANOVA for factorial non-repeated values and the Fisher test (Winer, 1971). In a given group of rabbits, the statistical significance ( $P < 0.05$ ) of the differences observed between the experimental meals (individual time points or areas under the curves (AUC)) or post-absorptive values were assessed by using ANOVA for repeated values and the Fisher test. Linear regression analyses were performed. The Stat-View II (Abacus Concepts Inc., Berkeley, CA, USA) micro-computer program was used.

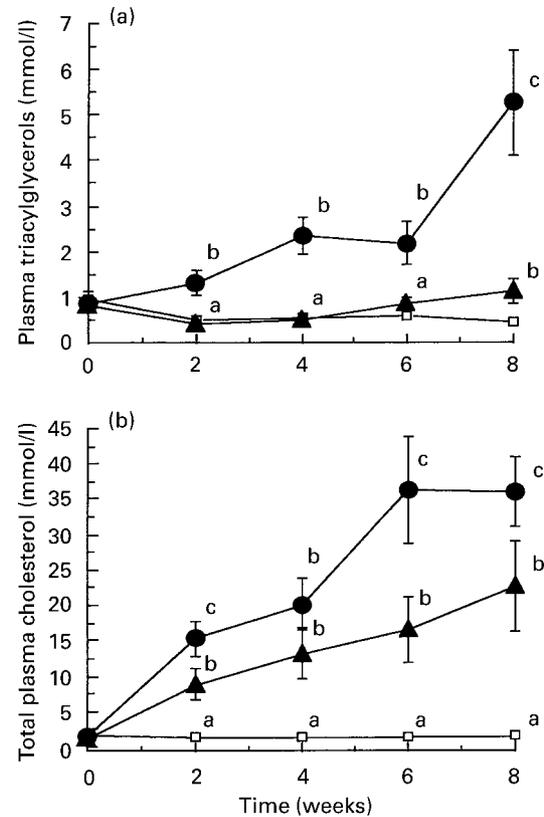
### Results

#### *Post-absorptive plasma lipids and lipoproteins*

As shown in Fig. 2, 18 h overnight post-absorptive plasma concentrations of triacylglycerols and total cholesterol measured in the rabbits fed on the LF diet remained unchanged throughout the 8 weeks nibbling. Conversely, in the two groups of rabbits chronically ingesting the HF diet, rapid and important changes were observed. After 2 weeks, the rabbits on the gorging diet (HF-G) showed significantly increased triacylglycerol levels which still increased markedly after 8 weeks (5.27 (SE 1.15) mmol/l). The rabbits on the nibbled diet (HF-N) maintained low triacylglycerol levels comparable to those of rabbits on the LF diet for 6 weeks which increased slightly after 8 weeks (0.91 (SE 0.24) mmol/l).

At the same time, plasma cholesterol levels increased in a stepwise fashion over time of feeding the HF diet as compared with the LF diet. After 8 weeks, post-absorptive plasma cholesterol levels were 1.6-fold higher in rabbits on HF-G (26.4 (SE 3.5) v. 14.8 (SE 5.8) mmol/l) than in those on HF-N. Overall changes in the two groups of rabbits were related to marked changes in cholesterol ester concentrations.

The post-absorptive lipoprotein lipid values are given in Table 2. After feeding the HF diet for 4 weeks and more markedly for 8 weeks, triacylglycerols in TCRL increased dramatically in rabbits receiving HF-G and only slightly but significantly in those receiving HF-N. Concentrations of phospholipids and total cholesterol in TCRL showed somewhat comparable values but with less marked differences



**Fig. 2.** Post-absorptive concentrations of (a) plasma triacylglycerols and (b) total plasma cholesterol in rabbits fed on a low-fat diet (□) or fed on a high-fat, high-cholesterol diet with the fat and cholesterol components ingested by nibbling (▲) or gorging (●) for 8 weeks. Values are means for seven rabbits, with their standard errors represented by vertical bars. <sup>a,b,c</sup>Mean values at a given time point not sharing a common letter were significantly different:  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test).

between HF-G and HF-N groups. Post-absorptive LDL- as well as HDL-triacylglycerol concentrations increased more markedly in the HF-G group than in HF-N after 4 and 8 weeks while comparable concentrations in phospholipids and total cholesterol were observed in LDL and HDL fractions in both groups.

#### *Postprandial plasma lipids and lipoproteins*

As shown in Fig. 3 (a and b), the relative changes in plasma triacylglycerol levels for 35 h postprandially after the bolus or the nibbled test-meals in rabbits fed on LF were comparably low. Conversely, a higher rise in triacylglycerols was observed postprandially in the HF-N group after 4 and 8 weeks and even more markedly, in the HF-G group.

In rabbits given LF, the postprandial relative changes in plasma esterified and free cholesterol (Fig. 3 (c, d, e and f)) were comparably low after the bolus or nibbled test meals. Small postprandial changes were also observed postprandially in the HF-N group whereas significantly higher free and esterified cholesterol concentrations were observed in the HF-G group.

The 0–35 h AUC values, calculated by the trapezoidal method are presented in Fig. 4. Triacylglycerol 0–35 h AUC

**Table 2.** Lipoprotein lipid levels (mmol/l) in post-absorptive (0 h) and postprandial (20 h) blood samples from rabbits given fat and cholesterol either by nibbling (HF-N) or gorging (HF-G) after 4 and 8 weeks on a high-fat, high-cholesterol diet† (Mean values with their standard errors for seven rabbits per group)

		Lipoprotein lipids after 4 weeks				Lipoprotein lipids after 8 weeks			
		HF-N		HF-G		HF-N		HF-G	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Postabsorptive values									
TCRL‡ 0 h	TG	0.29 <sup>a</sup>	0.08	1.92 <sup>b</sup>	0.43	0.49 <sup>c</sup>	0.18	4.36 <sup>d</sup>	1.39
	TC	8.89	3.05	13.44	2.55	16.91 <sup>c</sup>	6.63	29.37 <sup>d</sup>	3.89
	PL	1.36 <sup>a</sup>	0.43	2.58 <sup>b</sup>	0.40	2.93 <sup>c</sup>	0.14	4.98 <sup>d</sup>	0.21
LDL 0 h	TG	0.11 <sup>a</sup>	0.02	0.35 <sup>b</sup>	0.07	0.17	0.07	0.37	0.09
	TC	3.62	0.69	5.62	2.01	4.65	0.74	5.55	1.81
	PL	0.64 <sup>a</sup>	0.10	1.15 <sup>b</sup>	0.38	0.69	0.12	0.79	0.18
HDL 0 h	TG	0.09 <sup>a</sup>	0.01	0.43 <sup>b</sup>	0.07	0.19 <sup>c</sup>	0.06	0.39 <sup>d</sup>	0.13
	TC	0.66	0.11	1.09	0.29	1.14	0.19	1.11	0.23
	PL	0.36 <sup>a</sup>	0.04	0.75 <sup>b</sup>	0.11	0.42	0.05	0.49	0.08
Postprandial values									
TCRL‡ 20 h	TG	1.67 <sup>a*</sup>	0.55	4.75 <sup>b*</sup>	1.46	2.5 <sup>c*</sup>	0.93	6.75 <sup>d*</sup>	1.93
	TC	12.17 <sup>a</sup>	3.23	18.47 <sup>b</sup>	2.89	21.17 <sup>c</sup>	6.92	35.01 <sup>d</sup>	3.76
	PL	1.90 <sup>a</sup>	0.51	3.63 <sup>b*</sup>	0.34	3.81 <sup>c</sup>	1.00	5.86 <sup>d</sup>	0.52
LDL 20 h	TG	0.28	0.05	0.36	0.06	0.14 <sup>c</sup>	0.03	0.41 <sup>d*</sup>	0.11
	TC	1.99 <sup>a*</sup>	0.44	3.54 <sup>b</sup>	1.57	2.13 <sup>c</sup>	0.56	4.19 <sup>d</sup>	1.08
	PL	0.52 <sup>a</sup>	0.08	0.75 <sup>b</sup>	0.05	0.41	0.07	0.74	0.31
HDL 20 h	TG	0.25 <sup>*</sup>	0.05	0.46	0.13	0.20 <sup>c</sup>	0.03	0.38 <sup>d</sup>	0.11
	TC	0.67 <sup>a</sup>	0.13	0.96 <sup>b</sup>	0.16	0.84	0.13	1.11	0.13
	PL	0.44	0.06	0.44 <sup>*</sup>	0.14	0.36	0.03	0.47	0.08

TCRL, triacylglycerol- and cholesterol-rich lipoproteins; TG, triacylglycerol; TC, total cholesterol; PL, phospholipid.

<sup>a,b</sup> and <sup>c,d</sup> Mean values within a row with different superscript letters were significantly different at 4 (<sup>a,b</sup>) and 8 (<sup>c,d</sup>) weeks:  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test at a probability of 95%).

Mean values were significantly different from the corresponding post-absorptive (0 h) values: \*  $P < 0.05$  (ANOVA for factorial repeated values and Fisher's test at a probability of 95%).

† For details of diets and procedures, see Table 1 and pp. 550–551.

‡ TCRL represents the  $d < 1.09$  g/ml fraction and includes chylomicrons, VLDL and  $\beta$ -VLDL particles.

(Fig. 4(a)) were markedly and significantly higher in the HF-G group than in the HF-N group after 4 and 8 weeks feeding. Total cholesterol 0–35 h AUC (Fig. 4(b)) were also significantly higher in the HF-G group than in HF-N and LF groups.

TCRL, LDL and HDL lipid values obtained at the postprandial peak (20 h) after the different test meals are given in Table 2. TCRL triacylglycerol concentrations were significantly higher postprandially over post-absorptive values in both groups. Postprandial TCRL lipid concentrations reached dramatically higher values in the HF-G group after 4 and 8 weeks whereas the HF-N group showed lower values, especially after 4 weeks. The postprandial increase in total TCRL-cholesterol was not statistically different between HF-G and HF-N groups. The LDL as well as the HDL lipid moieties did not show noticeable postprandial changes in either HF group.

#### Postprandial accumulation of dietary lipids in plasma

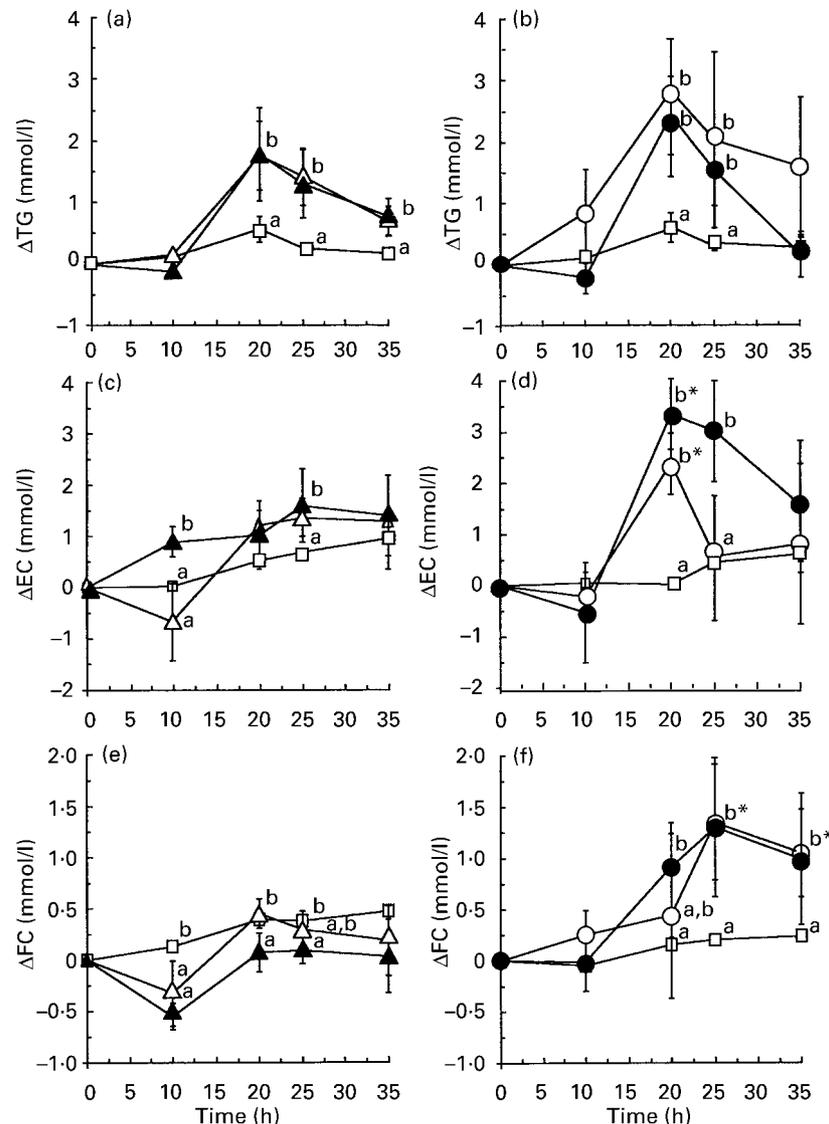
The addition of labelled esterified fatty acids and cholesterol to the test meals allowed the post-meal follow-up of plasma enrichments in lipids of dietary origin as illustrated in Figs. 4(d) and 4(e). A low accumulation of radiolabelled fatty acids was observed after the bolus or the nibbled test meals in the LF group while a slightly but significantly higher accumulation was observed in the HF-G group. A much

more dramatic rise was shown by the HF-G group after 4 and 8 weeks feeding. The overall 0–35 h AUC of labelled fatty acid concentration (Fig. 4(d)) were 2.1- and 5.9-fold higher in HF-N and HF-G groups respectively, than in the LF group after 8 weeks. In HF-N and HF-G groups, the differences observed in the accumulation of labelled fatty acids in plasma were essentially due to those found in plasma- and TCRL-triacylglycerols (Table 2).

At the same time (Fig. 4(e)), the accumulation of labelled dietary cholesterol in plasma was low in the LF group after either test meal, whereas a marked accumulation of dietary cholesterol was observed postprandially in HF-N and HF-G group plasmas. The overall 0–35 h accumulations of dietary cholesterol in the plasma (Fig. 4(e)) were 6.9- and 8.5-fold higher in HF-N and HF-G groups respectively, than in the LF group after 8 weeks.

#### Post-heparin plasma lipase activities

Minimal levels of hepatic and lipoprotein lipase activities (1.5 (SE 0.2) and 7.5 (SE 0.9)  $\mu$ mol free fatty acids/h per ml respectively) were measured in the plasma of rabbits fed on LF. After 4 weeks feeding the HF diet no marked change was observed except a significant increase in hepatic lipase activity in the HF-G group only (1.9 fold). After 8 weeks, hepatic and lipoprotein lipase activities were significantly increased in the HF-N group (4.9 (SE 0.3) and 19.7 (SE 1.2)  $\mu$ mol free



**Fig. 3.** Plasma 0–35 h triacylglycerol (TG), esterified cholesterol (EC) and free cholesterol (FC) responses to test meals in rabbits fed on a low-fat diet or fed on a high-fat, high-cholesterol diet with the fat and cholesterol components ingested by nibbling or gorging. (a,c,e), Nibbled test meals performed on week 4 with the low-fat diet ( $\square$ ) and on weeks 4 ( $\triangle$ ) and 8 ( $\blacktriangle$ ) with the high-fat diet. (b,d,f), Gorged test meals performed on week 8 with the low-fat diet ( $\square$ ) and on weeks 4 ( $\circ$ ) and 8 ( $\bullet$ ) with the high-fat diet. Results are expressed as concentration changes from post-absorptive values. Values are means for seven rabbits, with their standard errors represented by vertical bars. <sup>a,b</sup> Mean values at a given time point not sharing a common letter were significantly different:  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test). Values for rabbits nibbling and gorging the fat-cholesterol part of the diet were significantly different at a given time point: \*  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test).

fatty acids/h per ml respectively) over the LF group while significantly higher values were found for both enzymes in the HF-G group (7.1 (SE 1.0) and 28.4 (SE 4.0)  $\mu\text{mol}$  free fatty acids/h per ml respectively).

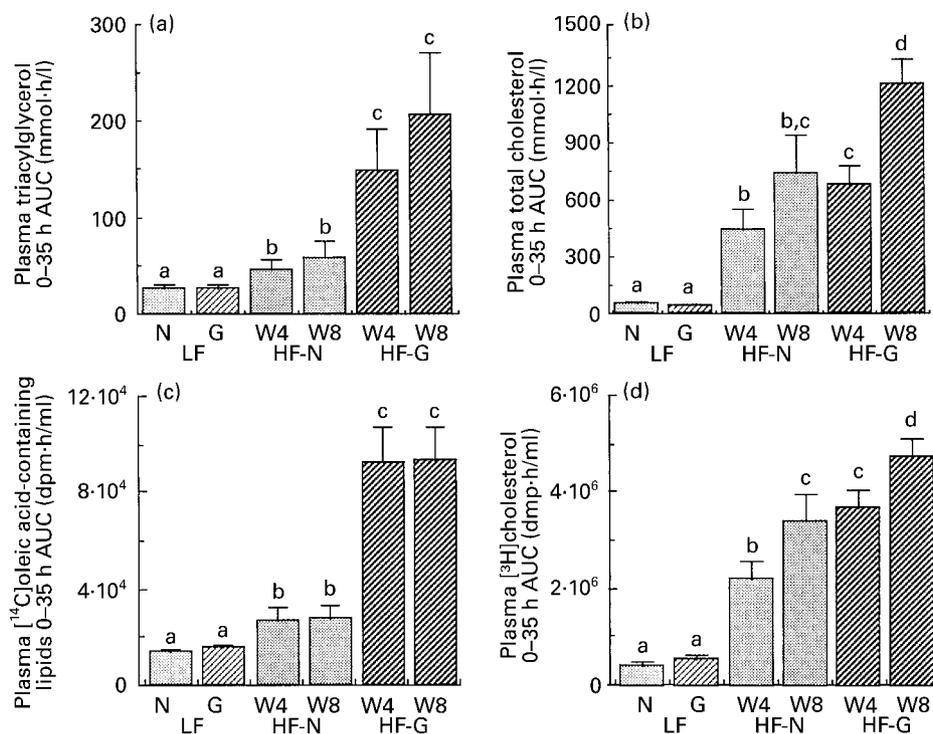
#### Accumulation of liver lipids

After 8 weeks, the HF diets, as compared with the LF diet, did not induce marked changes in liver triacylglycerol contents (11.2 (SE 1.8), 9.2 (SE 1.3) and 9.4 (SE 0.7) mg/g in LF, HF-N and HF-G groups respectively). Conversely,

the HF diets led to a marked 4–5-fold increase in liver cholesterol content as compared with the LF diet (5.0 (SE 0.5) mg/g), which was significantly higher in the HF-G group (25.4 (SE 0.9) mg/g) than the HF-N group (22.3 (SE 1.2) mg/g).

#### Uptake of postprandial triacylglycerol- and cholesterol-rich lipoproteins by cultured liver cells

The time-courses of uptake of postprandial TCRL by cultured rabbit hepatocytes are shown in Table 3. In these



**Fig. 4.** The 0–35 h area under the curve (AUC) values for (a) triacylglycerols, (b) total cholesterol, (c) [<sup>14</sup>C]oleic acid-containing lipids and (d) [<sup>3</sup>H]cholesterol in the plasma of rabbits fed on a low-fat (LF) diet or a high-fat, high-cholesterol (HF) diet with the fat–cholesterol component ingested by nibbling (N) or gorging (G). Nibbled test meals were given on week (W) 4 for the LF diet and on W4 and W8 for the HF diet. Bolus (gorged) test meals were given on W8 for the LF diet and on W4 and W8 for the HF diet. Values are means for seven rabbits, with their standard errors represented by vertical bars. <sup>a,b,c</sup>Mean values not sharing a common letter were significantly different:  $P < 0.05$  (ANOVA for factorial repeated or non-repeated values and Fisher's test).

**Table 3.** Triacylglycerols and total cholesterol in triacylglycerol- and cholesterol-rich lipoproteins (TCRL) (% remaining), and bile acid concentration (mg/l) in the culture medium of liver cells from rabbits fed on fat and cholesterol, after 4 and 8 or 20 incubation\* (Mean values with their standard errors for six donor rabbits)

	TCRL addition†			
	Simple dose		Fractionated doses	
	Mean	SE	Mean	SE
TCRL triacylglycerols				
4 h	73.6 <sup>a</sup>	3.8	57.5 <sup>b</sup>	2.1
8 h	68.5 <sup>a</sup>	2.5	53.8 <sup>b</sup>	2.3
TCRL total cholesterol				
4 h	91.1	1.6	86.2	3.0
8 h	83.6 <sup>a</sup>	1.3	74.3 <sup>b</sup>	2.6
Total bile acid				
20 h	38.8 <sup>a</sup>	5.4	66.8 <sup>b</sup>	5.7

<sup>a,b</sup>Mean values within a row not sharing a common superscript letter were significantly different:  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test at probability of 95%).

\*Hepatocytes from rabbits that had nibbled the fat and cholesterol were incubated with postprandial TCRL from corresponding nibbling rabbits, while hepatocytes from rabbits that had gorged the fat and cholesterol were incubated with postprandial TCRL from gorging rabbits. As no difference was observed in TCRL uptake or bile salt secretion by the two groups, results were pooled for clarity. Equal quantities of postprandial TCRL were introduced into the medium either once (simple dose) at 0 h or in stepwise fractionated doses (0.25 total amount) at 0, 1, 2 and 3 h. For details of procedures, see pp. 550–551.

experiments, HF-N rabbit hepatocytes were incubated with postprandial TCRL-N while postprandial TCRL-G were incubated with HF-G rabbit hepatocytes. As data concerning the uptake of TCRL-N or TCRL-G by respective hepatocytes did not show any difference under given incubation conditions, the pooled results are presented in Table 3. Conversely, when added once v. stepwise the disappearance of TCRL-N or TCRL-G triacylglycerols was significantly delayed. Concomitantly, the time course of uptake of total TCRL-cholesterol (Table 3) was different, but to a less marked extent, in the two incubation conditions.

The bile salt secretion in the culture medium was not different between N and G conditions (results not shown) and thus data obtained from HF-N and HF-G hepatocytes were pooled for clarity. As shown in Table 3, the concentration of bile salt secreted by hepatocytes after 20 h incubation was significantly higher in the culture medium when TCRL were added as fractionated doses as compared with a single dose.

#### Atheroma deposition

At the completion of the experiment (8 weeks), the percentage of aorta surface covered by lesions was quantified. The development of lesions was hardly detectable in rabbits from the LF group (lower level for quantification: 0.1 % of arterial surface). On the contrary, all rabbits in the HF groups developed atheromatous lesions on the aorta wall (Table 4).

**Table 4.** Aortic wall areas (% of lesioned surface) covered with atherosclerotic lesions in rabbits fed on a high-fat, high-cholesterol diet given either by nibbling (HF-N) or gorging (HF-G) for 8 weeks (Mean values with their standard errors for seven rabbits per group)

Aorta	HF-N group		HF-G group	
	Mean	SE	Mean	SE
Thoracic	14.7 <sup>a</sup>	3.9	26.4 <sup>b</sup>	3.3
Abdominal	9.4	2.6	13.9	3.1
Total	13.7 <sup>a</sup>	2.7	19.9 <sup>b</sup>	3.0

<sup>a,b</sup> Mean values within a row with different superscript letters were significantly different,  $P < 0.05$  (ANOVA for factorial non-repeated values and Fisher's test at probability of 95%).

Determination of the extent of lesions in the abdominal aorta did not show any significant difference between HF-N and HF-G groups. In contrast, the fraction of thoracic as well as the entire aorta surface covered by atheromatous lesions was significantly higher in the HF-G group (1.8 fold and 1.5 fold respectively) than in the HF-N group.

When data from HF-N ( $n = 7$ ) and HF-G ( $n = 7$ ) groups were analysed by regression analysis, several postprandial plasma and TCRL lipid variables were correlated with the percentage of aorta surface area covered by lesions (Table 5). No correlation was found for LDL and HDL lipids, except the plasma HDL-total cholesterol concentrations in the postprandial state (20 h).

## Discussion

The aim of the present study was to evaluate the effects of nibbling or gorging the fat and cholesterol components of

**Table 5.** Correlations of postprandial plasma and lipoprotein lipid responses with atherosclerotic lesions in rabbits fed on a high-fat, high-cholesterol diet

Postprandial variables	Aorta total lesioned surface	
	$r^2$	$P$
Plasma triacylglycerols*	0.68	0.012
Plasma [ <sup>14</sup> C]oleic acid-containing lipids	0.76	0.001
Plasma [ <sup>14</sup> C]oleic acid 0–35 h AUC	0.80	0.001
Plasma TCRL-triacylglycerols*	0.49	0.075
Plasma TCRL [ <sup>14</sup> C]oleic-acid*	0.68	0.008
Plasma LDL-triacylglycerols*	0.38	0.180
Plasma LDL [ <sup>14</sup> C]oleic-acid*	0.35	0.220
Plasma HDL-triacylglycerols*	0.18	0.520
Plasma HDL [ <sup>14</sup> C]oleic-acid*	0.49	0.071
Plasma total cholesterol*	0.51	0.070
Plasma cholesterol 0–35 h AUC	0.99	0.001
Plasma [ <sup>3</sup> H]cholesterol*	0.82	0.001
Plasma [ <sup>3</sup> H]cholesterol 0–35 h AUC	0.80	0.001
Plasma TCRL-total cholesterol*	0.88	0.001
Plasma TCRL-[ <sup>3</sup> H]cholesterol*	0.80	0.001
Plasma LDL-total cholesterol*	0.36	0.210
Plasma LDL-[ <sup>3</sup> H]cholesterol*	0.35	0.224
Plasma HDL-total cholesterol*	0.56	0.035
Plasma HDL-[ <sup>3</sup> H]cholesterol*	0.49	0.070

AUC, area under the curve; TCRL, triacylglycerol- and cholesterol-rich lipoproteins.

\* Plasma or lipoprotein lipid values were measured 20 h after test-meal intake.

the diet in the rabbit. The daily intakes of fat and cholesterol were high but usual for this kind of study (Van Heek & Zilversmit, 1990; Bocan *et al.* 1993; Daley *et al.* 1994a,b; Juhel *et al.* 1997). The two kinds of high-fat, high-cholesterol regimen were compared with a regular nibbled LF diet for 4 or 8 weeks. The postprandial effects of bolus or nibbled meals were monitored. To take into account the physiological needs of the rabbit, a natural nibbler, only fat and cholesterol were given as a bolus or not while most other nutrients were nibbled over 24 h. Thus, this experimental design does not exactly mimic the usual human dietary patterns.

As recently reviewed (Mann, 1997), it has been shown that increasing meal frequency attenuates the postprandial response to nutrient intake and improves post-absorptive lipid variables (Jenkins *et al.* 1989, 1992; Arnold *et al.* 1993; McGrath & Gibney, 1994) during studies performed in healthy or non-insulin-dependent diabetic human subjects. Nevertheless, these human studies were performed with diets containing low or moderate amounts of fat and the link between metabolic changes induced by nibbling *v.* gorging and progression of atherosclerosis was not evaluated. This is experimentally feasible using suitable animal models. The atherogenic nature of the postprandial state has been suggested for two decades (Zilversmit, 1979) and more recent data have established that exaggerated and/or delayed postprandial lipaemia (Weintraub *et al.* 1987; Miesenböck *et al.* 1992; Karpe *et al.* 1993; Lairon, 1996; Bergeron & Havel, 1997) could be related to coronary events in man. To study more mechanisms involved, we selected the rabbit model because of its ability to develop atheroma deposition when fed on a lipid-rich diet (Bocan *et al.* 1993; Daley *et al.* 1994a,b). From the limited number of postprandial studies already performed in the rabbit (Van Heek & Zilversmit, 1990; Juhel *et al.* 1997), it appeared that this species is characterized by a particularly long postprandial hypertriacylglycerolaemic response lasting about 20–30 h after ingestion of a bolus meal providing 6–14 g triacylglycerols that is primarily related to a slow rate of lipid gastric emptying (Fekete *et al.* 1990; Van Heek & Zilversmit, 1990; Juhel *et al.* 1997). In addition, a limited clearance capacity of dietary lipids could explain this protracted postprandial event which in turn was suggested to account for the well-known outstanding capability to develop atheroma in this species (Juhel *et al.* 1997).

In the control group fed on a regular low-fat, cholesterol-free diet (LF diet), post-absorptive plasma lipid levels did not vary noticeably over time and a minimal postprandial response was observed irrespective of the way (gorging *v.* nibbling) the lipids were ingested, as already observed after gorging a LF meal (Juhel *et al.* 1997). In these animals, there was limited accumulation of dietary fatty acid-containing lipids and cholesterol postprandially. The rabbits chronically fed on this LF diet did not accumulate triacylglycerol and cholesterol moieties in the liver and had no apparent clearance defect (Mamo *et al.* 1991; Demacker *et al.* 1992) and thus, exhibited a comparable capacity to handle dietary lipids as a single bolus or a nibbled test meal. The extent of atheroma deposition in the aorta was hardly detectable in these rabbits.

When rabbits were chronically fed on a moderately high-fat (10 g/d), high-cholesterol (0.5 g/d) diet (HF diet), the postprandial response to the test meals was amplified as compared with the LF-fed controls, in agreement with previous observations (Juhel *et al.* 1997). Nevertheless, a striking observation was that the postprandial rise in triacylglycerols was significantly exacerbated in the rabbits chronically fed on the HF diet under a gorging regimen (HF-G) as compared with the nibblers (HF-N). The exacerbated post-meal hypertriacylglycerolaemic response elicited by the HF-G regimen was essentially due to a 5.2-fold increased concentration of TCRL-triacylglycerols in which dietary fatty acid-containing lipids and cholesterol dramatically increased (results not shown). Isolated TCRL particles are a mix of endogenous (VLDL and  $\beta$ -VLDL) and exogenous (chylomicrons and remnants) particles but we did not aim to make separate evaluations of their behaviour.

Although overall dietary fat and cholesterol intakes were not different in the two groups of rabbits, it appeared that rabbits who were nibbling had a less marked accumulation of TCRL in the circulation 0–35 h postprandially and logically, in the post-absorptive state after overnight food deprivation. This observation probably suggests that the clearance ability of the gorgers had been reduced. Several mechanisms could have contributed to the different responses observed in gorgers and nibblers.

As the existence of a nutritional adaptation of the endovascular lipases has been reported in the rabbit (Van Heek & Zilversmit, 1990; Warren *et al.* 1991; Juhel *et al.* 1997), we checked whether the two ways of ingesting lipids would influence the enzyme levels. In the nibblers, ingesting the HF diet for 8 weeks led to a significant increase in the activities of post-heparin plasma lipoprotein lipase and hepatic lipase whereas such an adaptation occurred in the gorgers with an even more marked amplitude for both enzymes and much faster (i.e. at 4 weeks) in the case of hepatic lipase. It thus appears that repetition of lipid bolus ingestions, as compared with nibbled meals, leads to a greater nutritional adaptation of enzymes governing the plasma clearance of lipids. Nevertheless, such enhanced enzyme activities resulting from this adaptive process are unable to limit TCRL accumulation as observed in the gorgers postprandially. The saturation of lipoprotein lipase by the number and the cholesterol enrichment of TCRL particles might limit the rate of lipolysis of TCRL particles as reported in the hypercholesterolaemic rabbit (Connelly *et al.* 1994). Another role of these enzymes could be implicated. Both lipoprotein lipase (Mann *et al.* 1995; Takahashi *et al.* 1995) and hepatic lipase (Shafi *et al.* 1994; Ji *et al.* 1995; Karpe *et al.* 1996) have been shown to promote quantitatively the binding of TCRL remnants to the hepatocyte membranes via linkages to proteoglycans, thus facilitating the receptor-mediated uptake of these particles (Ji *et al.* 1995). In the rabbit, the hepatic lipase level is definitively low and thus, could be a limiting factor for binding and clearance even after maximal expression due to adaptation to the lipid diet. In both groups of rabbits fed with fat and cholesterol, it is possible that the relatively low level of plasma HDL could reduce normal exchanges of lipids and apoproteins and thus slow TCRL clearance (Tall *et al.* 1982).

A second mechanism possibly involved in the differences observed is the receptor-mediated uptake of TCRL particles by the liver. Although the TCRL fraction is heterogeneous with different sub-populations of particles with possible different clearance rates, we aimed to use a global test for the hepatic uptake of TCRL particles accumulated postprandially. Thus, we investigated how cultured hepatocytes from LF, HF-N and HF-G groups of rabbits handled postprandial TCRL isolated from rabbits fed on HF-N or HF-G regimens. To mimic the postprandial state *in vivo*, a given amount of TCRL lipids was added once (gorging pattern) or in four portions over 3 h (nibbling pattern). Postprandial TCRL obtained from HF-N or HF-G groups had comparable lipid composition. Hepatocytes from HF-N or HF-G groups of rabbits had a comparably low rate of uptake of TCRL which was highly dependent on the rate of supply, i.e. a lower rate of uptake when provided as a single dose. It has already been shown that cholesterol-rich diets reduce the expression of the LDL receptor (Kovanen *et al.* 1981; Meijer *et al.* 1991), a phenomenon linked to the accumulation of free cholesterol in hepatocytes, as observed here but more markedly in HF-G than HF-N rabbit livers. Thus, this suggests that the feedback regulation of the hepatic LDL receptor could be exacerbated when a higher flux of particles enters the liver as occurring under gorging. In addition, LDL-receptor related protein could play an important role in the clearance by liver of apolipoprotein B<sub>E</sub>-containing lipoproteins, such as  $\beta$ -VLDL, as suggested by studies in mice (de Faria *et al.* 1996), but the possible regulatory mechanisms involved are not yet clearly understood. With the hepatic receptors potentially involved, a higher flux of supply of TCRL particles is expected to exacerbate competition for binding and clearance in the space of Disse as suggested by other authors (Hussain *et al.* 1995). In the rabbit, extra-hepatic VLDL receptors (Takahashi *et al.* 1995) and bone marrow macrophages (Hussain *et al.* 1995) also play a role in TCRL clearance *in vivo*.

We observed that a single dose of TCRL as compared with a fractionated supply reduced the amounts of bile salts secreted. These data suggest that a higher flux of TCRL entering the liver might limit *de novo* synthesis of bile salts and thus reduce elimination of cholesterol from the liver into bile secretion. On the contrary, as shown by others in a cholesterol concentration-dependent manner (Meijer *et al.* 1992), the secretion of VLDL and  $\beta$ -VLDL by the liver in HF-G should be more pronounced than in HF-N animals. In fact, we observed such an accumulation of TCRL particles postprandially and in the post-absorptive state in the HF-G group. This TCRL fraction was enriched postprandially in dietary radiolabelled fatty acid-containing lipids (correlation with plasma triacylglycerols:  $r=0.84$ ,  $P=0.0001$ ) and dietary cholesterol (correlation with cholesterol esters:  $r=0.81$ ,  $P=0.0001$ ) which are probably present in the form of both intestinally-derived chylomicrons and endogenous VLDL and  $\beta$ -VLDL. Both kinds of particles are normally competing for lipolysis, binding and uptake by hepatic receptor pathways and the much greater postprandial accumulation in the plasma of lipid moieties of dietary origin in the gorging animals well illustrates the exacerbated competition occurring in this situation.

After 4 weeks of dietary regimen, markedly higher

postprandial TCRL triacylglycerol and cholesterol ester concentrations were found in gorging animals as compared with nibbling ones. The two main mechanisms involved in the difference observed thus seem to be the exacerbated secretion of  $\beta$ -VLDL from the liver and the lowered TCRL remnant clearance in the HF-G group rabbits. It is also interesting to note that LDL-cholesterol ester concentrations were 1.6–2.7-fold higher in the gorging *v.* nibbling animals postprandially. The fact that the difference in TCRL lipid concentration tended to be less marked between gorgers and nibblers after 8 weeks leads us to suggest that the difference between the two groups would tend to be lessened with time of feeding the HF diet.

The accumulation of TCRL and their remnants (Zilversmit, 1979; Tall *et al.* 1982) as well as LDL enrichment in cholesterol esters (Patsch *et al.* 1992) in the circulation postprandially and in the post-absorptive state are seen as potentially atherogenic factors (Gianturco & Bradley, 1994). The building of atheroma plaque on the aorta wall surface in lipid-fed rabbits has been reported previously (Daley *et al.* 1994a,b) as here, whereas no evident lesion development was observed in the control rabbits fed on the LF diet for 8 weeks. A worthwhile observation was that the entire extent of atheroma deposition was significantly less important (–37%) in the nibbling animals than in the gorgers. In the New Zealand White rabbit chronically fed on a lipid-rich diet, atheromatous lesions especially accumulate in the thoracic aorta (Daley *et al.* 1994a,b) as observed here. The rate of atheroma deposition has already been shown to be related to exposure to  $\beta$ -VLDL (Daley *et al.* 1994b) but also to chylomicrons (Mamo & Wheeler, 1994) that penetrate the artery wall in the same way as LDL particles, especially small dense LDL. In line with previous observations obtained in nibbling rabbits only (Juhel *et al.* 1997), linear regression analyses of the data obtained in both HF groups of rabbits clearly established a link between aorta lesions and triacylglycerol or cholesterol concentrations in plasma and TCRL postprandially as well as the postprandial accumulation of lipids and cholesterol of dietary origin (Table 5). These data thus support the concept that nibbling, as compared with gorging, reduces the accumulation of TCRL particles of intestinal and hepatic origin which, in turn, lowers the extent of atheroma deposition.

Considering that the overall changes in plasma lipids observed in rabbits under nibbling or gorging regimens fit well with some metabolic data obtained in human subjects in the short term (Jenkins *et al.* 1989, 1992), one can suggest that nibbling a fat- and cholesterol-containing diet in the long term could have less detrimental effects than gorging in human subjects. It should be emphasized that feeding the rabbit with fat and cholesterol leads to a specific accumulation of  $\beta$ -VLDL which does not generally occur in human beings. This hypothesis thus remains to be demonstrated in human subjects.

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