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DENSITY PROFILE AND CHOLESTEROL CONCENTRATION OF SERUM LIPOPROTEINS IN EXPERIMENTAL ANIMALS AND HUMAN SUBJECTS ON HYPERCHOLESTEROLAEMIC DIETS

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Abstract—1. The density profile of Sudan black stained serum lipoproteins was studied in human subjects and various animal species on diets supplemented with cholesterol.

2. In the animals studied (rabbits, calves, mice, chickens, rats and guinea-pigs), the feeding of cholesterol resulted in an elevation of serum cholesterol levels together with marked changes in the density profile and the cholesterol concentration of the serum lipoproteins. Large differences between animal species in their response to dietary cholesterol were found.

3. In a human subject, an increased concentration of serum cholesterol due to the consumption of a diet supplemented with six egg yolks per day was reflected in an elevated level of LDL cholesterol, while changes in the density profile of stained serum lipoproteins were not observed.

4. In subjects with familial type III and type IV hyperlipoproteinaemia, marked differences in the density profile of lipoproteins were found when compared with that of normolipoproteinaemic subjects.

5. The density profile of stained lipoproteins in the type III patients was remarkably similar to that in cholesterol-fed chickens and lean Zucker rats.

INTRODUCTION

We have recently developed a method for the visualization and localization of lipoprotein bands in density gradient ultracentrifugation (Terpstra *et al.*, 1981). Prior to ultracentrifugation, the lipoproteins in the serum are stained with Sudan black. After ultracentrifugation, the lipoprotein bands are visible in the gradient and can be photographed. We have used this method in order to compare the lipoprotein profiles in the sera of various experimental and domestic animals (Terpstra *et al.*, 1982a). Further, we have studied the effects of the type and proportion of dietary casein and soybean protein on the serum lipoprotein pattern in several animal species, such as rabbits, rats, chickens and guinea-pigs (Terpstra *et al.*, 1982b, 1983a,c; Mol *et al.*, 1982; West *et al.*, 1983). In the present communication we describe the effects of hypercholesterolaemic diets, generally diets enriched with cholesterol, on the density profile and the cholesterol concentration of serum lipoproteins in various experimental animals and man. Parts of this work have been published previously as aspects of studies on the effects of dietary proteins on serum cholesterol (Scholz *et al.*, 1982; Beynen *et al.*, 1983b; Mol *et al.*, 1982; Terpstra *et al.*, 1982c, 1983b,c).

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Abbreviations used: VLDL, very-low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins.

MATERIALS AND METHODS

Analytical methods

Serum lipoproteins were isolated by density gradient ultracentrifugation exactly as described previously (Terpstra *et al.*, 1981). The shape of the density gradient was found to be highly reproducible, thus it was possible to perform comparative studies using this technique. The cholesterol concentration of whole serum and serum lipoprotein fractions was determined enzymatically (Röschlau *et al.*, 1974), using the kit (catalase method) supplied by Boehringer Mannheim GmbH, FRG.

Experiment with humans

During the study, the subjects (three men and three women, aged 27–41 yr) consumed their habitual diets, with the exception that during the first 10 days of the study cholesterol-rich products were forbidden, whereas during the second 10 days of the study the diets were supplemented with six egg yolks per day, which is equivalent to about 1500 mg of cholesterol. The subjects were informed by a dietitian about the cholesterol-rich products; the products which were not to be consumed during the first 10 days were: eggs or egg containing products, crustaceans, liver and kidney. The subjects were also asked to limit their intake of meat and fish to 100 g/day. During the cholesterol-rich period, the egg yolks were supplied under supervision of a dietitian as whole eggs, homogenized with orange juice, or added to salads and desserts. The subjects generally avoided monotony by choosing different items. The 24-hr recall method was used to estimate food intakes during the experiment; food intake data were converted into nutrients using the computerized Dutch food table (Hautvast, 1975).

Blood samples were taken from an antecubital vein after an overnight fast on the last two days of the low- and high-cholesterol periods.

Experiment with rabbits

Male rabbits of the New Zealand White strain were used. At the age of about 11 weeks they were fed a semipurified diet containing soy isolate as a protein source for 4 weeks. Then (day 0), on the basis of their serum cholesterol concentrations and body weights, 9 animals were allocated to the diet supplemented with cholesterol while 9 other animals remained on the cholesterol-free soy-isolate diet. The composition of the semipurified diets containing soy isolate (20.8%, w/w) has been described earlier (Scholz *et al.*, 1982). Cholesterol (0.2%, w/w) was added to the semipurified diet at the expense of sawdust. Blood samples were taken at day 29 of the experiment between 8.00 a.m. and 10.00 a.m. after the removal of any remaining food at 4.00 p.m. the previous day.

Experiment with calves

Male, Dutch Friesian–Holstein calves were used. On arrival in the calf house, the animals were divided into two groups of 12 calves. One group was fed a milk replacer containing 60% (weight%, on the basis of air-dry matter) skim milk and 20% crude fat. The composition of this control diet and the feeding schedule can be found elsewhere (Beynen *et al.*, 1983b). The other group was fed the experimental diet which was identical to the control diet, except that 0.5% (w/w) cholesterol was added at the expense of the fat source. The experimental period lasted 21 weeks. Cholesterol in the diets was assessed by gas–liquid chromatography of the non-saponifiable fraction; the control diet and the experimental diet were found to contain 0.04 and 0.49% (w/w) of cholesterol, respectively. At the end of the experiment blood samples were taken from the jugular vein between 10.00 a.m. and 11.00 a.m., which was about 4 hr after feeding. In previous work we have demonstrated under similar experimental conditions that in control and cholesterol-fed calves diurnal variations in the level of serum cholesterol are absent (Beynen and Van Gils, 1983).

Experiment with chickens

In this experiment (Mol *et al.*, 1982), two groups of 7 week-old male Shaver Starbro broiler chickens were used, which were raised on a commercial diet. A group consisting of 4 animals was transferred to a commercial diet containing 1% (w/w) cholesterol, whereas 3 other animals continued to receive the cholesterol-free commercial diet. After feeding the diets for a period of 6 weeks, blood samples were taken from a wing vein without prior fasting.

Experiment with rats

Two groups of male lean Zucker rats and two groups of female genetically obese Zucker rats were used. The groups consisted of 8 and 5 animals, respectively. Their age at the beginning of the experiment was 40 days. The male lean Zucker rats were fed a commercial diet with or without 1.2% (w/w) cholesterol (Terpstra *et al.*, 1982c). One group of the genetically obese Zucker rats was fed a cholesterol-free commercial diet, whereas the other group was provided with a semipurified diet containing 50% (w/w) casein and 1.2% cholesterol (Terpstra *et al.*, 1983c). At the end of the experimental period, blood samples were taken after an overnight fast by orbital puncture under light anesthesia with ether.

Experiment with guinea-pigs

A group of 7 female guinea-pigs (weighing 568 ± 16 g, mean ± SEM) had been fed a cholesterol-free semipurified diet containing 40% (w/w) casein for 12 weeks. Then, the diet was enriched with 1% cholesterol (at the expense of the sawdust component), and the animals received this diet for a further period of 25 days. A group of 3 female guinea-pigs (weighing 678 ± 9 g, mean ± SEM) were provided with a cholesterol-free commercial diet during the entire experimental period (Terpstra *et al.*, 1982b). At the end of the experimental period, blood samples were taken after an overnight fast by orbital puncture under light anesthesia with diethyl ether.

Experiment with mice

Two groups of 16 female Swiss mice, aged 28 days, were fed a semi-purified diet containing 20% (w/w) soybean protein with or without 1% cholesterol. The composition of this diet was similar to that described previously and used in an experiment with rabbits (West *et al.*, 1982). After feeding the experimental diets for a period of 98 days, blood samples were taken after an overnight fast by orbital puncture under light anesthesia with diethyl ether.

RESULTS AND DISCUSSION

Figure 1 shows 9 density profiles of human serum lipoproteins. In apparently healthy subjects, 3 major lipoprotein classes are separated: VLDL at the top of the gradient, and LDL and HDL further down. At the base of the tube, residual stain is located. The lipoprotein classes can be observed as distinct bands and often the HDL_2 and HDL_3 (or rather the HDL_{2b} and HDL_{2a}) are present as single bands.

During the low- and high-cholesterol periods the volunteers consumed on average 207 and 1803 mg cholesterol/day. The consumption of 6 egg yolks daily in addition to the habitual diet caused an increase in the percentage of fat calories (from 39 to 46%) and a decrease in the energy derived from carbohydrates (from 44 to 37%). The increase in energy percentage from fats was almost exclusively due to monounsaturated fatty acids, while the energy percentage derived from polyunsaturated and saturated fats remained constant. Thus it can be concluded that only the increase in dietary cholesterol would affect the level of serum cholesterol (Keys *et al.*, 1965a,b).

In the 6 subjects we have studied, the consumption of 6 egg yolks per day for a period of 10 days elicited a pronounced inter-individual variation in the cholesterolaemic response. Changes in the level of total cholesterol of −3% (nonresponder) to 27% (hyperresponder) were observed (Katan and Beynen, 1983). However, visual inspection of the stained lipoprotein profile showed no changes upon egg yolk consumption, neither in a nonresponder nor in a hyperresponder (Fig. 1). In contrast, the cholesterol concentration of the LDL fraction in the hyperresponder was markedly increased (+47%) after cholesterol feeding (Table 1). In the nonresponder, dietary cholesterol only slightly affected the cholesterol concentration of lipoproteins, except for the VLDL fraction where a fall in cholesterol concentration was seen (Table 1). Based on the intensity of the Sudan black staining, it would appear that in humans the lipoprotein pattern is quite stable. At least, under the conditions of the present study, dietary cholesterol does not affect the lipoprotein profile.

In subjects with disorders of lipoprotein metabolism, the profile was quite different from that in normolipidaemic subjects. Visual inspection of the density profile of the Sudan black stained serum lipoproteins of patients with type III hyperlipoproteinaemia revealed a tremendous accumulation of IDL and VLDL particles (Fig. 1), irrespective of the

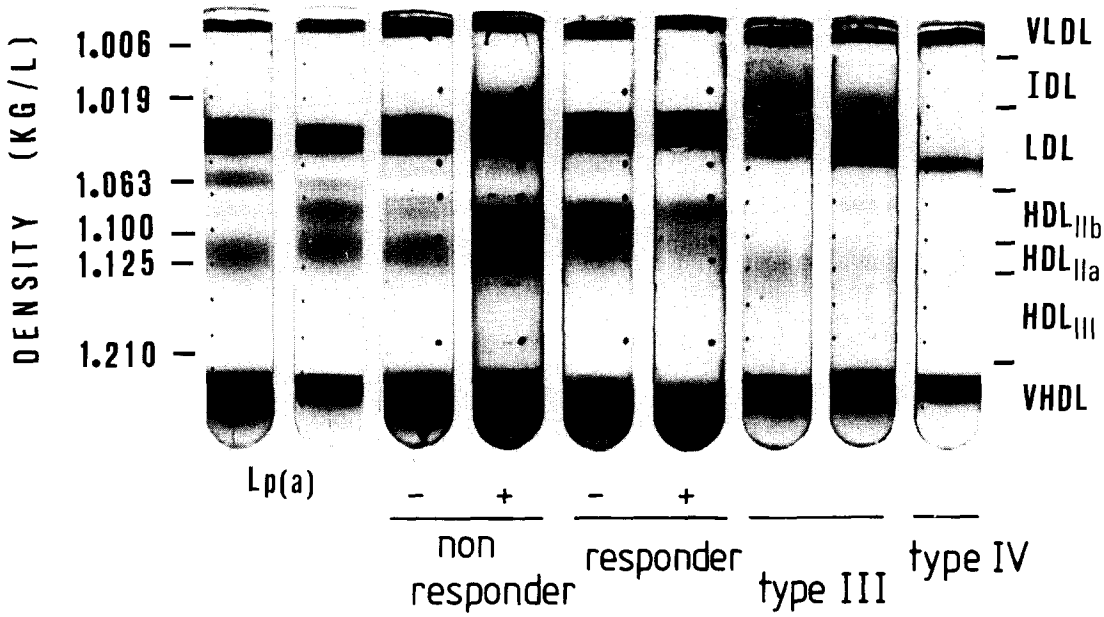

Fig. 1. Photograph of the density profile of Sudan black stained serum lipoproteins observed after density gradient ultracentrifugation of serum samples from subjects with a Lp(a) band, subjects on a low (−) or a high (+) cholesterol diet and from hyperlipidaemic patients.

level of total serum cholesterol (Table 1). This visual impression was reflected in an increased cholesterol concentration in these fractions. In the patient with type IV hyperlipoproteinaemia, it would seem that the LDL fraction had decreased (Fig. 1). However, the cholesterol concentration in this fraction was not different from that in normolipidaemic subjects, and was higher than that in one of the type III patients (Table 1). The cholesterol concentration of the VLDL fraction was markedly elevated in the type IV

Table 1. The cholesterol concentration in whole serum and serum lipoprotein fractions in man and various animal species on a low and high-cholesterol diet[1]

	Cholesterol concentration (mmol/l serum)									
		LDL		HDL			Whole serum (mean ± SEM)	Amount of cholesterol in diet	Number of probands	Days on diet
	VLDL	IDL	LDL	HDL_2	HDL_3	VHDL				
Human subjects										
Nonresponder[2]	0.28	0.16	2.75	0.39	0.72	0.19	4.85	low cholesterol diet	1	10
	0.12	0.12	3.01	0.46	0.76	0.21	5.12	six egg yolks per day		10
Responder[2]	0.28	0.16	2.54	0.64	0.75	0.12	4.77	low cholesterol diet	1	10
	0.22	0.17	3.73	0.76	0.81	0.13	5.93	six egg yolks per day		10
Type III patient	1.82	1.04	1.26	0.37	0.58	0.13	5.74		1	
Type III patient	1.73	0.94	3.87	0.65	0.41	0.10	8.04		1	
Type IV patient	2.81	0.45	2.52	0.37	0.42	0.08	6.65		1	
Rabbit	0.25	0.31	1.00	0.72	0.22	0.00	2.40 ± 0.33	—	9	29
	2.19	2.45	4.84	0.70	0.27	0.00	10.31 ± 1.48	0.2%	9	29
Calf	0.00	0.01	2.18	2.04	0.18	0.00	4.52 ± 0.26	—	11	147
	0.15	1.06	5.07	2.34	0.41	0.02	9.80 ± 0.71	0.5%	12	147
Mouse	0.14	0.00	0.27	2.10		0.00	2.48 ± 0.10	—	16	98
	0.59	0.08	0.23	2.30		0.00	3.25 ± 0.19	1.0%	16	98
Chicken	0.06	0.08	1.01	1.98		0.09	3.33 ± 0.24	—	3	29
	2.43	1.04	0.39	1.88		0.08	6.56 ± 1.73	1.0%	4	29
Lean Zucker rat	0.05	0.01	0.08	1.52		0.06	2.13 ± 0.04	—	8	98
	0.91	0.44	0.22	0.88		0.08	2.94 ± 0.16	1.2%	8	98
Obese Zucker rat	0.48	0.33		1.32		0.03	2.23 ± 0.10	—	5	77
	2.25	1.93		2.90		0.09	8.40 ± 0.59	1.2%	5	77
Guinea-pig	0.08	1.06		0.03		0.00	1.25 ± 0.11	—	3	25
	0.54	9.72		0.80		0.03	12.64 ± 1.36	1.0%	7	25

[1]Lipoprotein fractions were collected by aspiration using the conventional density limits for serum lipoproteins: VLDL, very-low density lipoproteins ($d < 1.006$ kg/l); IDL, intermediate density lipoproteins ($1.006 < d < 1.019$ kg/l); LDL, low density lipoproteins ($1.019 < d < 1.063$ kg/l); HDL, high density lipoproteins (HDL_2: $1.063 < d < 1.10$ kg/l; HDL_3: $1.10 < d < 1.21$ kg/l); VHDL, very high density lipoproteins ($d > 1.21$ kg/l). However, $d = 1.075$ kg/l was used as density limit between the LDL and HDL for human subjects, the guinea-pig and the mouse; further, in the rat, the density limits of $d = 1.042$ kg/l and $d = 1.182$ kg/l were used for the separation of the HDL fraction. For chickens and guinea-pigs, the mean values of the individual lipoprotein analyses are given; for the other animals, the values of a pool serum are presented. For whole serum, the mean values are given.

[2]The subjects consumed successively a low and high-cholesterol diet; the cholesterol concentrations of lipoprotein fractions and whole serum are the means of days 9 and 10 of both dietary periods (see Methods section).

patient. It would appear from Fig. 1 that the two type III patients and the type IV patient had reduced levels of HDL_2 and HDL_3. However, as can be seen in Table 1, the cholesterol concentration in the HDL_2 lipoprotein fraction, in contrast to that of the HDL_3 fraction, does not significantly differ between the patients and the healthy subjects. Apparently, the intensity of Sudan black staining of a lipoprotein band does not always correlate with its cholesterol content. The data on human lipoprotein profiles (Fig. 1) indicate that dietary cholesterol does not alter the Sudan black stained lipoprotein profile. However, in dyslipoproteinaemic states marked alterations can be observed.

The various animal species have a serum lipoprotein density profile which is quite different from that in man. In contrast to man, the animals studied exhibit only one single HDL band (Figure 2). Further, in normolipidaemic man the LDL is the main carrier of serum cholesterol, whereas in the normolipidaemic animals, with the exception of the guinea-pig, and possibly the calf, most of the cholesterol in the serum is transported by the HDL fraction (Table 1).

In rabbits, calves, chickens and guinea-pigs, the feeding of cholesterol resulted in a marked elevation of serum cholesterol levels. In mice and lean Zucker rats, however, this increase was relatively small, indicating that these rodents are rather resistant to exogenous cholesterol. In the female obese Zucker rat, severe hypercholesterolaemia can be induced by a semipurified cholesterol-enriched diet (Table 1).

The feeding of cholesterol was associated with considerable changes in the density profile and cholesterol concentration of Sudan black stained lipoproteins. In the chicken and the lean Zucker rat, a clear cut increase of VLDL and IDL particles was found (Fig. 2). This was accompanied with a decrease of LDL and HDL particles, respectively. This pattern, as induced by dietary cholesterol, showed a close similarity to the pattern observed in the familial type III hyperlipoproteinaemic patients. As would be anticipated from the lipoprotein profiles, in the chicken and the rat cholesterol feeding effected a marked increase in the cholesterol concentration of the VLDL and IDL fractions (Table 1). Simultaneously, a decrease in the cholesterol content of the LDL and HDL fractions was seen in the chicken and lean Zucker rat, respectively. These findings in cholesterol-fed rats are consistent with those reported by Mahley and Holcombe (1977). Similarly, in cholesterol-fed rhesus monkeys, Rudel *et al.* (1979) found that increased levels of VLDL and LDL cholesterol were associated with a decrease in HDL-cholesterol.

In the genetically obese Zucker rat, an increase in serum cholesterol due to cholesterol feeding was reflected in a markedly elevated cholesterol level in all the 3 major lipoprotein classes (Table 1). In the cholesterol-fed guinea-pig and calf, most of the increase in serum cholesterol was found in the LDL fraction. The elevation in serum cholesterol in the cholesterol-fed mouse was mainly attributable to an increased level of VLDL cholesterol.

In the genetically obese Zucker rat, the calf and the guinea-pig, the increased level of cholesterol in the LDL fraction was associated with a lower mean density of this lipoprotein band in the gradient (Fig. 2). This phenomenon was not observed in the cholesterol-fed rabbit (Fig. 2). In the chicken, the feeding of cholesterol resulted in an increase of the mean density of the HDL band (Fig. 2); this was associated with only minor changes in HDL-cholesterol (Table 1). However, in a subsequent study with chickens, we found that this can probably be explained by a decrease in HDL-phospholipids (Terpstra *et al.*, 1983b).

In the calf, dietary cholesterol did not affect the cholesterol concentration of the HDL_2 fraction (Table 1). This may be due to the fact that cholesterol feeding caused a decrease in the density of these lipoprotein particles, which in turn caused that the HDL particles were also recovered in the LDL fraction.

Dietary cholesterol induced an increase in the cholesterol concentration of the HDL fraction in the humans, the calves, the obese Zucker rats and the guinea-pigs (Table 1). It is possible that part of this increase was due to the appearance of so-called HDL_c particles. HDL_c lipoproteins are dietary-cholesterol-induced high density lipoproteins, which are rich in cholesteryl esters and apoprotein E. These lipoproteins have been shown to appear in cholesterol-fed dogs, swine, rats and monkeys, and possibly also in man (Mahley, 1978). The density of HDL_c ranges from 1.03 to 1.10 kg/l. It is uncertain that these lipoproteins can be unequivocally detected in the Sudan black stained lipoprotein profiles. Dietary cholesterol also induces the formation of the so-called β-VLDL particles in rabbits, dogs, rats and monkeys (Mahley *et al.*, 1980), and possibly also in man (Mistry *et al.*, 1976). These lipoproteins contain large amounts of cholesteryl esters and migrate on electrophoresis with β-lipoprotein mobility (Mahley *et al.*, 1980). Furthermore, the protein moiety of these particles consists of large quantities of arginine-rich protein (apoprotein E) (Shore *et al.*, 1974). The β-VLDL particles float at densities < 1.006 kg/l, and thus would not be separated from normal VLDL after density gradient ultracentrifugation (cf. Figs 1 and 2).

In order to visualize the serum lipoproteins in the gradient, the lipoproteins were stained with Sudan black prior to density ultracentrifugation. Sudan black binds to the lipid moiety of lipoproteins, but its affinity may depend on the lipid composition. Therefore, the intensity of the colour of the lipoproteins in the gradient not always gives a good impression of the concentration of cholesterol in the lipoproteins. For example in the cholesterol-fed rabbit, a faint HDL band was observed in comparison with the rabbit fed a cholesterol-free diet (Fig. 2), while the HDL-cholesterol was not changed. Similarly, as mentioned before, in the type IV patient, the intensity of the Sudan black stained LDL band was decreased compared with the LDL band of a normolipidaemic subject. However, the concentration of cholesterol in the LDL did not considerably differ.

With the exception of the human subjects, the guinea-pigs and the chickens, sera from pools of animals were used for studying the serum lipoproteins. It should be noted that in cholesterol-fed

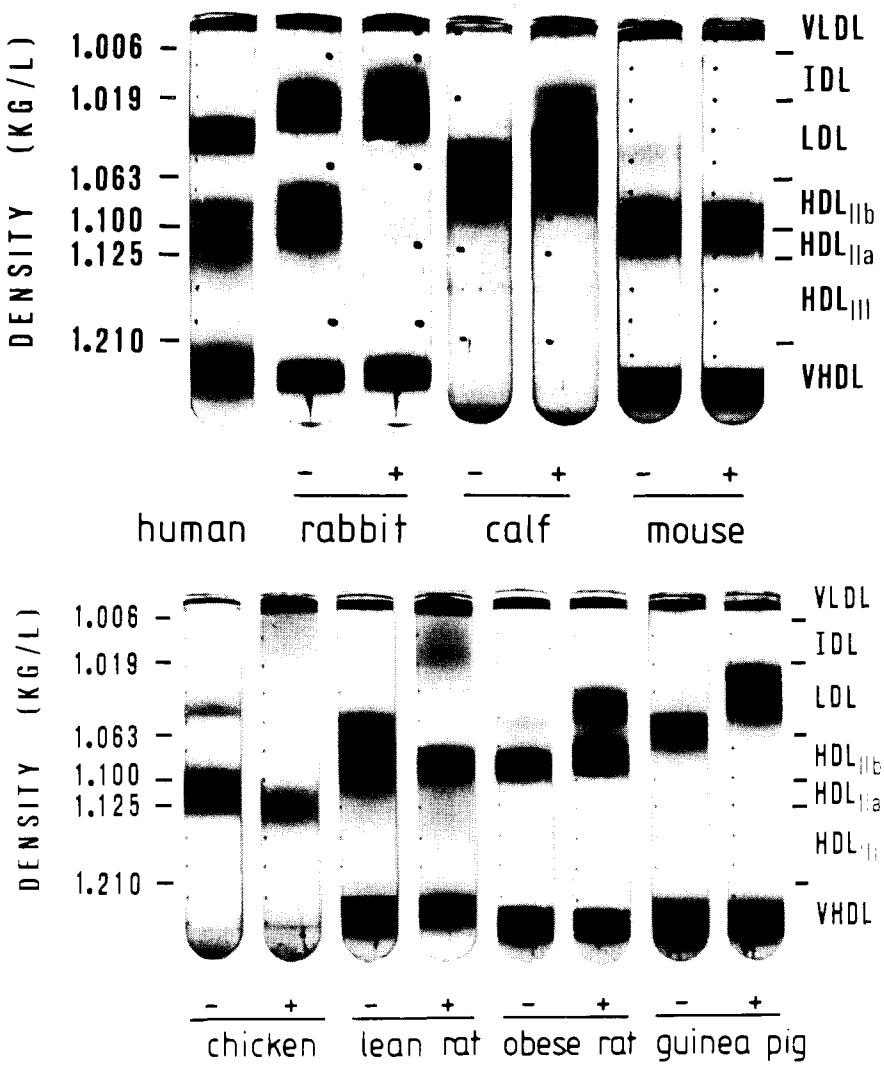

Fig. 2. Photograph of the density profile of Sudan black stained serum lipoproteins observed after density gradient ultracentrifugation of serum samples from various animals fed a cholesterol-free (−) and cholesterol-enriched (+) diet.

guinea-pigs large inter-individual variations were found in the density profile of the serum lipoproteins. The higher the cholesterol concentration of the LDL band, the lower the mean density of this band in the gradient (Terpstra *et al.*, 1982b). Similarly in rabbits made hypercholesterolaemic by feeding a cholesterol-free semipurified casein diet, marked inter-individual differences in the density profile were found (Terpstra and Sanchez-Muniz, 1981; Beynen *et al.*, 1983a). In cholesterol-fed chickens, however, such inter-individual differences were not found (Mol *et al.*, 1982).

Among healthy, normocholesterolaemic subjects, inter-individual variations in the density profile of lipoproteins have also been observed. Striking inter-individual differences are caused by the presence or absence of the so-called Lp(a) or sinking pre-β lipoprotein band. The density of this lipoprotein, which is located in the gradient between the LDL and HDL_2 lipoprotein band, shows a considerable between-person variability (Knuiman and West, 1982). In Fig. 1, we have selected two different human lipoprotein profiles with Lp(a). The identity of this lipoprotein was verified by polyacrylamide gel electrophoresis (Beynen A. C., unpublished).

In conclusion, the present report gives an overview of the effect of dietary cholesterol on Sudan black stained density profile and the cholesterol concentration of serum lipoproteins in man and various animal species. The results indicate that large differences exist between animal species in their response to dietary cholesterol. These findings might be useful, as such differences between man and animal species might provide clues as to elucidation of the regulation of cholesterol metabolism and etiology of atherosclerosis.

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