Hyperlipoproteinemia in fasting ponies

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Abstract Ponies fasted for up to 8 days showed, both by agarose electrophoresis and preparative ultracentrifugation, the appearance of a pre-β-migrating, very low density lipoprotein fraction in plasma. This lipoprotein differs from the very low density lipoprotein found in humans and rats in that it contains a relatively smaller amount of total cholesterol, 85% of which is present in the unesterified form. By the 8th day of fasting, plasma triglyceride concentrations had increased from a prefasting level of 20 mg/dl to as high as 1000 mg/dl. The increase in plasma lipid concentrations as a result of fasting was highly variable. Accumulation of plasma cholesterol and triglyceride after injection of Triton WR 1339 was not related to the degree of fasting hyperlipidemia. This suggests that the hyperlipoproteinemia of fasting may result from an impaired utilization of very low density lipoproteins.

Supplementary key words agarose electrophoresis · β-lipoproteins · pre-β-lipoproteins · α-lipoproteins · very low density lipoproteins · high density lipoproteins · free fatty acids · cholesterol · cholesteryl ester · triglyceride · phospholipid · Triton WR 1339

THE INITIAL TREATMENT of patients with hypertriglyceridemia usually consists of weight reduction by restriction of caloric intake. It is surprising, therefore, that equines brought to the clinic for treatment of a variety of diseases associated with food deprivation frequently show a hyperlipidemia (1). Bartley (2) describes several features of the hyperlipidemia of fasting equines, such as increased serum total and free fatty acids and increased cholesterol concentrations. Weik and Altmann (3) recently reported changes in fatty acid composition of serum lipids in four fasting Fjord pony geldings and provided information on serum cholesterol, free fatty acids and triglyceride concentrations.

The effect of fasting on blood lipids in various species has been studied, but many of the older studies were done on whole blood or with methods that lacked specificity. This literature has been reviewed by Deuel (4) and by Keys et al. (5). More recently, Rubin and Aladjem (6) reported significant increases in the S_f 12–100 lipoproteins of four out of five fasting humans and an increase in S_f 100–400 in two of these. Havel (7) reported variable effects of fasting on the $S_f > 10$ fractions in seven healthy adults, whereas no changes took place in the S_f 0–10 fractions.

The present studies were undertaken to define the hyperlipoproteinemia of fasting ponies by standard methods of electrophoresis and ultracentrifugation and to delineate the time of onset and duration of the hyperlipoproteinemic response. In addition, we have investigated whether overproduction of lipoproteins could account for the pronounced hyperlipidemia of fasting ponies.

MATERIALS AND METHODS

Care of ponies

Six gelding ponies which had been in the Cornell herd for at least 6 months, and whose estimated ages were 1–8 yr, were used for the first of these experiments. Ponies 1, 3, 46, and 52 were kept in individual stalls and were allowed to exercise with other ponies, in an outside concrete floored lot, about 2 hr a day. Ponies 50 and 51 were housed together in an inside pen which provided opportunity for some exercise. Ponies 50, 51, and 52 had been hyperimmunized with *Streptococcus equi* A 3 wk before the beginning of this experiment. Ponies 1, 3, 17, 22, and 46 were not hyperimmunized previously. All were clinically healthy at the initiation of the fasting experiments.

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Drinking water was available at all times to ponies 50 and 51, whereas the other ponies were watered twice daily. The animals were fed a pelleted diet, consisting of 40% timothy hay, 20% beet pulp, 17% corn, 16% wheat bran, 5% soybean meal, and 2% molasses. Each pony received 2.8–3.0 kg of the diet in each of two equal daily feedings.

Analytical procedures

Lipids and lipoproteins were measured in plasma separated from blood which was taken from the jugular vein, collected in Vacutainer (Becton, Dickinson and Co. Columbus, Neb.) tubes containing EDTA, and kept below 5°C at all times. Cholesterol was determined by saponification of the plasma (8) or by saponification after extraction of plasma or lipoproteins with chloroform–methanol (9); color development was with $FeCl_3$ (10). Phospholipids were analyzed on aliquots of purified chloroform–methanol extracts (11). Triglycerides were measured, after separation from polar lipids by silicic acid column chromatography (12), by the method of Sardesai and Manning (13), which was modified by the inclusion of a hexane-extraction step after color development. To each tube 1 ml of hexane was added, and the contents were shaken for 30–60 sec. The tubes were centrifuged for 5 min and the upper hexane layer was withdrawn.[1] The separation of the lipoproteins was carried out by centrifugation at 4°C in a 40.3 rotor at 40,000 rpm for 16 hr (14). The very low density lipoproteins of the hyperlipidemic ponies, 17 and 22 (Table 3), were purified by an additional gradient density centrifugation in an SW 41 rotor at 18°C for 16 hr at 41,000 rpm. The density of the lipoprotein solution was adjusted to 1.14, and 3 ml of this mixture was overlayered with equal volumes of density 1.08, 1.04, and 1.006 NaCl–NaBr salt mixtures. All solutions contained 1 mM EDTA and 0.4 mg of thimerosal/dl of solution.

Protein of the lipoprotein fractions was determined by the method of Lowry et al. (15). Lipids were removed, after color development, by a single extraction with an equal volume of chloroform, which contained 0.5% methanol.

Free fatty acids were determined by the colorimetric procedure of Novák (16). Agarose electrophoresis was carried out by the procedure of Noble (17), with 0.5% agarose and no agar.

[1] This modification is essential for the removal of turbidity in lipid samples which contain cholesterol (unpublished observation by M.D.M.).

RESULTS

The initial concentrations of plasma cholesterol and triglycerides for each of the ponies in this study, presented in Table 1, reveal no major differences between the ponies. 16 hr before the first blood samples were drawn, ponies 46, 50, 51, and 52 were fed their last meal. Subsequent plasma samples were obtained at 24-hr intervals. In the fed ponies (1 and 3, Table 1), the concentrations of both cholesterol and triglycerides were quite constant during the experiment. The plasma cholesterol concentrations in ponies 50 and 51 increased by 20 mg/dl between 16 and 40 hr after the last meal, whereas the hypercholesterolemic response in pony 52 was somewhat slower. By day 8 of the experiment the

TABLE 1. Effect of fasting on plasma cholesterol and triglycerides in ponies

Pony no.		46	50	51	52	1	3
Estimated age (yr)		8	1	4	5	4	4
Initial wt (kg)		237	118	169	171	173	139
Final wt (kg)		223	105	151	153		
Dietary treatment		Fasted	Fasted	Fasted	Fasted	Fed	Fed
Day of Expt.	Hr Post-feeding[a]	Total Cholesterol					
		mg/dl					
1	16	79	70	62	59	94	71
2	40	75	93	84	64	94	76
3	64	80	116	98	79	93	74
4	88	83	118	121	83	90	75
5	112	88	131	143	86	93	78
6	136	90	136	167	86	95	83
7	160	88	144	177	96	93	80
8	184	86	145	169	102		
		Triglycerides					
		mg/dl					
1	16	29	26	26	15	20	12
3	64	18	534	497	114	24	16
8	184	49	554	983	314	18	22

[a] Applies to fasted animals only.

plasma cholesterol had about doubled in ponies 50, 51, and 52. In the same three ponies an 8–30-fold increase in plasma triglycerides had occurred by day 3 of the experiment. Thereafter, a continued increase in plasma triglycerides was noted, so that by day 8 of the experiment each of the three ponies had plasma triglyceride concentrations at least 20 times greater than their respective control samples.

Pony 46 did not respond to fasting as did the other three fasted ponies. By day 8 of the fast a slight increase in plasma cholesterol had taken place, but this was no greater than was observed in control pony 3. Likewise, the triglycerides had increased only slightly.

The separation of the lipoproteins by agarose electrophoresis, shown in Fig. 1, revealed two major lipoproteins in 16–18-hr postfeeding samples: a definite oil red O-staining band corresponding to β-lipoprotein and a more intensely staining single α-lipoprotein band, which migrated just behind albumin. An invariable finding in association with increased plasma cholesterol and triglycerides during the fast was the appearance of a new lipid-staining band in the pre-β region of the electrophoretogram. The staining intensity of this band roughly correlated with the increases in plasma lipids.

A pronounced pre-β-lipoprotein band appeared by 40 hr postfeeding in ponies 50 (Fig. 1) and 51, and by 64 hr in pony 52; it was present in each subsequent plasma sample in these three ponies during the fast. The presence of the pre-β-lipoprotein did not influence the staining intensity of the α-lipoprotein band, but it did lead to a smearing in the β-lipoprotein region of the electrophoresis strip, making it difficult to evaluate any changes that might have taken place in this lipoprotein fraction. Fig. 1 also shows that in pony 46 the pre-β-lipoproteins did not increase during the 8-day fast. This is in agreement with the lipid analyses shown in Table 1.

According to the data in Table 2, except for pony 51, more than 95% of the phospholipid and more than 65% of the cholesterol were present in the $d > 1.063$ fractions on day 1 of the experiment. The absolute concentrations of the various lipids in these fractions varied only slightly during the 8-day fast. In spite of the low amounts of phospholipid and cholesterol present in the $d < 1.006$ lipoprotein fractions, 30–60% of the plasma triglyceride was present in these fractions in the postabsorptive ponies. During the period of fasting more than 90% of the plasma triglyceride was present in $d < 1.006$ lipoproteins.

From the data in Table 2 it was not possible to calculate reliable lipid and protein compositions of the $d < 1.006$ lipoprotein fractions because we did not purify these fractions by further ultracentrifugation. Therefore, in two additional ponies (17 and 22), fasting hyperlipidemia was induced. Lipoproteins of $d < 1.006$ were centrifuged an additional time in a discontinuous salt gradient. The final product moved as a single band on agarose with pre-β mobility. Disc electrophoresis showed that less than 1% of the total protein of the lipoprotein preparation was albumin. The composition of the $d < 1.006$ lipoproteins of ponies 17 and 22 is shown in Table 3. The protein concentration was about 5% of the total lipoprotein. Total cholesterol comprised about 7% of the total lipoprotein; less than 15% was present as esterified cholesterol. The triglyceride comprised about 75% of the total, and the triglyceride to cholesterol ratio was about 11. This closely resembles the ratios reported in Table 2.

The free fatty acid concentrations of pony plasma 16 hr after the last meal ranged from 0.04 to 0.2 μmole/ml. This had increased by day 8 to more than 0.6 μmole/ml in each of the fasted ponies. Low values for free fatty acids in postabsorptive ponies were also reported by Weik and Altmann (3), but not by Bartley (2) nor by Schotman and Wagenaar (1).

To obtain some insight into the mechanism of the fasting hyperprebetalipoproteinemia, lipoprotein secre-

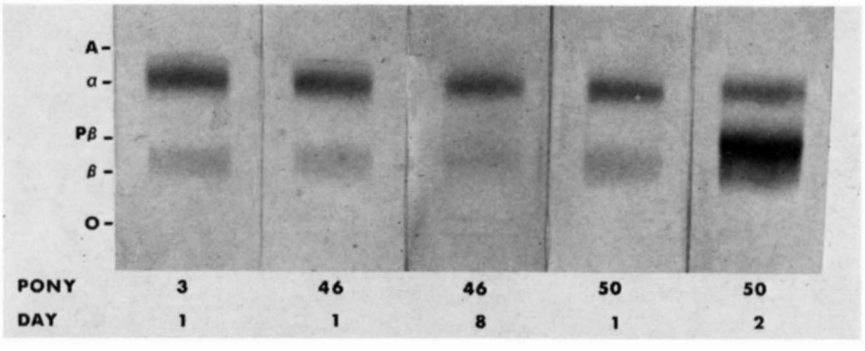

FIG. 1. Agarose electrophoresis patterns of lipoproteins in pony plasmas. Pony 3, a fed control pony; pony 46, a nonresponding pony on days 1 and 8 of the fast; pony 50, a responding pony on days 1 and 2 of the fast. *A*, albumin (shown on the original before staining with oil red O on the left margins as a spot of bovine serum albumin prestained with bromophenol blue); α, α-lipoprotein; *P*β, pre-β-lipoprotein; β, β-lipoprotein; *O*, origin.

TABLE 2. Lipid concentration in separated lipoproteins of fasted and fed ponies

Pony No.	Dietary Treatment	Lipoprotein Fraction	Total Cholesterol Day 1	Total Cholesterol Day 8	Phospholipids Day 1	Phospholipids Day 8	Triglycerides Day 1	Triglycerides Day 8	TG/TC[a] Day 8
			mg/dl		*mg/dl*		*mg/dl*		
46	Fasted	d < 1.006	1.1	2.5	1.3	4.9	11	32	13
		d > 1.006	80	80	120	132	8	7	
		Plasma	79	88	107	160	29	49	
		d > 1.063	56	58	105	117	4	3	
50	Fasted	d < 1.006	0.81	54	1.3	110	11	502	9
		d > 1.006	69	92	117	144	9	20	
		Plasma	70	149	118	279	26	554	
		d > 1.063	50	59	112	112	3	6	
51	Fasted	d < 1.006	0.89	99	0.90	187	11	1046	11
		d > 1.006	60	70	94	121	10	24	
		Plasma	62	169	94	332	26	983	
		d > 1.063	46	44	79	93	5	9	
52	Fasted	d < 1.006	0.80	24	0.62	52	9	285	12
		d > 1.006	60	70	90	128	7	11	
		Plasma	59	103	85	202	15	314	
		d > 1.063	47	55	85	113	5	8	
1	Fed	d < 1.006	0.41	0.87	0.69	0.56	7	8	9
		d > 1.006	—	87	—	121	—	7	
		Plasma	92	91	131	132	20	18	
		d > 1.063	60	52	109	97	4	3	
3	Fed	d < 1.006	0.35	0.76	0.28	0.42	6	7	9
		d > 1.006	70	80	116	119	5	6	
		Plasma	69	80	110	134	13	22	
		d > 1.063	58	61	108	106	3	3	

[a] TG/TC is the ratio (w/w) of triglyceride to total cholesterol.

TABLE 3. Composition of hyperlipidemic pony very low density lipoproteins

Pony No.	Protein	Cholesterol Free	Cholesterol Esters[a]	Phospholipids	Triglycerides	EC/TC[b]	TC/PL[c]	TG/TC[d]
	%	%	%	%	%			
17	6.2	5.8	1.9	14.0	72.1	16.6	0.49	10.4
22	4.8	5.7	1.1	12.7	75.6	10.7	0.50	11.8

[a] Esterified cholesterol × 1.67. Free and esterified cholesterol were separated by thin-layer chromatography with hexane–diethyl ether–acetic acid 80:20:1 (v/v/v).
[b] Ratio of esterified to total cholesterol (w/w).
[c] Ratio of total cholesterol to phospholipid (w/w).
[d] Ratio of triglyceride to total cholesterol (w/w).

tion was estimated by injection of Triton WR 1339 (18). Triton was administered intravenously as a 20% solution in 0.9% NaCl at a dose of 200 mg/kg. By 24 hr an acute hemolysis, accompanied by hemoglobinuria, was observed in pony 51, and by 48 hr pony 50 had severe intravascular hemolysis and hemoglobinuria. All four ponies eventually developed anemia, but they recovered spontaneously. Since the nonfasting ponies were used for other diet studies they were not injected with Triton. Data for the four ponies injected with Triton are shown in Fig. 2 and Table 4. The animals responded to the injection of Triton with increased total cholesterol and triglyceride concentrations after a delay of about 6 hr. The almost linear portion of the curve between 6 and 42.5 hr was used to calculate the secretion rate of the plasma lipids. Secretion of triglyceride into the plasma did not appear to be related to the magnitude of the fasting hypertriglyceridemia. Pony 46, for example, showed the lowest degree of fasting hypertriglyceridemia (Table 1) but appeared to secrete as much triglyceride as pony 50, with 10 times as much fasting triglyceridemia. Similarly, pony 52 secreted the lowest amount of plasma triglyceride, although its fasting triglyceride concentration was nearly six times as high as that of pony 46.

Plasma triglycerides increased at a rate about 10 times higher than that observed for cholesterol (Table 4). This observation is compatible with the secretion of very low density lipoproteins in which the ratio of triglyceride to cholesterol is about 10 to 1 (Tables 2 and 3). Identification of the plasma lipoprotein by agarose electrophoresis in the animals injected with Triton was attempted, but it was not possible because of the alterations in the lipoprotein patterns brought about in the presence of Triton (19).

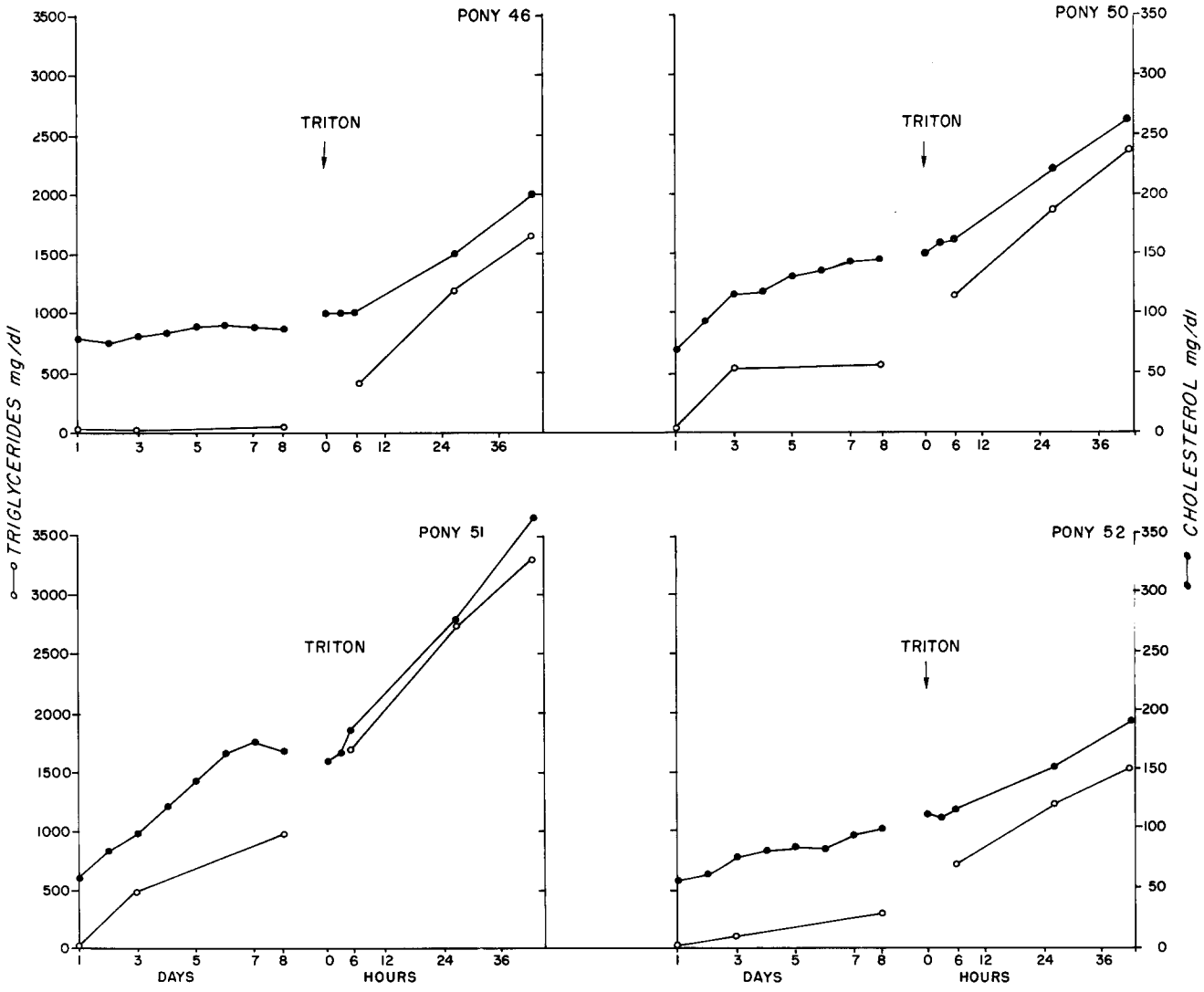

FIG. 2. Effects of fasting and Triton injection on the concentration of plasma triglycerides and total cholesterol. Changes in plasma triglycerides and total cholesterol concentration during the 8-day fast are shown on the left side of each figure. Responses to Triton injection between 0 and 42.5 hr are shown on the right side of each figure. ○——○, plasma triglycerides, mg/dl; ●——●, plasma cholesterol, mg/dl.

DISCUSSION

The present studies reveal that the alteration in serum lipids in response to fasting is variable. In ponies 50, 51, and 52 there was a marked hyperlipidemia, whereas pony 46 had only a slight change in cholesterol and triglycerides during the 8 days of the experiment. The response to fasting did not appear to be related to absolute weight loss, since all ponies lost about the same amount of weight (Table 1). In addition to the studies reported here, we have attempted to produce fasting hyperlipidemia in eight other ponies and have obtained inconsistent responses or no response following a fast of 88 hr. Two ponies initially selected as hyperlipidemic responders have, on two subsequent occasions, given minimal or no increase in plasma cholesterol during an 88-hr fast. Our experience has suggested that the hyperlipidemic response to fasting is not consistent in the same pony from one time to another.

The tremendous increase in plasma very low density lipoproteins in some of the fasting ponies appears to be unique for the equine species. Human subjects appear to have a variable response to fasting. In a few individuals a slight increase in very low density lipoproteins has been observed, whereas in others no change or a decrease was noted (6, 7). In fed ponies, very low density lipoproteins are not normally present to any significant extent, whereas in some of the fasting ponies very low density lipoprotein concentrations exceeded by far those found in normal fed or fasting humans. In male rats, Otway and Robinson (18) observed no change in plasma triglycerides, whereas in females the triglycerides were doubled during a 48-hr fast. Aladjem and Rubin (20) measured the serum lipoprotein changes in rabbits fasted for 7

TABLE 4. Effect of Triton[a] on plasma triglycerides and total cholesterol after a 9-day fast

Pony No.	Triglycerides: Hr after Triton 6	26	42.5	ΔTG[b]	Total Cholesterol: Hr after Triton 0	6	26	42.5	ΔTC[b]	ΔTG/ΔTC
	mg/dl			*mg/dl/hr*	*mg/dl*				*mg/dl/hr*	
46	383	1184	1654	34.8	98	102	150	200	2.58	13
50	1125	1869	2389	34.6	150	161	223	264	2.82	12
51[c]	1697	2730	3323	44.5	159	186	279	366	4.93	9
52	722	1206	1514	21.7	113	117	154	192	2.05	11

[a] Triton WR 1339, 200 mg/kg iv.

[b] ΔTG and ΔTC represent the rates of increase in concentrations of plasma triglycerides and total cholesterol, respectively. They were calculated from the differences in concentrations at 42.5 and 6 hr after Triton injection.

[c] At the time of Triton injection an abscess in the neck was observed. Subsequent drainage and culture of the organisms revealed *Streptococcus equi* A. Hematologic studies 2 days later showed a marked leukocytosis.

days and found a fourfold increase in the low density lipoproteins and a twofold increase in the very low density lipoproteins.

In view of the results obtained after Triton injection, i.e., that lipoprotein secretion was not related to the magnitude of the fasting hyperlipoproteinemia, one is tempted to postulate that the mechanism of the hyperprebetalipoproteinemia resides in the suppression of lipoprotein degradation.

The very low density lipoproteins of the fasting pony, obtained in pure form by density gradient ultracentrifugation, have both a low cholesterol content and an unusual distribution of cholesterol between free and ester. Only about 7% of the lipoprotein was cholesterol, of which 85% or more was in the free form. In agreement with these observations are the findings of Weik and Altmann (3), who observed a 3–4-fold increase in the plasma free cholesterol concentration in two ponies at the end of a 5-day fast with only a 20% or less change in plasma cholesteryl esters. Mahley, Hamilton, and LeQuire (21) studied the very low density lipoproteins of the rat liver, Golgi particles, and plasma and found that the esterified cholesterol comprised more than 50% of the total cholesterol. Likewise, in human very low density plasma lipoproteins most of the cholesterol is present as cholesteryl esters (22).

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