Influx of cholesterol into plasma in rabbits with fasting hyperbetalipoproteinemia

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Abstract New Zealand white rabbits exhibited as much as a threefold increase in plasma cholesterol but no change in hepatic cholesterol when fasted for 7–9 days. Agarose electrophoresis and ultracentrifugation of plasma samples showed that only low density lipoprotein increased during fasting. Fasting changed the composition of the low density lipoprotein by increasing the percentage of cholesterol and decreasing the percentage of triglyceride while protein and phospholipid remained the same. Rates of cholesterol secretion into plasma, measured by Triton WR 1339 injection, and rates of plasma cholesteryl ester synthesis, determined by [2-^{14}C]mevalonate injection, were similar for fed and fasted rabbits. These findings suggest that fasting hypercholesterolemia in rabbits did not result from increased production of low density lipoproteins. Triton WR 1339 was shown to inhibit plasma cholesterol esterification in vitro.

Supplementary key words Triton WR 1339 · mevalonate · lipoprotein composition · cholesterol esterification · low density lipoprotein

The effect of fasting on circulating cholesterol varies between different species of animals. Humans and rats do not consistently show an increase in plasma lipid levels in response to fasting (1–4), but a recent study from this laboratory has demonstrated an increase in plasma VLDL in fasting ponies (5). During a prolonged fast the rabbit exhibits hypercholesterolemia (2, 6), which appears to be associated primarily with an elevation in LDL (7). High concentrations of LDL in patients has been attributed to an impairment in the removal mechanism (8). Using two different methods we have sought to determine the mechanism whereby fasting leads to an elevation of plasma cholesterol concentrations in rabbits.

MATERIALS AND METHODS

Animal care and sample collection

Individually caged adult rabbits (New Zealand white) were fed 100 g of Purina laboratory rabbit chow daily at about 4 p.m., except when fasted. Water was supplied at all times. The rabbits weighed 3.5–4.9 kg and had an average weight loss of 0.4 kg during a 7-day fast.

Animals were bled from the marginal ear vein, and the blood was collected into tubes containing 0.01 ml of 15% EDTA/ml of blood, unless serum was desired. The blood was kept cold at all times; plasma was analyzed immediately or frozen for later analysis. Livers were removed from ether-anesthetized rabbits and perfused with saline to remove the blood.

Analytical procedures

Unless otherwise indicated, lipids were extracted from plasma or 50% aqueous liver homogenates with 20 vol of chloroform–methanol 2:1 (v/v), and the extract was washed with 0.2 vol of water (9). Plasma triglycerides were determined by the modified method (5) of Sardesai and Manning (10) after removal of the phospholipids with zeolite (50 mesh, Taylor Chemicals, Inc., Baltimore, Md.) (11). Silicic acid chromatography (12) was used to separate triglycerides and phospholipids when phospholipids were also determined (13). The proportion of plasma free and esterified cholesterol was determined by thin-layer chromatography on silica gel H (hexane–diethyl ether 1:1, v/v). Cholesterol was measured after saponification and hexane extraction (14) by the $FeCl_3$ method of Zak et al. (15). To determine radioactivity the hexane extract was evaporated under nitrogen and counted (Packard Tri-Carb, model 3375) in toluene scintillator (0.4% 2,5-diphenyloxazole, 0.01% *p*-bis-[2-(5-phenyloxazolyl)]benzene) to a standard deviation of less than 1.5%. The external standard showed no quenching. Counting efficiency was determined in a typical sample with an internal [^{14}C]toluene standard (New England Nuclear, Boston, Mass.).

Separation of serum lipoproteins was performed by centrifugation at 17°C in a Beckman L2-65 ultracentrifuge

Abbreviations: LDL, low density lipoprotein; VLDL, very low density lipoprotein.

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with a 40.3 rotor (16). Serum was centrifuged for 16 hr at 40,000 rpm (1.09×10^8 *g*-min, avg) to float the $d < 1.006$ fraction, which was removed by tube slicing. The density of the infranatant fraction was adjusted to 1.063 and centrifuged for 20 hr at 40,000 rpm (1.37×10^8 *g*-min, avg) to give the $1.006 < d < 1.063$ and $d > 1.063$ fractions. These density fractions have been established in the rabbit to give distinct separation of lipoprotein classes (17). In one experiment the $1.006 < d < 1.063$ fraction was washed by diluting it 7.5-fold with $d = 1.063$ salt solution and recentrifuging for 1.37×10^8 *g*-min (avg). Lipoproteins were analyzed for protein (18) and lipid content by extraction with 40 vol of chloroform–methanol as outlined for plasma. The protein values were adjusted for differences in tyrosine content of β-lipoprotein and bovine serum albumin, which was used as a standard (19). Agarose electrophoresis was performed on plasma (20). Levels of Triton WR 1339 (Ruger Chemical Co., Irvington, N.J.) in plasma were determined after extraction with 10 vol of isopropanol by measuring absorbance at 280 nm (21).

Experimental procedures

Fasting and fed animals were injected intravenously with Triton WR 1339 (200 mg/kg) as a 20% solution in 0.9% NaCl. Blood samples were taken before injection and periodically for the next 40 hr, during which time none of the animals received food. Plasma cholesterol and triglycerides were extracted with chloroform in the presence of zeolite (11).

In vitro studies on cholesterol esterification were performed with plasma from fed rabbits. Plasma was incubated with agitation for 24 hr at 37°C in the presence of various amounts of Triton WR 1339. All incubations were run in duplicate, and esterification was stopped by immediate chilling followed by chloroform–methanol extraction.

The procedure used to study plasma cholesteryl ester turnover was similar to that used by Nestel and Monger (22) in humans. DL-[2-^{14}C]Mevalonic acid lactone (12.9 mCi/mmole; Amersham/Searle, Arlington Heights, Ill.) was converted to its sodium salt by incubating the lactone for 1.25 hr at 37°C in 0.1 M $NaHCO_3$. 1 or 2 ml (10 μCi) was injected into the marginal ear vein of rabbits that were fasted or fed. Serial blood samples (2–3 ml) were taken for periods up to 56 hr. At the end of the sample collections, animals were injected with Triton WR 1339 as outlined above.

Plasma volume was determined by Evans blue dye dilution (23) 1–3 months after the experiments were performed. At this time, body weights were equal to prefasting weights, and separate experiments showed that the plasma volume of fed rabbits did not change after 7 days of fasting. The plasma pool of esterified cholesterol was calculated from the plasma volume and the average concentration of plasma cholesteryl ester during the time that specific activity was measured.

Theoretical considerations

The fractional turnover rate of a product can be determined by following the change in specific activities of the product and its precursor with time (24). Application of this idea to the turnover of plasma esterified cholesterol has been made in man (22) and rabbit (25). In our studies the precursor (free cholesterol) specific activity was consistently higher than the product (esterified cholesterol) specific activity when the latter reached its maximum value. This meant that either a certain amount of unlabeled cholesteryl ester was diluting the labeled plasma cholesteryl ester or that the plasma free cholesterol pool was not homogeneous. In view of the rapid exchange of free cholesterol between lipoprotein fractions, a nonhomogeneity in the free cholesterol pool appears less likely than dual sources of plasma cholesteryl ester: one derived from a precursor in equilibrium with plasma free cholesterol and one derived from an unlabeled source.

If this unlabeled cholesteryl ester entered the plasma cholesteryl ester pool at a rate p while labeled cholesteryl ester entered at a rate q, total efflux would equal $p + q$ in the steady state,[2] and the change in radioactive cholesteryl ester (dQ/dt) could be represented by the following equation:

$$dQ/dt = qX - (p + q)Y \qquad \text{Eq. 1}$$

where X and Y are the specific activities of plasma free and esterified cholesterol, respectively. Since dQ/dt at the time of cholesteryl ester maximum specific activity ($t\ max$) is zero, Eq. 1 can be written as follows:

$$(Y/X)_{t\ max} = q/(p + q) = f \qquad \text{Eq. 2}$$

Thus, the fraction (f) of plasma cholesteryl ester synthesized from the labeled free cholesterol pool should be equal to the ratio of the specific activities of esterified and free cholesterol at $t\ max$.

From Eqs. 1 and 2 we obtain

$$dQ/dt = (p + q)fX - (p + q)Y \qquad \text{Eq. 3}$$

Division of both sides of Eq. 3 by the quantity of cholesteryl ester in the entire pool changes Eq. 3 to

$$dY/dt = kfX - kY \qquad \text{Eq. 4}$$

where k is the fractional turnover rate of the cholesteryl ester pool.

[2] Cholesteryl ester concentrations were nearly constant after mevalonate injection (see footnote *c*, Table 4).

If the free cholesterol specific activity curves are fitted by a triexponential equation,

$$X = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} \quad \text{Eq. 5}$$

where A, B, C, α, β, and γ are constants, then Eq. 4 becomes

$$dY/dt + kY = kf(Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}) \quad \text{Eq. 6}$$

Integrating this linear differential equation and setting $Y = 0$ at $t = 0$ gives the following equation:

$$Y = kf[(A/\alpha - k)(e^{-kt} - e^{-\alpha t}) + (B/\beta - k)(e^{-kt} - e^{-\beta t}) + (C/\gamma - k)(e^{-kt} - e^{-\gamma t})] \quad \text{Eq. 7}$$

With the aid of a computer, a nonlinear least squares program was used to fit the specific activity time points of free and esterified cholesterol to Eqs. 5 and 7. First, the constants of Eq. 5 were determined, and then the fractional turnover rate (k) and the fraction of plasma cholesteryl ester (f) synthesized from the labeled free cholesterol pool were obtained from Eq. 7 by computer fitting of all cholesteryl ester specific activity time points. Thus, f could be calculated even in those experiments that were stopped before the maximum cholesteryl ester specific activity was attained. In experiments in which maximum cholesteryl ester specific activity was attained, a manual calculation of f and k gave values similar to those obtained with the computer, even when different integration intervals were used. The cholesteryl ester synthesis rate was then calculated from the product of k and the pool size of plasma esterified cholesterol (concentration × plasma volume).

RESULTS

Although the degree of response was variable, all rabbits, regardless of sex, showed an increase in plasma cholesterol in response to fasting, whereas animals maintained on the normal dietary regimen during this time showed only small changes in plasma cholesterol (**Table 1**). Comparison of the hepatic cholesterol of fasted and fed rabbits revealed that fasting did not alter total liver cholesterol in spite of a 26% reduction in liver weight (Table 1). The concentration of cholesterol was therefore higher in the fasted liver. Plasma cholesterol generally rose uniformly throughout the fast and decreased soon after feeding was resumed (**Fig. 1**). After the initial rapid decline in cholesterol levels with refeeding, plasma cholesterol fluctuated. Separation of circulating lipoproteins by agarose electrophoresis showed a definite increase in the lipid staining intensity of the β-lipoprotein band during fasting. In some animals the intensity of the α- and pre-β-lipoprotein bands decreased.

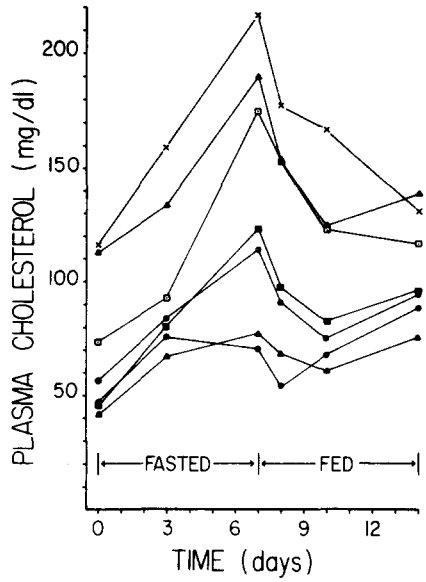

Fig. 1. Changes in rabbit plasma cholesterol during 7 days of fasting followed by 7 days of refeeding.

Serum lipoproteins were isolated by centrifugation at different stages of fasting and refeeding. Analysis of serum (**Table 2**) showed a drop in triglyceride levels during fasting (day 0 through day 7) while cholesterol levels were rising in three out of four animals. Refeeding produced the opposite effect. Determination of the cholesterol content of

TABLE 1. Changes in plasma and hepatic cholesterol levels in rabbits during fasting

Days Fasted	Sex	No. of Rabbits	Plasma Cholesterol			Liver	
			Initial	Final	Change	Cholesterol	Weight
			mg/dl			*mg*	*g*
0	M	4	24.4 ± 3.3	22.2 ± 2.7	−2.2 ± 3.1		
7	M	4	20.3 ± 2.5	65.0 ± 20.0	44.7 ± 17.6		
0	F	5	65.1 ± 13.4	61.7 ± 12.4	−3.4 ± 1.5		
7	F	7	70.8 ± 11.9	114 ± 19	43.0 ± 13.0		
0	F	5		75.3 ± 13.2		230.5 ± 10.1	82.2 ± 3.8
9	F	4	64.9 ± 12.5	188 ± 15	123 ± 8	216.1 ± 8.6	60.5 ± 4.1

Plasma samples were obtained at the beginning (initial) and end (final) of 7- or 9-day experiments with fasted or fed rabbits. Livers were removed in one experiment at the end of 9 days of fasting or feeding. Values are means ± SEM.

TABLE 2. Changes in composition of rabbit serum and lipoprotein fractions during fasting and refeeding

Rabbit No.	Sex	Lipoprotein Fraction	Component	Serum and Lipoprotein Composition[a] Fasted Day 0	Fasted Day 3	Fasted Day 7	Refed Day 9	Refed Day 14
						mg/dl		
1190	F	Serum	Cholesterol	121	121	190	156	148
			Triglycerides	91.8	—[b]	36.3	203	115
		d < 1.006	Cholesterol	16.0	19.0	14.6	54.4	21.1
		1.006 < d < 1.063	Cholesterol	63.1	64.9	144	72.6	93.9
			Protein[c]	38.1	34.7	71.5	56.9	54.6
		d > 1.063	Cholesterol	42.7	34.8	23.9	22.3	34.2
1185	M	Serum	Cholesterol	40.6	84.1	123	71.5	82.6
			Triglycerides	60.4	54.3	44.3	45.9	98.7
		d < 1.006	Cholesterol	7.6	7.3	4.6	7.4	11.4
		1.006 < d < 1.063	Cholesterol	11.5	46.8	96.4	40.6	37.2
			Protein[c]	12.1	34.9	63.4	25.6	28.0
		d > 1.063	Cholesterol	19.6	23.4	19.0	20.5	32.8
1180	F	Serum	Cholesterol	65.7	70.0	119	86.5	64.7
			Triglycerides	105	29.8	47.9	61.4	75.0
		d < 1.006	Cholesterol	10.3	4.0	12.2	8.6	8.2
		1.006 < d < 1.063	Cholesterol	23.8	33.9	80.5	48.5	30.5
			Protein[c]	17.2	20.1	43.9	34.6	29.6
		d > 1.063	Cholesterol	29.3	30.7	22.7	24.4	23.4
1154	F	Serum	Cholesterol	42.0	29.5	37.7	57.0	59.1
			Triglycerides	291	51.2	—[b]	223	249
		d < 1.006	Cholesterol	23.8	6.3	8.0	27.2	27.8
		1.006 < d < 1.063	Cholesterol	3.0	6.6	10.4	10.6	6.6
			Protein[c]	7.5	6.2	9.6	12.3	9.2
		d > 1.063	Cholesterol	14.1	15.1	14.5	15.2	21.0

[a] Rabbits were fasted on day 0 and refed after the day-7 samples were taken.
[b] Insufficient serum remained for triglyceride analysis in these cases.
[c] Protein values may be overestimated because the fraction was not purified by recentrifugation (see Table 3).

the various lipoprotein fractions demonstrated that the rise and fall of the serum cholesterol levels during fasting and refeeding reflected changes in the lipoprotein fraction $1.006 < d < 1.063$ or LDL. In all cases, the LDL cholesterol increased with fasting and decreased with refeeding. There was also a corresponding rise and fall in LDL protein except for rabbit 1154. The amount of cholesterol in lipoprotein fraction $d > 1.063$ generally remained constant throughout the experiment, whereas cholesterol in the $d < 1.006$ fraction tended to decrease during fasting and increase during refeeding. The fluctuations of $d < 1.006$ were especially noticeable in rabbit 1154, which carried most of its serum cholesterol in this lipoprotein fraction, had the highest total serum triglycerides, and had an intensely stained pre-β band. This different plasma lipoprotein pattern of rabbit 1154 suggests abnormal lipoprotein metabolism in this rabbit, which may also account for its unique response to fasting.

TABLE 3. Composition of washed low density lipoprotein from fed and 7-day-fasted rabbits

Rabbit No.	Dietary State	Cholesterol	Protein	Phospholipids	Triglycerides
		%	%	%	%
1284	Fed	29.7	20.6	24.8	24.8
	Fasted	38.0	19.5	30.4	12.2
1304	Fed	29.3	20.4	28.6	21.7
	Fasted	43.3	19.6	28.4	8.7

Serum was taken from rabbits just before fasting (fed) and after 7 days of fasting (fasted); the $1.006 < d < 1.063$ fraction was isolated by ultracentrifugation. This fraction was diluted with $d = 1.063$ salt solution and recentrifuged.

In a separate experiment, the composition of washed LDL was determined before and after a 7-day fast (**Table 3**). Although the mass of protein and phospholipid in the LDL fraction tripled with fasting, protein and phospholipid as percentages of total LDL remained constant. On the other hand, the cholesterol percentage in LDL increased while the triglyceride percentage decreased during fasting. In this experiment a comparison of washed and unwashed LDL cholesterol-to-protein ratios showed an average increase of 9% with washing; therefore, the unwashed LDL of the previous experiment (Table 2) may have been contaminated with other serum protein.

In order to determine whether the increasing LDL concentration during fasting was caused by an increased influx of lipoprotein, the turnover of cholesteryl ester, the major form of cholesterol in LDL, was studied in fed and fasted rabbits. In vivo incorporation of [2-^{14}C]mevalonate into free cholesterol and conversion of labeled cholesterol to cholesteryl ester was followed by plotting cholesterol

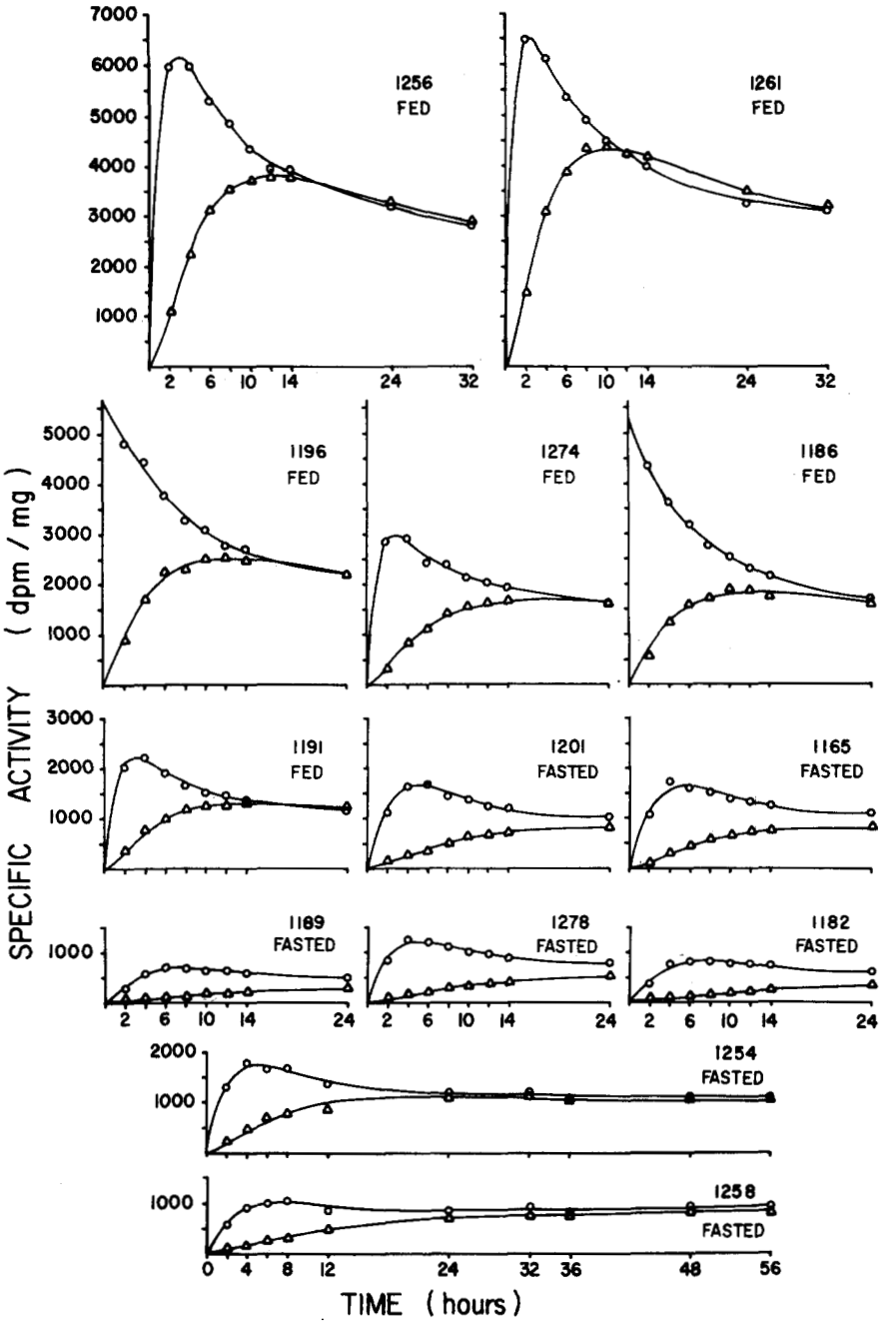

Fig. 2. Incorporation of [2-^{14}C]mevalonate into plasma free and esterified cholesterol after intravenous injection into fed and 5- or 6-day-fasted rabbits. The fed animals received their usual meal about 8 hr after mevalonate injection. Free (○) and esterified (△) cholesterol specific activity data were fitted by computer to give the smooth curves.

specific activity of plasma free and esterified cholesterol vs. time. The computer fit of the data to Eqs. 5 and 7 derived above are shown by smooth curves in **Fig. 2.** It can be seen from these curves that free cholesterol specific activity usually increased to a maximum rapidly, with fed animals reaching this maximum approximately 2 hr earlier than fasted animals. Esterified cholesterol specific activity peaked at about 12 hr for fed rabbits and in 24 hr or more in fasted ones. In two cases (rabbits 1186 and 1196) maximal specific activities of free cholesterol had been reached by 2 hr; therefore, a biexponential equation gave the best fit. Comparison of fed and fasted rabbits showed that although the average pool size had doubled with fasting, the average fractional turnover rate was halved, giving equivalent rates of cholesteryl ester synthesis (**Table 4**). Rabbits 1201, 1254, and 1258 showed relatively small increases in their plasma cholesteryl ester pools during the 5–6-day fast and significantly ($P < 0.05$) lower synthesis

TABLE 4. Plasma cholesteryl ester kinetic parameters for fed and fasted female rabbits[a]

Rabbit No.	Dietary State	Correction Factor[b]	Plasma Pool Size[c]		Fractional Turnover Rate[d]	Synthesis Rate[e]
			Initial	Final		
			mg		*hr*$^{-1}$	*mg/hr*
1191	Fed	0.95	107	95	0.13	12.4
1196	Fed	0.90	62	57	0.12	7.0
1274	Fed	0.95	89	78	0.11	8.3
1261	Fed	0.97	63	54	0.20	10.7
1256	Fed	0.92	45	40	0.16	6.4
1186	Fed	0.82	45	41	0.11	4.3
Avg ± SE	Fed	0.92 ± 0.02	68 ± 10	61 ± 9	0.14 ± 0.02	8.2 ± 1.2
1201	Fasted	0.86	49	64	0.070	4.5
1189	Fasted	0.82	138	174	0.043	7.5
1278	Fasted	0.76	92	177	0.059	10.4
1165	Fasted	0.72	52	119	0.105	12.5
1182	Fasted	0.82	79	187	0.043	8.0
1254	Fasted	0.92	55	65	0.107	7.0
1258	Fasted	0.97	41	60	0.072	4.3
Avg ± SE	Fasted	0.84 ± 0.03	72 ± 13	121 ± 22	0.071 ± 0.010	7.7 ± 1.1

[a] Kinetic parameters were determined from free and esterified cholesterol specific activity vs. time curves (Fig. 2) obtained after injection of [2-^{14}C]mevalonate.

[b] This factor was used to correct for heterogeneity of the labeled cholesteryl ester pool (f, see section on theoretical considerations). The standard error of estimate for fed animals was 3% and for fasted animals was 13%.

[c] Plasma pool size for cholesteryl ester was calculated from the product of the plasma esterified cholesterol concentration and the plasma volume as determined by Evans blue dilution. The average plasma volume for fed or fasted rabbits was 146 ml. The initial pool size of esterified cholesterol refers to the beginning of the experiment. The final value is the average during the 24–56-hr period after mevalonate injection (see Fig. 2). During this period the cholesteryl ester concentrations decreased on the average by about 8%.

[d] The fraction of the cholesteryl ester pool turning over per hour (k, see section on theoretical considerations). The standard error of estimate for fed animals was 7% and for fasted animals was 22%.

[e] Synthesis rate of cholesteryl ester was calculated from the product of the final cholesteryl ester pool size and its fractional turnover rate.

rates (5.3 mg/hr, avg) than the other fasted rabbits. If rabbits 1201, 1254, and 1258 are excluded from the fasting data, the average cholesteryl ester pool size of fasted animals increases to 164 mg and the average fractional turnover rate decreases slightly to 0.062 hr^{-1}, giving a synthesis rate of 9.6 mg/hr, which is not significantly different from that found in the fed animals.

As a second means of studying lipoprotein influx, we measured the secretion of triglyceride and cholesterol into plasma after the injection of Triton WR 1339 in these same animals immediately after the mevalonate experiment (**Table 5**). Initial secretion rates (0–4 hr) of cholesterol were similar to rates determined at later time periods after Triton WR 1339 injection, but triglyceride accumulation decreased after 16 hr. Measurement of Triton WR 1339 plasma concentrations at the end of each period (Table 5) revealed similar levels of Triton for fed and fasted rabbits throughout the experiment and a slow rate of clearance of Triton from plasma. Total cholesterol was separated into its free and esterified components. Although

TABLE 5. Rate of rise of plasma lipid content after Triton WR 1339 injection into fed and 7-day-fasted female rabbits[a]

Pre-Triton Condition	No. of Rabbits	Post-Triton Period	Plasma Triton Levels	Rate of Rise of Plasma Lipid			
				Total Cholesterol	Free Cholesterol	Esterified Cholesterol	Triglycerides
		hr	*mg/ml*	*mg/hr*			
Fed	7	0–4	3.7 ± 0.2	9.3 ± 1.1	7.1 ± 0.7	2.2 ± 0.7	82.7 ± 13.7
		4–16	3.3 ± 0.2	8.4 ± 0.4	6.2 ± 0.4	2.1 ± 0.3	84.2 ± 14.1[b]
		16–40	3.1 ± 0.2	8.0 ± 0.9	6.5 ± 0.7	1.5 ± 0.3	63.2 ± 11.4[b]
Fasted	7	0–4	3.9 ± 0.2	7.4 ± 1.1	5.8 ± 0.4	1.7 ± 1.0	103.6 ± 8.1
		4–16	3.2 ± 0.2	7.0 ± 0.9	5.7 ± 0.4	1.3 ± 0.6	106.7 ± 9.0[c]
		16–40	3.1 ± 0.1	7.3 ± 0.4	5.5 ± 0.3	1.7 ± 0.2	65.6 ± 4.5[c]

[a] Plasma lipid content was calculated from the product of plasma lipid concentration and plasma volume as determined by Evans blue dilution. The average plasma volume for all rabbits was 146 ml. The Triton was injected into the rabbits immediately after their use in the mevalonate experiments (Table 4). Values are means ± SEM.

[b] Difference significant at $P < 0.055$.

[c] Difference significant at $P < 0.001$.

70% of the plasma cholesterol before Triton injection was cholesteryl ester for either fed or fasted animals, free cholesterol accounted for 78% of the cholesterol increment after Triton treatment. In view of this disproportionate increase in free cholesterol, the effect of Triton WR 1339 on net plasma cholesterol esterification was measured in vitro (**Table 6**). This study showed that Triton inhibited plasma cholesterol esterification 60–80% at concentrations similar to those reached in the in vivo studies.

TABLE 6. Effect of Triton WR 1339 on rabbit plasma cholesterol esterification in vitro

Expt. No.	Triton Addition	Final Free Cholesterol	Cholesterol Esterified	Inhibition
	μg/ml	*μg/ml*	*μg/ml/24 hr*	*%*
1	0	50	152	0
	2000	175	27	82.2
	5000	179	23	84.9
2	0	32	158	0
	100	40	150	5.1
	500	104	86	45.6
	1000	146	44	72.2
	5000	134	56	64.6
	10000	130	60	62.0

Duplicate samples were incubated at 37°C for 24 hr in the presence or absence of Triton WR 1339. Cholesterol esterification was determined from the difference in the average initial (202 μg/ml for expt. 1 and 190 μg/ml for expt. 2) and final free cholesterol concentrations.

DISCUSSION

Changes in circulating lipid levels have been noted previously in the fasting rabbit. Iacono and Ammerman (6) observed significant increases in serum cholesterol, phospholipid, and triglycerides after a 6-day fast. Rabbit serum lipoproteins of S_f 0–100 (especially S_f 0–12) have also been shown to increase during a 7-day fast (7). We have observed a somewhat different response to fasting in our rabbits. Plasma cholesterol increased throughout the fast, but triglyceride levels decreased. This increase in plasma cholesterol was associated primarily with an increase in LDL cholesterol and protein concentrations. Fasting also led to cholesterol-enriched LDL in which cholesterol, probably as cholesteryl ester, was substituted for triglyceride. When the fasted animals were refed, both plasma cholesterol and LDL decreased. The response of rabbits to fasting differs from that observed in fasting ponies (5) in which VLDL concentrations increase with fasting.

The elevation of plasma cholesterol during fasting could be due to either increased cholesterol influx into plasma or decreased efflux. We have used two methods to measure influx. One method involved the use of Triton WR 1339 to determine plasma lipid secretion rates (26). Triton WR 1339 was cleared from plasma very slowly. The levels dropped from 4 to 3 mg/ml over a postinjection period of 4–40 hr. In rats, similar doses of Triton were cleared at a faster rate: levels dropped from 3 to 1 mg/ml over a similar period (21). This slower clearance of Triton could explain the prolonged linear increases of plasma cholesterol after Triton injection in rabbits (>24 hr) compared with that observed in other species (<8 hr) under similar conditions (21, 27, 28). Comparison of plasma cholesterol secretion rates in fed and fasted rabbits after Triton WR 1339 injection did not show an increase in cholesterol secretion after prolonged fasting, suggesting that elevated cholesterol during fasting was not due to increased cholesterol secretion into the plasma.

In the presence of sufficient Triton to inhibit plasma cholesterol esterification, we have found that 78% of the total cholesterol is secreted into rabbit plasma as free cholesterol. The small increment in plasma cholesteryl ester in response to Triton could be accounted for by direct secretion of ester or by incomplete inhibition of plasma cholesterol esterification by Triton. If it is assumed that Triton does not inhibit esterification of cholesterol in the liver, a substantial amount of the secreted free cholesterol must normally be esterified after entering the plasma. This conclusion does not support the suggestion that in the rabbit the liver is the major source of plasma cholesteryl esters (25).

We have studied the turnover of cholesteryl ester as a second method for estimating the influx of cholesterol into plasma. Our findings suggest that most of the cholesteryl ester was derived from a free cholesterol pool in rapid equilibrium with plasma free cholesterol but that some of the ester appeared to originate from a lower specific activity source. Assuming that this source was completely unlabeled, 8% of the plasma ester in fed animals or 16% in fasted ones came from the unlabeled source. This source might well be adipose tissue that is depleted of triglyceride during fasting. Since most of adipose tissue cholesterol is present as free cholesterol (29), a mechanism would be needed to esterify the free cholesterol before it equilibrated with the plasma free cholesterol. Adipose tissue cholesterol could be transferred directly to erythrocytes when these cells come in contact with capillary endothelium. Esterification of this cholesterol could occur before equilibration with plasma, as has been suggested by d'Hollander and Chevallier (30).

When we calculated the turnover of plasma cholesteryl ester taking into account a small influx of unlabeled ester, we obtained a rate of 145 μmoles/l/hr, which is similar to the 165 μmoles/l/hr that was observed by Rose (25). Rose did not correct for influx of unlabeled ester, and he measured the free and esterified cholesterol specific activity for only the first 4 hr after injection of labeled mevalonate. For the fasting rabbit, in which a larger correction for in-

flux of unlabeled cholesteryl ester must be made, cholesteryl ester synthesis rates will be underestimated unless a correction factor is applied.

The use of cholesterol secretion rates obtained from the Triton experiments and the cholesteryl ester synthesis rates from mevalonate injection to measure influx of cholesterol into plasma can be questioned. In rats, Triton has been shown to stimulate liver cholesterol synthesis during later stages of Triton treatment but not in the initial phase (31). This stimulated synthesis could lead to increased secretion of cholesterol. The inhibition of cholesterol esterification by Triton could also alter cholesterol secretion rates. Although we have seen similar rates of cholesterol secretion at several time intervals after Triton injection, this does not prove that the estimates for secretion are reliable. We felt that the rates of cholesteryl ester synthesis from mevalonate would give an additional estimate of total cholesterol influx if either the free and esterified cholesterol of plasma lipoproteins had similar fractional turnover rates or if all lipoprotein free cholesterol were esterified before it was cleared from plasma. The fact that both the Triton and the cholesteryl ester turnover studies gave similar values for cholesterol influx (8 mg/hr) suggests that in the rabbit these two methods give reliable estimates of influx of cholesterol into plasma. It also suggests that plasma free cholesterol and plasma esterified cholesterol are metabolized sequentially or at similar fractional rates.

Analysis of the cholesteryl ester turnover data showed that the synthesis of cholesteryl ester was not altered by fasting but that the fractional turnover rate was halved. If cholesteryl ester were removed from plasma by a first-order reaction, the fractional turnover rate would be a measure of the degradation constant of plasma cholesteryl ester. In that case, one would conclude that fasting rabbits showed a decreased capacity to degrade cholesteryl ester. Since measurements of both cholesterol secretion rates and cholesteryl ester synthesis rates show that fasting does not affect influx of cholesterol into plasma, either these methods were not sensitive enough to detect any increases or fasting hypercholesterolemia in the rabbit is caused by decreased degradation of plasma cholesterol, which leads to the accumulation of LDL. This conclusion is supported by evidence from sterol balance studies in rabbits[3] that showed a marked decrease in fecal excretion of steroids during fasting.

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[3] Klauda, H. C., and D. B. Zilversmit. Unpublished experiments.

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