

Fate of intravenously administered particulate and lipoprotein cholesterol in the rat

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Abstract Unesterified radioactive cholesterol, both bound to serum lipoproteins and dispersed in ethanol-saline, was injected into bile fistula and intact rats. Due to phagocytosis, mainly by the liver macrophages, intravenously injected cholesterol in ethanol-saline disappears from the bloodstream significantly faster than lipoprotein-bound cholesterol.

Soon after the initial phagocytosis, the particulate isotopic cholesterol started to reappear in blood, reaching a maximal radioactivity in blood 10-24 hr after injection. Although the radioactive cholesterol reappears in serum in both esterified and unesterified form, it is likely that cholesterol is released from the phagocytic cells as unesterified cholesterol which is then esterified intravascularly or at other sites. In the bile fistula rats, somewhat more of the lipoprotein cholesterol than of the particulate cholesterol appeared in bile early after injection. However, cholesterol turnover calculated from a two-pool model was the same for rats injected with lipoprotein-bound or particulate cholesterol.

Supplementary key words cholesterol particles · serum lipoprotein · colloidal chromic phosphate · phagocytosis · cell isolation · liver macrophages · serum cholesterol · cholesteryl ester · bile acids · bile cholesterol

THE INTRAVENOUS INJECTION of labeled cholesterol dissolved in ethanol or dispersed in ethanol-saline is widely used in studies of cholesterol turnover (1, 2). Cholesterol administered in this form or as an emulsion, stabilized with detergent, is rapidly removed from the bloodstream (3-5), but direct evidence that this is due to uptake by phagocytic cells is lacking. In the present study the fate of this kind of cholesterol preparation was compared with that of cholesterol bound to serum lipoproteins labeled in vitro. The studies were designed

to increase our understanding of the mechanisms by which phagocytized cholesterol is metabolized and released by reticuloendothelial cells. In addition, the data help to evaluate kinetic data obtained with various forms of intravenously injected cholesterol preparations.

MATERIALS AND METHODS

[1,2-³H]Cholesterol and [4-¹⁴C]cholesterol were obtained from New England Nuclear Corp., Boston, Mass. [³H]Cholesterol was purified by thin-layer chromatography on silica gel H developed in benzene-ethyl acetate 7:2 (v/v) or diethyl ether-hexane¹ 50:50 (v/v) and eluted with chloroform-methanol 2:1 (v/v). [¹⁴C]Cholesterol (98.5% pure or better) in the same system and on AgNO₃-impregnated silica gel H (6) was used as furnished by the supplier. When [³H]cholesterol purified by thin-layer chromatography was added to carrier cholesterol (which had previously been purified by dibromination) and then converted to cholesterol dibromide (7, 8), the specific radioactivity decreased by 6.2%. After the same treatment the specific activity of the [¹⁴C]cholesterol remained unchanged. In several experiments [³H]cholesterol purified by dibromination was used; in others, colloidal and lipoprotein cholesterol preparations were labeled with [³H]- and [¹⁴C]cholesterol interchangeably in order to avoid isotope effects due to small amounts of impurities or resulting from possible lability of the ³H label.

Colloidal ³²P-labeled chromic phosphate was obtained from Abbot Laboratories, Chicago, Ill. The preparation was diluted with 25% glucose and centrifuged at 300 *g* for 10 min before use. The supernatant was used for injection experiments. Pronase, B grade, was obtained from Calbiochem, Los Angeles, Calif.

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¹ Skellysolve B, Skelly Oil Co., Kansas City, Mo., bp 60-70°C.

Labeled cholesterol preparations

Rat serum was obtained from rats fasted overnight. Chylomicrons, if present, were removed by centrifuging at 20,000 *g* for 30 min. A tracer dose of radioactive cholesterol was applied to a piece of filter paper (Whatman no. 1) from a solution of hexane and dried under nitrogen. Rat serum was incubated with the coated filter paper for 3 hr at 37°C. After filtering the incubated serum through a Millipore filter (pore size, 0.2 μ), 35–60% of the added radioactivity was recovered in the filtrate. After agarose electrophoresis in 0.05 M barbiturate buffer, pH 8.6 (9), 3–6% of the serum radioactivity stayed at the origin, indicating that the labeled lipoprotein preparation contained some radioactive cholesterol that was not bound to native lipoprotein. Erythrocytes were labeled with [¹⁴C]cholesterol by incubating with *in vitro* labeled serum for 2 hr at 37°C. The labeled serum was removed after incubation by centrifugation and four washes with 0.9% NaCl.

A dilute suspension of labeled cholesterol was prepared immediately before injection by adding 50 μ l of radioactive cholesterol (20 μ g) dissolved in ethanol to 2 ml of 0.9% NaCl. A more concentrated colloidal suspension of radioactive cholesterol was prepared by dilution of a saturated cholesterol solution in ethanol with warm water according to Day, Gould-Hurst, and Wilkinson (10) as modified by Wurster and Zilversmit (11). This suspension is not stable in 0.9% NaCl, and 4% bovine serum albumin was added before making the suspension isotonic for injection.

Animal preparations

Labeled serum (0.5 ml, 0.4 μ Ci) and the labeled cholesterol suspension (1 ml, 2 μ Ci) were mixed and immediately given intravenously to white male Holtzman rats weighing 250–325 g, either immediately after or 36–48 hr after cannulation of their bile ducts. The bile was collected and blood samples were taken from the tail. The animals had free access to a solution containing 0.5% NaCl, 0.05% KCl, and 2.5% glucose. Animals which were injected 36–48 hr after cannulation were given a constant intragastric infusion (1 ml/hr) of 9.2% glucose and 6.4% casein hydrolysate in 0.5% NaCl–0.05% KCl from 4–6 hr after surgery until the end of the experiment (12). Intact animals used for long-term studies of cholesterol turnover were injected intravenously with three times as much labeled cholesterol as was used for the bile fistula animals. All intravenous injections were made under light ether anesthesia.

Preparation of Kupffer cells

To quantitate the initial uptake of [³H]cholesterol by reticuloendothelial cells, liver macrophages were isolated

from liver essentially according to Mills and Zucker-Franklin (13). Rats were bled by heart puncture during ether anesthesia 30 min after the injection of colloidal chromic [³²P]phosphate and radioactive cholesterol suspensions. The heart and lungs were removed, and the abdominal viscera were cleared of blood by perfusion through the thoracic aorta with 100–200 ml of cold saline. In some experiments the perfusion was terminated with 10 ml of 0.25% pronase in Gey's solution (227 mg of NaHCO₃/liter), pH 7.4 (14). After mincing the liver in Gey's solution, 30–40 ml of 0.25% pronase in Gey's solution, pH 7.4, was added and the mixture was stirred at 4°C on a magnetic stirrer. At 20–30-min intervals about 25 ml of the digest was decanted, and 25 ml of fresh enzyme solution was added to the remaining pieces. After 90–120 min, when most of the liver pieces were completely disintegrated, the pooled digests were filtered through silk, and the pH, which decreased during digestion, was readjusted to 7.4. The preparation was stirred at moderate speed for another 20 min to minimize contamination of Kupffer cells with large pieces of debris and intact parenchymal cells. In the experiments in which the liver was perfused with pronase, stirring times could be shortened, but the cell yields, normally exceeding 2×10^7 cells/10g of liver, were less consistent. Cells were sedimented from the digest by centrifugation at 300 *g* for 3 min. In many experiments this cell preparation, which contained very few parenchymal cells, was washed three times to remove various membrane fragments. When parenchymal cells were present in significant numbers, they were removed by repeated centrifugations at 50 *g* for 1 min or by centrifugation in pointed test tubes at approximately 140 *g* for 10–15 min. The parenchymal cells then formed a firm pellet under a gelatinous mass consisting primarily of Kupffer cells. Each preparation was examined by phase contrast microscopy and found to contain few or no intact parenchymal cells and little or no debris.

Tissue lipids

Lipids were extracted from tissues and bile with chloroform-methanol and washed according to Folch, Lees, and Sloane Stanley (15). The two first upper phases from extracts of bile were combined for the measurement of radioactivity in bile acids. In human bile from patients injected with [¹⁴C]cholesterol, 95.9% of the radioactivity of the wash has been shown to be in conjugated bile acids (16). In view of this and the findings of others (17, 18) that more than 90% of the radioactivity secreted in rat bile after [¹⁴C]cholesterol injection is in bile acids, we used the radioactivity in the wash as a measure of bile acid secretion in our animals.

Cholesterol and cholesteryl ester from blood plasma extracts were isolated by silica gel H thin-layer chromatography in hexane-diethyl ether-acetic acid 70:30:1 (v/v/v). When specific activities were measured, the spots were eluted with chloroform-methanol 2:1 (v/v). Radioactivity was measured by liquid scintillation counting in toluene containing 0.5% PPO (2,5-diphenyl-oxazole) and 0.01% POPOP (*p*-bis[2-5-phenyloxazolyl]-benzene). When thin-layer scrapings containing cholesterol or cholesteryl ester were counted directly, 1 ml of methanol was added to 10 ml of scintillation mixture. Bile acids were dissolved in 1 ml of ethanol before adding the scintillator solution.

Cholesterol was determined according to Abell et al. (19) with the color reagent of Zak et al. (20). Nitrogen was determined according to Minari and Zilversmit (21).

RESULTS

Three bile fistula rats were injected intravenously with [¹⁴C]cholesterol-labeled lipoproteins and [³H]cholesterol in ethanol-saline immediately after surgery. During the labeling of serum lipoproteins in vitro some of the labeled cholesterol was esterified, whereas all the cholesterol injected in ethanol-saline was in nonesterified form. The disappearance from plasma of the two preparations differed markedly. The cholesterol in ethanol-saline was completely cleared from plasma within 20 min. The unesterified and esterified portions of the lipoprotein-bound cholesterol must be considered separately. Whereas the unesterified portion rapidly equilibrated with the readily exchangeable cholesterol in various tissues, the cholesteryl ester had a much slower disappearance rate. For example, 1 hr after injection, when the first blood sample was taken, 5% of the injected cholesteryl ester was found per milliliter of plasma. Only one-tenth as much of the unesterified cholesterol was present in plasma at the same time (Fig. 1).

In a different set of experiments, the uptake by whole liver and by Kupffer cells of cholesterol injected in ethanol-saline was measured 30 min after injection. A comparison was made with colloidal ³²P-labeled chromic phosphate and with a cholesterol preparation with known colloidal properties, prepared according to Wurster and Zilversmit (11). All three preparations were efficiently removed from the bloodstream primarily by liver, which contained 75–85% of all three isotopes 30 min after injection. The Kupffer cells contained ten times as much radioactive chromic phosphate per milligram of nitrogen as whole liver. The cholesterol radioactivity per milligram of nitrogen was, on the average, 7–8 times as high in the Kupffer cells as in the whole liver. If it is assumed that all the chromic phos-

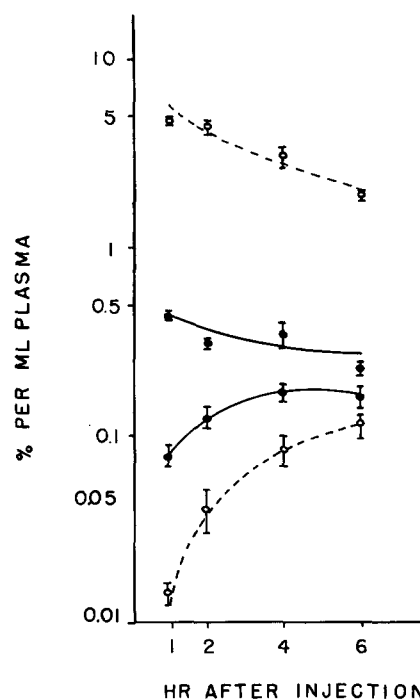


FIG. 1. Blood plasma radioactivity in cholesteryl ester (---) and unesterified cholesterol (—) after injecting ¹⁴C-labeled serum (upper curves) and [³H]cholesterol particles (lower curves). The injected labeled serum contained 16.7–22.3% of the radioactivity as cholesteryl ester. For [¹⁴C]cholesterol the curves indicate % of injected radioactive ester and free cholesterol, respectively. For [³H]cholesterol the esterified and nonesterified cholesterol is given as % of the total injected dose. Means and SEM are given for three bile fistula rats injected immediately after operation.

phate taken up by the liver was in Kupffer cells, then, on the average, 70–80% of the liver cholesterol radioactivity was present in the Kupffer cells (Table 1). The difference in uptake of the chromic phosphate and the cholesterol preparations might mean that some labeled cholesterol had been released from the phago-

TABLE 1. Uptake of particulate cholesterol and chromic phosphate by liver macrophages

Injected Material	(cpm/mg N in Kupffer cells)/(cpm/mg N in Whole Liver)		% Liver Radioactivity Present in Kupffer Cells
	Range	Mean	
Colloidal chromic [³² P]phosphate	6.50–13.19 (6)	10.0	100
20 μg [³ H]cholesterol in ethanol-saline	4.15–11.80 (6)	8.2	82
~0.5 mg [³ H]cholesterol as a colloidal suspension	4.27–10.30 (4)	7.3	73

Livers and Kupffer cells were obtained from rats killed 30 min after injection of the radioactive cholesterol and the inert colloidal marker, ³²P-labeled chromic phosphate. Radioactivities are expressed as counts per minute per milligram of total tissue nitrogen. Numbers in parentheses reflect the number of animals per group.

cytes and had entered parenchymal cells within the 30-min time period. It is clear that cholesterol administered in ethanol-saline is cleared from the circulation primarily by phagocytosis by the liver macrophages. Therefore, the subsequent appearance of radioactivity in bile and blood reflects the release of cholesterol from these cells.

In the next experiment, the release of cholesterol from macrophages was compared with that from labeled red cells. Rat erythrocytes, labeled with [¹⁴C]cholesterol, and [³H]cholesterol in ethanol-saline were injected intravenously into the same animal. Fig. 2 shows the appearance of the respective isotopes in serum free cholesterol and cholesteryl ester 1-24 hr after injection. By 1-2 hr after injection, mainly unesterified [³H]- and [¹⁴C]-cholesterol were present in plasma. Thereafter, a notable increase in plasma cholesteryl ester radioactivity occurred. The proportion of isotopic cholesterol appearing in plasma cholesteryl ester was about the same for the cholesterol administered in red blood cells as that given in ethanol-saline.

In order to find out whether phagocytized cholesterol might be preferentially excreted in bile, we examined the biliary excretion of particulate and lipoprotein cholesterol. Experiments were performed both immediately after and 36-48 hr after bile duct cannulation. In the former, most of the feedback mechanisms were probably still in existence, but some effects of surgery on bile excretion might have been present. At the later time intervals the effects of surgery were probably less, but the rates of cholesterol oxidation were no longer controlled by physiological concentrations of bile acids in plasma and liver. Fig. 3 shows the appearance of labeled bile acids in bile of bile fistula rats. Nearly 20%

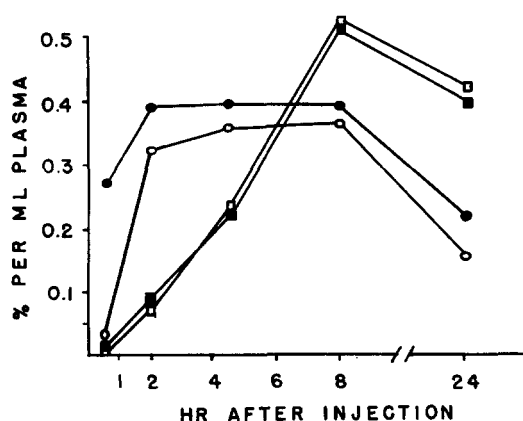


FIG. 2. Plasma cholesterol (circles) and cholesteryl ester (squares) radioactivity after simultaneous injection of [¹⁴C]-cholesterol-labeled erythrocytes (● and ■) and [³H]cholesterol in ethanol-saline (○ and □). The [³H]cholesterol used was purified by thin-layer chromatography plus bromination-debromination. The figure represents one of two similar experiments.

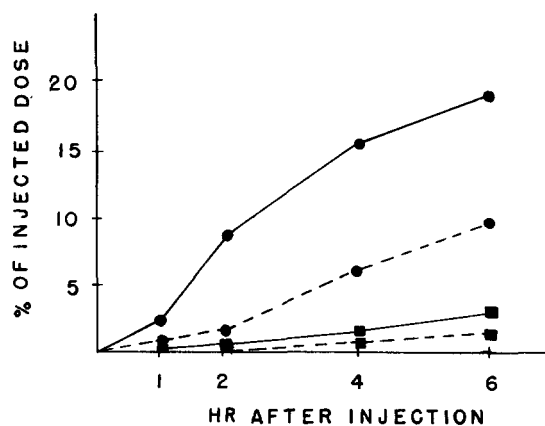


FIG. 3. Cumulative recoveries of radioactive biliary bile acids from rats injected with both lipoprotein-bound (—) and particulate (---) cholesterol. Four animals were injected 36-48 hr after the bile fistula operation (●), and three animals were injected immediately after surgery (■). The figure represents mean values. The [³H]cholesterol used in these experiments was purified by thin-layer chromatography. Two of the rats, operated 36-48 hr before injection, received ³H-labeled lipoprotein and ¹⁴C-labeled particles; in the other two the isotopes were reversed.

of the lipoprotein cholesterol appeared in biliary bile acids within 6 hr in rats injected 36-48 hr after bile drainage was begun, but in the same interval only about 10% of the cholesterol injected in ethanol-saline was converted to bile acids. In rats injected immediately after surgery, relative rates of appearance of lipoprotein-bound and of particulate cholesterol in bile acids also differed by a factor of two, but the fraction of the injected dose excreted in bile was much less than that in the more chronic preparations.

Although less than one-tenth of the label secreted in bile was present in neutral sterols, the cholesterol injected in the form of serum lipoprotein appeared somewhat faster in this fraction than did the sterol injected as a saline suspension. In both instances the specific radioactivity of biliary cholesterol was slightly less or equal to that of plasma free cholesterol, which probably means that cholesterol released by Kupffer cells reaches bile by way of plasma.

In rats injected intravenously with [¹⁴C]cholesterol bound to lipoproteins and [³H]cholesterol in ethanol-saline, the disappearance curves followed a similar course after the first 24-hr period. In a semilogarithmic plot of radioactivity per ml of blood vs. time (Fig. 4), the values formed a straight line from 14 to 38 days after injection, when most experiments were terminated. The disappearance curve could be described in terms of a two-pool model similar to that described by Goodman and Noble for the cholesterol turnover in man (22). The slopes (α , β) and intercepts (C_A and C_B) in the equation, radioactivity per ml of blood = $C_A e^{-\alpha t} + C_B e^{-\beta t}$, for cholesterol injected in ethanol-saline and in

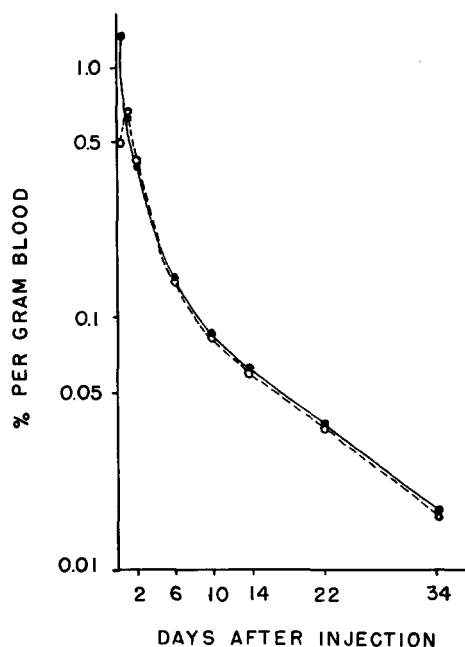


FIG. 4. Blood radioactivity after the simultaneous injection of [^3H]cholesterol-labeled serum (—) and [^{14}C]cholesterol in ethanol-saline (- - -). The figure is typical for a series of six animals, two of which received the ^{14}C as lipoprotein and ^3H as particles. The [^3H]cholesterol was purified by preparing the dibromide derivative.

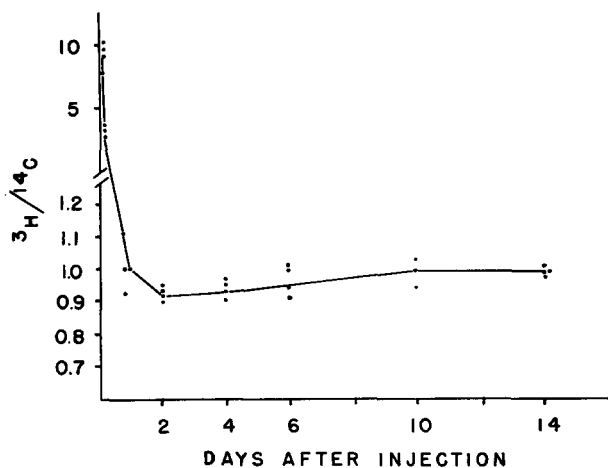


FIG. 5. Normalized $^3\text{H}/^{14}\text{C}$ ratios in a series of four rats receiving ^3H as lipoprotein and ^{14}C as particles. In blood samples taken 38 days after injection, the ratio was 0.97–1.02, with an average of 0.99. Values are the $^3\text{H}/^{14}\text{C}$ ratios of the cholesterol dibromide formed from the nonsaponifiable blood lipid fractions and the injected material. The $^3\text{H}/^{14}\text{C}$ ratio of the injected dose equals one.

lipoprotein, were measured. The data in Table 2 show excellent agreement for the two cholesterol preparations.

Fig. 5 shows data from four rats injected with [^3H]cholesterol-labeled lipoprotein and [^{14}C]cholesterol in ethanol-saline. The ratio of ^3H to ^{14}C in the whole blood cholesterol fraction is seen to decrease rapidly

TABLE 2. Disappearance of cholesterol injected as lipoproteins and dispersed in ethanol-saline

Rat	Lipoprotein-bound Cholesterol				Particulate Cholesterol			
	% / ml		Days $^{-1}$		% / ml		Days $^{-1}$	
	C_A	C_B	α	β	C_A	C_B	α	β
1	1.10	0.198	0.58	0.092	1.10	0.191	0.62	0.090
2	0.65	0.180	0.58	0.064	0.65	0.160	0.58	0.069
3	0.98	0.156	0.71	0.067	0.97	0.160	0.71	0.067
4	0.75	0.149	0.56	0.064	0.80	0.143	0.53	0.068
5	0.66	0.160	0.47	0.062	0.65	0.151	0.52	0.063

The disappearance curves conformed to a two-pool model and C_A , C_B , α , and β are the constants in the equation: radioactivity per ml blood = $C_A e^{-\alpha t} + C_B e^{-\beta t}$. In animals 1, 2, and 3 the lipoprotein-bound cholesterol was labeled with ^3H and the particulate cholesterol with ^{14}C ; in animals 4 and 5 the isotopes were reversed.

within the first 24 hr. This reflects the relatively constant [^3H]cholesterol concentration in blood while the [^{14}C]cholesterol is being rapidly released from the macrophages. It is of interest, however, that the $^3\text{H}/^{14}\text{C}$ ratio does not reach the value of the injected material, which is unity, until sometime between 6 and 10 days after cholesterol injection.

DISCUSSION

The foregoing studies demonstrate that the rapid clearance of an injected tracer dose of cholesterol in ethanol-saline is mainly due to phagocytosis by the reticuloendothelial system. The fate of this cholesterol preparation after the initial clearance from the bloodstream is therefore pertinent to the mechanism by which phagocytic cells metabolize and secrete cholesterol. Earlier studies indicated that cholesterol injected as an ethanol solution or emulsified in water with a detergent, after its initial rapid clearance (3–5) reappears in blood as both free cholesterol and cholesteryl ester (4). Since exchange of cholesteryl ester between tissues and serum lipoproteins presumably is slow or does not occur at all (23), the appearance of both unesterified and esterified sterol in blood fairly early after injection could indicate either secretion of cholesteryl ester in the form of soluble lipoprotein by the phagocytic cells or esterification after release in a nonesterified form. Glomset (24), for example, has indicated that lecithin-cholesterol acyltransferase in serum might aid cholesterol transport by accelerating the esterification of cholesterol released from various tissues. Other investigators (25, 26) have emphasized the possibility that esterification of cholesterol ingested by phagocytic cells may be linked to an excretory process, and they have postulated that cholesterol is released from these cells primarily in the esterified form as lipoprotein.

In our own experiments we used the well-known exchange of free cholesterol between labeled red cells and serum lipoproteins as a base line against which to judge whether or not, in the intact animal, cholesterol released from phagocytic cells was secreted primarily in the free or the esterified form. Our data show a close parallelism between the release of phagocytized labeled cholesterol and cholesterol exchanged from red blood cells. Both show early appearance of unesterified labeled cholesterol in serum and a gradual appearance of labeled cholesteryl ester. Our data, therefore, suggest that phagocytized cholesterol is released primarily in the unesterified form, which is then gradually converted to the esterified form after the cholesterol has reached the plasma.

The question remains, however, whether the cholesterol in Kupffer cells of the liver is transferred directly to parenchymal tissue or bile canaliculi, or whether the phagocytized cholesterol reaches the biliary apparatus only by way of the circulating plasma. Our data confirm the observations of others (17, 18), who have shown that in the rat, labeled cholesterol is excreted primarily in the form of bile acids. In addition, our data show that this route is the major excretory pathway for both injected lipoprotein and particulate cholesterol preparations. If particulate cholesterol had been transferred directly from Kupffer cells to liver parenchyma, one would have expected in bile relatively higher amounts of isotopic cholesterol derived from particles than from injected lipoprotein. If anything, the opposite was the case early after injection. In addition, the observation that the specific activity of biliary cholesterol almost equaled but never exceeded that of plasma free cholesterol, irrespective of the form in which the cholesterol was injected, makes it unlikely that a significant amount of cholesterol is transferred directly from Kupffer cells to bile. The data obtained from the bile fistula experiments are compatible with the hypothesis that cholesterol ingested by Kupffer cells is released into the bloodstream as free cholesterol and that this is the principal mechanism by which cholesterol leaves the reticuloendothelial cells.

The data in Fig. 4 indicate that in the intact rat the preferential loss of lipoprotein cholesterol in bile during the first hours after injection is not quantitatively important. This may be so, because the loss of biliary sterols during the first few hours after injection is small when the enterohepatic circulation is intact. As the curves indicate, after 24 hr the amount of labeled cholesterol in blood is almost the same whether the cholesterol was injected as particles or bound to lipoprotein. This must mean that the release of cholesterol from the reticuloendothelial cells is essentially complete within 24 hr. In the two-pool model described by

Gurpide, Mann, and Sandberg (27) and by Goodman and Noble (22), very similar values were obtained for the slopes and intercepts of the curves, independent of the physical form in which the cholesterol was administered. Apparently, the injection of a tracer dose of cholesterol in ethanol-saline gives valid data for the calculation of cholesterol turnover in intact rats and probably also in other animals and in man.

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