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The use of cholesterol-4-C^{14}-labeled lipoproteins as a tracer for plasma cholesterol in the dog*

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SUMMARY

A method is described for labeling lipoprotein cholesterol with cholesterol-4-C^{14}. The specific activity of the labeled cholesterol is equal in low and high density lipoproteins, and none of the label is associated with other serum proteins. Lipoproteins labeled in this manner were used to define the quantity of cholesterol that exchanges with plasma free cholesterol in 30 hours. This quantity is approximately three times the amount of cholesterol contained in blood plasma, red blood cells, and liver.

Study of the fraction of total body cholesterol that is in equilibrium with cholesterol in blood plasma has been hampered by lack of a tracer whose behavior is identical with that of plasma cholesterol. Since cholesterol exists in blood plasma as a constituent of lipoproteins, artificial cholesterol emulsions are not suitable for this purpose. Emulsified or suspended cholesterol labeled with C^{14} is rapidly removed from the circulation, and reappears later as labeled free and ester cholesterol associated with lipoproteins (1, 2, 3).

In 1955 Eckles and her collaborators (4) demonstrated the rapid exchange of isotopically labeled free cholesterol between plasma, liver, and red blood cells. This observation led to the concept of a rapidly exchangeable liver-blood pool of cholesterol. The limits of this pool have not been determined as yet, nor has its physiologic significance been evaluated.

This paper describes a method of incorporating isotopically labeled free cholesterol into plasma lipoproteins *in vitro* and the utilization of this material to study the kinetics of the early phases of plasma free cholesterol turnover in the dog.

METHODS

Incorporation of Cholesterol into Lipoproteins. Two to five microcuries of cholesterol-4-C^{14} (specific activity 28 μc/mg)[1] was dissolved in 1 ml of absolute ethanol. The solution was rapidly injected from a syringe through a 23-gauge needle under the surface of 10 ml of a 5% solution of human serum albumin in 0.15 M NaCl. The mixture was allowed to stand for 20 minutes. Alcohol was then evaporated from the solution at 25° under an air stream. Volume was maintained at 10 ml by frequent small additions of distilled water. When the odor of alcohol could no longer be detected, the solution was mixed with 10 ml of whole blood serum from the experimental animal, and allowed to stand for 1 hour. The protein-free density of the mixture was raised to 1.019 by the addition of a saline solution of density 1.083. The solution was centrifuged for approximately 15 hours at 4° in the 40.3 rotor of a Spinco Model L ultracentrifuge at 109,000 × *g*. The supernatant material was removed with a tube slicer (5) and discarded. The sedimented material was redispersed prior to use.

Administration of Labeled Lipoproteins. One male and one female mongrel dog, maintained on a diet of nine parts Friskies dog meal and one part lean horse meat, were used. A measured amount (15 to 20 ml) of labeled solution was rapidly injected from a calibrated syringe into a foreleg vein. Samples were withdrawn at timed intervals from a vein in the opposite foreleg.

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[1] Obtained commercially from Nuclear-Chicago Corporation, Chicago, Ill.

Food was withheld for 16 hours before and 48 hours after the injection; access to water was allowed at all times. In dog #1 red blood cell and plasma volumes (6, 7) were determined one week later. At this time both animals were sacrificed. The liver of each was back-perfused through the vena cava with physiologic saline, blotted and weighed.

Analytical Procedures. Blood specimens were collected in tubes containing 1.5 mg sodium oxalate per ml, mixed, and chilled immediately in ice water. Within 15 minutes the samples were centrifuged at 500 × *g* for 20 minutes at 4°. The plasma was removed and stored at 4°. The red cells were washed three times with 25 volumes of ice-cold physiologic saline solution, and centrifuged at 18,000 × *g* for 15 minutes in the SW-39 rotor of a Spinco Model L ultracentrifuge. The plastic tubes were cut with a tube slicer just below the top of the packed red blood cells. One-milliliter aliquots of the cells were drawn into a calibrated syringe through a large bore needle, discharged into tubes containing 2 ml of distilled water, mixed to achieve hemolysis, and stored at 4°.

The lipids were extracted from 2 ml of plasma or hemolyzed red blood cells in 50 ml of alcohol-acetone 1:1 (v/v). Liver lipids were extracted similarly in a Waring Blendor. Aliquots of the extracts were taken for determination of free and total cholesterol in plasma and liver and for free cholesterol in red blood cells[2] (8). The supernatant fluids and washings from the free cholesterol digitonide precipitates were saved. Duplicate aliquots of free cholesterol digitonides were prepared (8) and dissolved in 1.2 ml of methanol. One milliliter of the methanolic solution was counted in a Packard Tri-Carb liquid scintillation spectrometer, with 10 ml of 0.3% diphenyloxazole (PPO) in toluene as phosphor. No quenching correction was used, since quenching was found to be equal in all samples. The supernatant fluids and washings from the free cholesterol digitonide precipitates were pooled, taken to dryness under an air stream, and extracted three times with 4 ml of heptane. The heptane was evaporated and the residue dissolved in 10 ml of 0.3% PPO in toluene for counting.

Distribution of Labeled Cholesterol in Lipoproteins. To define the nature of the binding of the labeled material *in vitro* and *in vivo*, selected samples of the material to be injected and of the blood serum after injection were separated into lipoprotein classes at densities 1.019, 1.063, and 1.21, according to the method of Havel *et al.* (9). The lipids were extracted from these fractions and analyzed as described for whole blood plasma.

Calculations. In dog #1 the total amounts of free and ester cholesterol in blood were computed by multiplying the determined red cell and plasma volumes by the concentrations of plasma free and ester cholesterol and red blood cell free cholesterol. In dog #2 plasma and red blood cell volumes were estimated on the basis of the volumes determined in dog #1. These estimated figures were used in similar computations.

RESULTS

Lipoprotein Incorporation. After an incubation period of 1 hour the labeled serum was adjusted to a density of 1.063 and centrifuged for 1 hour at 109,000 × *g*. The tubes were sliced so that the top fraction contained approximately one-third the total volume. Under these conditions there is little stratification of cholesterol-containing lipoproteins in the tube. The specific activities of the top and bottom fractions isolated from the tubes were approximately equal (Table 1), demonstrating that the labeled cholesterol was not concentrated in any one layer in the tube. Further centrifugation at this same density for 16 hours separated the high and low density lipoproteins. The specific activities of the two fractions were practically equal, despite marked differences in cholesterol concentrations (Table 1).

Further fractionation (Table 2) showed no detectable C^{14}-labeled cholesterol in proteins of d > 1.21, and approximately equal specific activities in the d =

TABLE 1. DISTRIBUTION AND SPECIFIC ACTIVITY OF FREE CHOLESTEROL IN LIPOPROTEIN FRACTIONS SEPARATED AT DENSITY 1.063

Sample	Centrifugation Time	Fraction	Free Cholesterol	
			Distribution	Specific Activity
	hours		*mg/fraction*	*cpm/mg*
1	1	Top	0.45	33,000
		Bottom	0.68	36,100
	16	Top (low density)	0.23	34,700
		Bottom (high density)	0.86	32,400
2	16	Top (low density)	0.38	20,300
		Bottom (high density)	0.61	20,400

[2] Preliminary work with red blood cells demonstrated that this method of extraction gives the same results as a 24-hour extraction with alcohol-acetone in a Soxhlet apparatus.

TABLE 2. DISTRIBUTION AND SPECIFIC ACTIVITY OF FREE CHOLESTEROL IN LIPOPROTEIN FRACTIONS

Fraction	Free Cholesterol		
	Distribution	Radioactivity	
	mg/100 ml	*cpm/100 ml*	*cpm/mg*
d < 1.019	*	1,290	*
d = 1.019 to 1.063	16	17,100	1,140
d = 1.063 to 1.21	27	32,300	1,200
d > 1.21	0	0	0
Whole serum	45	50,800	1,130

* Fraction contained too little cholesterol for accurate measurement.

1.063 to 1.21 and d = 1.019 to 1.063 fractions. Fasting dog plasma contains so little lipoprotein material of d < 1.019 that specific activities could not be determined accurately, but less than 2% of total plasma radioactivity was present in this fraction. Because the specific activity of this fraction may have been higher than that of the other two fractions, material for injection was first centrifuged at d = 1.019, after which the supernatant material was discarded. The incorporation of cholesterol-4-C^{14} into lipoproteins varied from 40% to 80%. Most of the loss was probably attributable to adherence of the labeled material to glass or plastic tubes, particularly during alcohol evaporation.

In Vivo Behavior of Labeled Lipoprotein. Table 3 summarizes pertinent data on both dogs. Figure 1 shows the specific activity-time curve of blood cholesterol fractions in dog #1, plotted to give the best visual fit. A similar curve was drawn for dog #2. In the case of dog #2, there were only three points beyond 24 hours, so that the slope of the terminal portion of the curve is subject to appreciable error. To allow comparison of the two sets of values, the slowest component of the curve was extrapolated to the ordinate, and this point was assigned a value of one. Figure 2 presents the data on both dogs, with the cholesterol specific activities expressed as a fraction of this intercept. The values for plasma free cholesterol in the two animals appeared to fall on the same line. Resolution of the entire free cholesterol curve by successive subtraction of each semilogarithmic component gave five exponential terms with half times of 4 minutes, 30 minutes, 65 minutes, 7 hours, and 96 hours (Fig. 3). Because of the few experimental points beyond 30 hours, the assumption that all the data fall on the same curve is somewhat arbitrary. However, the large number of points in close agreement prior to 30 hours prompted the use of this method of analyzing the data.

As shown in Figures 1 and 2, the red cell free, plasma free, and plasma ester cholesterol reached equilibrium in about 30 hours. The quantity of cholesterol which apparently equilibrated in 30 hours was arbitrarily designated C_e. This value was computed by dividing the quantity of injected radioactivity by the intercept of the slowest component of the curve for each animal. The measured quantity in the anatomic blood-liver pool was found to account for less than one-half of C_e (Table 4).

In two sera from each dog, taken 3 and 5 hours after injection and separated into lipoprotein fractions, virtually no activity was found in the fraction d > 1.21. The specific activity of free cholesterol in the fraction d = 1.063 to 1.21 was the same as that of free cholesterol in whole plasma.

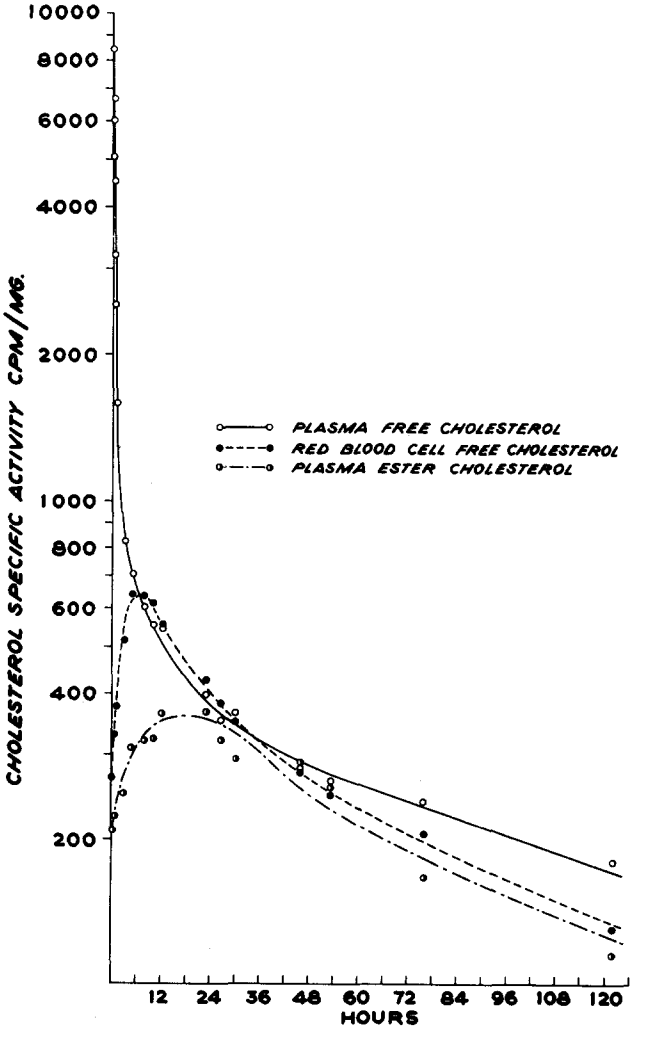

FIG. 1. Specific activities of blood cholesterol fractions after intravenous administration of lipoprotein-bound free cholesterol-4-C^{14} in dog #1.

TABLE 3. QUANTITY OF CHOLESTEROL IN PLASMA, RED BLOOD CELLS, AND LIVER OF DOGS

	Dog #1 (25.6 Kg)	Dog #2 (18.6 Kg)
Plasma		
Volume (ml)	1,100	800*
Free cholesterol (mg/100 ml)	30	56
Ester cholesterol (mg/100 ml)	73	136
Red blood cells		
Volume (ml)	688	500*
Free cholesterol (mg/100 ml)	157	172
Liver		
Weight (g)	760	590
Total cholesterol (mg/g)	2.08	2.45

* Estimated volume.

DISCUSSION

In Vitro Labeling. Because the precise location of cholesterol in lipoprotein molecules is not known, no absolute criterion is available for judging whether the material labeled by our method is identical with biosynthetically labeled free cholesterol. Several characteristics of the *in vivo* behavior of our labeled lipoprotein cholesterol strongly suggest that there is a close resemblance: (*a*) We found a smoothly curvilinear venous disappearance curve of labeled free cholesterol, whereas labeled cholesterol in emulsions characteristically disappears almost completely from the circulation within 1 hour and subsequently reappears, reaching maximal specific activity 24 to 48 hours after injection (1, 2, 3). Such behavior has been attributed to rapid phagocytosis of the emulsified lipid (1) and its subsequent slow release in lipoprotein form. (*b*) The injected cholesterol equilibrated rapidly with red cell free cholesterol. Hagerman and Gould (10), in studies using cholesterol labeled biosynthetically from C^{14}-acetate, reported a half time for this equilibrium of 70 minutes *in vitro*, which would result in 97% equilibration in slightly less than 6 hours. The average time for equilibration of plasma and red cell free cholesterol specific activities in our experiments was 5 hours, and graphical subtraction revealed a component with a half time of equilibration of 65 minutes (see below). (*c*) Specific activities in the major lipoprotein fractions were equal. Biggs and Nichols (11) and others (12) have shown that C^{14}-labeled free cholesterol in chylomicrons harvested from the thoracic duct of animals fed cholesterol-C^{14} rapidly equilibrates with unlabeled serum lipoprotein cholesterol during *in vitro* incubation.

TABLE 4. COMPONENTS OF EXCHANGEABLE CHOLESTEROL (C_e)

Cholesterol	Dog #1	Dog #2
	mg	*mg*
C_e	11,180	10,050
Plasma		
Free	330	450
Ester	800	1,090
Red blood cell	1,080	860
Liver	1,580	1,470
Blood-liver pool	3,790	3,870
C_e minus blood-liver pool	7,390	6,180

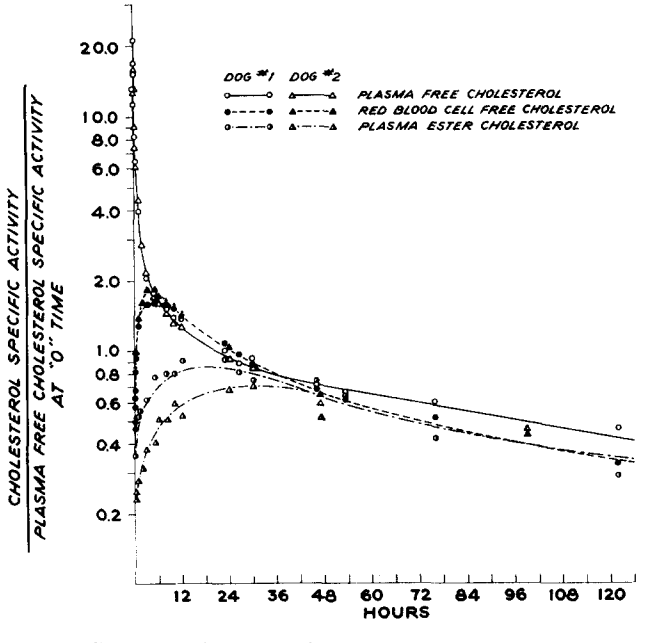

FIG. 2. Specific activities of blood cholesterol fractions after intravenous administration of lipoprotein-bound free cholesterol-4-C^{14} in two dogs. The extrapolated intercept of the slowest component of the plasma free cholesterol specific activity-time curve has been assigned a value of one.

Avigan (3) recently reported a similar method for incorporation of C^{14}-labeled sterols and steroids into lipoproteins; however, in contrast to our results, he found appreciable quantities of C^{14}-labeled material in the $d > 1.21$ fraction. In our studies the amount of C^{14}-labeled cholesterol taken up by low and high density lipoproteins was a function of the quantity of cholesterol already present, which suggests that the incorporation resulted from molecular exchange. The ease of introduction of the labeled material and its subsequent rapid interchange with red cell and liver cholesterol support the concept of a peripheral location for the free cholesterol normally contained in the lipoprotein molecule.

The ability to label lipoprotein cholesterol should permit more definitive studies of plasma cholesterol

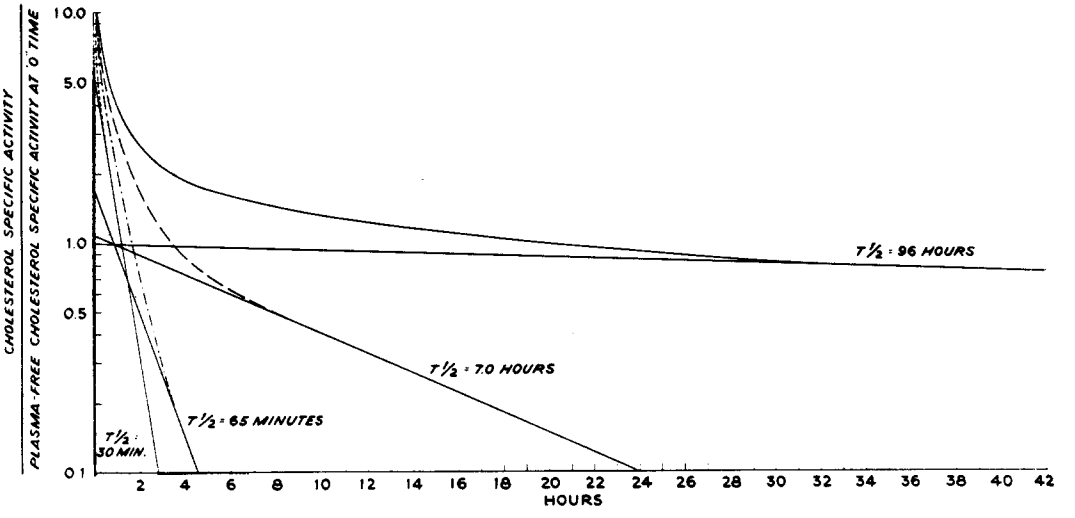

FIG. 3. Graphic resolution of the specific activity-time curve of plasma free cholesterol in Figure 2.

metabolism, including pathways of conversion to ester cholesterol and mechanisms of excretion.

Venous Disappearance Curves. The exponential rate constants derived from the disappearance of labeled plasma free cholesterol must be interpreted with caution, as a small error in the determination of the slope of the slowest component of the curve can result in magnified errors in the other components. With this in mind, however, we have compared the rate constants we observed with cholesterol turnover times reported in the literature. The second rate constant (half time 30 minutes) corresponds most closely to that reported by Eckles *et al.* (4) for exchange between free cholesterol of liver and plasma in dogs. The third rate constant (half time 65 minutes) is almost identical with that reported for exchange of free cholesterol between plasma and red blood cells *in vitro* (10). It is likely, therefore, that these two exchange reactions are major determinants of the second and third rate constants. The fourth rate constant (half time 7 hours) must be determined in part by formation of plasma ester cholesterol, since its specific activity was approaching that of plasma free cholesterol during the first 30 hours. The slowest component (half time 96 hours) includes metabolic turnover plus other slowly equilibrating compartments. This is a somewhat faster turnover time than that reported previously in the dog (13), but complete equilibrium with total body cholesterol undoubtedly did not occur in 4 days. The most rapid component (half time 4 minutes) cannot be related to any known physiological compartment.

Three possibilities could account for the finding that C_e was considerably larger than the anatomic blood-liver pool. First, labeled cholesterol could be lost irreversibly by excretion or chemical alteration prior to 30 hours. We have calculated the extent to which such loss could contribute to C_e based on the following considerations. Maximum fecal excretion of endogenous sterols and of bile acids is said to be 22 mg (14) and 15 mg (15), respectively, per kilogram body weight per day in dogs, and the specific activity of these compounds could not have exceeded the mean specific activity of plasma free cholesterol during this period. In dog #1, which weighed 25.6 kg, the mean specific activity of plasma free cholesterol during the first 30 hours was 730 cpm/mg. Calculated excretion of sterols and bile acids during this period should not have exceeded 1180 mg ($[22 + 15] \times 25.6 \times 30/24$), so that not more than 0.86×10^6 cpm (1180×730) could have been excreted during this time. This is only about 20% of the quantity of radioactivity injected, and the actual amount probably was considerably less than this. Similar calculations lead to equivalent results in dog #2. Second, there may be anatomical sites other than red blood cells and liver in which equilibrium is complete in 30 hours. To our knowledge, such sites have not been described. Third, incomplete equilibration with cholesterol may take place in still other sites. Gould (16) administered C^{14}-acetate to dogs and injected their blood containing C^{14}-cholesterol into recipient animals. After 30 hours the specific activity of cholesterol of visceral organs and muscle and connective and adipose tissues was 30% to 50% that of plasma free cholesterol. Therefore the third possibility appears to offer the best explanation of the magnitude of C_e. It is clear that C_e does

not correspond to any anatomical compartment and includes cholesterol not in isotopic equilibrium at 30 hours.

Special note should be made of the fact that the specific activity of plasma ester cholesterol, despite its greater experimental variability, never exceeded that of plasma free cholesterol. This finding differs from those of previous studies performed after feeding isotopically labeled cholesterol or acetate or injecting labeled acetate (17, 18, 19). Our results, in agreement with those of other workers (20, 21), suggest that liver free cholesterol, which is in isotopic equilibrium with plasma free cholesterol, is not the only precursor of plasma ester cholesterol.

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