ABSTRACT
Position 4 labeled carbon-14 cholesterol was placed in abomasums (stomachs) of two lactating goats. Blood and milk samples were collected from the animals for 5 to 13 days. Specific activities of cholesterol and cholesteryl esters in various fractions of blood serum and milk were determined to reveal pathways by which dietary cholesterol enters milk. Results with the two animals showed similar trends. Within 24 h both cholesterol and cholesteryl esters of the three principal serum lipoproteins of the goat were labeled, and this labeling persisted in substantial degree for the 13-day experiment. Specific activities for cholesteryl esters in milk fat globules exhibited several remarkable attributes: they fluctuated in intensity with a 3-to 4-day cycle reaching a maximum at 7 to 8 days after tracer injection; they exceeded cholesteryl ester specific activity in the skim milk by an order of magnitude; and at their maximum they exceeded all specific activities for serum components. The results of this investigation exemplify the ease with which dietary cholesterol enters and crosses membranes in the animal body.

INTRODUCTION
The transport of dietary cholesterol into milk has been investigated in the rat (4, 5, 9), the rabbit (6), and the guinea pig (6, 16). Blood is only one source of milk cholesterol, the other is de novo synthesis within the mammary gland (19). When \(^{14}\text{C}-\text{cholesterol was fed to lactating}

rats, Easter (9) found maximum specific activity of total cholesterol in the blood at 8 to 10 h and maximum activity in the milk at 25 to 50 h. In the goat, labeled dietary cholesterol was in the blood and milk in increasing specific activity over an 8-day experiment (D. J. Easter, unpublished). Arterio-venous difference studies across the mammary gland in vivo indicated no net uptake of serum cholesterol in the goat (15). However, cholesterol concentration in goat serum is 10 to 20 times that in the milk which could make removal of significant amounts from serum into milk difficult to detect. Also, it is possible that only an exchange of cholesterol takes place between serum and mammary tissue.

Previous work from this laboratory has demonstrated that high density lipoproteins (HDL, 1.063<\(\rho<1.21\)) are the major cholesterol bearing lipoprotein in the bovine; the low density lipoproteins (LDL, 1.006<\(\rho<1.040\)) and very low density lipoproteins (VLDL, \(\rho<1.006\)) contained lesser concentrations of total cholesterol (21). However, triglycerides of VLDL contribute fatty acids to triglycerides of milk fat (3), and it is possible that VLDL cholesterol also is exchanged in this transport process. Bovine HDL are essentially devoid of triglyceride (21).

Although each lipoprotein class is unique in its cholesterol and cholesteryl ester content (21), the possible contribution of each lipoprotein class to milk cholesterol has not been defined. In this study radioactive cholesterol was injected into the abomasum of two lactating goats. Milk and blood samples were collected and analyzed to examine cholesterol transport mechanisms involved.

MATERIALS AND METHODS
Two lactating goats from the University herd were used in this study. Both animals were at mid-lactation and were maintained on hay and grain ad libitum throughout the experiments.
Labeled cholesterol was obtained from New England Nuclear Corporation, Boston, MA; 200 \( \mu \text{Ci} \) of \([4-\text{\textsuperscript{14}C}]\) cholesterol were dissolved in 5 ml of corn oil and administered by injection directly into the abomasum (true stomach) of the fed, lactating goat.

Milk was collected at 12-h intervals. In the first experiment, 40-ml samples of blood were collected by jugular venipuncture at 24, 72, 120, 168, 216, and 312 h following injection of the tracer. In the second experiment blood was collected similarly by venipuncture at 3, 6, 12, 24, 48, 96, and 144 h.

Blood samples were allowed to clot for 2 h at room temperature. Serum was obtained subsequently according to the procedures of de Lalla and Gofman (7). An inhibitor of cholesterol – cholesteryl ester interactions (14), 5, 5'-dithio-bis-(2-nitrobenzoic acid) was added.

Lipoprotein fractions were separated in an International Preparative Ultracentrifuge (Model B-35 with A-269 rotor) by the method of Puppione et al. (20). Serum lipoproteins were resolved into three density classes, VLDL, LDL, and HDL.

Lipids of whole serum and lipoprotein fractions were extracted according to the method of Folch et al. (13). The lipid extract was dried under a nitrogen atmosphere, percolated over a sodium sulfate column in ethyl ether to remove residual water, again evaporated to dryness, and weighed.

The freshly drawn milk was cooled to 10 C, centrifuged (International Centrifuge, Model K) in 50 ml tubes at 2,500 rpm for 15 min to obtain layers of compacted milk fat globules and infranatant skim milk. After hardening the globule layers by placing the tubes in ice water for 30 min, the two phases were separated mechanically and then extracted (2) to obtain the lipids. Extracting solvents were removed from the lipid samples with a rotating evaporator at 40 C.

Aliquots of the lipid extracts from blood and milk were separated to yield cholesterol and cholesteryl ester fractions by thin layer chromatography on plates of silica gel G (Supelco, Bellefonte, PA). The solvent system was petroleum ether, ethyl ether, and acetic acid (80:20:1). The developed plates were placed in an iodine saturated atmosphere for a few seconds to facilitate spot identification. Free and ester cholesterol were quantitated by the colorimetric method of Searcy and Bergquist (22). Cholesterol and cholesteryl ester spots were scraped and eluted with ethyl ether for analysis. Radioactivity was measured by scraping the appropriate cholesterol or cholesteryl ester spots into vials and counting in scintillation fluid \([2, 5\text{-diphenyloxazole, } 5\text{ g/liter and } 1,4\text{-bis-2-(5-phenyloxazolyl)-benzene, } 100\text{ mg/liter in toluene}]\). Data are reported graphically as cpm/mg cholesterol.

**RESULTS**

**Serum**

Specific activity values for cholesterol of the serum lipoproteins in the first experiment are summarized in Fig. 1. With the exception of the high initial value in the LDL fraction at 24 h, the three lipoprotein classes show activities of the same order of magnitude gradually declining over the 13-days. The cholesteryl ester specific activities of the serum lipoproteins for the same experiment are in Fig. 2. VLDL demonstrated peak cholesteryl ester specific activity at 24 h while HDL and LDL did not reach peak activity until 72 h.

Data from the second experiment (not shown) confirmed the general activity for the 2

![FIG. 1. Specific activities of free cholesterol in serum lipoproteins of a lactating goat following injection of \([4-\text{\textsuperscript{14}C}]\) cholesterol (200 \( \mu\text{Ci} \)) into the abomasum. VLDL \( \circ--\circ \), LDL \( \bullet--\bullet \), HDL \( \square--\square \).](image-url)
to 6 day period in Fig. 1 and 2. In addition they revealed that specific activities at 3 h in cholesteryl esters of VLDL (5500 cpm/mg) and in free cholesterol of HDL (4700 cpm/mg) and LDL (2400 cpm/mg) fell to lower values at 6 h. Activities of 1 to $2 \times 10^5$ cpm/mg prevailed for all components in the 6 and 12 h samples with all exhibiting higher values at 24 h. These data suggest the existence of serum lipoproteins with substantial radioactivity within 3 h of tracer injection.

The transport of dietary $[1^{14}C]$ cholesterol into milk in the first experiment is shown in Fig. 3. Cholesterol associated with milk fat globules exhibited maximum activity at 2 days whereas that in the skim milk reached a peak at 3 days. Results in the second experiment (not shown) were similar to those of the first. The trend of the data in these two experiments with maxima at 48 to 72 h closely resemble those presented by Connor and Lin (6) for the guinea pig and our more recent data (unpublished) for the rat.

Specific activities of cholesteryl esters in fat globules and skim milks for the first and second experiments are in Fig. 4 and 5, respectively. These data are notable in several respects. One is the remarkable cyclic phenomenon in specific activity of cholesteryl esters of the milk fat globules. This effect is also observable in previously cited data of Easter for specific activity of total cholesterol in goats milk (see Fig. 6 in reference 18). Another unique aspect of the data of Fig. 4 and 5 is that the specific activity of the cholesteryl esters in the two phases of milk differ by an order of magnitude. Specific activities of the cholesteryl esters of fat globules emerging in milk required 7 to 8 days after injection to achieve maxima, and at that time they were higher than any values encountered at any time for free or esterified choleste...
Discrimination of cholesterol and cholesteryl esters in any of the serum lipoproteins during either experiment.

The distribution of cholesterol and cholesteryl esters between milk fat globules and skim milk is summarized in Table 1 for the 15 milkings collected during the 13-day period of the first experiment. The range of cholesterol content in esterified form (1.6 to 5.2%) is lower than the 5 to 10% reported for cow's milk (8).

**DISCUSSION**

Efforts to quantify the contribution of dietary cholesterol, serum cholesterol, and cholesterol synthesized de novo in mammary tissue as sources of milk cholesterol have encountered the difficulty of defining which vehicle(s) of the serum supplies cholesterol to milk. For example in studies of the rat by Clarenburg and Chaikoff (5) it was assumed that chylomicrons are the principal donors of cholesterol from plasma to milk. This enabled them to calculate from radioactivity data that 11% of milk cholesterol arose from the diet. On the other hand assuming that serum cholesterol, in total, is a uniform precursor of milk cholesterol, their data imply that 80% of milk cholesterol is derived from the diet.

The assumption that chylomicrons and VLDL would contribute cholesterol to milk is reasonable. Triglycerides of these lipoproteins supply fatty acids for the synthesis of milk fat (1, 3, 21). In lactating ruminants VLDL is an important substrate because their serum rarely contains significant numbers of chylomicrons. At the time that lipoprotein lipase of the mammary capillaries acts on these substrates (16, 23) it is to be expected, as for the guinea pig (16), that cholesterol would be taken up by the tissue. Free and esterified cholesterol of the chylomicrons may be removed by mammary tissue without prior passage through the liver.

**TABLE 1. Cholesterol distribution in milk (goat).**

<table>
<thead>
<tr>
<th></th>
<th>Free</th>
<th>Esterified</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Fat globules</td>
<td>8.49 (65.7%)</td>
<td>4.76 - 12.37</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.44 (34.3%)</td>
<td>1.95 - 6.39</td>
</tr>
<tr>
<td>Whole milk</td>
<td>12.93 (97.4%)</td>
<td>8.80 - 17.79</td>
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Analysis of 15 milkings during 13 days by the method of Searcy and Bergquist (22).
Certain of the findings in our investigation are consistent with this latter observation. In both of our experiments VLDL exhibited the highest specific activity in cholesteryl esters among the serum lipoproteins during the first 24 h. In fact a high cholesteryl ester activity in this lipoprotein is implied shortly after tracer injection since in the second experiment this activity appears to be falling between 3 and 6 h post-injection. The very high specific activities in cholesteryl esters of milk fat globules in both experiments might also be explained by rapid transport of cholesteryl ester from intestinal mucosa to mammary tissue via VLDL or chylomicrons followed by incorporation of the ester intact into milk fat globules. The remarkable cycling in the specific activity of these latter cholesteryl esters appeared to have no precursor pattern in the blood. Some rhythmic functioning of cells, membranes, or capillaries of the mammary gland seems required to explain the phenomenon.

Information on the forms and distribution of cholesterol in milk has been reviewed (18). Both free and esterified cholesterol of goat's milk occur mainly in membrane material derived from the lactating cell. That in the skim milk is contained in membranous vesicles; that of milk fat globules is derived by their envelopment with plasma membrane in the secretion process. However, some cholesteryl ester appears to be associated with glyceride core of the fat globule, and some free cholesterol may be associated with the surface of the fat globule as it exists in the cell prior to secretion.

In our experiments, trends in the specific activity of cholesterol and cholesteryl esters among the serum lipoproteins over time show an extensive equilibration of label but do not establish clearly precursor-product relationships. However, the data can be considered in the light of a number of predictable events and other published evidence. The labeling of VLDL and chylomicron at the time of their synthesis in intestinal mucosa probably represents the earliest incorporation of dietary cholesterol into serum lipoprotein. The action of lecithin-cholesterol acyl transferase enzyme, which has been studied in ruminants (17), may account for the early and intense labeling of cholesteryl esters in VLDL.

The various drops in cholesterol and cholesteryl ester specific activities after 3 h followed by rises some hours later suggest clearance of labeled species from the blood followed by their partial reemergence, particularly from the liver. The removal of triglycerides from VLDL by lipoprotein lipase in various tissues of the body including the mammary gland would be expected to increase their density and possibly transform them to LDL. Recent evidence indicates that both LDL and HDL components are derived from VLDL (10, 11). With respect to exchange of cholesterol and cholesteryl esters among serum lipoproteins, in vitro experiments by Evans and Patton (12) showed that both lipid species tended to move from bovine low density to high density serum lipoprotein. In general we interpret our results as indicating an initial labeling of VLDL by dietary \([^{14}\text{C}]\) cholesterol followed by equilibrium of this label in free and esterified form to the other serum lipoproteins.

Additional experiments will be required to define more clearly which serum vehicle(s) donate cholesterol to milk. In a preliminary experiment in which individual serum lipoprotein classes of a fasted rat were isolated after in vivo labeling with \([^{14}\text{C}]\) cholesterol, and then these lipoproteins were injected individually into second (lactating) rats, we observed significant labeling of free cholesterol in milk by all three of the classical serum lipoproteins, i.e., VLDL, LDL, and HDL. While serum of the fasted rat contains no chylomicrons, we presume that these too would be capable of donating cholesterol to milk.

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