Evidence for a contribution by the intestinal wall to the serum cholesterol of the rat

CHARLES A. LINDSEY, JR., and JEAN D. WILSON*

Department of Internal Medicine, The University of Texas Southwestern Medical School, Dallas, Texas

SUMMARY Rats were fed cholesterol in order to block hepatic cholesterol synthesis, and their intestinal lymph ducts were cannulated. Experiments with these rats showed that cholesterol synthesized in the intestinal wall enters into the circulating cholesterol pool. The quantitative significance of this source of serum cholesterol has not been established.

KEY WORDS cholesterol · serum · lymph · biosynthesis · intestinal wall · lymph duct cannulation · rat · Δ^7-cholestenol

ALTHOUGH IT WAS established as early as 1950 that cholesterol is synthesized in virtually every tissue of the body (1), the possible role of extrahepatic tissues in contributing synthesized cholesterol to the blood has never been resolved. Indeed, Hotta and Chaikoff (2) concluded on the basis of studies of cholesterol-C^{14} turnover in intact versus functionally hepatectomized rats that the liver is the main endogenous site of origin of serum cholesterol and that tissues other than liver contribute little or no cholesterol to the serum. However, even in rats fed a diet containing 2% cholesterol for periods of 4–6 weeks, a regimen which is known (3) to suppress hepatic cholesterol synthesis in this animal, Morris et al. (4) found that 10–18% of serum cholesterol was of endogenous origin. The purpose of the present study was to reinvestigate the origin of this nonhepatic fraction of serum cholesterol.

Of the nonhepatic tissues that synthesize cholesterol the intestine was thought to be a likely source of serum cholesterol. Not only does this tissue synthesize cholesterol (1, 5), but in addition, it lacks a negative feedback system and consequently synthesizes cholesterol even in the cholesterol-fed rat (3, 6). Furthermore, as the site of cholesterol absorption into lymph the intestine contains a potential route for the release into the circulation of endogenous as well as dietary cholesterol. The experiments to be described in the present report were designed to investigate the possibility that the intestinal wall represents a source of serum cholesterol.

PROCEDURE

Male rats of the Sprague-Dawley strain, weighing 200–300 g, were allowed free access to a diet containing 0.5% cholesterol[1] for periods varying from 5 days to 2 weeks. Intestinal lymph duct cannulations were performed under ether anesthesia by a modification of the method of Bollman, Cain, and Grindlay (7), and the animals were placed in restraining cages and allowed free access to food and water. In most experiments the animals were allowed to recover for 12–16 hr before further study. Acetate-2-C^{14} in normal saline (0.5–1.0 ml) was injected intravenously into the leg vein. Blood samples were obtained by intermittently bleeding the tail, and total lymph and bile samples were collected at intervals for periods of 4–12 hr. At the end of the experimental period the animals were killed, and in some experiments the carcasses were dissected for further processing.

In two experiments acetate-2-C^{14} was infused over a 9–13 hr period into the central tail vein at a rate of 0.45 ml/hr by an infusion pump. In some instances high bile duct cannulations were also performed at the time of

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[1] To a prepared diet obtained from General Biochemicals Inc. (Chagrin Falls, Ohio), cholesterol dissolved in oleic acid was added so that the final composition of the diet was as follows: casein, 20%; sucrose, 56%; nonnutritive fiber, 3.8%; USP XIV salt mix, 3.8%; oleic acid, 5%; cholesterol, 0.5%; and added vitamins.

lymph duct cannulation, and bile samples were collected in addition to lymph and blood; in one experiment bile previously collected from a donor animal was infused into the distal half of the bile duct at a rate of 1.0 ml/hr by means of an infusion pump for 9 hr, while endogenous bile was drained from the proximal duct. Finally, in one experiment a cannula was implanted into the ileum at the time of operation, and intestinal contents were periodically washed into collecting vials during the experiment.

METHODS AND MATERIALS

The tissue and fluid samples were saponified in an excess of KOH at 150° and 15 psi for 30 min. An equal volume of ethanol was added, and the samples were extracted with pentane. The alcoholic KOH layer was aspirated, and the samples were backwashed with 5 ml of aqueous ethanol. The pentane layer was taken to dryness by heating, and cholesterol digitonide was precipitated from the residue and washed by the method of Sperry and Webb (8). The digitonide was dissolved in methanol; one aliquot was assayed for C^{14} in a Packard Tri-Carb Liquid Scintillation Spectrometer, and cholesterol content was determined on another portion by the Liebermann-Burchard reaction (8).

In order to determine whether the C^{14} of the washed digitonide was actually in cholesterol, free sterols were regenerated from pooled digitonide as described by Sperry (9). Dibromination was performed on one aliquot of the recovered sterol after the addition of carrier cholesterol by the method of Fieser (10); the specific activity was 106 cpm/mg before bromination and 78 cpm/mg after bromination. Another portion was chromatographed by thin-layer chromatography in benzene–ethyl acetate 2 : 1. The entire chromatogram was divided into fractions, which were eluted with ethyl ether and assayed for C^{14}; 96% of the C^{14} was found in the cholesterol area. Finally, an aliquot was analyzed by gas–liquid chromatography on a 1% SE-30 column (230°, with an argon flow of 96 ml/min); 97% of the recovered C^{14} was present in the area corresponding to the cholesterol peak. A small radioactive noncholesterol peak, about 5% as large as the cholesterol area and with the R_F of Δ^7-cholestenol, was demonstrated in this system but was not further identified.

In experiments in which free and esterified cholesterol were separated, chloroform–methanol (2 : 1) extracts of lymph were taken to dryness, dissolved in benzene, and chromatographed on silicic acid columns by a previously described modification (11) of the method of Frantz et al. (12). After saponification of the esterified fraction, digitonide was formed, washed, and assayed for cholesterol and C^{14} as before.

RESULTS

The results of experiments designed to determine whether cholesterol synthesized in the intestinal wall does enter the circulating pool of cholesterol are shown in Fig. 1. Rats which had cannulae in the intestinal lymph duct were placed in restraining cages, and acetate-2-C^{14} (2×10^7 cpm) was injected intravenously. Lymph and blood samples were collected at hourly intervals. Cholesterol was extracted from blood and lymph; specific activities of blood and lymph cholesterol were determined and plotted against time. The specific activities of blood cholesterol are shown by the broken line, and the specific activities of lymph cholesterol are shown by the solid lines. It is apparent that the specific activity of blood cholesterol remains zero while the specific activity of lymph cholesterol rises to 400–1300 cpm/mg within 4–5 hr. In each instance the specific activity of lymph cholesterol is greater than that of blood cholesterol at all times studied, which excludes the possibility that the lymph cholesterol-C^{14} was derived from the blood.

Although these experiments suggested that the intestine serves as a source of serum cholesterol, the dilution of the lymph cholesterol-C^{14} was too great to allow detection of cholesterol-C^{14} in blood. Therefore, in order to prove that the intestine does contribute cholesterol into the blood, an amount of acetate-C^{14} sufficient to label the blood was administered (Fig. 2). Acetate-2-C^{14} (5×10^8 cpm in each animal) was infused for 9 or 13 hr into the tail veins of rats that had been fed cholesterol for 5 days; in each of the experiments one of the two rats (No. 1) had a lymph duct cannulation, and blood and lymph were collected from this animal. From the other rat (No. 2), which served as the control, only blood was collected. Radioactive cholesterol in the lymph of the rats with cannulae in the intestinal lymph ducts rose to peaks of about 3,000 and 15,000 cpm/mg by the end of the experiments, whereas, as before, the blood cholesterol remained unlabeled. In the control animal with the intact lymphatic circulation, however, blood cholesterol reached a specific activity of 440 and 892 cpm/mg; this is approximately the degree of dilution one would expect if about 2 ml of the respective radioactive lymphs were diluted by the entire blood volume. These experiments were interpreted as providing evidence that the intestine does contribute cholesterol of endogenous origin into the blood of the rat.

In order to exclude the possibility that the cholesterol-C^{14} in lymph was arising from a small but rapidly turning over pool which was reaching the intestine via the bile instead of from the intestinal wall itself, the experiment described in Fig. 3 was performed. Again, the intestinal lymph duct was cannulated, and a double bile duct cannulation was also performed. The cannula proximal to

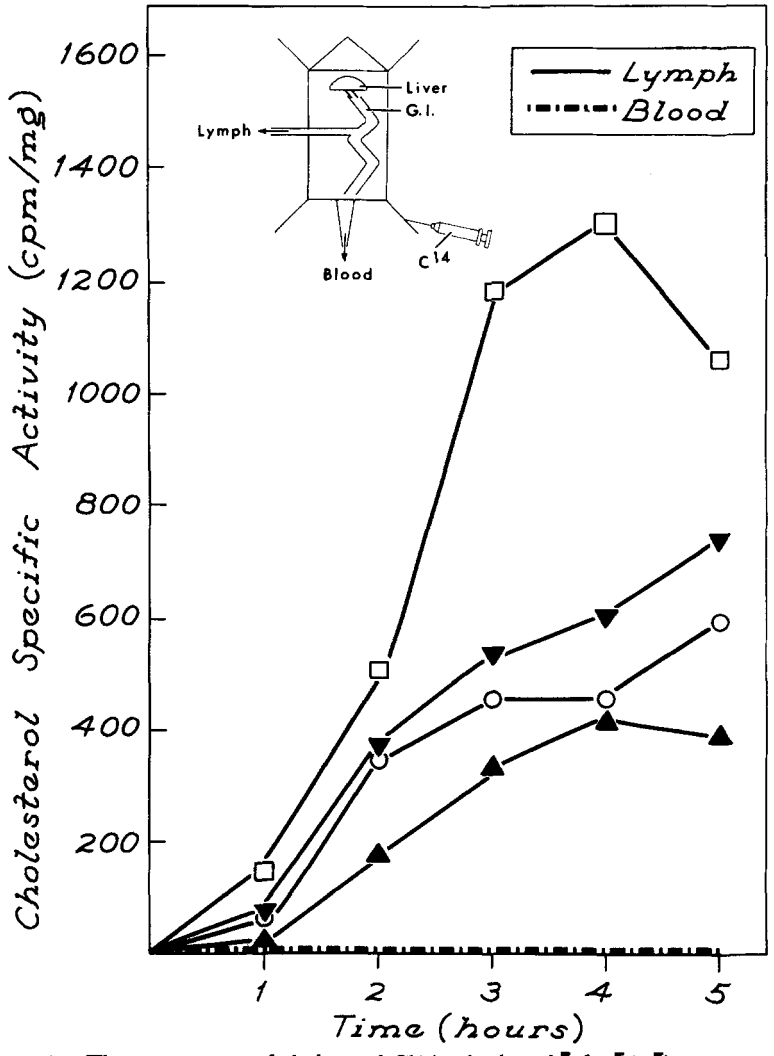

FIG. 1. The appearance of cholesterol-C^{14} in the lymph after the injection of acetate-2-C^{14}. Four male rats (250–290 g) were allowed free access to diets containing 0.5% cholesterol[1] for 8–14 days. Following cannulation of the intestinal lymph duct, each animal was placed in a restraining cage and given by injection into the leg vein acetate-2-C^{14} (2×10^7 cpm; 22.5 μc/μmole). Blood and lymph samples were collected as indicated and assayed for cholesterol content and C^{14}.

the liver drained the animal's bile away, while bile from a donor animal was infused into the distal portion of the bile duct via a constant infusion pump. This preparation was designed to maintain the enterohepatic circulation in a physiological state. The animal was placed in a restraining cage and injected with acetate-2-C^{14}. Hourly lymph, blood, and bile samples were collected, and the cholesterol specific activities plotted against time. As shown on the upper portion of the figure the specific activity of lymph cholesterol showed the same type of rise as in the previous group of animals, while both blood and bile cholesterol remained essentially unlabeled. The bottom half of the figure contrasts the cumulative excretion of cholesterol in lymph and bile, the cumulative excretion of radioactivity into bile also being virtually zero. These experiments, then, have demonstrated that the cholesterol-C^{14} in the lymph of the cholesterol-fed animal must have originated in the intestinal wall itself.

These data, however, do not yield any information as to the form in which cholesterol is contributed by the gut wall to the circulation. The experiment described in Fig. 4 was designed to determine whether the C^{14} from acetate appeared preferentially in the esterified or free cholesterol of lymph. The lymph cholesterol was separated by silicic acid chromatography into esterified and free fractions, and both the cumulative excretion of C^{14} (upper panel) and the specific activity of the two cholesterol fractions (lower panel) were plotted against time. More radioactive cholesterol appeared in the esterified fraction of lymph than in the free fraction; the percen-

tages of esterified cholesterol-C^{14} in lymph in these two rats (71 and 72%) were similar to the percentage of esterified cholesterol-4-C^{14} in lymph following the feeding of cholesterol-4-C^{14} (13). As demonstrated by the specific activity curves in the lower two panels, the specific activities of free and esterified cholesterol-C^{14} were similar at each time studied, suggesting that both the free and esterified fractions are derived from a common pool.

Two different types of experiments have been performed in an attempt to elucidate the mechanism by which the endogenously synthesized cholesterol reaches the lymph. In Fig. 5 two experiments are shown in which one animal had only lymph duct cannulation whereas the other animal had cannulae placed in both bile and lymph ducts. Acetate-C^{14} was injected into each animal, and lymph was collected at intervals and ana-

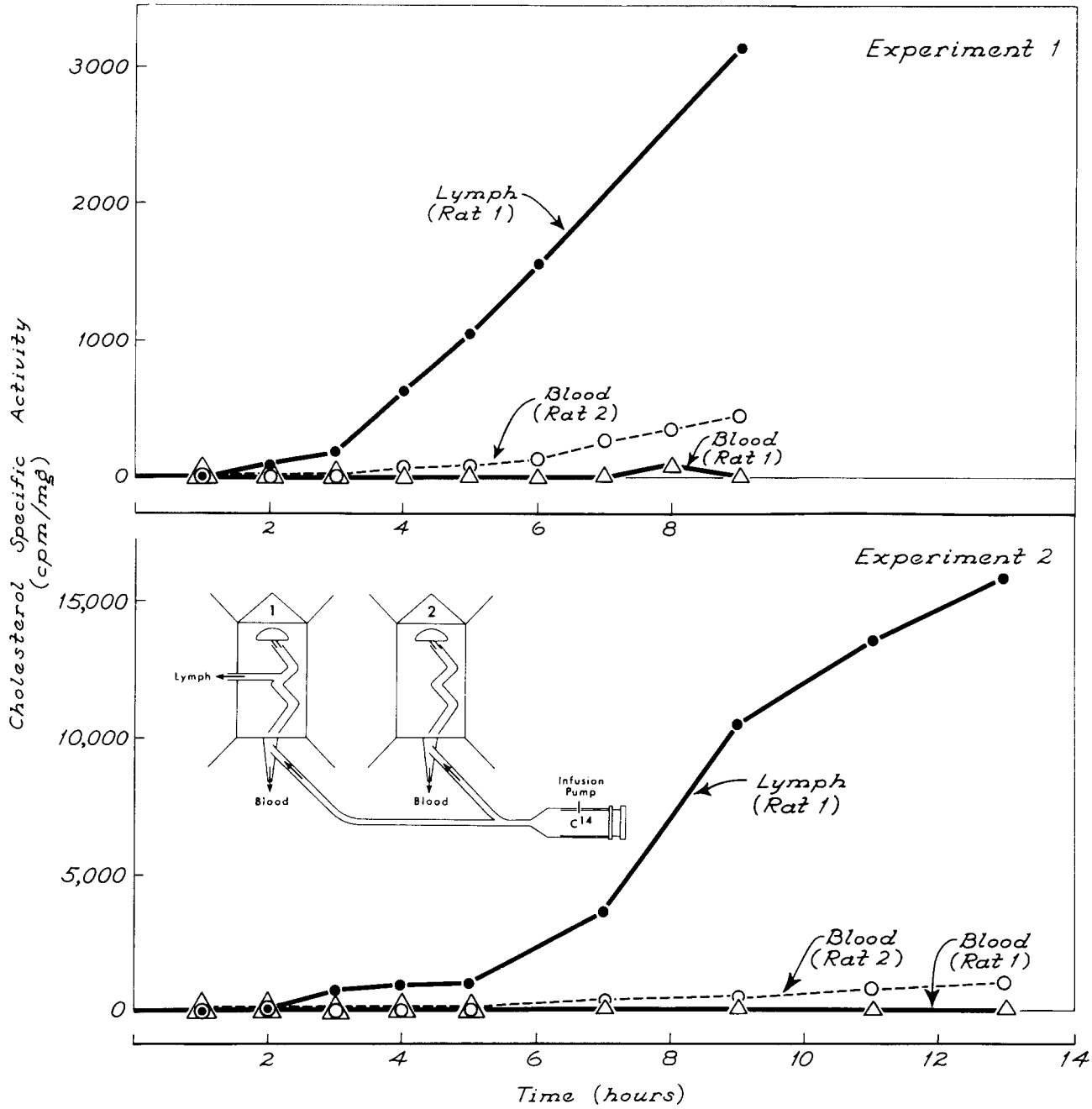

FIG. 2. The influence of lymph duct cannulation on the appearance of cholesterol-C^{14} in the blood of rats fed cholesterol and injected with acetate-2-C^{14}. Rats weighing 300–440 g were allowed free access to the 0.5% cholesterol diet for 2 weeks. In each experiment a lymph-cannulated (rat 1) and control rat (rat 2) were administered by constant infusion into the tail vein 0.45 ml/hr of normal saline containing 5×10^8 cpm acetate-2-C^{14} (22.5 μc/μmole) for either 9 or 13 hr. Blood or lymph and blood samples were collected from each animal as indicated and assayed for cholesterol content and cholesterol-C^{14}.

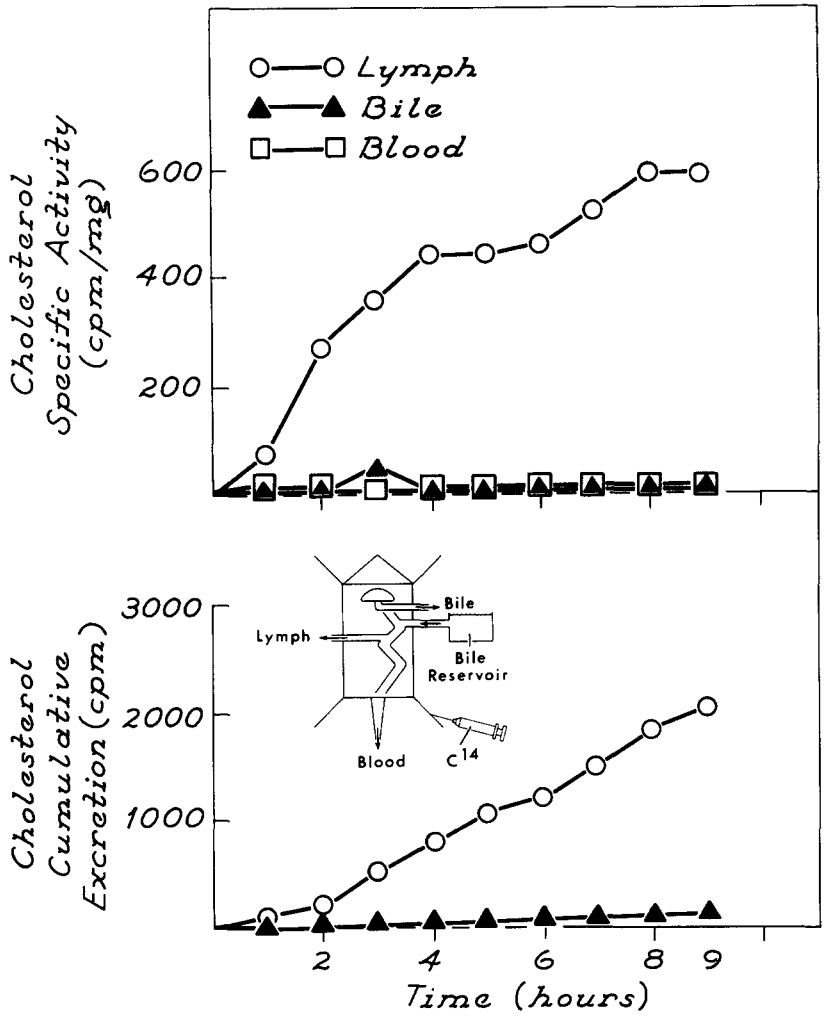

FIG. 3. Comparison of the appearance of cholesterol-C^{14} in lymph and bile after the administration of acetate-2-C^{14} to a cholesterol-fed rat. A rat (240 g) which had been fed the 0.5% cholesterol diet for 2 weeks had cannulae placed in both the intestinal lymph duct and the distal and proximal portions of the bile duct. Unlabeled bile from a donor animal was infused into the distal portion of the bile duct at a rate of 1 ml/hr via a constant infusion pump. Acetate-2-C^{14} (2×10^7 cpm; 22.5 $\mu c/\mu$mole) was injected into the leg vein, and lymph, blood, and bile samples were collected as indicated. Both the change in the specific activity of cholesterol-C^{14} in lymph, bile, and blood (upper portion) and the cumulative excretion of cholesterol-C^{14} into lymph and bile (bottom portion) have been plotted against time.

lyzed for cholesterol-C^{14} specific activity. In both instances the deprivation of bile markedly depressed the appearance of cholesterol-C^{14} in the lymph, suggesting that bile is required for the transfer of endogenous cholesterol from the wall into the lymph. However, in these experiments lymph flow averaged 0.5 ml/hr in the animals with an intact bile flow and only 0.15 ml/hr in the animals with both bile and lymph duct cannulations. Therefore, the enhancement of the appearance of radioactive cholesterol in the lymph of these animals may be secondary to an acceleration of lymph flow by bile rather than to a specific effect of bile on the transport of cholesterol from the gut wall to the lymph. At the end of the experiments, the intestines were removed and the contents were washed out and analyzed for cholesterol-C^{14}. The total counts recovered in the digitonin-precipitable material of lymph, intestinal wall, and intestinal contents are listed in Table 1. Of the cholesterol-C^{14} recovered in these fractions about 15% had been released into lymph 10 hr following the injection of acetate-2-C^{14} into animals with lymph duct cannulations, whereas only about 1% was released into lymph in the animals with the double cannulation. Consequently, it was clear that the effect of short-term bile duct cannulation was on the release of cholesterol-C^{14} into lymph rather than on cholesterol synthesis.

Finally, in an attempt to determine whether or not cholesterol-C^{14} was excreted into the lumen and reabsorbed along with the dietary cholesterol or secreted directly into lymph, the experiment shown in Fig. 6 was performed. In addition to the cannulation of the intestinal lymph duct an indwelling cannula was placed in the lumen of the jejunum so that hourly samples of intestinal contents as well as of lymph could be studied after the injection of acetate-2-C^{14}; the assumption was made that the lower jejunum would constitute a representative fraction of the intestinal wall for this study. In this experiment the specific activities of cholesterol of

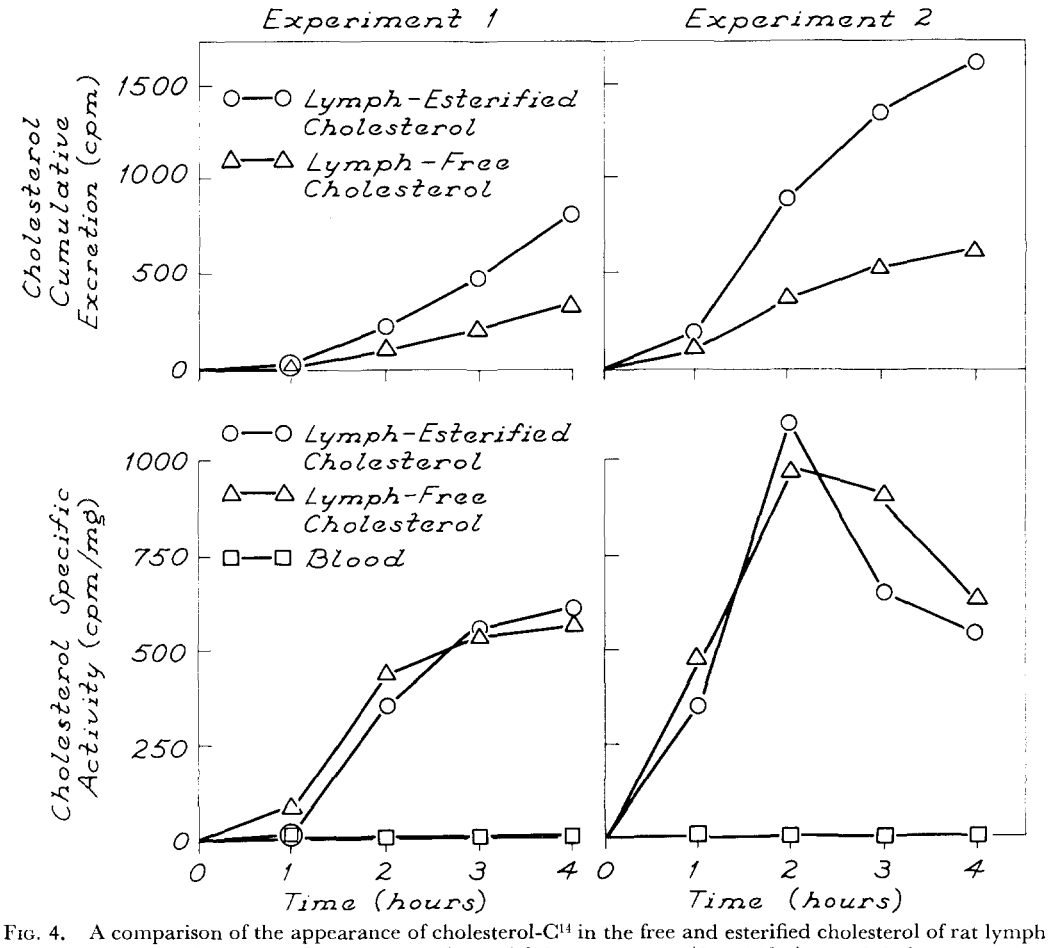

FIG. 4. A comparison of the appearance of cholesterol-C^{14} in the free and esterified cholesterol of rat lymph after the injection of acetate-2-C^{14} into the cholesterol-fed rat. Two rats (280–345 g) were fed diets containing 0.5% cholesterol for 3 weeks. Following cannulation of the intestinal lymph duct, the animals were given acetate-2-C^{14} as described in the legend to Fig. 1. Both the cumulative excretion of free and esterified cholesterol-C^{14} into lymph (upper portion) and the change in the specific activity of esterified and free cholesterol of lymph and of total blood cholesterol (bottom portion) have been plotted against time.

TABLE 1 DISTRIBUTION OF CHOLESTEROL-C^{14} AMONG INTESTINAL WALL, LYMPH, AND INTESTINAL CONTENTS 10 HR AFTER THE INJECTION OF ACETATE-2-C^{14}

Expt. No.	Rat No.	Weight	Treatment	Cholesterol-C^{14} Recovered in: Intestinal Lymph	Lymph	Intestinal Contents	Total	Percentage of the Intestinal Cholesterol Released into Lymph
		g		*cpm*	*cpm*	*cpm*	*cpm*	
1	1	210	Lymph duct cannulated	127,740	25,064	13,940	166,744	15.0
	2	215	Lymph and bile ducts cannulated	154,580	2,220	20,600	177,180	1.2
2	1	195	Lymph duct cannulated	214,580	49,170	19,890	283,640	17.3
	2	235	Lymph and bile ducts cannulated	242,280	2,699	39,810	284,789	0.9

The treatment of the animals is described in the legend to Fig. 5.

lymph and intestinal contents were similar at each time interval studied. The object of the experiment was not accomplished, and the results are merely compatible with the possibility that it is secreted in both directions, since at the earliest time interval studied (1 hr) the two specific activities were similar.

DISCUSSION

The present studies were designed to determine whether cholesterol synthesized in the intestinal wall contributes to the circulating cholesterol of the rat. In designing the study advantage was taken of two previous demonstrations, namely that the feeding of cholesterol to the rat suppresses the synthesis of cholesterol by the liver but not by the intestine (3, 6), and that the intestinal lymph represents the sole means by which the intestine contributes cholesterol into the circulation (14). Therefore, the cholesterol-fed, lymph-cannulated rat has been utilized for a study of the conversion of acetate-C^{14} to circulating cholesterol-C^{14}. The results of these experiments clearly demonstrate that cholesterol-C^{14} does appear in the intestinal lymph of cholesterol-fed rats injected with acetate-C^{14}. Furthermore, evidence has accrued showing that this cholesterol-C^{14} did not reach the lymph via either blood or bile and must, therefore, have arisen in the gut wall itself. Consequently, it can be concluded that in contrast to previous assumptions (2), cholesterol synthesized in the intestinal wall does contribute to the circulating cholesterol pool.

The quantitative importance of this source of circulating cholesterol cannot be assessed by the present studies. The demonstration in the blood of cholesterol that was synthesized in the intestine does not necessarily imply that the intestine makes a net contribution to the blood cholesterol; this contribution may be of trivial impor-

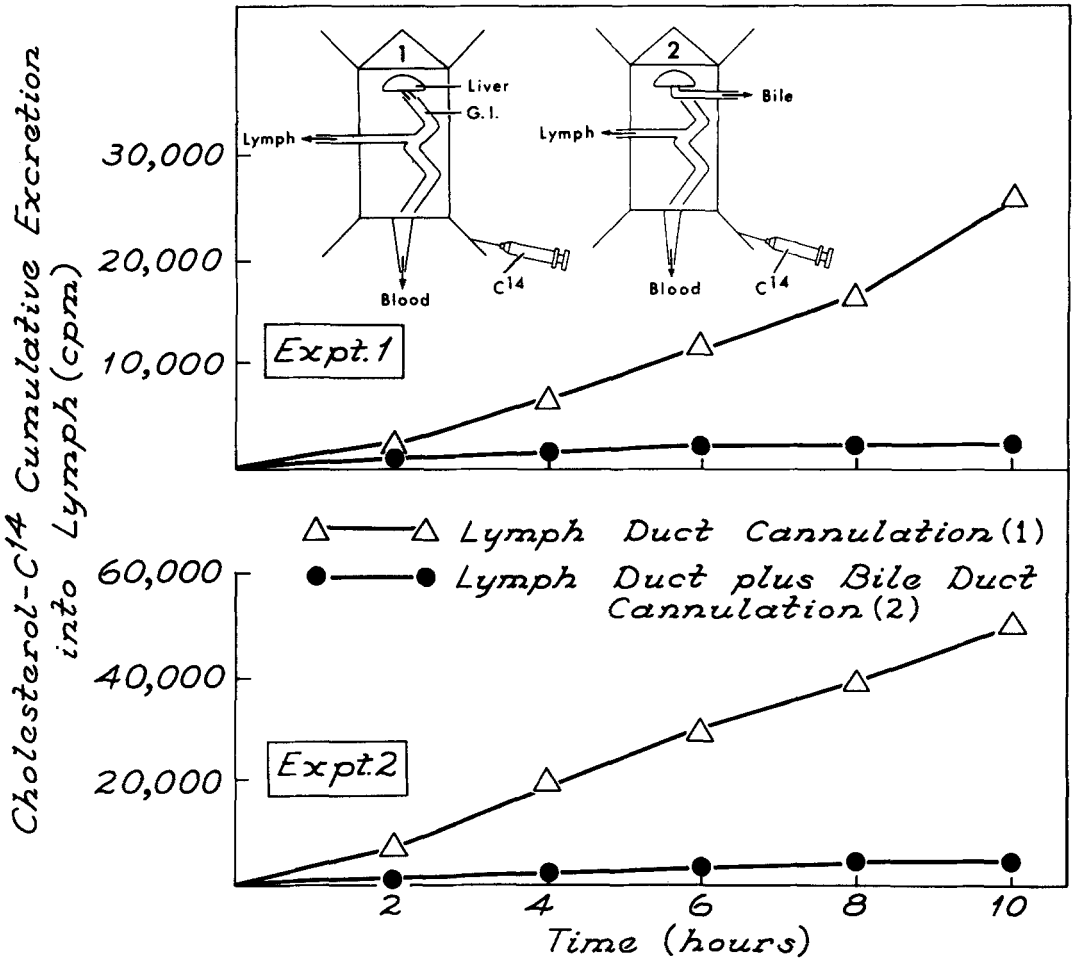

FIG. 5. The effect of bile duct cannulation on the appearance of cholesterol-C^{14} in the lymph of rats fed cholesterol and given acetate-2-C^{14}. Two pairs of rats (200–240 g) were fed 0.5% cholesterol for either 6 days (Expt. 1) or 12 days (Expt. 2). In each experiment one of the two rats had lymph duct cannulation (rat 1), and the other had both lymph and bile duct cannulations performed (rat 2). The animals were placed in restraining cages, and acetate-2-C^{14} was injected (5×10^7 cpm; 22.5 μc/μmole) intravenously. Lymph or bile and lymph samples were collected as indicated and assayed for cholesterol content and C^{14}.

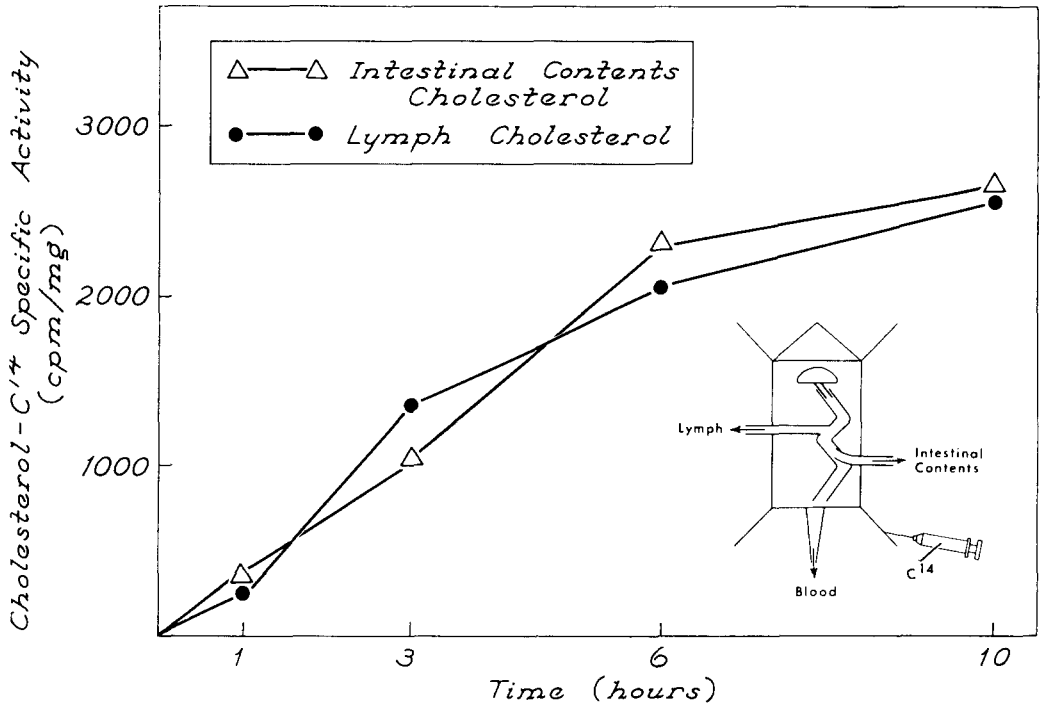

FIG. 6. Comparison of the change of specific activity of cholesterol-C^{14} with time in lymph and intestinal contents of a rat fed cholesterol and given acetate-2-C^{14} by intravenous injection. Cannulae were inserted into the gut lumen and intestinal lymph duct of a rat (250 g) which had been fed 0.5% cholesterol for 2 weeks. The animal was placed in a restraining cage and fed only 10% sucrose in water overnight. Following the injection of acetate-2-C^{14} (5×10^7 cpm, 22.5 μc/μmole), lymph and intestinal contents were collected and assayed for cholesterol content and C^{14}.

tance quantitatively, or, on the other hand, the intestine might account for the 10–18% of serum cholesterol of nonhepatic endogenous origin (4). The demonstration by Hotta and Chaikoff that turnover of serum cholesterol ceases following functional hepatectomy in dogs has been interpreted as suggesting that the liver is the only significant endogenous site of origin of plasma cholesterol (2); however, the functional hepatectomy which they utilized included evisceration, and it is likely that the role of an organ such as the intestine which serves both as a site of cholesterol synthesis (1) and cholesterol excretion (15, 16) would be significantly underestimated by such a technique.

The present studies also do not furnish evidence as to the possible significance of other tissues in contributing endogenous cholesterol to the circulation. No radioactivity appears in the blood stream of the cholesterol-fed, lymph-cannulated rat for as long as 13 hr after the administration of acetate-C^{14}, and it is possible that the intestine is the only nonhepatic tissue that contributes endogenous cholesterol to the circulating pool. However, the duration of these experiments was not sufficiently long to exclude the possibility that other tissues, although slow in the rate of exchange, might contribute in the steady state to serum cholesterol.

The dependence of cholesterol-C^{14} appearance in lymph upon the presence of bile and the similarity in the ratio of esterified to free cholesterol in lymph between dietary and endogenously synthesized cholesterol suggest that cholesterol synthesized by the gut wall mixes with the dietary cholesterol. Whether such mixing occurs, however, in the mucosa or in the lumen (via desquamation of cells) cannot be answered. The similarity of the specific activity of cholesterol of lymph and intestinal contents after acetate-C^{14} administration suggests, however, that the cholesterol may be secreted in both directions, some being desquamated and some transferred into lymph.

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