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### Publication Date
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UCRL-2586
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Contract No. W-7405-eng-48

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Max W. Biggs and Alexander V. Nichols

May 12, 1954

Berkeley, California

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# THE METABOLISM OF CHYLE CHOLESTEROL IN THE RAT*

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May 12, 1954

## ABSTRACT

Observations on the metabolism of chyle cholesterol in the rat show that exogenous cholesterol entering the systemic circulation in chyle exists in lipoproteins of low density (including chylomicrons) migrating with a high $S_f$ rate (i.e. $>400$) in the ultracentrifuge. Following entry into the systemic circulation these molecules are rapidly removed from the plasma. This "clearing" of serum chyle cholesterol is a tissue phenomenon, the liver being the predominant site. Within the liver the chyle cholesterol esters are at least partially hydrolyzed; hydrolysis apparently does not occur in the plasma to any appreciable extent. After its entry into the liver exogenous cholesterol, if normally metabolized, presumably mixes with and becomes indistinguishable from cholesterol produced by endogenous synthesis.

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* This work was supported in part by the U.S. Atomic Energy Commission.

+ Dr. Biggs is a Research Fellow of the San Joaquin County Heart Association.

# THE METABOLISM OF CHYLE CHOLESTEROL IN THE RAT

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## INTRODUCTION

The rate of appearance of tritium-cholesterol in the cholesterol-containing components of the serum of various patients following a single dose of tritium-cholesterol by mouth varies markedly, but in a manner that can be predicted accurately by the lipoprotein spectrum as determined ultracentrifugally [1]. This observed difference in the dynamics of exogenous cholesterol metabolism in man involves primarily the quantitative aspects of the partition of newly absorbed cholesterol into the free and esterified cholesterol pools of the serum. Patients having a normal lipoprotein spectrum with predominantly $S_f$ 0-12 lipoprotein molecules* show a high free cholesterol specific activity after a tritium-cholesterol meal while patients who show pathological lipoprotein spectra with large concentrations of $S_f$ 12-400 lipoprotein molecules have a high ester-cholesterol specific activity. Toward the ultimate understanding of these differences in cholesterol metabolism, experiments on the metabolism of chyle cholesterol in the normal rat have been done as described below.

Earlier studies in the rat indicate that newly absorbed cholesterol enters the systemic circulation via the lymph of the thoracic duct, [3,4] and that the majority of this newly absorbed cholesterol is esterified. [3,5] In spite of this fact, the serum esterified-cholesterol specific activity is not higher than the free-cholesterol specific activity during active tracer cholesterol absorption in normal men, dogs, [6] and rats (see below). This suggests either a differential hydrolysis of chyle cholesterol esters or a differential removal of chyle cholesterol esters from the serum early in normal exogenous cholesterol metabolism.

A study of certain aspects of chyle cholesterol metabolism has been made in the normal rat, using tritium-cholesterol. It has been possible to

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* Lipoproteins are classified in the ultracentrifuge according to Gofman [2]. Units are Svedbergs of flotation ($S_f$). One $S_f$ unit equals a migration rate of $10^{-13}$ cm/sec/dyne/g at 26°C in a medium of NaCl solution of density 1.063.

demonstrate: that the cholesterol in chyle is in lipoprotein molecules of high $S_f$ rate ($>400$); the chyle cholesterol exchanges with the cholesterol of the other serum lipoproteins in vitro; that newly absorbed chyle cholesterol is quickly taken up by the liver, where at least a portion of the chyle cholesterol esters undergo hydrolysis.

## I. THE LIPOPROTEIN VEHICLE FOR NEWLY ABSORBED CHOLESTEROL IN LYMPH AS DEFINED WITH THE ULTRACENTRIFUGE

Following the demonstration that newly absorbed cholesterol is carried to the systemic circulation via the lymph of the thoracic duct, rat chyle was submitted to ultracentrifugal analysis. A 24-hour lymph collection was made from the thoracic duct of a rat fed 30 mg of tritium cholesterol (sp. act. =1.81 μc/mg). This lymph contained 91.4 mg % total cholesterol with a specific activity of 0.32 μc/mg; and 30.7 mg% free cholesterol with a specific activity of 0.31 μc/mg. The lymph was processed as follows. Six ml of the lymph was centrifuged at 20,000 rpm for 30 minutes. The top 1 ml of the centrifuge tube's contents was removed by pipetting and submitted to analytical centrifugation at 40,000 rpm. A continuum of large particles with very rapid flotation rates was observed; the slowest moving molecules in this spectrum migrated at an $S_f$ rate greater than 400,* the majority at a rate much faster than this. Molecules with a slower flotation rate than this were not present in detectable amounts. The bottom 5 ml of solution from the original 6 ml sample above was adjusted to a solution density of 1.063 with NaCl solution. The mixture was then submitted to the routing low-density lipoprotein analysis used for serum lipoproteins in this laboratory[2]. This procedure analyzes for lipoproteins ranging in density from less than 1.0 to 1.04 g/ml. Traces of lipoproteins of $S_f$ rate 400-minus** were observed, but they were in too small concentration to be measured quantitatively.

To ensure that the newly absorbed cholesterol was in the rapidly flotating lipoproteins (i.e. $S_f$ 400-plus) and not in the trace amounts of more dense lipoproteins (i.e. $S_f$ 400-minus, or in high-density lipoproteins of density greater than 1.05), the chyle was treated as follows. Three 6-ml samples of chyle were centrifuged at 20,000 rpm for 30 minutes. Then the top 1-ml portion from each tube was pipetted off and the 3 portions were combined. Three ml of saline ($\rho$ =1.0073) was added and thoroughly mixed. Again this tube was centrifuged at 20,000 rpm for 30 minutes. The top 1 ml was removed and analyzed for tritium activity. The above centrifugation was calculated to move most of the rapidly flotating lipoproteins of the 18 ml of chyle into 1 ml

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* Calculated for a solution density of 1.063 g/ml at 26°C.

** $S_f$ 400-minus lipoproteins is a designation which includes all lipoproteins migrating at an $S_f$ rate of less than 400 (i.e. $S_f$ 0-12; $S_f$-400) in a solution density of 1.063 at 20°C.

at the top of the tube, and at the same time dilute out most of the $S_f$ 400-minus lipoproteins that might be present.

Calculation of the percent of the total amount of lipoproteins in the original 18 cc of chyle of the various $S_f$ classes which would be contained in the final 1 ml analyzed gives: $S_f$ 1000-plus = 100%; $S_f$ 400 = 29%; $S_f$ 100 = 7%; $S_f$ 20-minus = 3%.* This final 1 ml was found to contain 4.76 μc of tritium-cholesterol or 90.5% of the total tritium-cholesterol in the original 18 ml of chyle (5.26 μc). The experiment was repeated a second time, using a different labeled lymph collection from a different rat. The final 1 ml contained 86% of the total label in this case. These values of 90.5% and 86% are minimal values inasmuch as the mild centrifugation used did not move all the $S_f$ 400-plus molecules into the top 1 ml and, too, there was some remixing during the removal of this top fraction by pipetting.

In summary, then, it can be concluded that more than 85%, perhaps all, newly absorbed cholesterol in chyle enters the systemic circulation in low-density lipoproteins, including chylomicrons, which flotate in the ultracentrifuge with an $S_f$ rate greater than 400. This is of additional interest when we consider that the serum cholesterol (man, rat, dog) is predominantly in $S_f$ 0-12 and $S_f$ 12-400 and high-density lipoprotein molecules. Some of the metabolic events between the entry of exogenous cholesterol in chyle into the systemic circulation in lipoproteins of $S_f$ 400-plus and its appearance in lipoproteins of lower $S_f$ rate is considered in the following sections.

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* Spinco preparatory rotor No. 40.3.

## II. IN VITRO EXCHANGE OF SERUM LIPOPROTEIN CHOLESTEROL AND CHYLE CHOLESTEROL

It has been known for some time from the work of Gould[7] and Hagerman[8] and from work in our laboratory[9] that cholesterol of various lipoprotein molecules and cholesterol of red blood cells are not stably bound, and undergo exchange in vitro as well as in vivo. Thus it was initially thought that labeled cholesterol entering the plasma in chyle would be diluted quickly by exchange with cholesterol of the various other lipoproteins of the plasma and by rbc cholesterol. If it were, then the cholesterol specific activities of the plasma after a tritium cholesterol meal would be determined by the absolute amount of labeled cholesterol entering in the chyle, free and esterified, and by the size of the blood cholesterol pools, free and esterified. That this is not so can be easily shown.

Thirty mg of tritium-cholesterol, sp. act. = 1.81 μc/mg, was fed to an unfasted male rat (Long-Evans) 350 grams in weight. Chyle was collected from the thoracic duct for the period 2 to 8 hours after the labeled-cholesterol feeding. This lymph contained 41.4 mg% free cholesterol, sp. act. = 0.47 μc/mg, and 69.6 mg% esterified cholesterol, sp. act. = 0.57 μc/mg. The serum of this rat contained 12.0 mg% free cholesterol and 48.8 mg% esterified cholesterol. If we estimate the relative sizes of the free cholesterol pool of the blood, including rbc cholesterol, and the esterified-cholesterol pool of the blood in such a rat, we find that free cholesterol pool to be about 1.9 times as large as the ester pool.* Thus, addition of the collected chyle to the whole blood would result, after exchange of cholesterol between the various lipoprotein molecules and rbc's had reached completion, in a serum esterified-cholesterol specific activity nearly 4 times as great as the serum free cholesterol specific activity. However, 2 rats given 30 mg of tritium-cholesterol by mouth and sacrificed in 17 hours had serum free-cholesterol values of $0.87 \times 10^{-1}$ μc/mg and $1.34 \times 10^{-1}$ μc/mg, serum total-cholesterol values of $0.89 \times 10^{-1}$ μc/mg and $1.31 \times 10^{-1}$ μc/mg, and rbc cholesterol values of $0.77 \times 10^{-1}$ μc/mg and $1.23 \times 10^{-1}$ μc/mg, respectively.

Before proceeding it was decided that information on the speed of in vitro cholesterol exchange between chyle cholesterol and cholesterol of the other blood pools was needed. The two following in vitro experiments provided such information.

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* Using a hematocrit of 40 and a rbc cholesterol content of 120 mg %, all free.

1. A sample of 0.3 ml of the labeled chyle obtained above was added to 25 ml of pooled rat whole blood heparinized in vitro and incubated for 30 minutes with gentle stirring. Following incubation, the rbc's were removed immediately by centrifugation and washed three times with normal saline. The separated plasma was slightly pink from hemolysis. The plasma solution density was adjusted to 1.063 g/ml with NaCl and the plasma was centrifuged at 25,000 rpm for 20 minutes. Such treatment was calculated to move all $S_f$ 400-plus lipoproteins into the top 1 ml of the centrifuge tube.* The centrifuge tube (9 ml volume) was frozen in liquid nitrogen and the top 2 ml sliced off. The bottom 7 ml was used for determination of cholesterol specific activities. The values obtained were as follows:

Free cholesterol $= 1.11 \times 10^{-2}$ μc/mg
Ester cholesterol $= 0.29 \times 10^{-2}$ μc/mg
RBC cholesterol $= 0.08 \times 10^{-2}$ μc/mg

Plasma from the pooled rat blood contained 11.3% free cholesterol and 54.4 mg% esterified cholesterol. The lymph used contained 41.4 mg% free cholesterol, sp. act. = 0.47 μc/mg; and 69.6 mg% ester cholesterol, sp. act. = 0.57 μc/mg. Thus approximately 54% of the labeled free cholesterol introduced in a small amount of chyle (0.3 cc) was to be found in the free-cholesterol pools of the $S_f$ 400-minus lipoproteins and rbc's in 30 minutes.* Approximately 11% esterified cholesterol introduced in this chyle was to be found in the $S_f$ 400-minus lipoproteins after 30 minutes.

2. The experiment was repeated using 0.5 ml of chyle and 25 ml of pooled rat blood, containing 14.8 mg% free-cholesterol and 48.0 mg% esterified cholesterol, and the incubation was continued for 1 hour. The chyle was removed in the ultracentrifuge as before. The cholesterol specific activities were as follows:

Free cholesterol $= 1.50 \times 10^{-2}$ μc/mg
Ester cholesterol $= 0.66 \times 10^{-2}$ μc/mg
RBC cholesterol $= 0.31 \times 10^{-2}$ μc/mg

Thus in 1 hour** approximately 72% of the free cholesterol introduced in 0.5 ml of chyle was to be found in the $S_f$ 400-minus lipoproteins and rbc's, while about 25% of the esterified cholesterol so introduced was to be found

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* Spinco preparatory rotor No. 30.2

** The ultracentrifugal separation of chyle from the blood required about 30 minutes at 20°C and is not included in these times.

in the $S_f$ 400-minus lipoproteins.

Thus the morphology of the serum lipoproteins is such that there is a continuous and relatively rapid exchange of the cholesterol moieties between the various lipoprotein species as defined with the ultracentrifuge. This exchange is more rapid for free cholesterol than for esterified cholesterol.

## III. OBSERVATIONS ON IN VITRO "CLEARING" OF CHYLE BY POST-HEPARIN PLASMA

In the introduction we suggested that a specific hydrolysis of cholesterol esters of chyle early in exogenous cholesterol metabolism may produce the observed cholesterol specific-activity absorption curves. From Part 2 we see that this must occur rapidly (in less than 1 hour or so) if it is to occur before excessive cholesterol exchange has occurred. The clearing of lipemic serum by intravenous heparin, occurring within minutes after the injection, would fulfill this requirement for speed. Several workers [10, 11] have shown that the clearing of lipemic sera by post-heparin plasma in vivo and in vitro involves the liberation of fatty acids with a concurrent decrease in neutral fats. Attempts in our laboratory by ordinary analytical methods have failed, however, to demonstrate any change in cholesterol ester content in experiments in vitro involving post-heparin plasma clearing. The following experiment was done in an attempt to demonstrate the hydrolysis of chyle cholesterol esters during this clearing reaction.

The chyle used was obtained from the thoracic duct in the usual way after the feeding of 30 mg of tritium-cholesterol (sp. act. = 1.81 μc/mg) by mouth. The 2-to-8-hour sample was collected and showed a free-cholesterol content of 27.4 mg%, sp. act. = 0.326 μc/mg and an ester-cholesterol content of 43.6 mg%, sp. act. = 0.44 μc/mg.

A series of rats were given 10 mg of heparin intravenously, and 10 minutes later exsanguinated from the abdominal aorta. The various bloods were pooled and the plasma was removed by centrifugation. This pooled plasma contained 10.3 mg% free cholesterol and 45.3 mg% esterified cholesterol, and of course contained no radioactive cholesterol.

To ensure that the post-heparin plasma was "active", its lipemic serum "clearing" capacity was measured as follows. By turbidometric methods, comparable to those used by French, [12] it was found that 0.01 ml of chyle was 100% cleared in 11 minutes by 1.0 cc of the post-heparin plasma, a result to be expected from the work of French. Further evidence of the plasma's activity was obtained as follows: Two ml of this plasma was added to 1.0 ml of the chyle, thoroughly mixed, and incubated for 1 hour at 37°C with occasional stirring. Then the fatty acid content of the incubated tube was compared to a control tube. The method used for this determination is that of Freeman, Lindgren, and Nichols, [13] and involves chromatographic separation of the neutral fats and fatty acids followed by intrared absorption

analysis. Prior to incubation the mixture had 12. 1 mg of glycerol fatty acid esters and 0. 8 mg of free fatty acids. Following incubation the glycerol fatty acid esters were reduced to 9. 7 mg and the free fatty acid content had increased to 4. 5 mg. Colorimetric determination (Schoenheimer-Sperry) of the esterified and free cholesterol with and without incubation showed no significant change.

Thus convinced that the post-heparin plasma was "active," we placed 10. 5 ml in each of 4 tubes. Two tubes were inactivated at 65°C for 20 minutes and used as controls. To each of the four tubes 0. 2 ml of the chyle containing radioactive cholesterol was added. Following incubation at 37°C for 1 hour the free- and total-cholesterol values and the free- and total-cholesterol specific activities were determined for each tube. The results are in Table I.

It is to be seen that the free-cholesterol specific activity is only approximately 10% (0. 13 x $10^{-2}$ μc/mg) higher on the average in the active plasma. This difference is not significant.

The sensitivity of the system employed for detecting the hydrolysis of chylous cholesterol esters can be estimated as follows. In the previous section it was shown that something less than 25% of the chylous cholesterol esters exchanged with cholesterol esters of the other lipoproteins of the serum in 1 hour at 37°C. Therefore, the chylous esterified cholesterol in the present experiment had a specific activity greater than 0. 336 μc/mg (0. 75 x 0. 447 μc/mg) throughout the 1-hour incubation. Only 0. 087 mg of this labeled, esterified cholesterol was added to the system. Hydrolysis of 20% of this amount (0. 017 mg), with the resultant shift of the contained radioactivity into the free-cholesterol pool, would have resulted in an increase of 0. 53 x $10^{-2}$ μc/mg in the free-cholesterol specific activity, an increase of approximately 40%.

In view of these results the statement seems justified that in vitro clearing of lipemic serum by post-heparin plasma in the rat does not involve a significant hydrolysis of the cholesterol esters of chyle. Because the amount of chyle added to the test system was small, it has been tentatively concluded that whatever differential hydrolysis of cholesterol esters of chyle takes place in normal exogenous cholesterol metabolism does not occur in the plasma.

Table I

| | Free Cs content (mg%) | Total Cs content (mg%) | Free Cs sp. act. $10^{-2}$ μc/mg | Total Cs sp. act. $10^{-2}$ μc/mg |
|---|---|---|---|---|
| Active Plasma | | | | |
| No. 1 | 10.2 | 56.0 | 1.44 | 0.85 |
| No. 2 | 10.1 | 55.2 | 1.42 | 0.89 |
| Inactive Plasma | | | | |
| No. 3 | 10.7 | 54.8 | 1.26 | 0.90 |
| No. 4 | 10.1 | 56.4 | 1.33 | 0.91 |

## IV. HEPATIC UPTAKE OF NEWLY ABSORBED CHYLE CHOLESTEROL

If hydrolysis of chyle cholesterol esters does not occur in the plasma as the data above suggest, any hydrolysis must be a tissue phenomenon. Six Long-Evans male rats, approximately 145 days old and weighing between 315 and 395 grams, each received an intravenous injection of 0.5 ml of chyle containing radioactive cholesterol. The chyle injected into these animals contained 26.8 mg% free cholesterol, sp. act. = 0.35 μc/mg and 75.0 mg% total cholesterol, sp. act. = 0.58 μc/mg. These animals were then killed serially by exsanguination at 10 minutes, 30 minutes, 45 minutes, 1 hour, 3 hours and 8 hours. The blood was heparinized in vitro so the plasma and rbc's could be separated immediately after collection. The total-cholesterol specific activities of the plasma and of the liver were determined for each rat. The results are recorded in Fig. 1. Each pair of points (liver and plasma cholesterol specific activities at each time interval) is from a different individual rat.

The percent of the injected radioactivity to be found in the liver and plasma at each time point* is given in Table II. The total plasma volume used for these calculations has been estimated at 15 ml for each animal.

The rise of liver-cholesterol specific activity values well above those of the plasma-cholesterol specific activity clearly indicates the chyle cholesterol is removed from the plasma by the liver.

The rapid arrival of liver cholesterol and plasma cholesterol at an equilibrium value in from 3 to 8 hours is entirely consistent with all the evidence to show liver cholesterol and plasma cholesterol undergo rapid exchange.[7] Thus exogenous cholesterol in chyle is in a distinct metabolic pool until its entry into the liver; once processed in normal fashion there, it apparently becomes indistinguishable from cholesterol of endogenous origin.

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* Of interest is the fact that if plasma chylous cholesterol and liver cholesterol turn over in exponential fashion with half times of 15 minutes and 30 minutes respectively (values consistent with the data in Fig. 1), the maximum percent of injected label to be found in the liver and cholesterol at any one time would have been about 60%. Such reasoning would lead one to suspect that all chylous cholesterol is "processed" in the liver.

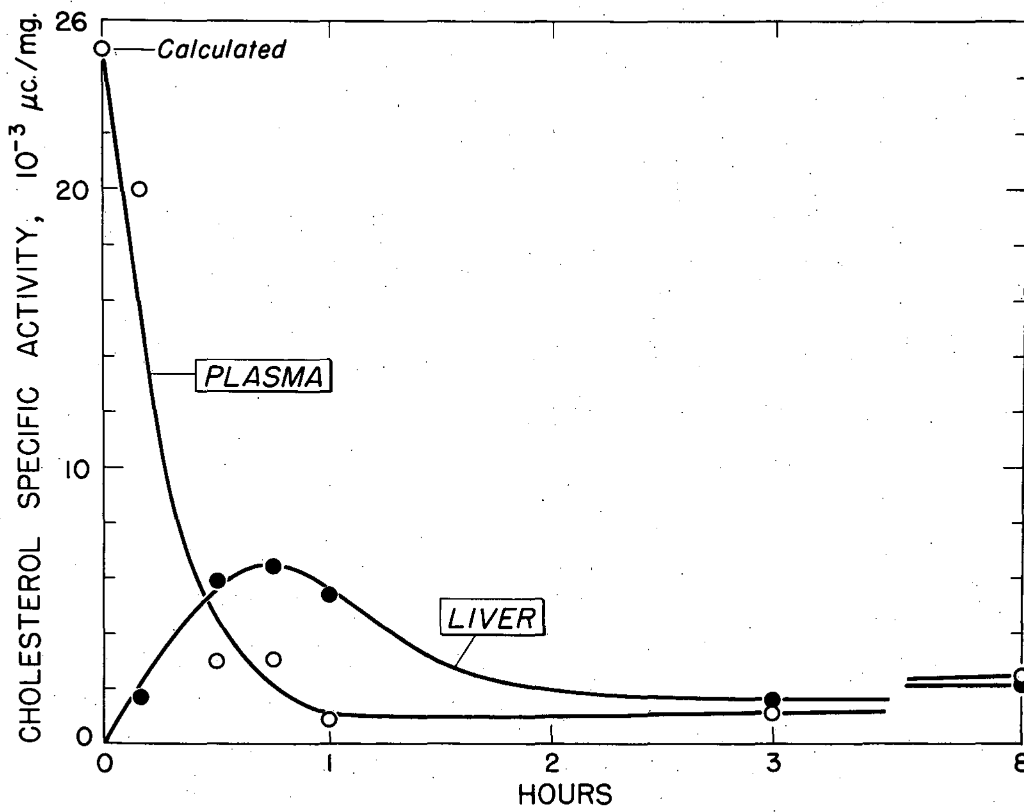


Fig. 1 Hepatic Uptake Intravenously Injected Chyle Cholesterol.

Table II

| Time after chyle injection | Percent of injected radioactivity in the plasma | Percent of injected radioactivity in the liver |
|---|---|---|
| 0 | 100% | 0 |
| 10 min | 81% | 12% |
| 30 " | 12% | 63% |
| 45 " | 13% | 59% |
| 1 hour | 4% | 58% |
| 3 hours | 5% | 17% |
| 8 " | 10% | 16% |

## V. HYDROLYSIS OF CHYLE CHOLESTEROL ESTERS IN THE LIVER

Two rats comparable to those used in Part 4 were given 0.5 ml of chyle intravenously and killed by exsanguination at 45 minutes. The livers were removed immediately, minced and frozen in acetone — dry ice, and freeze-dried. The livers were thus frozen within 2 or 3 minutes after death of the animal. The dried livers were extracted 3 times with absolute alcohol — ether, 3:1, made slightly acid with a drop of HCl. The extracts were combined and concentrated to a total volume of 100 ml. Portions of this volume were used for chemistries and specific activity determinations. The results are listed in Table III.

The chyle injected into each rat contained 26.8 mg% free cholesterol, which had a specific activity of 0.35 μc/mg. Thus 0.5 ml contained 0.134 mg of free cholesterol and $4.7 \times 10^{-2}$ μc of tritium in free cholesterol. Liver 1 above, however, contained $6.1 \times 10^{-2}$ μc of tritium in free cholesterol and Liver 2 contained $9.0 \times 10^{-2}$ μc of tritium in free cholesterol. Thus "127%" and "191" of the injected free labeled cholesterol was found in the liver. Therefore appreciable hydrolysis of chyle cholesterol esters has occured within 45 minutes, presumably in the liver.

Table III

| Rat No. | Total liver cholesterol | Total free cholesterol | Total-chol. sp. act. | Free-chol. sp. act. | Percent of Total radioactivity injected present in total liver |
|---|---|---|---|---|---|
| 1 | 24.3 mg | 19.8 mg | $5.6 \times 10^{-3}$ μc/mg | $3.05 \times 10^{-3}$ μc/mg | 62% |
| 2 | 20.1 mg | 15.5 mg | $6.4 \times 10^{-3}$ μc/mg | $5.8 \times 10^{-3}$ μc/mg | 59% |

## DISCUSSION

Thus it has been observed that chyle cholesterol is "processed" in the liver in the rat, and that this processing involves hydrolysis of cholesterol esters. It seems almost certain that this metabolic pathway functions to some degree in man; however, it should be pointed out here that the quantitative aspects of rat lipid metabolism differ in some respects from those of lipid metabolism in man. First, the lipoproteins in the normal rat are predominantly of density 1.05 and higher, with only small amounts of molecules of density less than 1.04. Man, on the other hand, has predominantly lipoproteins of the lower density classes, with smaller concentrations of high-density molecules. Thus the normal serum lipoprotein spectrum is quite different in the rat from that in man. Secondly, seventeen hours following a tritium-cholesterol meal in the rat the free- and esterified-cholesterol pools of the serum are already essentially identical in specific activity. In normal man the free-cholesterol specific activity at this time is higher than the ester-cholesterol specific activity, and persists so for some 48 hours. No explanation of these two quantitative differences in metabolism is at present available.

From the present observations (in the light of past studies) on chyle metabolism it would appear potentially profitable to test the hypothesis that the heparin content of the liver is operative in the initial "clearing" of chyle lipoproteins from the serum; that a subsequent step after "heparin action" is hydrolysis of chyle cholesterol esters by the cholesterases known in abundance in the liver; that normally much of the newly absorbed ester-cholesterol is hydrolyzed to give the specific activity absorption curves observed in normal man [1]; that when this hydrolysis is delayed and inefficient the pathological specific activity curves found in patients with high $S_f$ 12-400 lipoproteins (i.e. xanthoma tuberosum) occur.

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