Volume 1, Number 1, January 1964
Printed in U. S. A.

JOURNAL OF THE RETICULOENDOTHELIAL SOCIETY **1**, 40-49 (1964)

The Uptake and Metabolism of Cholesterol-H^3 Labeled Lipoprotein by Macrophages *in Vitro*[1]

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Received April 8, 1963

Lipoprotein, labeled with cholesterol-H^3, obtained from cholesterol fed rabbits was incubated with rabbit macrophages *in vitro* in order to investigate the uptake and subsequent metabolism of lipoprotein cholesterol by macrophages. Partial uptake of the lipoprotein by the cells occurred and this was followed by appreciable hydrolysis of the lipoprotein cholesterol ester.

Hydrolysis of lipoprotein cholesterol ester by macrophage homogenates was also demonstrated and the addition of either synthetic lecithin or purified animal lecithin to such homogenates was shown to accelerate significantly this hydrolysis.

INTRODUCTION

The uptake of cholesterol suspensions and of triglyceride emulsions by RE cells and the subsequent metabolism of these preparations by these cells have been recently reported (Day and French, 1959; Day, 1960; Day and Gould-Hurst, 1961; Day, 1961; Day and Fidge, 1962). However, the normal physiological form of transport of cholesterol and other lipids, and the form in which we might expect it to be presented to macrophages *in vivo* under physiological or pathological circumstances, is as soluble lipoprotein. In the present experiments, therefore, the uptake by macrophages of lipoprotein containing H^3-labeled cholesterol has been investigated and some aspects of the metabolism of this lipoprotein by RE cells have been described.

MATERIALS AND METHODS

H^3-Labeled cholesterol of specific activity 388 μc per milligram was obtained from the Radiochemical Center, Amersham, United Kingdom.

Lipoprotein labeled with cholesterol-H^3. Rabbits fed a diet containing

[1] This work was aided by a grant from the National Heart Foundation of Australia.

0.75 gm of cholesterol and 4.5 ml of peanut oil daily for 6 weeks were given a single dose of 2.5 mc of H^3-labeled cholesterol by intragastric intubation. Three days later the rabbit was exsanguinated by cardiac puncture and the labeled lipemic serum used as a source of lipoprotein.

The radiochemical composition of the lipid in the lipoprotein was verified by silicic acid chromatography (Hirsch and Ahrens, 1958) and the distribution of the cholesterol-H^3 in the serum lipoprotein determined by paper electrophoresis. The labeled lipoprotein was stored at —20° C prior to use.

Lecithin solutions. Synthetic D.L. α-lecithin and animal lecithin "90% pure" were obtained from Nutritional Biochemicals Corporation. Aqueous suspensions of these materials at a concentration of 10 mg per milliliter were prepared using Tween 20 as described for cholesterol by Meier *et al.* (1952).

Sodium oleate-albumin solution was prepared by dissolving 100 mg sodium oleate in 2.5% bovine albumin solution.

Rabbit macrophages were obtained from the peritoneal cavity of rabbits after the intraperitoneal injection of 40 ml of liquid paraffin using the method of Lucké *et al.* (1933) as modified by Mackaness (1952).

Uptake and Metabolism Using Intact Cells

In each experiment 70–100 $\times$ 10^6 macrophages, 2 ml of fresh normal rabbit serum, 0.5 ml of cholesterol-H^3 labeled lipemic rabbit serum (14.5 mg cholesterol per milliliter; sp. act., 1900 counts per second per milligram cholesterol) made up to 10 ml with Hanks solution were incubated at 37° C in siliconized vessels. Following incubation for 30 minutes to allow initial uptake of the labeled lipoprotein, the cells were centrifuged at 1000 rpm for 3 minutes, washed twice with 0.9% sodium choride solution, and recentrifuged. The washed cells were then resuspended in Hanks solution containing 7.5% serum, plated into 2 pairs of 100-ml McCartney bottles, and the bottles incubated on their sides for 1 hour at 37° C. At the end of this period the media was removed, the cells which had then become attached to the glass were washed with warm saline, and one pair reserved for extraction and estimation of labeled free and ester cholesterol. To the second pair was added 10 ml of media containing Hanks:serum (2:1) together with penicillin and streptomycin, and these were incubated for a further 20 hours at 37° C. After this period the cells were washed with saline and extracts prepared, the saline washings having been centrifuged to deposit cell debris and this debris included in the cell extracts. The

cells and cell debris were extracted with alcohol-ether (3:1) and the extracts taken to dryness prior to separation and counting of the ester and free cholesterol as described below.

Homogenate Experiments

Macrophage homogenates were prepared at a concentration of 1 in 5 in 0.2 *M* phosphate buffer at pH 6. Packed cells were suspended in buffer and homogenized for 5 minutes in a microblender at 5° C. Complete disintegration of cells was checked microscopically. Penicillin and streptomycin were added to the homogenate prior to incubation.

Cholesterol-H^3 labeled lipoprotein prepared as described above was diluted 1:10 to give an activity of 2700 counts per second per milliliter and 1.45 mg cholesterol per milliliter.

Incubations were set up containing 0.3 ml of macrophage homogenate, 0.1 ml of sodium oleate-albumin solution, 19 µl of 1:10 diluted H^3-labeled lipoprotein together with either an additional 0.1 ml of dilute Tween 20 solution equivalent to that present in the lecithin preparations, or 0.1 ml of either synthetic lecithin or animal lecithin solution prepared as above. Controls containing phosphate buffer alone, but no macrophages, were also set up. Tubes were stoppered and incubated at 37° C for 20 hours. After incubation lipid extracts of the preparations were prepared using chloroform-methanol (2:1) as described by Folch *et al.* (1957). The free and ester cholesterol in the extracts were separated on alumina columns and the H^3-labeled cholesterol in each fraction counted as described below.

Separation of Free and Ester Cholesterol

This procedure was carried out on alumina columns as described by Kerr and Bauld (1953) and the H^3-labeled free and ester cholesterol determined in the eluates by liquid scintillation counting.

Radioassay

H^3-labeled total cholesterol or free and ester cholesterol were counted in the alcohol-ether extracts or in the column eluates by liquid scintillation counting using an Ekco N664A Scintillation counter, at —20° C, counting at an efficiency of 31% for tritium.

Chemical Assay

Cholesterol in the lipoprotein-containing serum was determined after extraction of the serum with alcohol-ether (3:1) using the method of Zlatkis *et al.* (1953).

RESULTS

Properties of Cholesterol-H^3 Labeled Lipoprotein

Figure 1 shows the distribution of the H^3-label in the serum lipoprotein as determined by paper electrophoresis. It is apparent that almost all of

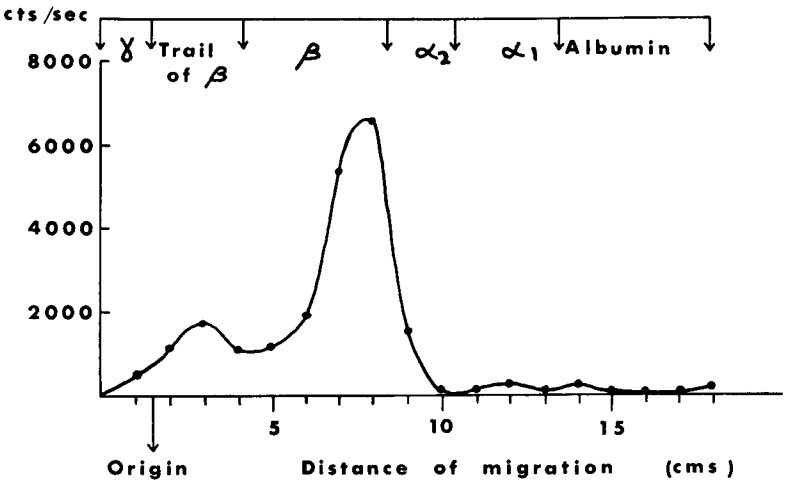

Fig. 1. Distribution of H^3-labeled cholesterol in the lipoprotein used for uptake and metabolism experiments. Identification of lipoprotein bands (by protein staining with bromophenol blue and lipid staining by Sudan black) is shown above the distribution of H^3-labeled cholesterol.

the H^3-labeled cholesterol has been incorporated in the serum into the β-lipoprotein, so that subsequent metabolic changes shown are indicative of changes in the cholesterol in this fraction.

Table 1 gives the distribution of the label in the various lipid fractions in the lipemic serum as determined by silicic acid chromatography. Of the

TABLE 1

Per cent Distribution of H^3 Label in Lipid Fractions of Hypercholesterolemic Rabbit Serum Following Administration of H^3-Labeled Cholesterol-Silicic Acid Chromatographic Separation

Lipid		Distribution (%)
Cholesterol ester	(Fraction III)	79.6
Triglyceride	(Fraction IV)	1.3
Free cholesterol	(Fraction V)	17.8
Di- and monoglycerides	(Fractions VI and VII)	0.6
Phospholipid	(Fraction VIII)	0.5

total label recovered, 79.6% was present as cholesterol ester, 17.8% as free cholesterol, and only 2.4% was recovered in all other lipid fractions.

Uptake and Metabolism of Lipoprotein by Macrophages in Vitro

The uptake of lipoprotein was determined as the amount of H^3-labeled cholesterol present in the cells after incubation in a media containing labeled lipoprotein, and subsequent washing, plating out the cells, and extraction. Data are given in Table 2 for five experiments. Between 0.2

TABLE 2

Uptake of H^3-Labeled Lipoprotein by Macrophages *in Vitro*

Expt.	No. of cells	Total uptake (cts/sec)	Uptake (%)
1	25×10^6	29.0	0.9
2	19×10^6	13.3	0.4
3	18×10^6	62.0	1.9
4	24×10^6	11.6	0.4
5	21×10^6	7.2	0.2

and 1.9% of the labeled lipoprotein presented to the cells was taken up and recovered in the washed cells.

The hydrolysis of cholesterol ester in the lipoprotein taken up is shown in Table 3. Following initial incubation and plating, the percentage

TABLE 3

Percentage Cholesterol Ester Present in H^3-Labeled Lipoprotein before and after Incubation with Macrophages *in Vitro*

Expt.	Before incubation (%)	After initial 90-min incubation (%)	After further 20-hour incubation (%)
1	—[a]	33	39
2	—[a]	34	23
3	—[a]	59	46
4	82	31	29
5	78	25	22

[a] Not separately determined.

ester present in the lipoprotein cholesterol had fallen from approximately 80% to a mean value of 36 ± 5.9%. During the subsequent 20-hour incubation period the relative amount of cholesterol ester present continued to fall to a mean of 32 ± 4.7%.

Metabolism of Macrophage Homogenates

Detailed data from one experiment in which macrophage homogenates were incubated with H^3-labeled lipoprotein are given in Table 4. The

TABLE 4

Hydrolysis of Lipoprotein H^3-Cholesterol Ester by Macrophage Homogenates and the Effect of Lecithin[a]

	Ester cholesterol (cts/sec)	Free cholesterol (cts/sec)	Ester (%)
Control; no homogenate (0 hour)	35.0	11.5	75.4
Control; no homogenate (20 hours)	37.3	12.6	74.8
Homogenate alone (20 hours)	29.0	15.7	65.0
Homogenate plus animal lecithin (20 hours)	24.5	27.0	47.7
Homogenate plus synthetic lecithin (20 hours)	29.2	22.2	56.9

[a] Detailed data from one experiment.

mean change in percentage ester together with the standard error for the six experiments carried out is shown in Fig. 2 with respect to the control group containing no homogenate and to groups containing homogenate alone or homogenate with synthetic or animal lecithin added. In the control where lipoprotein was incubated alone no significant change in percentage ester occurred. Where macrophage homogenates were present a fall in cholesterol ester content of the lipoprotein from a mean of $74.6 \pm 1.6\%$ at 0 hour to $65.7 \pm 1.5\%$ at 20 hours occurred. When either animal lecithin or synthetic lecithin were added, the hydrolysis was significantly increased so that the percentage of ester fell to $56.7 \pm 3.0\%$ and $57.6 \pm 2.2\%$, respectively.

DISCUSSION

The presence of H^3-labeled cholesterol in macrophages following their incubation with cholesterol-H^3 labeled lipoprotein, together with the fact that the lipoprotein cholesterol ester has been hydrolyzed within the cells establishes that some uptake by macrophages of lipoprotein has taken place. However, this uptake is relatively small and the lipoprotein is not concentrated in the cells much above the concentration in the media. It is probable, therefore, that the amount of lipoprotein recovered from the macrophages, under the circumstances investigated, is due to diffusion

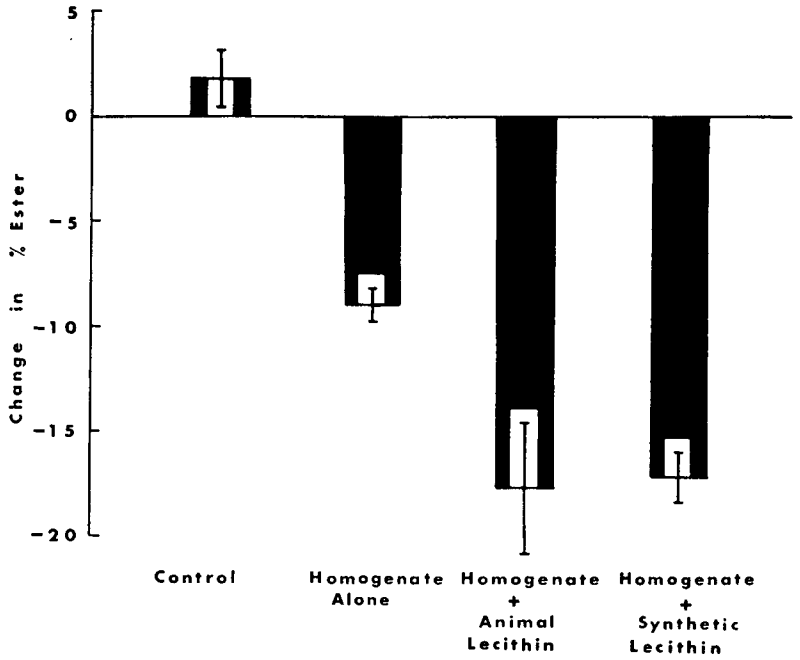

FIG. 2. Mean change in percentage ester together with the standard error of the mean of H^3-labeled cholesterol contained in lipoprotein incubated with macrophage homogenates alone or together with either animal lecithin or synthetic lecithin.

into them and not to active phagocytosis of the preparation. The uptake of lipid by macrophages in the arterial wall is a feature of atheromatous lesions, and it has been suggested that such lipid is taken up in an insoluble form following disruption of infiltrating serum lipoprotein (Buck, 1958). The present experiments were partly designed in order to determine whether unchanged lipoprotein as such can be taken up by macrophages, and within the limits of the present experiments there is no evidence for any significant phagocytosis of lipoprotein as such by macrophages. It must be borne in mind, however, that lipoprotein may be changed in the arterial wall following infiltration from the blood (Gerö *et al.*, 1961) and such changes may effect its uptake *in vivo*.

The hydrolysis of lipoprotein cholesterol ester was brought about readily by macrophages. The relatively rapid and more complete hydrolysis which occurred when intact cells were used is doubtless due to the relatively small amount taken up to be dealt with by these cells. When homog-

enates were used there was also significant hydrolysis of the lipoprotein cholesterol ester, but the reaction was relatively slow and less complete than was the case with intact cells. The hydrolysis of plasma lipoprotein by macrophages establishes the presence of hydrolytic cholesterol esterase in these cells. Thus both synthetic and hydrolytic cholesterol esterase activity of macrophages have been demonstrated (Day and French, 1959; Day and Gould-Hurst, 1961). The fatty acid pattern of the cholesterol ester synthesized by RE cells has been shown to be predominately saturated (Day *et al.*, 1963). It is probable, therefore, that, following the hydrolysis of lipoprotein, equilibrium between synthesis and hydrolysis will be set up with a changing fatty acid pattern in view of this capacity of RE cells to esterify cholesterol more readily with saturated fatty acid. Such changes brought about by macrophages which take up infiltrated lipoprotein cholesterol ester in arterial lesions would adequately explain the increased free to ester cholesterol ratio and the more saturated fatty acid pattern of the cholesterol esters present in such lesions (Weinhouse and Hirsch, 1940; Zilversmit *et al.*, 1961).

The role of phospholipid in cholesterol metabolism is of interest. Phospholipid has been shown to increase esterification by serum cholesterol esterase (Wagner and Rogalski, 1952; Wagner, 1959) and to decrease esterification of cholesterol by macrophages (Day and Gould-Hurst, 1963). In the present work lecithin has been shown to increase the hydrolysis of lipoprotein cholesterol ester. If, as seems likely, hydrolysis and synthesis are continuing as a result of two separate enzymes, either inhibition of synthetic activity or augmentation of hydrolytic activity would explain the apparent accelerated hydrolysis brought about by lecithin in the present experiments.

The effect of lecithin in promoting hydrolysis of cholesterol ester by macrophages also provides some basis for its favorable influence in removing lipid from atheromatous lesions (Friedman *et al.*, 1957; Maurukas and Thomas, 1960), since free cholesterol is more exchangeable in RE cells than is ester cholesterol (Day and Gould-Hurst, 1961).

RÉSUMÉ

Une lipoprotéine, contenant du cholestérol-H^3, obtenue du sérum de lapins nourris avec du cholestérol, a été incubée *in vitro* avec des macrophages de lapin. On a observé une absorption partielle par les macrophages du complexe lipoprotéine–cholestérol, suivie d'une hydrolyse appréciable de la liaison estérifiée. Une hydrolyse de l'ester cholestérol lecithine a été également observée avec l'homogénat des macrophages. L'addition de lecithine synthétique ou animale à de tels homogénats a accéléré, d'une manière significative, le processus d'hydrolyse.

ACKNOWLEDGMENTS

We wish to thank Mrs. G. K. Wilkinson for the electrophoresis pattern and the Misses M. Kleeman and D. Knapman-Jones for technical assistance.

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