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The uptake and metabolism of C^{14}-labeled fatty acids by macrophages *in vitro*

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SUMMARY

Macrophages obtained from the peritoneal cavity of rabbits were incubated *in vitro* in the presence of C^{14}-labeled sodium palmitate, and the uptake of fatty acid and its subsequent incorporation into other lipid fractions was determined. Up to 21% of the C^{14}-labeled sodium palmitate added to the incubation medium was taken up by the macrophages. Most of this was converted to triglyceride and phospholipid, but a small amount was converted to cholesterol ester and to mono- and diglyceride.

Evidence has previously been obtained that indicates that macrophages can participate actively in lipid metabolism. Esterification of cholesterol (1, 2) and oxidation of fatty acids and triglycerides (3, 4) can be brought about by macrophages *in vitro*, while several lipid-hydrolyzing enzymes have been demonstrated in macrophage homogenates (5). In the present study the metabolism of unesterified fatty acids by macrophages has been further investigated. The uptake of C^{14}-labeled fatty acid by these cells and its subsequent incorporation into triglyceride and phospholipid have been studied.

MATERIALS AND METHODS

C^{14}-labeled sodium palmitate-albumin. Palmitic acid-1-C^{14} (specific activity, 7.8 μc/mg) obtained from the Radiochemical Centre, Amersham, United Kingdom, was dissolved in 0.05 N sodium hydroxide to give a stock solution of sodium palmitate containing 20 μc (2.5 mg)/ml. Prior to its use, this was diluted one in ten with 5% bovine albumin solution.

C^{14}-labeled lipids used for paper chromatography standards. Cholesterol-4-C^{14} (specific activity 63.4 μc/mg) and tripalmitin-1-C^{14} (specific activity 16.2 μc/mg) were obtained from the Radiochemical Centre, Amersham. The tripalmitin-1-C^{14}, which contained appreciable amounts of di- and monoglyceride, was purified on silicic acid columns as described below, and the purified tripalmitin and the di- and monopalmitin were used as standards.

Rabbit macrophages were obtained from the peritoneal cavity of adult rabbits five days after the intraperitoneal injection of sterile liquid paraffin (Harrington) using the method of Lucké, Strumia, Mudd, McCutcheon, and Mudd (6) as modified by Mackaness (7). Forty ml of sterile liquid paraffin was injected intraperitoneally into adult rabbits. After five days, sterile 0.9% sodium chloride was introduced into the peritoneal cavity and the exudate removed into a separating funnel by suction through a sheathed tube. The washings were allowed to stand for five min to separate the paraffin, and then the aqueous lower fraction was passed through a gauze filter to remove giant cells, cellular aggregates, and debris. Macrophages were then deposited by centrifuging at 800 rpm for three min. Smears were prepared for differential counts and stained by Leishman's method.

Exudates obtained in this way contained 85 to 90% large mononuclear cells identical with tissue macrophages. These were motile, actively phagocytic, and metabolically active, but did not multiply *in vitro*. Some of these macrophages contained droplets of paraffin. In addition, the exudates contained approximately ten per cent small mononuclear cells, but rarely any polymorphs.

Incubation of macrophages and extraction of lipid. The macrophages were counted in a hemocytometer chamber, and a known number of cells (up to 100×10^6) were suspended in 45 ml of Hanks solution containing 0.5% bovine albumin and dispensed in 20-oz McCart-

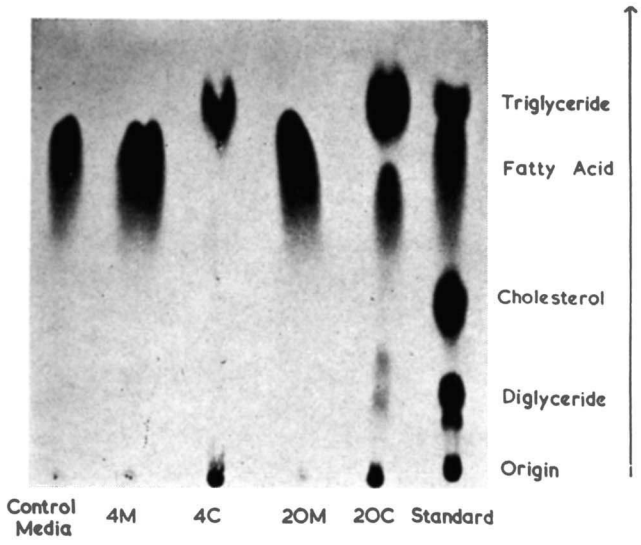

FIG. 1. Fractionation of lipids from macrophage and medium extracts on silicic acid-impregnated paper. Radioautogram prepared from the chromatogram in Exp. G2. Mobile phase: petroleum ether–diisobutyl methyl ketone 96:6. Control media: incubation of sodium palmitate-1-C^{14} in the absence of cells. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-C^{14}, 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-C^{14}. The presence of triglyceride and diglyceride in addition to unesterified fatty acid in the cell extracts is demonstrated.

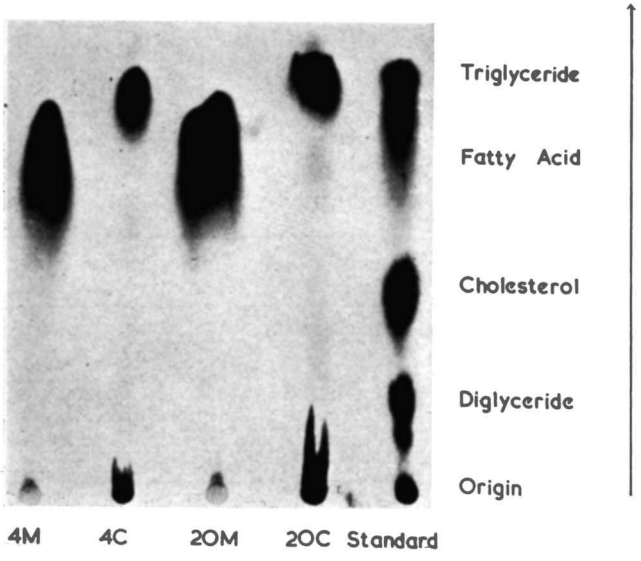

FIG. 2. Fractionation of lipids from macrophage and medium extracts on silicic acid impregnated paper. Radioautogram prepared from the chromatogram in Exp. G6. Mobile phase: petroleum ether–diisobutyl methyl ketone 96:6. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-C^{14}. 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-C^{14}. Mainly triglyceride is demonstrated in the cell extracts.

ney bottles. These were incubated on their side at 37° for 60 min. After this period, the macrophages had firmly adhered to the glass while any contaminating cells remained free in the fluid medium. The Hanks solution was removed and the adherent film of macrophages washed with 0.9% sodium chloride solution to remove any remaining contaminating cells. Thirty ml of medium containing Hanks solution–old serum–new serum 4:1:1 was then added to the cells together with a known amount (approx 2 μc) of C^{14}-labeled sodium palmitate-albumin solution. Streptomycin and penicillin were added to the medium to prevent bacterial growth. Controls were set up containing medium and C^{14}-labeled palmitate but no macrophages.

After incubation at 37° for 4 hr or 20 hr, the medium was removed and the cells still adherent to the glass were washed twice with 10 ml of warm 0.9% sodium chloride. The medium was centrifuged (2,000 rpm for 10 min) to deposit the cells that had become detached from the glass during incubation. These cells were washed with 0.9% sodium chloride and recentrifuged. The cells still adherent to the glass and the detached cells deposited from the medium were combined and extracted with 10 ml of alcohol–ether 3:1 at 70°. Five ml of the supernatant solution obtained after centrifuging the medium was extracted with 100 ml of alcohol–ether 3:1. A known amount of this extract was evaporated to near dryness, 2 ml of water added, and the lipid extracted with 3 × 10-ml washings of 15% alcohol in petroleum ether (b.p. 60–80°). Portions of both the cell and the medium extracts were taken for counting and for chromatography on silica-impregnated paper, as described below. In some cases the extracts were evaporated and the lipids fractionated on silicic acid columns as described below.

Paper chromatography on silicic acid-impregnated paper was carried out using the method of Marinetti and Stotz (8) but with the following two solvent systems: for general lipid separation, petroleum ether (b.p. 60–80°)–diiso-butyl ketone 96:6; for phospholipid separation, diiso-butyl ketone–acetic acid–water 40:20:3. The relative positions on the chromatogram of the various lipid classes were found to be the same as those described by Marinetti (9) (see Figs. 1 and 2).

Radioautographs were prepared using Ilfex X-ray film applied to the chromatograms in X-ray casettes. Exposure varied from five to ten days. In the case of the phospholipid runs, nonradioactive synthetic DL-dipalmitoyl lecithin (Nutritional Biochemicals, Ltd.) was run as a standard and its position determined by staining with Rhodamine 6 G (10), and marking the radioautographs.

Column chromatography. Fractionation of lipids on silicic acid columns was carried out using the method of Hirsch and Ahrens (11). Stepwise elution Scheme A was used and approximately 10 mg each of cholesterol ester, glyceryl tripalmitate, palmitic acid, cholesterol, and lecithin was added as carrier. The 20-ml fractions collected were evaporated to dryness and the tube contents washed into weighed planchettes. These were reweighed and counted using a thin mica end-window G.M. tube, sufficient activity being counted to give an error of less than ±3% in all but the very low-activity samples. All counts were corrected for self-absorption.

RESULTS

The data for the experiments performed are summarized in Table 1. After a 4-hr incubation, the macrophages had taken up and incorporated intracellularly between 5 and 7% of the C^{14}-labeled sodium palmitate added to the medium; while after a 20-hr incubation, up to 21% of the sodium palmitate in the medium had been taken up.

The synthesis of triglyceride from the C^{14}-labeled fatty acid taken up by the macrophages is shown in Figures 1 and 2. The medium extracts contained only C^{14}-labeled fatty acid whereas all the cell extracts contained both C^{14}-labeled triglyceride and diglyceride. In one experiment (G2, Table 1), appreciable C^{14}-labeled fatty acid was still present in the cell extracts in addition to the triglyceride and diglyceride formed (Fig. 1); but in all other experiments, there was little remaining C^{14}-labeled fatty acid in the cells, most being converted to triglyceride, diglyceride, and phospholipid (see further reference in this paper). Figure 2 is a representative radioautograph (Exp. G6, Table 1). Little detectable C^{14}-labeled fatty acid was present after either 4-hr or 20-hr incubation, but labeled triglyceride and some labeled diglyceride was present at both time intervals.

The conversion of C^{14}-labeled fatty acid to phospholipid after 4-hr and after 20-hr incubation with macrophages is shown in Figure 3. In each case, the fatty acid has been incorporated by the macrophages into 2 phospholipid fractions. The faster moving spot corresponds to lecithin, the other is possibly sphingomyelin but definite identification was not attempted in these experiments. No labeled phospholipid was present in any of the medium extracts.

Chromatography, on silicic acid columns, of the medium and cell lipid extracts was carried out in several of the experiments. In our hands, the elution of the palmitic acid peak occurred earlier in Fraction

TABLE 1. THE UPTAKE OF C^{14}-LABELED FATTY ACIDS BY MACROPHAGES *in vitro*

Experiment		No. of Cells	Substrate	C^{14} Added cps	Incubation Time h	Recovery of C^{14} Cells (cps)	Recovery of C^{14} Medium (cps)
G2		90×10^6	Palmitate 1-C^{14}	(3220)	(4)	214	2263
		"	"	"	20	589	1905
		No cells	"	"	20	...	2510
G4		37×10^6	Palmitate 1-C^{14}	2800	20	132	1877
		No cells	"	"	20	...	2410
G5	A.	63×10^6	Palmitate 1-C^{14}	3260	4	164	2455
		"	"	"	20	535	2075
		No cells	"	"	20	...	2680
	B.	63×10^6	Palmitate 1-C^{14} with 8 mg cholesterol suspension added	"	20	408	2200
		No cells	"	"	20	...	2750
G6	A.	90×10^6	Palmitate 1-C^{14}	3260	4	166	2090
		"	"	"	20	693	1850
		No cells	"	"	20	...	2420
	B.	90×10^6	Palmitate 1-C^{14} with 8 mg cholesterol suspension added	3260	20	389	2160
		No cells	"	"	20	...	2660
G8		100×10^6	Palmitate 1-C^{14}	3525	4	254	...*
		"	"	"	20	506	...

* Extraction of medium not carried out in this experiment.

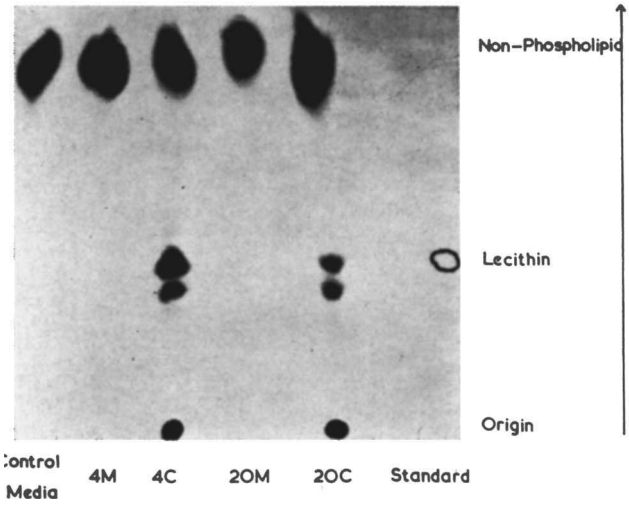

FIG. 3. Separation of phospholipids from macrophage and medium extracts on silicic acid impregnated paper. Radioautogram prepared from the chromatogram in Exp. G2. Mobile phase: diisobutyl methyl ketone–acetic acid–water 40:20:3. Control media: incubation of sodium palmitate-1-C^{14} in the absence of cells. 4M and 4C: corresponding medium and cell extracts after 4-hr incubation with sodium palmitate-1-C^{14}. 20M and 20C: corresponding medium and cell extracts after 20-hr incubation with sodium palmitate-1-C^{14}. The position of the nonradioactive DL-dimyristoyl lecithin as determined by staining with Rhodamine 6G is marked on the radioautogram. The presence of lecithin and one other unidentified phospholipid in the cell extracts is demonstrated.

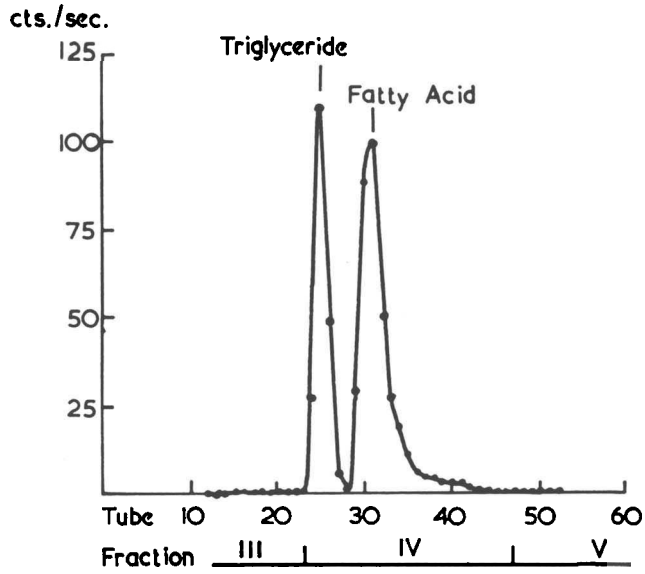

FIG. 4. Separation on an 18-g silicic acid column of a standard mixture of tripalmitin-1-C^{14} and palmitic acid-1-C^{14}. Ten mg of nonlabeled tripalmitin and 10 mg of nonlabeled palmitic acid added as carrier.

IV than that shown for mixed nonesterified fatty acids by Hirsch and Ahrens (11) (Fig. 4). We record here only the elution of the labeled tripalmitin and of the labeled palmitic acid. All other lipids were eluted from the columns as described by Hirsch and Ahrens.

The separation of lipid fractions of macrophage extracts and of medium extracts on silicic acid columns from one experiment (G6) is shown in Figures 5A and B. In the cell extracts, synthesis of relatively large amounts of triglyceride and phospholipid occurred, together with smaller amounts of cholesterol ester and mono- and diglycerides. Very little C^{14}-labeled fatty acid remained after its uptake by the macrophages. The medium extract contained traces of mono- and diglyceride and of phospholipid but 99% of the C^{14} was still present as unesterified fatty acid.

Figures 6 A and B show the separation on silicic acid columns of lipids from macrophage and medium extracts in an experiment in which 8 mg of nonlabeled cholesterol suspension was added to the incubation medium in addition to the C^{14}-labeled sodium palmitate. The experiment was carried out at the same time and with the same batch of macrophages as that for the chromatogram above (Exp. G6, Table 1). The total uptake of C^{14}-labeled sodium palmitate by the cells was less than in the absence of cholesterol (see Table 1) but the relative conversion to triglyceride and phospholipid was essentially the same. Incorporation of labeled fatty acid into cholesterol ester, and into mono- and diglyceride also occurred.

DISCUSSION

The uptake of fatty acid from the medium has been reported for a number of tissues incubated *in vitro*. Adipose tissue (12), liver slices (13), and ascites tumor cells (14) have all been shown to take up relatively large amounts of labeled fatty acid from fatty acid-albumin complexes. In the present experiments, up to 21% of the fatty acid added to the medium has been taken up by the macrophages. The enzymic conversion of the fatty acid to triglyceride and phospholipid, etc., which has been shown to occur in the cells, would probably facilitate this uptake by transferring fatty acid to the water-insoluble phase. In fact, the situation described for macrophages is very similar to that described by Shapiro, Chowers, and Rose (12) for adipose tissue incubated *in vitro*, where ready uptake of fatty acid and its conversion to triglyceride has been shown to occur.

Triglyceride and phospholipid synthesis in liver and other tissues proceeds along a common pathway via phosphatidic acid and diglyceride (15). The synthesis of both triglyceride and phospholipid by macrophages, together with the appearance of appreciable quantities

of diglyceride, suggests that a similar common pathway for phospholipid and triglyceride synthesis exists in the macrophage, but further work is clearly necessary to verify this. The small amount of monoglyceride found in the cells is possibly due to enzymic breakdown of some of the triglyceride with the formation of mono- and diglyceride in addition to unesterified fatty acid. That lipase is present in macrophage homogenates has already been shown (5).

It is significant that a small amount of the fatty acid taken up by the macrophages was incorporated into cholesterol ester. It is known (1, 2) that macrophages can bring about esterification of cholesterol, and the present finding that exogenous fatty acids can be incorporated into the cholesterol ester renders it likely that the fatty acid composition of the cholesterol ester, produced by macrophages, can be influenced by varying the fatty acid available to the cell.

The synthesis of phospholipid by macrophages is of interest for several reasons. Zilversmit, Shore, and Ackerman (16) have shown that phospholipid is synthesized in the arterial wall in cholesterol-fed rabbits. It has already been suggested (17) that such synthesis

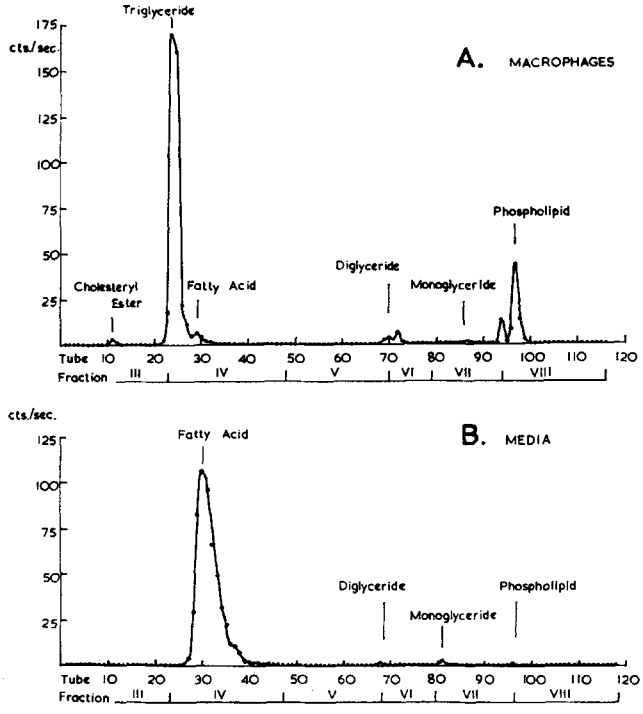

FIG. 5. Separation on silicic acid columns of the lipid components of macrophages and medium after 20-hr incubation with C^{14}-labeled sodium palmitate. A. *Macrophage extract.* Column load: 630 cps C^{14} with 10 mg cholesteryl ester, 10 mg tripalmitin, 10 mg palmitic acid, 10 mg cholesterol, 15 mg lecithin added as carrier. B. *Medium extract.* Column load: 500 cps C^{14} and carrier lipids as in A.

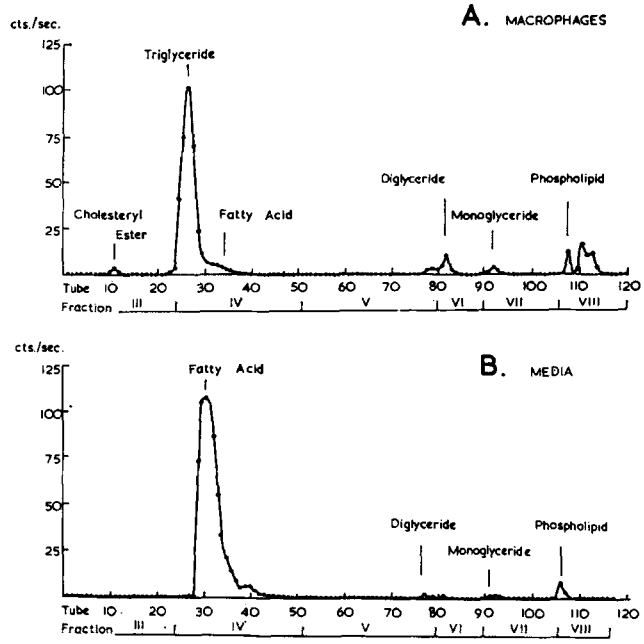

FIG. 6. Separation of silicic acid columns of the lipid components of macrophages and medium after 20-hr incubation with C^{14}-labeled sodium palmitate and nonlabeled cholestrol suspension. A. *Macrophage extract.* Column load: 570 cps C^{14}, with 11 mg cholesteryl ester, 11 mg tripalmitin, 11 mg palmitic acid, 11 mg cholesterol, 18 mg lecithin added as carrier. B. *Medium extract.* Column load: 480 cps C^{14} and carrier lipids as in A.

may be carried out by macrophages since it is known that the bulk of the lipid is present intracellularly in "foam cells", at least in early atheroma. The demonstration of phospholipid synthesis by reticuloendothelial (RE) cells is also of possible significance in the pathogenesis of the lipid storage diseases. Thannhauser (18) has suggested, but without experimental evidence, that abnormal lipid metabolism by RE cells is responsible for the accumulation of lipids in these cells in such conditions as Niemann-Pick disease. Previously there has been no experimental evidence that RE cells can synthesize phospholipid, so that such a finding may be of significance in support of Thannhauser's concepts.

The uptake of a cholesterol suspension by the macrophages failed to influence appreciably the uptake of fatty acid and the subsequent synthesis of either triglyceride or phospholipid by the cells. It has been shown (17) that the uptake of cholesterol suspensions by RE cells in rat lymph nodes was followed by triglyceride and phospholipid accumulation, presumably by its synthesis in the cells. The phospholipid synthesis that occurs in the arterial wall (16) is also accelerated in cholesterol-fed rabbits as compared with normally fed animals. In the present system, however,

no facilitation of synthesis was brought about by the uptake of cholesterol.

The significance of the uptake and metabolism of fatty acid by macrophages under physiological circumstances is not clear. It has been suggested (19, 20) that RE cells in the liver are involved in the uptake of lipid from the blood for passage to and metabolism by the liver and it is of interest to consider whether the metabolic reactions described in this paper, if true of RE cells in general, may influence the general metabolism of fatty acid in the body.

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