Quart. J. exp. Physiol. (1966) **51**, 11–17

Reprinted from the QUARTERLY JOURNAL OF EXPERIMENTAL PHYSIOLOGY, VOL. LI, 1966

UPTAKE AND METABOLISM OF ^{14}C-LABELLED TRIGLYCERIDE BY RETICULO-ENDOTHELIAL CELLS.*

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(*Received for publication* 26*th July* 1965)

The uptake and metabolism of ^{14}C-labelled triglyceride by R.E. cells in rat lymph nodes has been investigated. After the intraperitoneal injection of a corn oil emulsion containing glyceryl tripalmitate-1-^{14}C uptake of labelled triglyceride by the R.E. cells in the nodes occurs. The uptake is accompanied by hydrolysis of the triglyceride and reincorporation of the liberated fatty acid mainly into phospholipid. Where cholesterol suspension is injected together with the triglyceride emulsion the ^{14}C-labelled fatty acid is also incorporated into cholesterol ester in the nodes. Investigation by gas-liquid chromatography of the labelled fatty acid in each of the lipid moieties in the nodes showed that some chain elongation of the ^{14}C-labelled palmitic acid occurred, but that the stearic acid formed appeared only in the phospholipid fraction.

THE relatively rapid rate of removal of triglyceride from R.E. cells in rat lymph nodes compared with that of cholesterol [French and Morris, 1960; Day *et al.*, 1965 *a*] can be attributed in part to the oxidation of triglyceride [Day, 1960 *a*, 1961]. It is also possible that the breakdown of triglyceride and resynthesis of the fatty acid into other lipids may be contributing to the process of removal. There is evidence for this from previous work [Day *et al.*, 1965 *a*] and in addition other R.E. cells, such as rabbit peritoneal macrophages, have been shown to incorporate fatty acid into lipid moieties [Day and Fidge, 1962]. In the present study the uptake of ^{14}C-labelled triglyceride fatty acid by reticulo-endothelial cells in rat lymph nodes and its incorporation into phospholipid and cholesterol ester has been investigated.

MATERIALS AND METHODS

^{14}C-labelled Triglyceride Emulsion. – Glyceryl tripalmitate-1-^{14}C of specific activity 25·1 mc./mM. (Radiochemical Centre, Amersham, U.K.) was dissolved in a known amount (up to 400 mg.) of corn oil and the preparation emulsified in a Microblender using 15 mg. Tween 60/100 mg. of oil as emulsifying agent.

Non-Radioactive Cholesterol Suspension was prepared by dissolving cholesterol in acetone, adding the solution to water and removing the acetone by boiling. The suspension was concentrated by boiling to contain approximately 10 mg. of cholesterol/ml.

Experimental Procedure. – Male Norwegian hooded rats (180–200 g.) were each injected intraperitoneally with 2 ml. of a preparation containing ^{14}C-labelled triglyceride emulsion (5 mg. triglyceride, a known amount (21,000–64,000 c.p.s.) of glyceryl tripalmitate-1-^{14}C) and 50 mg. bovine albumin. In some experiments a second series of rats was injected with a mixture to which 4–5 mg. of non-labelled cholesterol suspension was added to the ^{14}C-labelled triglyceride emulsion. These preparations are absorbed *via* the diaphragm and taken up by R.E. cells in sternal lymph nodes.

* This work was aided by a Grant from the National Heart Foundation of Australia.

Printed in Great Britain

Groups with four rats in each were killed at intervals of 4, 12, 24 and 48 hr. after injection and the sternal lymph nodes removed from each group were combined and extracted with chloroform : methanol (2 : 1 v/v) by the method of Folch *et al.* [1957]. Homogenization prior to extraction was carried out using a Kontes all glass conical tissue grinder (Kontes Glass Co., Vineland, N.J.). The lipid extract of the nodes was counted and then separated into its components by thin layer chromatography as described below and the distribution of the ^{14}C-label determined either by scanning or by elution and counting. The phospholipid of some of the extracts was fractionated on silicic acid impregnated paper, while in others the ^{14}C-labelled fatty acids of the cholesterol ester, triglyceride and phospholipid fractions of the lipid extracts were examined by gas phase chromatography.

Thin Layer Chromatography. – Duplicate aliquots of the lipid extract were evaporated to dryness, taken up in chloroform and spotted onto thin layer plates coated with Merck Silica Gel G of 0·25 mm. thickness. The plates were developed with diethyl ether : acetic acid : hexane (25 : 2 : 73) for 15 min. over a distance of 10 cm. After drying at room temperature the spots were located with iodine vapour or radio autographs prepared and the Rf values compared with standards run simultaneously. The plates were then either scanned or, with the aid of a spatula and brush, the areas corresponding to each spot were directly transferred to scintillator containers for counting. To show that the label remaining at the origin was made up of phospholipid only, this spot was developed in the second dimension with chloroform : water : methanol (95 : 4 : 35).

Radioassay was carried out using an Ecko N664A scintillation counter with 0·3 per cent paraphenyl oxazole in toluene as scintillator and counting at an efficiency of 75 per cent for ^{14}C.

Phospholipid Separation. – An aliquot of the lipid extracts were spotted onto silicic acid impregnated paper and the phospholipids separated using the solvent system diisobutyl ketone : acetic acid : water (40 : 25 : 5) [Marinetti, 1962]. Radioautographs were then prepared to demonstrate the ^{14}C-labelled phospholipid. The phospholipids were identified by comparison of Rf values with standards and by spot tests as described elsewhere [Day *et al.*, 1966]. In order to determine the distribution of ^{14}C-labelled fatty acid between different phospholipids the papers were scanned using a Nuclear Chicago 4 π Actigraph Scanner. Measurements of peak areas were made for quantitation.

Gas Phase Chromatography. – Following separation of the lipid extract by thin layer chromatography the cholesterol ester, triglyceride and phospholipid fractions were eluted with chloroform : methanol (2 : 1 v/v) and the methyl esters of the fatty acids in each fraction prepared by the method of Stoffel, Chu and Ahrens [1959]. The fatty acid methyl esters were then separated using a Pye Argon Chromatograph with polyethylene glycol adipate (10 per cent on Embacel 100–120 mesh) at 180° C. as stationary phase. The ^{14}C-labelled fatty acids in the column effluent were monitored using the Pye Radio-chromatography attachment connected above the detector by means of a splitter [James and Piper, 1961]. Fatty acid mass was obtained by measuring peak area, while the amount of ^{14}C present in each peak was recorded linearly using the integrated range of the rate meter. Calibration with ^{14}C-labelled methyl palmitate of known specific activity was carried out.

Results

The amount of ^{14}C present in the sternal lymph nodes at intervals after the intraperitoneal injection of the triglyceride emulsion either with or without the cholesterol suspension is given for a typical experiment in Table I. The maximum amount of ^{14}C is present in the nodes at 4 hr. and represents

2–3 per cent of the ^{14}C-labelled triglyceride injected into the peritoneal cavity. After 4 hr. the ^{14}C label present in the nodes declines rapidly so that by 48 hr. only about a quarter of the amount present initially persists.

The incorporation of the triglyceride ^{14}C-labelled fatty acid into phospholipid and cholesterol ester is shown in fig. 1. In the radioautograph shown, it can be seen that 48 hr. after the injection and uptake of the labelled triglyceride by the R.E. cells in the nodes, an appreciable amount of the fatty acid has been incorporated into phospholipid. Where cholesterol was taken up with the triglyceride by the R.E. cells in the nodes the ^{14}C-labelled fatty acid has been incorporated into cholesterol ester as well as into phospholipid.

TABLE I. CTS./SEC. OF ^{14}C-LABELLED FATTY ACID PRESENT IN STERNAL LYMPH NODES AT INTERVALS AFTER THE INTRAPERITONEAL INJECTION OF ^{14}C-LABELLED TRIGLYCERIDE IN RATS.* †

Time (hr.)	^{14}C-labelled triglyceride injected	^{14}C-labelled triglyceride with cholesterol injected
4	2,642	2,675
12	1,638	1,623
24	788	888
48	610	758

* Each rat received 24,600 c.p.s. glycerol tripalmitate-1-^{14}C, 5·0 mg. triglyceride with or without 4·9 mg. cholesterol.

† Figures given are total content of the combined extract from four rats at each time interval.

These results are further illustrated in fig. 2 where the ^{14}C-labelled fatty acid present in the four lipid fractions separated by thin layer chromatography is plotted against the time which has elapsed since the intraperitoneal injections. The conversion from triglyceride to phospholipid is essentially the same for both injections but where cholesterol has been added to the labelled triglyceride emulsion there is a notable increase in the amount of cholesterol ester which becomes labelled. The rate at which it becomes labelled however is less than for the phospholipid.

The relatively rapid conversion of the label from triglyceride to phospholipid is shown in fig. 3, for an experiment in which the uptake of triglyceride was less than 1 per cent but in which after 24 hr., two thirds of the ^{14}C labelled fatty acid introduced as triglyceride is incorporated into phospholipid.

The incorporation of the ^{14}C-labelled triglyceride fatty acid into phospholipid by the R.E. cells is further illustrated in fig. 4, where incorporation of ^{14}C into individual phospholipids is shown. Most of the ^{14}C-labelled fatty acid has been incorporated into lecithin but incorporation into sphingomyelin and 'cephalin' has also occurred.

The distribution of the ^{14}C-labelled fatty acid between the various phospholipids as determined by scanning is shown in Table II. Over 60 per cent of the ^{14}C- is present as lecithin with smaller amounts in sphingomyelin and cephalin. While there was a trend in some experiments for an increased

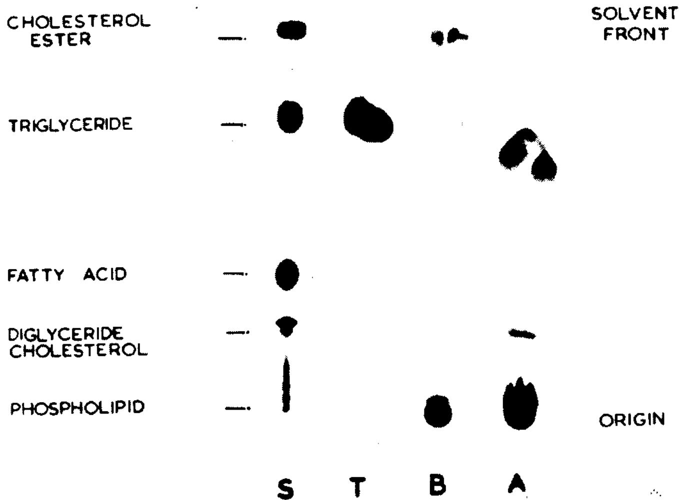

Fig. 1. Radioautograph showing the incorporation of ^{14}C-labelled fatty acid into phospholipid and cholesterol ester 48 hr. after an intraperitoneal injection of ^{14}C-labelled triglyceride with and without cholesterol. Lipid extract from rat nodes separated by thin layer chromatography.

S. Standard mixture.
T. Triglyceride emulsion injected.
B. Node extract 48 hr. after injection of 5 mg. labelled triglyceride (24,600 c.p.s.) and 4·9 mg. unlabelled cholesterol.
A. Node extract 48 hr. after injection of 5 mg. labelled triglyceride (24,600 c.p.s.).

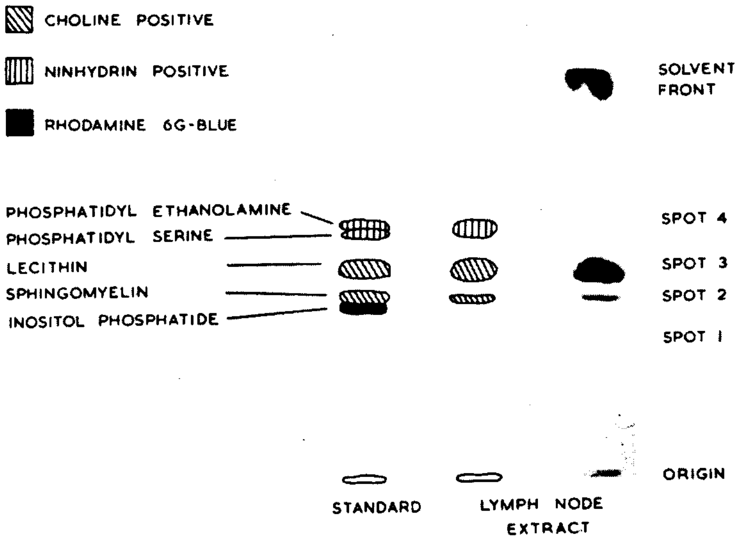

Fig. 4. Separation of phospholipids by paper chromatography. A radioautograph of node lipid extract from eight rats 24 hr. after intraperitoneal injection of 5·6 mg. ^{14}C-labelled triglyceride (36,800 c.p.s.) is shown on the right, together with a comparison of Rf values and staining characteristics of standard phospholipids.

Spot 1 is unidentified.

Triglyceride is shown at solvent front.

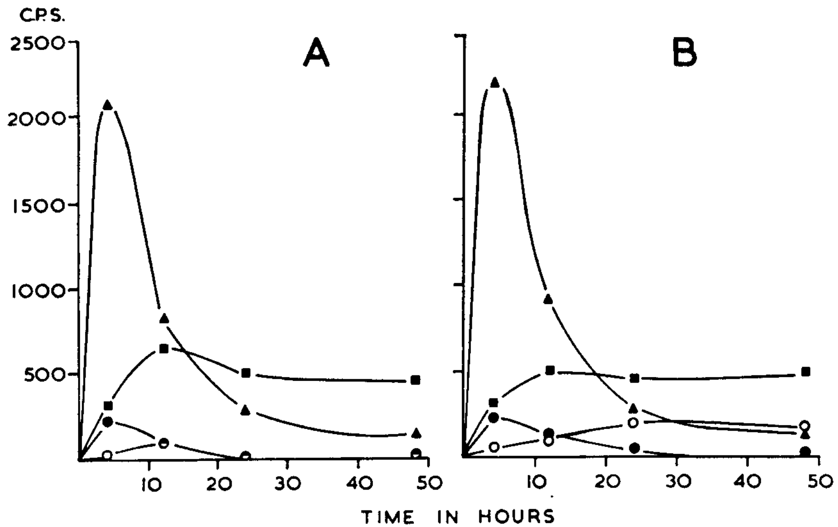

FIG. 2. Distribution of ^{14}C-labelled fatty acids in rat nodes at time intervals after intraperitoneal injection of labelled triglyceride with and without cholesterol. Label determined by scanning thin layer chromatography plates.

A. 5·0 mg. ^{14}C-labelled triglyceride (24,600 c.p.s.) injected.
B. 5·0 mg. ^{14}C-labelled triglyceride and 4·9 mg. unlabelled cholesterol injected.
▲ triglyceride.
■ phospholipid.
● diglyceride.
○ cholesterol ester.

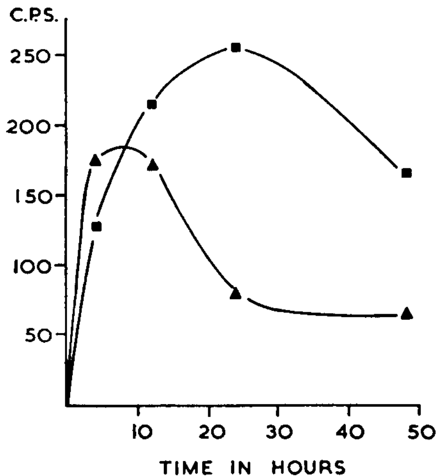

FIG. 3. Transfer of ^{14}C-labelled fatty acid from triglyceride to phospholipid in rat nodes at time intervals after the intraperitoneal injection of 5·0 mg. ^{14}C-labelled triglyceride (21,200 c.p.s.). Label determined by elution of spots from thin layer chromatography plates and counting.

▲ triglyceride.
■ phospholipid.

amount of sphingomyelin to be labelled where cholesterol was present than with triglyceride alone the difference was not significant.

The specific activity of the individual fatty acids in both the triglyceride and phospholipid fraction of the nodes together with that of the triglyceride emulsion used is shown in Table III. Only two labelled fatty acids were

TABLE II. PERCENTAGE DISTRIBUTION OF ^{14}C-LABELLED FATTY ACID BETWEEN PHOSPHOLIPID FRACTIONS 24 HR. AFTER UPTAKE OF ^{14}C-LABELLED TRIGLYCERIDE BY R.E. CELLS IN RAT LYMPH NODES.*

	^{14}C-labelled triglyceride	^{14}C-labelled triglyceride with cholesterol
Sphingomyelin	18·7 (2·4)	21·5 (1·5)
Lecithin	64·9 (4·2)	61·8 (2·0)
Cephalin	12·0 (0·7)	12·5 (0·6)
Unidentified	4·5 (2·7)	4·2 (0·3)

* Data gives the mean together with the standard deviation for three experiments.

present in the fractions. In the case of the triglyceride fraction all of the labelled fatty acid remained as the palmitic acid injected. In the phospholipid fraction, however, a significant proportion of the palmitic acid had been converted to stearic acid. The amount of radioactivity present in the stearic acid in the phospholipid fraction amounted to about 6 per cent of the total label present in this fraction. Since methods were sensitive enough

TABLE III. SPECIFIC ACTIVITY OF FATTY ACIDS (c.p.s./mg) FROM TRIGLYCERIDE AND PHOSPHOLIPID FRACTIONS OF RAT NODE LIPID EXTRACTS AT TIME INTERVALS AFTER AN INTRAPERITONEAL INJECTION OF ^{14}C-LABELLED TRIGLYCERIDE.*

		Node extracts							
		Triglyceride fraction				Phospholipid fraction			
Fatty acid	Triglyceride emulsion	4 hr.	12 hr.	24 hr.	48 hr.	4 hr.	12 hr.	24 hr.	48 hr.
Palmitic .	67,459	9,999	1,856	693	294	588	5,958	2,616	935
Stearic .	nil	nil	nil	nil	nil	nil	nil	353	223

* Each rat received 5 mg. triglyceride, 55,200 c.p.s. glyceryl tripalmitate-1-^{14}C. Lipid extract prepared from nodes of six rats at each time interval.

to detect less than 1 per cent of the total label in an individual fatty acid, it can be concluded with confidence that no stearic acid appeared in the triglyceride fraction.

DISCUSSION

It is apparent from the present studies that triglyceride fatty acid taken up by R.E. cells in rat lymph nodes is actively metabolized by these cells. The amount of ^{14}C-labelled fatty acid present in the nodes 2 days after the

uptake of triglyceride is in itself relatively small, but a considerable proportion of this labelled fatty acid has by this time been incorporated into phospholipid and, where cholesterol is also taken up, into cholesterol ester. These observations imply prior hydrolysis of fatty acid, removal of much of this rapidly turning over pool together with reincorporation of the fatty acid into other lipids. Lipase activity has been demonstrated in peritoneal cavity macrophages [Day and Harris, 1960] and electron microscopical evidence [Casley-Smith and Day, 1966] has indicated that hydrolysis occurs following the uptake of triglyceride emulsion by macrophages *in vitro*. It has been shown that macrophages from the peritoneal cavity [Day and Fidge, 1962] or alveolar macrophages [Elsbach, 1965 *b*] will incorporate fatty acid into phospholipid. The present observations indicate that with R.E. cells in lymph nodes active incorporation of fatty acid into phospholipid also occurs. It is of interest that fatty acid is not esterified with cholesterol present in the nodes, but that when cholesterol is added and taken up with the triglyceride emulsion that esterification of this cholesterol occurs. This may be due to enzymes secreted into phagocytic vesicles, so that hydrolysis and reincorporation of fatty acid into cholesterol ester all occur within phagocytic vesicles. [See Elsbach, 1965 *a*, *b*; Casley-Smith and Day, 1966].

Some reincorporation into triglyceride would seem likely. This does occur when fatty acid is taken up by macrophages *in vitro* [Day and Fidge, 1962; Elsbach, 1965 *b*]. However, in the present study the specific activity of the phospholipid fatty acid is higher than that of the triglyceride fatty acid after 4 hr. Although this may be explained by the existence of different size pools, it is consistent with increased phospholipid in response to the presence of other lipids That this occurs when cholesterol ester is taken up by such cells has been demonstrated previously [Day, 1960 *b*; Day *et al.*, 1965 *b*] Such phospholipid synthesis might then effect a protective function, itself facilitating solubilization and removal of other lipids embarrassing the cell.

It is evident from the gas phase chromatography data that the R.E. cells in the nodes are able to effect the chain addition of 2C to palmitic acid to form stearic acid. What is perhaps surprising is the absence of stearic acid in the triglyceride fraction. There are several possible explanations for this. There may be no reincorporation of fatty acid into triglyceride. Certainly, from data where cholesterol ester fatty acid was taken up by R.E. cells [Day *et al.*, 1965 *b*] relatively little of the fatty acid was incorporated into triglyceride. It may well be that metabolism in the present experiments is in favour of phospholipid synthesis, rather than triglyceride resynthesis. The higher specific activity of the phospholipid palmitic acid relative to the triglyceride palmitic acid at all but the 4 hr. intervals would support this concept, although the possibility of several pools cannot be excluded. Preferential esterification of stearic acid to form phospholipid rather than triglyceride *via* the diglyceride pathway may be a possibility but there is no evidence to suggest that such preferential esterification is occurring. One interesting possibility, however, is that fatty acid may be incorporated into phospholipid

via lysolecithin by the pathway described by Lands [1958, 1960]. Most of the label is present in lecithin in the present experiments and it might be noted that the lysolecithin + fatty acid→lecithin pathway for lecithin synthesis has been desciibed for polymorphs [Elsbach, 1965 *a*] and for alveolar macrophages [Elsbach, 1965 *c*]. Preferential esterification of stearic acid to phospholipid by this pathway would explain the presence of stearic acid in the phospholipid but not in the triglyceride.

It can be concluded, therefore, from the present work that R.E. cells in lymph nodes take up triglyceride emulsions and that such preparations are hydrolyzed, the fatty acid being partly removed and partly incorporated into phospholipid and into cholesterol ester. The rise in specific activity of the phospholipid fatty acid and the appearance of stearic acid in the phospholipid fraction suggest strongly that in the R.E. cells of the nodes phospholipid is being turned over more rapidly than other lipids.

Acknowledgments

We are indebted to Misses M. Kleeman and M. Wynbergen for technical assistance.

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