J. Lipid Research, April, 1962
Volume 3, Number 2

Incorporation of palmitate-1-C^{14} into neutral lipid of rat diaphragm*

E. M. Neptune, Jr., H. C. Sudduth, M. Colodzin, and J. J. Reish, Jr.

Naval Medical Research Institute, National Naval Medical Center, Bethesda 14, Maryland

[Received for publication September 8, 1961]

SUMMARY

When excised rat diaphragms are incubated with palmitate-1-C^{14}, radioactivity is found in triglyceride, diglyceride, and monoglyceride. The specific activity of diglyceride exceeds that of triglyceride after the various time periods studied. With increasing incubation time, the specific activity of triglyceride gradually approaches that of diglyceride. When diaphragm is incubated with labeled palmitate and then transferred to a second medium containing unlabeled palmitate, there is a marked decrease in the specific activity of the diglyceride fraction. There is formation of labeled triglyceride when a cell-free, particulate preparation of diaphragm is incubated with labeled dipalmitin and palmitoyl-CoA. After very short incubation periods with labeled palmitate, the specific activity of the total neutral lipid is significantly higher than that of the total phospholipid. In addition, the values obtained in these experiments were compared with the values obtained in a second study of individual phosphatides of diaphragm. In all instances, the specific activity of the diglyceride exceeded that of the glycerol phosphatides that were isolated. The data are consonant with the hypothesis that diglyceride is a precursor of triglyceride and glycerol phosphatides.

In an earlier communication from this laboratory (1), it was reported that labeled palmitate was incorporated more rapidly into unfractionated neutral lipid than into phospholipid during incubation of rat diaphragm *in vitro.* This raised the possibility that neutral lipid might be a precursor of phospholipid or might transfer fatty acid to phospholipid. If neutral lipid is a precursor of phospholipid in diaphragm, then the most likely possibility is that labeled palmitate is incorporated into a diglyceride fraction of neutral lipid as shown by Kennedy and co-workers for various other tissues (2). A number of other workers, as cited earlier (1), have presented evidence to support transesterification as the mechanism for incorporation of fatty acid into neutral lipid; if transesterification were operative in diaphragm, then it would be expected that labeled palmitate would appear predominantly in the triglyceride fraction. A number of experiments were designed to investigate these two possibilities, and the results are reported here.

* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

EXPERIMENTAL METHODS

Rat diaphragms were prepared as described previously (1) with the following exceptions: Diaphragms were extracted by grinding with chloroform—methanol 2:1 with the aid of sea sand. The smooth paste was mixed with more than 50 volumes of chloroform—methanol and centrifuged. The supernatant extract was decanted, and the debris of tissue and sand was re-extracted with a similar volume of chloroform—methanol. The pooled lipid extract was taken just to dryness at 70° under N_2, was immediately redissolved in approximately 3 ml of chloroform, and was again dried and redissolved in 3 ml of chloroform.

The lipid mixture in chloroform was placed on a 2-g silicic acid column, packed in an upright 10-ml syringe; a disk of lipid-extracted filter paper prevented escape of silicic acid into the receiving vessel. The silicic acid used was Mallinckrodt 100 mesh, preheated

Downloaded from www.jlr.org by guest, on June 19, 2012

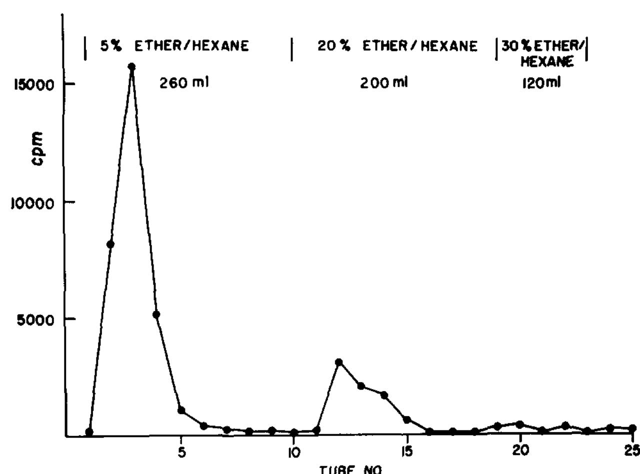

FIG. 1. Silicic acid chromatography of neutral lipid obtained from the diaphragms of 10 rats after a 10-minute *in vitro* incubation with palmitate-1-C^{14}. The values are observed counts per minute per tube of eluate. See text and Table 3 for other details.

at 125° for 12 to 24 hours. The neutral lipids were eluted with 30 ml of chloroform and the phospholipids with 30 ml of absolute methanol. The elution technique was checked by chromatographing varying amounts of neutral lipid and phospholipid obtained from liver and muscle. Both fractions labeled with C^{14} were available from earlier *in vivo* experiments, and unlabeled fractions were prepared as necessary. It was shown that up to 5 mg of phospholipid and 10 mg of neutral lipid could be cleanly separated. In five experiments, 96 ± 3.2% of both fractions was recovered.

Neutral lipids were fractionated on a 4-g, 1 × 11-cm silicic acid column by using the elution sequence recommended by Barron and Hanahan (3). The elution volumes shown in Figure 1 were satisfactory for up to 40 mg of neutral lipid.

Figure 1 represents the results from a typical experiment. Diaphragms from 10 rats were incubated with labeled palmitate for 10 minutes, and the neutral lipids were separated and chromatographed. The material in the first peak is triglyceride plus free fatty acid; the material in the second peak is diglyceride. The elution sequence continues with 120 ml of 30% ether in hexane, and a small monoglyceride peak occurs here. The preliminary elution sequence of 30 ml of hexane and then 100 ml of 15% benzene in hexane, as recommended by Barron and Hanahan, is not shown in the figure.

The identity of these fractions was first established by eluting synthetic, saturated neutral lipids singly and then in mixtures. These compounds were eluted very sharply with the appropriate solvents, whereas the muscle neutral lipids were less sharply eluted, as shown in Figure 1. This may represent an effect of the partial unsaturation of the muscle lipids as contrasted with the saturated, synthetic standards.[1] In addition, the identity of these fractions was checked repeatedly by glycerol—fatty acid ratios. Free fatty acids, eluted with the triglyceride, were extracted by the Borgström technique (4).

The flow rate was approximately 2 ml per minute and was controlled by nitrogen under positive pressure. On several occasions, the column was inadvertently allowed to run dry; the elution was continued, and the separations did not appear to be disturbed. The reproducibility of the column is so excellent that batch collection should be possible, although it is not difficult to follow the elution by measuring the radioactivity of each fraction. This column is excellent for neutral lipid from diaphragm, but other tissues containing appreciable amounts of sterols or other compounds may not be successfully chromatographed on this column.

Esters of the fatty acids were determined by the method of Rapport and Alonzo (5). Glycerol was determined by the method of Van Handel and Zilversmit (6). "Mitochondria"[2] were prepared by modifications of the techniques of Schneider (7) and of Hogeboom *et al.* (8). Dipalmitin-carboxyl-C^{14} was prepared by the action of pancreatic lipase on tripalmitin-carboxyl-C^{14} as described by Hanahan *et al.* (9) with the aid of the emulsion technique reported by Balls *et al.* (10). Palmitoyl-CoA was prepared by the technique of Seubert (11).

RESULTS AND DISCUSSION

The results of these studies clearly indicate that the designation of the neutral lipid fraction as "triglyceride" was incorrect in the earlier report from this laboratory (1) and that the hypothesis proposed from the earlier data is not tenable.

At the earliest incubation period studied (10 minutes) in the previous report (1), there was some ambiguity as to whether or not the specific activity of "triglyceride" (neutral lipid) exceeded that of phospholipid. In this study, therefore, experiments were performed in which diaphragms were incubated with palmitate-1-C^{14} for 1 and 5 minutes. The results, shown

[1] The synthetic, authentic dipalmitin used was a gift from Dr. F. H. Mattson, Procter and Gamble Co., Cincinnati, Ohio.

[2] Recent work in this laboratory indicates that the "mitochondrial" fraction contains an appreciable amount of the microsome fraction. Therefore, the enzymatic activity reported here for the particulate fraction cannot be attributed specifically to mitochrondria.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 1. INCORPORATION OF PALMITATE-1-C^{14} INTO LIPIDS OF DIAPHRAGM*

Incubation Time	Incorporation into Tissue FFA†	Neutral Lipid		Phospholipid	
		Sp. Act.‡	Incorporation†	Sp. Act.‡	Incorporation†
min	*mμmoles/g*	*cpm/mg*	*mμmoles/g*	*cpm/mg*	*mμmoles/g*
1	4.0 ± 0.87	27 ± 5.1	1.9 ± 1.14	0	0
5	4.1 ± 1.14	199 ± 0.91	11.7 ± 0.96	7.5 ± 29.7	3.0 ± 0.87

* Both hemidiaphragms from one rat were incubated at 37° in 5 ml Ringer-phosphate, pH 7.4, containing 0.5 μmoles of palmitate-1-C^{14} and 7.5 mg of crystalline bovine albumin. Results shown represent the mean and standard error of four experiments at each time interval.

† Minimum incorporation calculated from the specific radioactivity of added palmitate-1-C^{14} and the total count incorporation into each fraction.

‡ Specific radioactivity of the fatty acid moiety.

in Table 1, clearly indicate that the specific activity of the total neutral lipid fraction exceeds that of the phospholipid fraction during these brief periods of incubation.

From the data in Table 2, it is evident that there is radioactivity in all three fractions of the neutral lipid after a 10-minute incubation. The specific activity of the diglyceride, however, is 9-fold greater than that of the triglyceride or monoglyceride. After increasingly longer periods of incubation, the specific activity of the triglyceride gradually approaches that of the diglyceride. Although the chemical concentration of these glycerides varied somewhat, the triglyceride represented about 95%, the diglyceride 5%, and the monoglyceride 0.5% to 1.0% of the total neutral glycerides. When the neutral lipids were chromatographed on the large column of Barron and Hanahan (3), no appreciable radioactivity appeared in the eluate fractions that contain sterols and sterol esters; the glyceride fractions appeared exactly as described by these workers.

In a further attempt to gain information about the relative turnover rates of the diglyceride and triglyceride fractions, a transfer type of experiment was performed in which diaphragms were first incubated with labeled palmitate, then rinsed in buffered salt solution and transferred to flasks containing unlabeled palmitate. The assumption is that, with compounds having a rapid turnover with respect to palmitic acid, the labeled fatty acid would be rapidly replaced by the unlabeled fatty acid in the second incubation. The data from two such experiments are shown in Table 3. In the first experiment in which the incubation times were 20 minutes in labeled palmitate and then 20 minutes in unlabeled palmitate, the specific activity of the diglyceride increased during the second incubation. At the end of the first incubation, there was still a very appreciable amount of radioactivity in the free fatty acid fraction in the tissue, and after the second incuba-

TABLE 2. SPECIFIC ACTIVITIES OF DIAPHRAGM NEUTRAL LIPID COMPONENTS AFTER VARIOUS PERIODS OF INCUBATION WITH PALMITATE-1-C^{14}*

Time	Fraction	Specific Activity	Specific Activity Ratio (Diglyceride/Triglyceride)
min		*cpm/μmole*	
10	Triglyceride	273	
	Diglyceride	2340	8.5
	Monoglyceride	92	
20	Triglyceride	999	
	Diglyceride	7519	7.5
	Monoglyceride	474	
30	Triglyceride	1152	
	Diglyceride	4732	4.1
	Monoglyceride	2349	
40	Triglyceride	1041	
	Diglyceride	5542	5.3
	Monoglyceride	470	
60	Triglyceride	1137	
	Diglyceride	3184	2.8
	Monoglyceride	588	
120	Triglyceride	2593	
	Diglyceride	5192	2.0
	Monoglyceride	1380	

* Diaphragms from 7 to 10 rats were used for each set of values at any one incubation period. Each flask contained 0.5 μmoles of palmitate-1-C^{14} and 7.5 mg of crystalline bovine albumin in 5 ml of Ringer-phosphate, pH 7.4. Four hemidiaphragms were placed in each flask (of necessity, the final flask contained only two hemidiaphragms in some of the experiments). All radioactivity values have been normalized by proportional correction of the actual initial radioactivity per flask against a theoretical radioactivity of 100,000 cpm/flask.

Downloaded from www.jlr.org by guest, on June 19, 2012

TABLE 3. Specific Activity of Neutral Lipid after Transfer from Labeled Substrate to Unlabeled Substrate*

Lipid Fraction	Specific Radioactivity (cpm/μmole) Incubation with Labeled Substrate	Incubation with Unlabeled Substrate
	20 min	*20 min*
Triglyceride	651	768
Diglyceride	2636	3760

Lipid Fraction	Incubation with Labeled Substrate	Incubation with Unlabeled Substrate
	20 min	*60 min*
Triglyceride	999	957
Diglyceride	4334	1050

* In the 20 minute-20 minute experiments, hemidiaphragms from 14 rats were incubated in a medium containing palmitate-1-C^{14} (albumin-complex). There were four hemidiaphragms per flask, and each flask contained 0.36 μmoles of palmitate in 4 ml of Ringer-phosphate, pH 7.4. After 20 minutes, one-half of the diaphragms were rinsed and transferred to flasks containing an equivalent amount of unlabeled palmitate and incubated for another 20 minutes. Both sets of tissue were rinsed and immediately ground in chloroform–methanol after their respective incubations. The 20 minute-60 minute experiment was similar except that the second incubation was 60 minutes in duration and only 12 rats were used.

tion there was essentially no radioactivity in this tissue FFA fraction. The incorporation of this labeled tissue FFA into neutral lipid could well account for the marked rise in the specific activity of diglyceride and slight rise in the specific activity of triglyceride during the second 20-minute incubation. Furthermore, as shown by Zierler (12), there is a depression of respiration when diaphragm is transferred to fresh incubation medium. Such a depression of respiration and oxidative metabolism might very well slow the rate at which unlabeled fatty acid is esterified during the second 20-minute incubation. This would also account in part for the lack of evidence for a rapid replacement of labeled esterified fatty acid by unlabeled fatty acid. Therefore, the experiment was repeated but with the second incubation lasting one hour. Here the data clearly indicate that the specific activity of the diglyceride is markedly reduced, whereas the specific activity of the triglyceride is much less changed. This is consonant with the concept that, after transfer, the labeled fatty acids of diglyceride are replaced by the unlabeled palmitate.

TABLE 4. Radioactivity in Neutral Lipids after Incubation of Diaphragm Mitochondria with Dipalmitin-Carboxyl-C^{14} and Palmitoyl-CoA*

Lipid Fraction	Total Radioactivity
	cpm
Triglyceride	132,000
Diglyceride	324,000
Monoglyceride	20,000

* The reaction mixture contained 1.5 μmoles dipalmitin (500,000 cpm), 4 μmoles of palmitoyl-CoA, mitochondria from diaphragms of 5 rats in 1.0 ml of 0.25 M sucrose, 40 μmoles of cysteine, 60 μmoles of $MgCl_2$, 20 mg of Tween 20, 4 μmoles of palmitoyl-CoA, and 1000 μmoles of Tris buffer (tris[hydroxymethyl]aminomethane) pH 7.4, in a final volume of 10 ml. Before these additions, the dipalmitin was added to the flasks and the organic solvent was evaporated under a stream of nitrogen. The gas phase was nitrogen and the bath temperature was 37°. At the end of two hours, the total flask contents were extracted with chloroform–methanol and chromatographed as described in the text.

Additional experiments were performed in an attempt to observe whether or not diglyceride could be converted to triglyceride by diaphragm. Diaphragm "mitochondria" were incubated with dipalmitin carboxyl-C^{14} and palmitoyl-CoA in a system nearly identical to that reported by Weiss and Kennedy (13). The results, shown in Table 4, demonstrate appreciable formation of triglyceride.

Kennedy (2) has presented evidence that diglyceride is not only a precursor of triglyceride but is also a precursor of certain glycerol phosphatides. In the initial experiments described in this communication, the specific activity of the total neutral lipid exceeded that of the total phospholipid. Since the possibility remained that one fraction of the phospholipid mixture might be very highly labeled, the data obtained in these experiments were compared with the data obtained by Colodzin *et al.* (14) for individual components of the phosphatide mixture. In every comparison made, the specific acitivity of diglyceride was greater than that of any of the individual phosphatides isolated by Colodzin *et al.* Thus, the data in this report are compatible with diglyceride serving as precursor for either triglyceride or the glycerol phosphatides isolated from diaphragm muscle in agreement with the pathway proposed by Kennedy (2).

REFERENCES

1. Neptune, E. M., Jr., H. C. Sudduth, D. R. Foreman, and F. J. Fash. *J. Lipid Research* **1**: 229, 1960.
2. Kennedy, E. P. *Ann. Rev. Biochem.* **26**: 119, 1957.

Downloaded from www.jlr.org by guest, on June 19, 2012

3. Barron, E. J., and D. J. Hanahan. *J. Biol. Chem.* **231**: 493, 1958.
4. Borgström, B. *Acta Physiol. Scand.* **25**: 111, 1952.
5. Rapport, M. M., and N. Alonzo. *J. Biol. Chem.* **217**: 193, 1955.
6. Van Handel, E., and D. B. Zilversmit. *J. Lab. Clin. Med.* **50**: 152, 1957.
7. Schneider, W. C. *J. Biol. Chem.* **176**: 259, 1948.
8. Hogeboom, G. H., W. C. Schneider, and G. E. Palade. *J. Biol. Chem.* **172**: 619, 1948.
9. Hanahan, D. J., H. Brockerhoff, and E. J. Barron. *J. Biol. Chem.* **235**: 1917, 1960.
10. Balls, A. K., and M. B. Matlack. *J. Biol. Chem.* **123**: 679, 1938.
11. Seubert, W. *Biochem. Prep.* **7**: 80, 1960.
12. Zierler, K. L. *Am. J. Physiol.* **185**: 12, 1956.
13. Weiss, S. B., and E. P. Kennedy. *J. Am. Chem. Soc.* **78**: 3550, 1956.
14. Colodzin, M., E. M. Neptune, Jr., and H. C. Sudduth. *J. Lipid Research* **3**: 234, 1962.

Downloaded from www.jlr.org by guest, on June 19, 2012