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Phospholipid and triglyceride metabolism of excised rat diaphragm and the role of these lipids in fatty acid uptake and oxidation*

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SUMMARY

After a 48-hour fast, the phospholipid fatty acids of rat diaphragm are reduced much more than the triglyceride fatty acids. Phospholipid thus appears to contribute more to the labile fatty acids than triglyceride. Both long- and short-chain C^{14}-labeled fatty acids are incorporated into triglyceride but only the long-chain fatty acids are incorporated into phospholipid. After incubation with palmitate-1-C^{14} and transfer to substrate-free Ringer phosphate, triglyceride specific activity decreases but phospholipid specific activity increases. During incubation with palmitate-1-C^{14} the triglyceride specific activity rises, then remains constant. Phospholipid specific activity is initially the same as triglyceride but rises at a nearly linear rate. There is net synthesis of triglyceride but not of phospholipid. Combustion of long-chain fatty acids to CO_2 indicates the existence of an endogenous dilution pool. It is proposed that phospholipids and triglycerides participate in the oxidation of saturated fatty acids.

In a previous communication (1) labile fatty acid concentrations were measured in excised rat diaphragm. The fall in concentration of these fatty acids during a 4-hour incubation could, in theory, support nearly all of the respiration of this tissue. In the earlier study only total fatty acids were determined. The present studies were designed to investigate the contribution of the major lipid components, phospholipid and triglyceride, to the labile fatty acids and the interrelationship of these lipids.

MATERIAL AND METHODS

Incubation Techniques. Diaphragm muscle was obtained as described previously (2). In the transfer experiments additional diaphragm was obtained from donor rats and incubated in the transfer recipient flask; these were discarded just at the time of transfer. Zierler (3) has presented evidence that diaphragm is metabolically dependent on enzymes that leak out into the surrounding medium during *in vitro* incubation. This donor incubation was employed to minimize depression of metabolism upon transfer to another medium. The incubation medium was Ringer phosphate, calcium free, as described previously (2). The flasks were incubated in a Dubnoff shaking metabolic incubator at 37°C.

Extraction of Lipids. At the end of the experimental period the tissues were removed, blotted on filter paper, and ground in a porcelain mortar with 0.5 to 1.0 ml. of (3:1, v/v) alcohol-ether. The ether was freshly distilled each day from stock ether which contained a small quantity of hydroxylamine HCl. The muscle suspension was transferred to a 25-ml. volumetric flask with three or four washings of alcohol-ether to a total volume of 12 to 17 ml. The solution was brought to a boil three times in a boiling water bath, cooled, and made to 25 ml. with alcohol-ether. The contents of the flask were transferred to tubes and centrifuged at 3,000 rpm. for 2 minutes. One ml. of supernatant was transferred to an aluminum planchet for determination of radioactivity.

Triglyceride Determination. Three ml. was removed for determination of fatty acid-ester bonds by the hydroxylamine, ferric chloride method of Stern and Shapiro (4); the color from various concentrations of

* 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.

diaphragm phospholipid was determined separately and used to correct the total ester value after phospholipids were determined. The final value, compared to a tripalmitin standard, was called the triglyceride fraction; triglyceride fatty acid values were obtained by assuming that 94 per cent of the triglyceride was composed of fatty acids.

Phospholipid Determination. The remaining 21 ml. was taken to dryness at 100°C under a stream of nitrogen; 1 ml. of hexane was added and the tube warmed briefly. Then 14 ml. of acetone and 0.1 ml. of saturated $MgCl_2$ in ethanol were added, mixed for 5 minutes, and cooled at 10°C for 14 to 18 hours or at —10°C for 2 hours. The mixture was centrifuged, the supernate decanted, and the precipitate washed with 10 ml. of acetone. Since the acetone wash never contained over 1 per cent of the radioactivity present in the original acetone supernate, the acetone wash was routinely discarded in the isotope experiments.

The acetone-$MgCl_2$-insoluble precipitate was dried under nitrogen and treated in one of two ways:

(*a*) To the precipitate, 1.5 ml. of 60 per cent KOH and 1.5 ml. of absolute ethanol were added. The tube was capped, heated for 2 hours at 100°C, cooled to room temperature, and 1.5 ml. of 18 N H_2SO_4 was added. After cooling, 10 ml. of *n*-hexane was added and the tube shaken on a mechanical shaker for one-half hour. After centrifugation, 4 ml. of the hexane was taken to dryness under N_2 and titrated as reported previously (1). The titrated fatty acids in this fraction are referred to as phospholipid fatty acids. One ml. of the hexane was dried on a planchet for determination of radioactivity.

(*b*) In certain phases of the work, the radioactivity of the intact phospholipid molecule was determined. The acetone-$MgCl_2$ precipitate was dried and cooled. Four ml. of chloroform was added and, after warming, the tube was shaken and centrifuged. One ml. was transferred to a planchet for determination of radioactivity. The remaining 3 ml. was dried, digested, acidified, and extracted as in (*a*).

A detailed comparison of the acetone-$MgCl_2$ technique with silicic acid chromatography has been published by Borgström (5). Although separation by silicic acid chromatography yielded better recovery of phospholipid than the acetone-$MgCl_2$ precipitation, Borgström was able to precipitate 98 to 99 per cent of the lipid phosphorus. In the present study, experiments demonstrated that 96.4 ± 0.63 per cent of the total phosphorus present in the lipid extract was precipitated by acetone-$MgCl_2$. Although Borgström found that about 4 per cent of labeled free fatty acid added to phospholipid was coprecipitated by acetone-$MgCl_2$, our observations with silicic acid chromatography of precipitated phospholipid indicate that there is no appreciable contamination of the phospholipid fraction by free fatty acid.

Radioactivity and Manometric Determinations. Radioactivity and manometric CO_2 determinations were performed as described previously (2) with the following exceptions: Initial and residual activities of the incubation media were determined by direct plating of 0.1 ml. of a 1:10 dilution of the aqueous solutions. Radioactivity of material in organic solvents was determined by drying 1 ml. of the solution in a planchet. "Triglyceride" radioactivity was determined on the acetone-$MgCl_2$ supernate. An aliquot of the acetone supernate was also dried, taken up in hexane, transferred and extracted with alkaline ethanol as described by Borgström (6), and the extractable radioactivity determined and tabulated as free fatty acids. Triglyceride values were corrected by subtracting radioactivity and free fatty acids. Recovery experiments using palmitic acid-1-C^{14} were in excellent agreement with those reported by Borgström.

Preparation of Albumin-Fatty Acid Complexes. The long-chain fatty acid-albumin complexes were prepared in the following manner: Both labeled and unlabeled fatty acids of a measured weight were dissolved in alcohol-ether, and aliquots were dried under nitrogen at 100°C when using saturated acids, or at 60°C when using unsaturated acids. The sodium salts were prepared by adding a slight excess of NaOH in ethanol, and subsequent drying under nitrogen. The albumin was dissolved in approximately 10 ml. of Ringer phosphate and warmed to 40°C on a water bath. To the dry fatty acid salt, 0.5 ml. of Ringer phosphate was added and the tubes heated under nitrogen to the temperature listed above. The cloudy mixture was then transferred to the albumin solution with two more 0.5 ml. washes of Ringer phosphate and the optically clear albumin-fatty acid solution made to volume.

The albumin in these experiments was bovine albumin fraction V or crystalline bovine albumin. The fatty acid content of these proteins was determined by digestion, extraction, and titration, and its value incorporated into all calculations of fatty acid concentrations.

RESULTS

Incorporation of Labeled Fatty Acid. The values for phospholipid and triglyceride fatty acids before and after a 4-hour incubation with diaphragm from fed rats are given in Table 1. Both phospholipids and

TABLE 1. PHOSPHOLIPID AND TRIGLYCERIDE FATTY ACIDS OF DIAPHRAGM BEFORE AND AFTER A 4-HOUR INCUBATION COMPARED WITH TOTAL FATTY ACIDS *

	Number of Experiments	A Phospholipid Fatty Acids	Number of Experiments	B Triglyceride Fatty Acids	A Plus B as Per Cent of Total Fatty Acids †
		mg./g. wet wt.		*mg./g. wet wt.*	
Fed					
Before	5	7.19	11	13.16	91
After	5	5.38	11	12.18	96
Difference		−1.81 ±0.41		−0.98 ±0.21	69
p		<0.01		<0.01	
Fasted 48 Hours					
Before	5	0.84	5	10.30	87
After	5	0.34	5	9.48	87
Difference		−0.50 ±0.126		−0.82 ±0.05	87
p		<.02		<0.001	

* One-half diaphragm was used as a control; the opposite half was incubated for 4 hours. Total volume of medium was 3.0 ml.

† Total fatty acids from figures published previously (1). Variations are estimated standard errors.

triglycerides decrease during the incubation but there is a greater decrease in the phospholipid fatty acids. A more striking demonstration of the lability of phospholipid fatty acids can be seen in diaphragm from rats fasted for 48 hours. The preincubation level of phospholipids is only one-tenth the value for fed rats, whereas triglyceride fatty acids decrease by only 22 per cent. After a 4-hour incubation, these tissues from fasted rats have a somewhat greater fall in triglyceride fatty acids than phospholipid fatty acids, but the total fall in fatty acids is much less than noted with tissues from fed rats.

These results are in general agreement with the total fatty acid studies reported earlier (1). It is apparent that the phospholipid plus triglyceride values do not represent 100 per cent of the total fatty acids. Whether this is due to technical difficulties or to other unidentified lipid components has not been determined.

In Table 2 the results of studies are listed in which muscle was incubated with palmitate-, laurate-, oleate-, octanoate-, butyrate-, or acetate-1-C^{14}. All of the acids studied are incorporated into triglyceride; the greater incorporation of the short-chain acids may in part be due to the much higher concentration of these acids in the incubation medium. Although there is measurable incorporation of long-chain fatty acids in phospholipid, there is no incorporation of acetate, butyrate, or octanoate.

The great chemical lability of the phospholipids compared to that of triglycerides suggests that the phospholipids undergo a more rapid degradation than triglycerides and perhaps play a more active metabolic role. Therefore a series of experiments were performed in the following manner: Hemidiaphragms were incubated with C^{14}-labeled palmitate. At the end of 2 hours, one hemidiaphragm of each pair was removed for determination of lipids and radioactivity; the other hemidiaphragm was transferred to a flask containing unlabeled palmitate and incubated for 2 more hours, and then removed for determination of lipids and radioactivity. The results are shown in Table 3. Although during the second incubation there is no significant change in the concentrations of phospholipid, there is a significant fall in phospholipid specific activity. This indicates that there is a greater loss of labeled than of unlabeled fatty acid. The fall in specific activity of triglyceride indicates that this fraction is also losing labeled fatty acid faster than unlabeled. This experiment does not exclude transfer of labeled fatty acid from triglyceride to phospholipid, or the reverse.

To examine the interrelationship between triglyceride and phospholipid further, another series of experiments was performed in much the same way except that the tissues were transferred to Ringer phosphate without added substrate. The results are shown in Table 3. Although the fall in triglyceride concentration is paralleled by a fall in specific activity, there is a

TABLE 2. INCORPORATION OF C^{14}-LABELED PALMITATE, LAURATE, OLEATE, OCTANOATE, BUTYRATE, AND ACETATE INTO TRIGLYCERIDE, PHOSPHOLIPID, AND FREE FATTY ACID OF RAT DIAPHRAGM *

Added Substrate	Uptake	Triglyceride	Phospholipid	Free Fatty Acid
	mμmole/g.	*mμmole/g.*	*mμmole/g.*	*mμmole/g.*
Palmitate (5)	876 ± 86	260 ± 43	81 ± 8.2	42 ± 5.9
Laurate (4)	7121 ± 831	2250 ± 454	592 ± 129	920 ± 165
Oleate (6)	861 ± 120	191 ± 40	34 ± 6.5	86 ± 18.4
Octanoate (3)	———	4435 ± 1150	0	497 ± 291
Butyrate (5)	33,100 ± 3,400	167 ± 87	0	290 ± 106
Acetate (4)	16,800 ± 1,056	230 ± 69	0	490 ± 132

* One-half diaphragm from fed rat was incubated for 2 hours. Total volume was 2.7 ml. Substrates were present in the following concentrations: palmitate 0.9×10^{-4}, laurate 1.5×10^{-4}, oleate 1.0×10^{-4}, octanoate 22.2×10^{-4}, butyrate 44.4×10^{-4}, acetate 44.4×10^{-4}M. Palmitate, laurate, and oleate were all added as the albumin complex (see text). Figures in parentheses indicate the number of experiments. Uptake was calculated by measuring the residual activity in the medium and subtracting this from the initial activity. The percentage of radioactivity taken up was multiplied by chemical concentration to derive the mμmole values.

TABLE 3. CHANGES IN CHEMICAL VALUES AND SPECIFIC ACTIVITY OF TRIGLYCERIDE AND PHOSPHOLIPID OF DIAPHRAGM INCUBATED IN LABELED FATTY ACIDS AND THEN TRANSFERRED TO A SECOND MEDIUM *

Incubation Medium		Phospholipid		Triglyceride	
		Concentration	Specific Activity	Concentration	Specific Activity
		mg./g. wet wt.	*cpm./mg. fatty acid*	*mg./g. wet wt.*	*cpm./mg. fatty acid*
A. 2 hours in C^{14}-palmitate, then transferred to unlabeled palmitate (6)	2 hours	3.18	1209	16.3	1020
	4 hours	2.80	1025	13.7	718
	pair difference	−0.38 ±0.69	−177 ±50.8	−2.6 ±0.48	−302 ±75.6
	p value	>0.6	<0.01	<0.01	<0.01
B. 2 hours in C^{14}-palmitate, then transferred to Ringer phosphate (12)	2 hours	5.02	3196	19.0	3570
	4 hours	2.00	10,296	14.6	1226
	pair difference	−3.02 ±0.51	+7100 ±2110	−4.4 ±1.02	−344 ±98.4
	p value	<0.01	<0.01	<0.01	<0.01

* Incubation medium was Ringer phosphate, calcium free, pH 7.4, total volume 2.7 ml. One-half diaphragm was removed at 2 hours and the other half transferred and incubated for 2 more hours. Initial concentrations and radioactivity: A. = 0.9×10^{-4}M and 18,000 cpm./flask; B. = 1.2×10^{-4}M and 62,500 cpm./flask. The number of experiments in each series is in parentheses. Variations are expressed as standard errors.

striking rise in phospholipid specific activity despite a fall in concentration. This nearly threefold increase in specific activity is compatible with the concept that phospholipid is enriched from other lipid sources. There is again a depletion of labeled fatty acids in the triglyceride component.[1]

[1] These experiments were suggested by LCDR H. D. Baldridge, MSC, USN.

To elucidate the lipid component into which exogenous fatty acid is incorporated, some experiments were performed in which the early incorporation of labeled palmitate into phospholipid and triglyceride was studied. One hemidiaphragm served as a zero time control, while the opposite hemidiaphragm was incubated for a given time period with labeled palmitate. The results of this experiment are portrayed in Fig-

TABLE 4. OXIDATION OF FATTY ACID AND GLYCEROL SUBSTRATES TO $C^{14}O_2$ BY RAT DIAPHRAGM (2-HOUR OBSERVATION) *

Added Substrate	Concentration	$C^{14}O_2$ / Substrate C^{14} × 100	Total CO_2	CO_2 from Substrate / total CO_2 × 100
	$10^{-4}M$		μmole	
Laurate (8)	0.9	18	5.07	9.5 ± 0.60
Laurate (8)	1.8	28	5.99	26.0 ± 0.60
Palmitate (8)	0.9	7	5.90	5.4 ± 0.32
Palmitate (8)	1.2	9	4.76	10.1 ± 0.90
Oleate (12)	1.0	5	5.80	3.1 ± 0.30
Glycerol (12)	11.1	3	5.30	5.1 ± 0.60

* Quarter diaphragms were incubated in Dixson-Keilin flasks for 2 hours in 2.7 ml. of Ringer phosphate, calcium free, pH 7.4, 37°C. Substrate concentrations are listed in the Table. Techniques and calculations have been described elsewhere (2). Variations are estimated standard errors. Figures in parentheses indicate the number of individual experiments.

ure 1. At 10 minutes the specific activity of triglyceride is not significantly different from that of phospholipid, but although the triglyceride specific activity remains constant over the next 60 minutes there is a progressive rise in the specific activity of phospholipid. The concentrations indicate that there is an early net synthesis of triglyceride but not of phospholipid.

The Conversion of Fatty Acids to CO_2. In a separate series of experiments the extent to which muscle converts labeled long-chain fatty acids to $C^{14}O_2$ was determined. The results with palmitate-, oleate-, and laurate-1-C^{14} are shown in Table 4. Data for glucose have been reported elsewhere (2) as well as data for butyrate and octanoate. Contrary to the results for glucose and short-chain fatty acids, the conversion of long-chain fatty acids to $C^{14}O_2$ constitutes only a small fraction of the total CO_2. The labile fatty acids of diaphragm (1) are almost all esterified. If these fatty acids represent a dilution pool for exogenous long-chain fatty acids, then exogenous fatty acids such as palmitate should also be esterified if they are truly mixing with this endogenous pool. As shown in Table 2, added palmitate is incorporated into both triglyceride and phospholipid, which are the two sources of the endogenous labile fatty acids. Of course it is also possible that the free fatty acids might constitute a direct dilution pool. Although these fatty acids are present in low concentration (1), they could dilute directly and significantly the added labeled fatty acid if they were turning over rapidly.

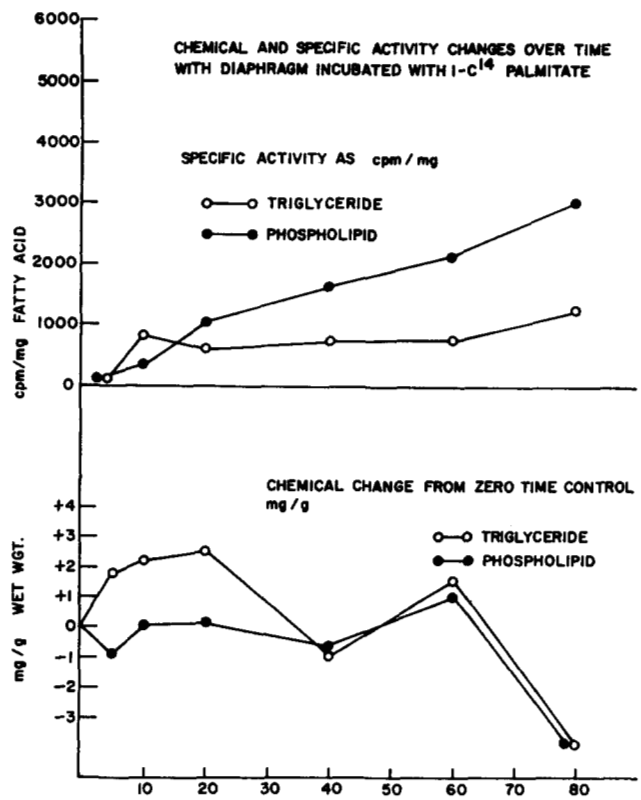

FIG. 1. One hemidiaphragm was used as a zero time control and the opposite hemidiaphragm was incubated for a given time period. The tissues were incubated in 2.7 ml. of Ringer phosphate. Palmitate-1-C^{14} was added in a concentration of 0.9 × 10^{-4}M. Initial activity was 29,000 cpm./flask. All values are the means of four separate experiments.

To test this pool concept, transfer experiments were performed in which muscle was incubated for 2 hours with labeled substrate, rinsed, and then transferred to vessels containing unlabeled substrate. The specific activities of CO_2 determined at different time intervals are shown in Figure 2. It is apparent that both glucose and octanoate show a definite fall in relative specific activity and the slopes of these curves are similar; the values for the specific activity of the CO_2 when diaphragm is incubated with palmitate do not fall. This indicates that the added labeled palmitate taken up by the tissue must be nearly completely equilibrated with the endogenous source of CO_2 over the time intervals studied and that entry of unlabeled fatty acid from the second medium is not rapidly diluting the endogenous pool. Furthermore, the constancy of the specific activity of the CO_2 after transfer to unlabeled substrate indicates that the dilution pool is large and this would not be compatible with the dilution pool existing in the form of free fatty acids; in the latter instance the specific activity of the CO_2 would rapidly decline after transfer to unlabeled substrate.

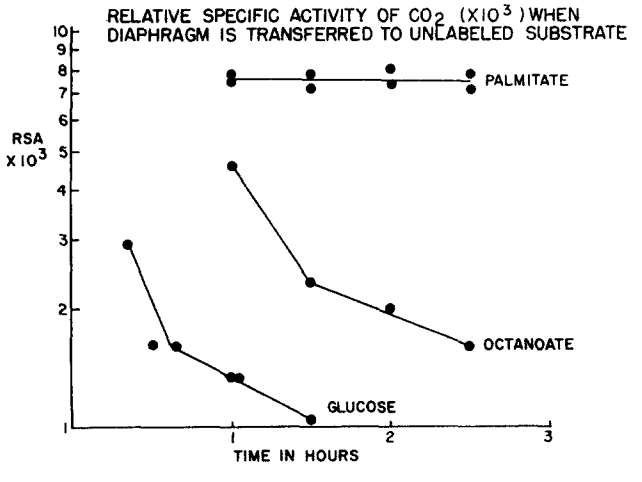

FIG. 2. Quarter diaphragms were incubated in Ringer phosphate, calcium free, pH 7.4 with one of the following substrates: glucose-U-C^{14} 82.6 $\times$ 10^{-4}M, octanoate-1-C^{14} 22.2 $\times$ 10^{-4}M, or palmitate-1-C^{14} 0.9 $\times$ 10^{-4}M for 1.5 hours. They were then transferred to Dixson-Keilin flasks containing the same amount of the same unlabeled substrates. Relative specific activity represents cpm./μmole CO_2/initial cpm.

DISCUSSION

It must be emphasized that in these initial studies phospholipids have been treated as a single group, whereas in actuality there are a variety of lipid compounds containing phosphorus, some of which undoubtedly have specific metabolic functions. The results of the present studies are sufficiently clear, however, to establish certain concepts.

It is evident from these studies that the endogenous labile fatty acids of rat diaphragm are contained in both phospholipid and triglyceride fractions. The phospholipid fraction is somewhat more labile during a 4-hour incubation and is strikingly reduced after a 48-hour fast.

Both long- and short-chain fatty acids labeled with C^{14} are incorporated into triglyceride, but of the acids studied only the long-chain acids, palmitate, oleate, and laurate, are incorporated into phospholipid. It is possible that the incorporation in triglyceride is mediated directly by lipase enzymes since the studies reported by Geyer *et al.* (7) show that striated rat muscle produces appreciable $C^{14}O_2$ when incubated with an emulsion containing carboxyl-labeled trilaurin, which presumably requires an active lipase. The failure of short-chain fatty acids to appear in the phospholipid fraction indicates a different mechanism which may depend, in part, on the difference in solubilities of short- and long-chain fatty acids. Bergström *et al.* (8) reported that in liver and intestine the incorporation of saturated acids C_{14}, C_{16}, and C_{18} into phospholipid was directly proportional to chain length. Hanahan and Blomstrand (9) found a much greater incorporation *in vivo* of palmitate than oleate into the lecithins of liver, intestine, and lymph. Stevens and Chaikoff (10) fed laurate-1-C^{14} and myristate-1-C^{14} and found 3 per cent of the laurate activity in carcass phospholipids as laurate and 19 per cent of myristate activity as myristate; most of the activity was found in the longer chain fatty acids and presumably represented elongation *in vivo* of the original 12 or 14 carbon fatty acids. Similarly, in our studies no incorporation of short-chain acids in phospholipid was observed. The monounsaturated acid, oleate, exhibited a lower proportion in phospholipid than laurate or palmitate.

In the study of the incorporation of palmitate with time, it appears that the specific activity of triglyceride remains at a constant level. There is an apparent net synthesis of triglyceride during the first hour, and during most of this period the specific activity is constant; this must mean that both labeled and unlabeled fatty acids are being incorporated in a constant proportion. The only obvious significant source of unlabeled fatty acid is phospholipid. The phospholipid specific activity values would indicate that labeled palmitate is incorporated more slowly in the very early part of the incubation, but that there is steady, almost linear, progressive enrichment.

The data presented do not clearly indicate the sequential pathway of incorporation of labeled palmitate entering the cell. It appears, however, that palmitate rapidly enters triglyceride and enters phospholipid more slowly; furthermore, fatty acid is apparently being transferred from phospholipid to triglyceride. If there is an active two-way shuttle of fatty acids between triglyceride and phospholipid, then palmitate may enter first into triglyceride and from there be more slowly incorporated into phospholipid. In experiments where diaphragm was transferred from labeled palmitate to buffered salt solution, it is possible to assume that low specific activity fatty acids are being removed from phospholipids with a net increase in their specific activity. The more tenable explanation, however, is that there is transfer to phospholipid of some high specific activity fraction of the triglyceride fatty acid.

The latter concept is supported by the experiments in which the tissues were transferred to unlabeled palmitate; in these there is no change in the amount of phospholipid, but there is a significant fall in specific activity; there is also a significant fall in the amount and specific activity of triglyceride. Therefore high specific activity fatty acids are leaving phospholipid and are also leaving triglyceride. It is most likely that these high specific activity fatty acids are being oxidized. It does not seem probable that, after transfer to unlabeled palmitate, fatty acids of high specific activity are removed from phospholipid, whereas after transfer to salt solution, fatty acids of low specific activity are preferentially removed. This, coupled with the observation that phospholipids fall more than triglycerides during an incubation or in starvation, would be compatible with the hypothesis that phospholipid fatty acids are transferred to oxidative enzyme systems for oxidative degradation.

The concept of transfer of saturated fatty acids from triglyceride to phospholipid was established in *in vivo* studies by Bergström *et al.* (8) when they fed triglyceride labeled with 1-C^{14} stearate. Although the specific mechanism was not elucidated by these workers, they suggested that transesterification might be involved. On the basis of the work of Stein and Shapiro (11) with liver preparations and also from the data of others (quoted by the authors) these investigators arrived at the "almost inescapable" conclusion that free fatty acid is first incorporated into triglyceride and then transferred to phospholipid. If this is the transfer mechanism in liver, intestine, and muscle, then this may represent a general role for phospholipids. The concept of such a role for phospholipid is not new and Artom has reviewed the historical background relating to this concept (12).

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