Uptake and esterification of palmitate by rat diaphragm in vitro

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ABSTRACT The contribution of exogenously supplied palmitate to the intracellular palmitate pool and its role in esterification were studied for the intact rat diaphragm in vitro. Palmitate-1-^{14}C attached to albumin in various molar ratios ($\bar{\nu}$) was taken up by the tissue in an initial rapid phase which led, after 4 min, to a steady-state level of tissue free fatty acid. The level was determined by $\bar{\nu}$ but also by the albumin concentration below 2 g/100 ml. The exogenous palmitate taken up constitutes one-quarter to three-quarters of the intracellular palmitate pool as $\bar{\nu}$ is increased from 0.5 to 5.5 (albumin concentration 2 g/100 ml).

Turnover rates of intracellular palmitate at a given $\bar{\nu}$ are stable over 35 min of incubation, as indicated by constant rates of esterification (largely to triglyceride, but also to phospholipids) and by the unchanging specific activity ratios between intracellular and medium palmitate-1-^{14}C. These ratios also indicate that only a portion of the palmitate taken up is available for exchange with the albumin complex in the medium.

Calculation of esterification rates at various $\bar{\nu}$ values suggests that not all of the palmitate is in a pool that is available for esterification.

KEY WORDS free fatty acid · muscle lipid metabolism · palmitate · fatty acid:albumin molar ratio

THE PRESENCE OF SMALL AMOUNTS of FFA has been reported in several tissues (1) including skeletal muscle (2, 3). It has been demonstrated that the FFA fraction of skeletal muscle is subject to modification by a variety of nutritional and hormonal factors. In the rat diaphragm, increases in FFA content of 200–400% in vivo are readily produced by starvation (2), epinephrine injection (4, 5), and the induction of alloxan diabetes (5). The sources and metabolic role of these FFA have not been clearly elucidated. The studies of Eaton and Steinberg (6) in a rat leg muscle, and of Spector, Steinberg, and Tanaka (7) in the Ehrlich ascites tumor cell suggest that the in vivo fluctuations may reflect the increased plasma FFA levels seen in the above conditions. In vitro studies in the rat diaphragm (4), on the other hand, suggest that the endogenous tissue lipids contribute materially to the size of the tissue FFA pool (8, 9). Measurement of the intracellular content of single FFA, which would allow the assessment of the relative contribution of the endogenous and exogenous lipids to the tissue FFA fraction, has not been undertaken in muscle.

The direct "intermediate" role of the tissue FFA in oxidation and esterification in the Ehrlich ascites tumor cell has been established (10). A similar role for muscle FFA is not clear since the kinetics of incorporation into the various tissue lipid fractions have not been studied in detail. The purpose of this study was to evaluate the contribution of exogenous palmitate to the tissue unesterified palmitate pool and to assess the role of intracellular palmitate as an intermediate in esterification.

METHODS

These experiments were performed on the intact (11) diaphragms taken from normal male rats of the Sprague-Dawley strain. The animals were fed Purina rat chow and tap water ad libitum, and weighed 200–250 g at the time of decapitation. To avoid cutting any of the muscle fibers, the diaphragm was removed en bloc with its surrounding rib cage and spine and incubated in 50 ml of Krebs–Ringer phosphate (KRP) buffer containing crystalline bovine serum albumin (Pentex, Inc., Kankakee, Ill.),

Abbreviations: FFA, free fatty acid(s); KRP, Krebs–Ringer phosphate; BSA, bovine serum albumin; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; TG, triglyceride; PL, phospholipid; TLC, thin-layer chromatography; $\bar{\nu}$, mole ratio of free fatty acid to albumin.

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palmitic acid (Calbiochem, Los Angeles, Calif.), and palmitic acid-1-^{14}C (Calbiochem) at 37°C with 100% oxygen. At the end of incubation the tissue was rinsed in ice-cold saline. The diaphragmatic leaflets were excised, blotted, frozen in liquid nitrogen, and weighed, and the lipids were extracted.

Bovine serum albumin (BSA) was usually used as a 2% solution in KRP buffer. The FFA concentrations of these solutions were 80–100 μeq/liter. Palmitate was added to albumin solutions as previously described (4). Sodium palmitate was stirred with a 15% solution of albumin (160 mg/5 g) at 50°C for 30 min. The mixture was stored at 4°C overnight and filtered. The FFA concentration of 2% "refatted" solutions varied between 1200 and 1800 μeq/liter. The two albumin solutions were then mixed to obtain any desired intermediate FFA concentrations. Palmitate represented >85% of the medium FFA at the lowest and >98% at the highest concentrations used in these studies. Sufficient palmitic acid-1-^{14}C was added to give a concentration of about 6×10^5 dpm/ml. Concentrations of FFA in the medium were determined by application of the colorimetric method of Duncombe (12) to extracts prepared as described by Dole (13). ^{14}C was counted in a Packard TriCarb model 3375 scintillation spectrometer either in Packard's Hyamine hydroxide (*p*-(diisobutylcresoxy-ethoxyethyl) dimethyl benzyl ammonium hydroxide) or with PPO–POPOP in toluene (14). Correction for quenching was made by the channels ratio method with Packard ^{14}C quench standards. The concentration and specific activities of medium FFA changed less than 10% during incubation.

Tissues were ground in chloroform–methanol 2:1 and extracted three times. The extracts (16 ml/200 mg of tissue) were pooled and washed once with 0.2 volume of 0.05% $CaCl_2$ (15). Triple extraction was used because it was found that about 15% of the triglyceride (TG) and 10% of the phospholipid (PL) were recovered in the second extraction, but the third extraction yielded negligible amounts of lipid. Aliquots of the solvent phase were dried under nitrogen and resuspended in a small volume of chloroform–methanol; one aliquot was counted directly for "total lipids"; another aliquot was applied to silica gel plates (Eastman Chromogram sheet, Rochester, N. Y.) and the FFA, TG, and PL fractions were separated by thin-layer chromatography (TLC) (5). The spots were scraped off the TLC plates and counted directly in PPO–POPOP and toluene.[1] Recoveries of palmitate-^{14}C and tripalmitin-^{14}C added to "cold tissue" and carried through the entire procedure were 93–97 and 87–92%, respectively. ^{14}C incorporation values are reported in mμeq/g wet weight of tissue and are based on the specific activity of the medium palmitate. Unesterified palmitate was determined after methylation of chloroform–methanol (2:1) eluates of the FFA spots (16) in a Barber-Coleman model 5000 gas chromatograph by the dual differential method with flame ionization detectors. The columns were packed with 15% (w/w) diethylene glycol succinate polyester on 80–100 mesh Gas-Chrom Q (Applied Science Laboratories, Inc., State College, Pa.). Temperature programming was at 5°C/min between 110 and 200°C. Peaks were identified by comparison of retention times with those of standard mixtures (Applied Science Laboratories) and quantified with the aid of a C_{15} internal standard (17).

Since FFA were exposed to the tissue in the albumin-bound form (18), it was necessary to determine the kinetics of albumin distribution in the diaphragm. Intact diaphragms were incubated for 1–30 min in 2% BSA, mixed with human serum albumin-^{131}I (Abbott Laboratories, North Chicago, Ill.) in KRP buffer at 37°C in 100% oxygen. At the end of incubation the tissue was rinsed in cold saline, the diaphragm leaflets were excised and blotted, and the ^{131}I contents of the tissue and incubation fluid were counted in a gamma spectrometer. More than 98% of the ^{131}I in the incubation medium and in the diaphragm after 30 min of incubation was precipitated by 5% trichloroacetic acid. Results are reported as mμmoles of albumin per g of tissue and are based on the specific activity of the incubating solution.

The reversibility of the binding of palmitate and of albumin to the tissue was assessed as follows. Diaphragms were preincubated in medium containing labeled palmitate, rinsed in saline, and reincubated either in KRP buffer or in a medium identical with the one used initially except for the absence of palmitate-^{14}C. At the end of incubation the tissues were processed as described above.

RESULTS

Relationship between Extra- and Intracellular Unesterified Palmitate

Palmitate-1-^{14}C was rapidly incorporated into the FFA fraction extracted from the tissue between 0 and 10 min of incubation. No further increase in uptake occurred thereafter (Table 1). As palmitate concentrations in the medium were raised from 150 and 500 and 1800 μeq/liter while BSA content was maintained at 2 g/100 ml (FFA:albumin molar ratios[2] [$\bar{r}$] of 0.5, 1.5, and 5.5), increasing amounts of label entered the FFA fraction.

[1] In order to ascertain that all of the PL were available to the fluor we eluted the phospholipid spots from the silica gel with chloroform–methanol 2:1, evaporated off the solvents under N_2, and counted the residue. The results differed less than 5% from those obtained by the "direct" counting methods.

[2] Calculated on the basis of BSA and FFA concentrations in the medium.

The tissue content of unesterified palmitate after 30 min also appeared to increase with $\bar{\nu}$ (Table 1); however, because of the great variability between animals, the means could not be shown to be different statistically. However, if the mean values are accepted as approximations, it is seen that palmitate-1-^{14}C accounted for less than 50% of the tissue unesterified palmitate at $\bar{\nu}$ = 0.5 and 1.5, whereas for $\bar{\nu}$ = 5.5 it accounted for more than 75% of the palmitate in the tissue FFA fraction. Mean tissue unesterified stearate, oleate, and linoleate contents remained unchanged as $\bar{\nu}$ was raised from 0.5 to 5.5 (Table 2).

When extracellular palmitate was kept at 800 μeq/liter (Table 3) and BSA concentration was decreased from 4 to 3 and 2 g/100 ml ($\bar{\nu}$ = 1.2, 1.8, and 2.4), incorporation varied with $\bar{\nu}$ during 10 min of incubation (Table 3). Other tissues were incubated for 4 min at $\bar{\nu}$ = 5.5 and 4.4 with albumin and palmitate contents being decreased in parallel by the dilution of the $\bar{\nu}$ 4.4 medium with KRP buffer. Incorporation into all fractions decreased (Table 4). Similar incubations prolonged to 30 min yielded parallel results.

TABLE 1 INCORPORATION OF PALMITATE-1-^{14}C INTO THE FFA FRACTION OF THE INTACT RAT DIAPHRAGM

Duration of Incubation	Concentration of Palmitate in Medium (μeq/liter) 150 ($\bar{\nu}$ = 0.5)	500 ($\bar{\nu}$ = 1.5)	1800 ($\bar{\nu}$ = 5.5)
min	*mμeq/g*		
0.5	4.1 ± 0.5		41.1 ± 3.7(5)
1	5.3 ± 0.4		55.6 ± 4.9(6)
2	6.0 ± 0.5		57.1 ± 13.7(4)
4	8.3 ± 2.8	17.2 ± 2.9 (53.5 ± 39.5)*	83.6 ± 16.6(6)
10	8.2 ± 2.9	40.3 ± 4.3	105.0 ± 15.6
20	6.7 ± 0.5		107.3 ± 26.8
30	8.5 ± 0.6 (36.7 ± 23.3)*	26.7 ± 2.2 (65.0 ± 44.8)*	93.0 ± 6.9 (121 ± 73.5)*
45			133.3 ± 32.0

Incubations were carried out in a 2% solution of BSA in KRP buffer, at 37°C in 100% O_2. Values are means ± SD (n = 3 except where otherwise indicated by the numbers in parentheses). $\bar{\nu}$ = mole ratio palmitate:albumin.

* Total tissue content of unesterified palmitate after incubation.

TABLE 2 EFFECT OF INCUBATIONS IN ALBUMIN–PALMITATE MEDIA ON THE TISSUE CONTENTS OF STEARATE, OLEATE, AND LINOLEATE

Incubation Fluid [BSA]	[Palmitate]	$\bar{\nu}$	Tissue FFA Contents Stearate	Oleate	Linoleate
g/100 ml	*μeq/liter*		*mμeq/g*		
2	150	0.5	48.4 ± 22.1	37.0 ± 19.1	41.4 ± 24.2
2	1800	5.5	52.4 ± 27.3	31.4 ± 17.2	19.2 ± 15.3

Incubations were carried out for 30 min at 37°C in 100% O_2. Results are means ± SD (n = 3).

TABLE 3 EFFECT OF $\bar{\nu}$ ON PALMITATE-1-^{14}C INCORPORATION IN THE INTACT RAT DIAPHRAGM

Incubating Fluid [BSA]	[Palmitate]	$\bar{\nu}$	Total Lipids	FFA
g/100 ml	*μeq/liter*		*mμeq/g*	
4.0	800	1.2	60.3 ± 13.0	14.0 ± 0.5
3.0	800	1.8	74.3 ± 8.4	18.5 ± 0.7
2.0	800	2.4	130.0 ± 1.6	40.9 ± 1.5

Incubation at 37°C in 100% O_2 for 10 min. Means ± SD (n = 3).

TABLE 4 INCORPORATION OF PALMITATE-1-^{14}C INTO TISSUE LIPIDS AT CONSTANT $\bar{\nu}$

Incubating Fluid [BSA]	[Palmitate]	$\bar{\nu}$	Palmitate-1-^{14}C Incorporation FFA	TG	PL
g/100 ml	*μeq/liter*		*mμeq/g*		
2	1800	5.5	83.6 ± 16.6(6) (53.0)*	56.4 ± 7.0	22.5 ± 2.5
1	720	4.4	41.5 ± 2.0(3) (30.5)*	24.8 ± 3.1	9.9 ± 2.0
0.5	360	4.4	17.0 ± 0.7(3)	13.7 ± 2.0	5.3 ± 0.9

Incubation at 37°C in 100% O_2 for 4 min. Means ± SD (n in parentheses).

* "Intracellular" unesterified palmitate-1-^{14}C, calculated as in Table 6.

TABLE 5 RATE OF ALBUMIN-^{131}I UPTAKE BY INTACT RAT DIAPHRAGM

Duration of Incubation	Tissue Albumin Content
min	*mμmoles/g*
1	1.7 ± 0.3(3)
4	3.5 ± 0.4(3)*
10	5.9 ± 1.2(3) (2.5 ± 0.3)†
30	4.7 ± 1.3(5)

Incubation at 37°C in 100% O_2 in 2% BSA solution, with human albumin-^{131}I as tracer. Means ± SD (n in parentheses).

* When this tissue was reincubated in unlabeled 2% albumin for 1 min at 37°C, 30% of the label was lost (2.5 ± 0.3 [n = 3] mμeq/g left); when the reincubation proceeded at 4°C, no label was returned to the medium (3.5 ± 0.3 [n = 3] mμeq/g remaining).

† Incubation carried out in 1% BSA solution.

Increasing amounts of albumin-^{131}I were taken up by the diaphragm between 1 and 10 min of incubation in 2% BSA. Distribution then remained stable between 10 and 30 min (Table 5). When incubation was carried out for 10 min in a 1% BSA solution, only half the amount of label was taken up. Thus albumin and palmitate uptake were both characterized by a rapid initial uptake and the attainment of steady-state levels by 5–10 min.

When the amount of albumin distributed in the tissue at various times is multiplied by $\bar{\nu}$, the amount of unesterified palmitate-1-^{14}C taken up by the tissue by virtue of being bound to albumin can be estimated (assuming that $\bar{\nu}$ remained unchanged at or near the surface of the tissue). The unesterified palmitate-1-^{14}C taken up in excess of that calculated in this way may then have represented palmitate molecules attached to cellular sites (Table 6, values derived from Table 1). For the $\bar{\nu}$ values tested, about one-third of the label in the FFA fraction was "albumin-bound" and two-thirds "cell-bound." The latter was considered to be a part of the intracellular FFA.

As the $\bar{\nu}$ of the incubating fluid was increased from 0.5 to 1.5 and 5.5, the specific activity of the palmitate in the medium decreased from 4100 to 1370 and 350 dpm/mμeq. Concomitantly the intracellular palmitate specific activity fell from 320 to 300 and 220 dpm/mμeq (intracellular palmitate levels were obtained by subtracting the "albumin-bound" from the tissue unesterified palmitate levels presented in Table 1). Thus the specific activity ratios between intracellular palmitate and medium palmitate rose from 0.08 to 0.21 and 0.63 and remained constant between 10 and 30 min of incubation.

20–40% of the label present in the FFA fraction of tissue prelabeled with ^{14}C was removable by reincubation in palmitate–albumin for 1 min at 37°C (Table 7). This represented a net loss of label from the tissue (see total lipid column). Reincubations in KRP buffer or in palmitate–albumin at 4°C did not result in any such loss of label. When tissue loaded with albumin-^{131}I was reincubated in BSA at 37°C (Table 5), about 30% of the label was lost from the tissue in 1 min. When reincubations were carried out at 4°C, ^{131}I label was not lost from the tissue.

TABLE 6 "ALBUMIN-BOUND" AND "CELL-BOUND" UNESTERIFIED PALMITATE-1-^{14}C AFTER INCUBATION

Duration of Incubation	$\bar{\nu}$	Palmitate-1-^{14}C "Albumin-Bound"*	Palmitate-1-^{14}C "Cell-Bound"†
min		*mμeq/g*	
1	0.5	0.9	4.4
4		1.8	6.5
10		3.0	5.2
30		3.0	5.7
5	1.5	5.3	12.3
10		9.0	31.3
30		9.0	17.7
1	5.5	9.9	45.0
4		19.3	64.3
10		33.0	72.0
30		33.0	60.0

* Obtained by multiplying tissue albumin content by $\bar{\nu}$.

† Obtained by subtracting albumin-bound palmitate-1-^{14}C from the total palmitate-1-^{14}C extracted in the FFA fraction.

TABLE 7 EFFECT OF REINCUBATION IN ALBUMIN SOLUTION ON THE UNESTERIFIED PALMITATE-1-^{14}C CONTENT OF THE TISSUE FFA FRACTION

First Incubation	Second Incubation	Second Incubating Fluid*	Palmitate-1-^{14}C Content: Total Lipid	Palmitate-1-^{14}C Content: FFA
min			*mμeq/g*	
0.5	0		39 ± 3.7(7)	24.8 ± 3.2
0.5	1	2% Albumin	35 ± 3.7	10.3 ± 2.3
1.0	0		52 ± 7.3(9)	33.8 ± 3.4
1.0	1	2% Albumin	42 ± 3.9	13.8 ± 2.7
4.0	0		44 ± 4.5	23.3 ± 1.0
4.0	1	2% Albumin	34 ± 3.7	12.4 ± 1.1
			(43 ± 3.9)†	(22.7 ± 1.1)†
4.0	0		48 ± 4.5	18.2 ± 1.2
4.0	1	KRP buffer	47 ± 5.6	13.7 ± 1.8

Values are means ± SD (n = 3 except where otherwise indicated by the numbers in parentheses). The first incubation was carried out at 37°C in 100% O_2 in a 2% BSA solution; the medium (palmitate) was 1400 for the 0.5 min and 560 μeq/liter for 1-min and 4-min incubations.

* At the end of the first incubation tissues were rinsed twice in saline at 37°C and rapidly transferred to beakers containing media identical with the first but lacking palmitate-1-^{14}C.

† Reincubation carried out in 2% albumin–palmitate at 4°C.

Role of Tissue Unesterified Palmitate as an "Intermediate" in Esterification

Diaphragms prelabeled with palmitate-1-^{14}C were reincubated in palmitate–albumin solution for 1, 4, and 8 min (Table 8). During the 1st min of reincubation label was lost from FFA fraction of the tissue. In the following 7 min no more label was lost (see total lipids) but label moved from the FFA to the triglyceride fraction quantitatively, i.e., all of the decrease in FFA label content was recovered in the triglycerides. The label content of the phospholipids remained constant. When reincubations were carried out in KRP buffer (Table 8), the total label content of the tissue (see total lipids) was unchanged. Label, however, moved from the FFA to the triglyceride fraction. Phospholipid label content again remained constant. This suggests that both the "albumin-" and "cell-bound" fractions were incorporated directly into the triglycerides, but does not rule out a small but undetectable incorporation into the phospholipids during the reincubations, since in direct incubations (Figs. 1, 2) the incorporation of palmitate-1-^{14}C into triglycerides greatly exceeded the incorporation into the phospholipids. The incorporation of label into the esterified fractions was rapid (Figs. 1, 2) and proceeded linearly between 0.5 and 45 min of incubation. As the palmitate content of the medium was increased, while albumin levels were held constant ($\bar{\nu}$ = 0.5, 1.5, and 5.5), the rates of incorporation into triglyceride (calculated from the 10–45 min slope of the lines) increased from 1.6 to 5.4 and 14.6. The comparable rates for the phospholipids were 0.7, 0.8, and 1.6. When the palmitate concentration was fixed while al-

TABLE 8 UTILIZATION OF PALMITATE-1-^{14}C DURING REINCUBATION IN ALBUMIN AND KRP BUFFER

Duration of Second Incubation	Second Incubation Medium	Palmitate-1-^{14}C Content			
		Total Lipid	TG	PL	FFA
			mμeq/g		
0	Albumin*	38.2 ± 4.1	2.0 ± 0.8	3.3 ± 0.6	24.8 ± 5.5
1	"	21.2 ± 3.6	4.0 ± 1.1	2.6 ± 0.5	10.3 ± 2.3
2	"	21.9 ± 2.5	7.1 ± 0.9	3.2 ± 0.6	9.4 ± 1.2
4	"	21.1 ± 2.5	9.7 ± 1.0	3.1 ± 0.3	6.4 ± 1.3
8	"	20.7 ± 1.9	11.0 ± 2.0	4.3 ± 0.4	4.3 ± 0.8
0	KRP buffer†	47.7 ± 4.5	13.6 ± 1.5	6.8 ± 0.3	18.3 ± 1.3
1	"	46.7 ± 5.6	22.7 ± 2.9	6.2 ± 0.8	13.7 ± 1.8
8	"	51.3 ± 6.2	28.4 ± 4.1	6.9 ± 0.8	4.5 ± 0.4

Results are means ± SD (n = 3).

* Initial and second incubations were carried out at 37°C in 100% O_2 in KRP buffer, 2% in BSA and 1400 μeq/liter in palmitate; first incubation was for 0.5 min and medium included 6 × 10^5 dpm of palmitate-1-^{14}C per ml.

† Initial incubations were carried out for 4 min at 37°C in 100% O_2 in KRP buffer, 2% in BSA and 560 μeq/liter in palmitate with 6 × 10^5 dpm/ml in palmitate-1-^{14}C; second incubation was in KRP buffer alone at 37°C in 100% O_2.

bumin levels were decreased ($\bar{\nu}$ = 1.2, 1.8, and 2.4), incorporation into total lipids varied with $\bar{\nu}$ (Table 3) (9).

DISCUSSION

Palmitate-1-^{14}C moved rapidly from the incubating fluid into the FFA fraction of the diaphragm and within 4–10 min reached stable levels determined by the $\bar{\nu}$ of the surrounding fluid (Table 1). It is apparent, however, that low albumin concentrations in the medium play an important modifying role in this process (Table 4). The limited data suggest that the steady-state distribution of the albumin–palmitate complex in the tissue was determined by the levels of albumin in the medium, at least below 2 g/100 ml. These findings differ from those reported in the Ehrlich ascites tumor cell (7) and erythrocytes (19). There are other differences as well: (*a*) a 5–10 min lag period before steady-state levels of unesterified palmitate-1-^{14}C are attained in the diaphragm (Table 1)—probably a reflection of the kinetics of distribution of albumin within the tissue (Table 5); (*b*) reincubation of prelabeled tissues in albumin solution at 0–4°C removed the "reversibly-bound" FFA in the erythrocyte (19), whereas at similar temperatures neither albumin-^{131}I (Table 5) nor unesterified palmitate-1-^{14}C (Table 7) was lost from the diaphragm. Clearly the interaction between albumin and tissue is affected by the state of dispersion of the cells. The data indicate that variations in albumin levels in the medium above an undetermined concentration would probably not have affected the tissue steady-state distribution. In this range, $\bar{\nu}$ would be expected to determine the steady-state contents of tissue unesterified palmitate-1-^{14}C independently of albumin concentration in the medium (Fig. 3).

The utilization of palmitate-1-^{14}C depends not only on $\bar{\nu}$ but also on the method of preparation of the FFA–albumin complex (20). This may be a potential factor in

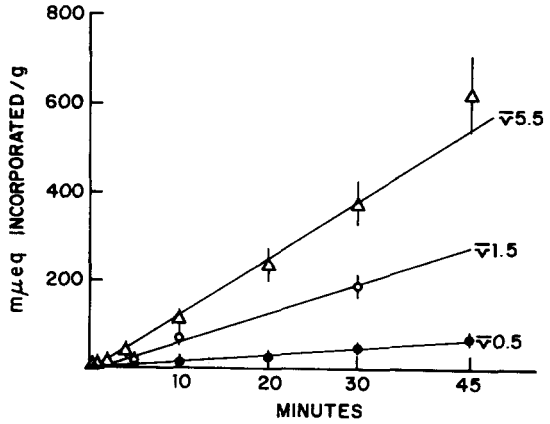

FIG. 1. Incorporation of palmitate-1-^{14}C into TG of rat diaphragm (mμeq/g of wet tissue). Incubations were carried out at 37°C in 100% O_2 in KRP buffer, 2% in albumin, and 6 × 10^5 dpm/ml in palmitate-1-^{14}C; palmitate concentrations were 150, 500, and 1800 μeq/liter ($\bar{\nu}$ = 0.5, 1.5, and 5.5). Each point represents the mean ± SD (n = 3–6).

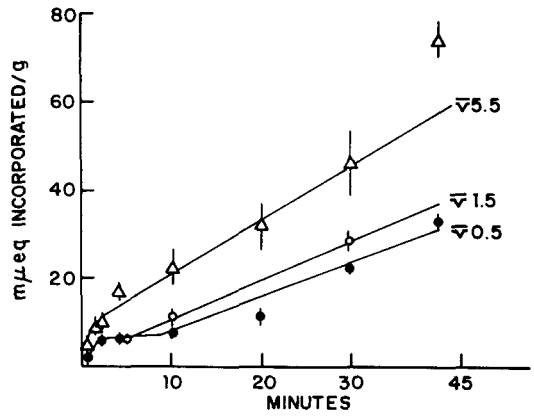

FIG. 2. Incorporation of palmitate-1-^{14}C into PL of rat diaphragm. Incubation as in Fig. 1.

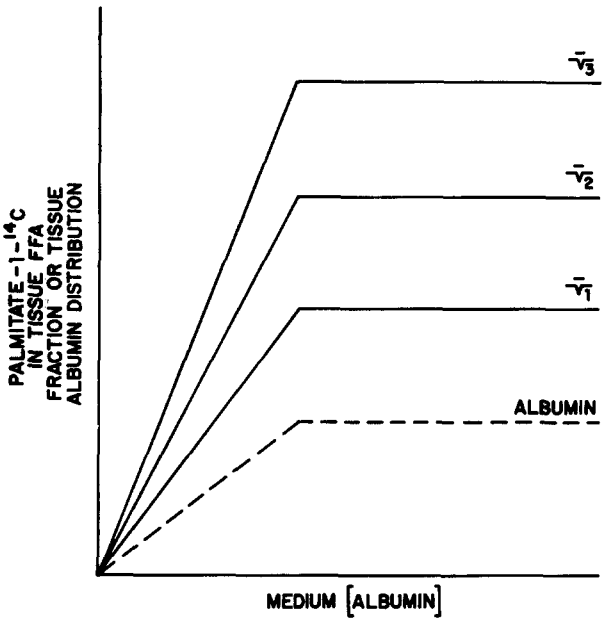

FIG. 3. Proposed effect of the albumin concentration in the medium on tissue contents of unesterified palmitate-1-^{14}C and albumin-^{131}I.

determining the tissue steady-state levels of labeled palmitate as well. This factor was not evaluated in our studies.

One of the difficulties in this work, not encountered in the ascites tumor cell where no FFA are detectable at the start of incubation (7), has been the evaluation of the relative contributions of the endogenous esterified lipids and the exogenous FFA to the intracellular FFA pool. To assess the contribution of the latter, one must know the changes in quantity of the component molecules of the intracellular FFA as $\bar{\nu}$ is varied. We have attempted to assess this parameter in the case of palmitic acid.

At the end of incubation the palmitic acid extracted from the tissues represented molecules present prior to incubation as well as those newly acquired during the incubation. Of those newly acquired, some were trapped on albumin ("albumin-bound"), a very small amount was in free solution (18), and the rest were bound to the cells ("cell-bound"). The last may be assigned to the intracellular pool. The validity of this division rests on the assumption that albumin remained extracellular during incubation. On the other hand, the accuracy of the size given to the "albumin-bound" and hence to the intracellular fraction depends upon the further assumption that $\bar{\nu}$ remained unchanged at or near the surfaces of the tissue. The first assumption is supported (*a*) by the unvarying content of ^{131}I label in the tissue between 10 and 30 min of incubation, while under identical conditions the ^{14}C content of the tissue increased linearly, and (*b*) by the rapid loss of ^{131}I label from the tissue during reincubation in albumin. The second assumption was not tested directly, since this would have necessitated the isolation and characterization of the palmitate–albumin complex from the tissue. The second alternative, of assaying the FFA content and composition of the incubating fluid, is not informative in the intact diaphragm preparation. But the fact that about 35% of the steady-state level of unesterified palmitate-1-^{14}C has been called "albumin-bound" (Table 6), and 20–40% of the label of the FFA fraction was removed by reincubation in albumin (Table 7), suggests that the estimate was accurate within the limits of the methods. It is quite likely from the studies of others (10, 19) that some of the ^{14}C label lost to the albumin solutions represented "intracellular" unesterified palmitate-1-^{14}C. However, all of the label which was to be lost from the tissue was gone at the end of 1 min of reincubation. No more was lost to the medium during the subsequent 7 min of reincubation in spite of the continued presence of unesterified palmitate-1-^{14}C in the tissue. Thus, it is likely that most of the lost label represented the "albumin-bound" fraction.

The variability of results precludes any firm conclusions, but the data are compatible with the idea that the tissue contents of unesterified palmitate are increased as $\bar{\nu}$ is raised from 0.5 to 1.5 and 5.5. In the rat, three- to four-fold short-term elevations of plasma $\bar{\nu}$ values in vivo are not followed by increased tissue FFA levels, while prolonged similar elevations yield definite increases of 200–400% in tissue FFA contents. Nevertheless, the results of these studies do not allow the conclusion that a disparity exists between the in vitro and short-term in vivo results, for it has been shown that the erythrocyte exhibits definite affinities for the various FFA with respect to both their uptake and esterification (25) and there is no reason to assume that an analogous situation does not exist in muscle. On the basis of the available data, then, it is not possible to forecast how the total intracellular FFA contents of the diaphragm in vitro would be affected were the tissue to be presented with a mixture of FFA such as is present in vivo. The in vitro results may therefore still be compatible with the conclusions derived from the in vivo studies (8), namely, that acute changes in plasma or medium FFA levels are not reflected by large changes in tissue FFA contents.

The specific activities of intracellular unesterified palmitate and the rate of esterification remained constant between 10 and 30 min of incubation, which suggests that the rate of transit through the tissue FFA pool at any given $\bar{\nu}$ was constant. Specific activity ratios did not exceed 0.3 in the physiologic range for $\bar{\nu}$ of 0.5 to 1.5 and less than 50% of the unesterified intracellular palmitate was accounted for by unesterified palmitate-1-^{14}C: this indicates that not all of the intracellular unesterified palmitate was available for equilibration with the medium.

The movement of label from the "cell-bound" to the triglyceride fraction suggests a destination for the molecules traversing the tissue FFA pool (Fig. 1). It is impossible to decide on the basis of our experiments whether incorporation into phospholipids proceeds via this pool or directly from the "albumin-bound" fraction. The rapid incorporation of label into PL (Fig. 2) makes it more likely, however, that incorporation is direct from exogenous sources rather than via any "labile fatty acids" arising from the triglycerides (21). It is also clear that not all of the tissue unesterified palmitate pool serves as a precursor for esterification since if the specific activities of the pools are used in calculating esterification rates, unreasonable answers are obtained, e.g., 30 min TG points (Fig. 1) become 640, 822, and 795 mμeq/g for $\bar{\nu}$ = 0.5, 1.5, and 5.5, respectively. Thus the tissue unesterified palmitate pool appears to be functionally heterogeneous.

The intact diaphragm preparation is not suitable for the assessment of gas exchange. It was, therefore, not possible to assess the role of either the "albumin-" or "cell-bound" fractions as direct intermediates in oxidation. The work of others, however, suggests that the exogenous FFA contribute increasingly to CO_2 production as $\bar{\nu}$ is increased (22), and during starvation (9) and muscular contraction (23–25). It appears, therefore, that exogenous or intracellular FFA can serve as direct intermediates for both esterification and oxidation.

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