Free Fatty Acid Oxidation by Forearm Muscle at Rest, and Evidence for an Intramuscular Lipid Pool in the Human Forearm

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ABSTRACT The objects of these experiments were to determine to what extent oleic acid, removed from plasma by forearm muscles, was oxidized immediately, and to search for evidence of an intramuscular lipid pool which may be composed of triglycerides synthesized from plasma free fatty acids and which may supply substantial portions of lipid substrates for oxidation by muscle. To these ends [1-^{14}C]oleic acid was infused at constant rate into the brachial artery of seven healthy young men at rest in the postabsorptive state. Results were: (*1*) muscle respiratory quotient (0.76) implied that about 80% of the oxygen consumed was for the oxidation of lipid. (*2*) Muscle free fatty acid uptake, had it been oxidized directly, could account for more than 100% of observed oxygen uptake. (*3*) There was a lag of at least 30 min before $^{14}CO_2$ appeared in forearm venous blood. (*4*) $^{14}CO_2$ release from forearm muscle tended to reach an apparent plateau after 3 h of infusion of [^{14}C]oleic acid. (*5*) During the time of plateau $^{14}CO_2$ release, oleic acid extracted from plasma could account for only 20% of oxygen consumption; most of the oleic acid taken up was not oxidized directly. (*6*) $^{14}CO_2$ release persisted at a high level during the 1–3 h follow-up period after the infusion ended. (*7*) Neither the delay in initial appearance of $^{14}CO_2$ nor its continued release after cessation of infusion was due to delay and distribution in a forearm CO_2 pool, since intra-arterial infusion of $NaH^{14}CO_3$ in two additional subjects demonstrated much more rapid distribution of $^{14}CO_2$ in the forearm. Results show that most, if not all, free fatty acids taken up by resting muscle are not oxidized directly, but probably enter an intramuscular pool which has a slow turnover during resting metabolism and is the immediate source of oxidized lipid substrate.

INTRODUCTION

On the basis of experience in this laboratory (1–6) and of reports by others (7–16) we adopted or formulated the following hypotheses: oxidative maintenance of skeletal muscle uses lipid as the major substrate, accounting for at least 80% of O_2 consumption. Lipid substrate is supplied by hydrolysis of triglycerides inside muscle fibers. The quantity of intramuscular triglycerides is protected so that, in the steady state, for each molecule of fatty acid leaving to be oxidized another molecule of fatty acid just removed from circulating plasma enters the pool. Intramuscular triglycerides are not a well-mixed pool.

To examine these proposals a series of studies were undertaken in which [1-^{14}C]oleate, tracing the most abundant free fatty acid (FFA)[1] in plasma, was injected continuously for 3–4 h at constant rate into the branchial artery of normal subjects at rest. Tracer oleate uptake and local forearm $^{14}CO_2$ production were measured during and after the infusion to examine both immediate and delayed oxidation. Since part of the

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[1] *Abbreviations used in this paper:* FFA, free fatty acids; Ox*, fraction of [^{14}C]oleic acid uptake appearing as $^{14}CO_2$ production; RQ, respiratory quotient; TLC, thin-layer chromatography.

delay and distribution of $^{14}CO_2$ appearance in venous blood must lie in the contribution made by residence of label in local tissue CO_2 pools, an estimate of the role played by distribution of residence times in CO_2 pools was made by studies of the response to intra-arterial infusion of $NaH^{14}CO_3$. The results are compatible with the hypothesis that most, if not all, of the fatty acid oxidized by muscle is supplied by intramuscular lipid pools, which are replenished by simultaneous uptake of FFA from plasma.

METHODS

Subjects and procedures. Nine healthy men, age 21–31 yr (mean 23.2), volunteered for these experiments. After a 12-h overnight fast, each subject reported to the laboratory and rested for 30 min. The experiment began at 9:00 a.m. The subject remained in the supine position with his arm supported comfortably throughout the study. The experiment was performed in an air-conditioned room at 25°C. At the end of the experiment one subject (L. J.) performed forearm muscle exercise for 20 min as described previously (5). The exercise was flexion of the hand upon the wrist against gravity and return to the extended position once every 2 s.

Procedures for catheterization of the forearm and measurements of blood flow have been reported elsewhere (2, 17). In brief, after local procaine infiltration, a deep and a superficial vein of the same arm were catheterized percutaneously in a retrograde direction. The ipsilateral brachial artery was cannulated with an 18 gauge, thin-walled Riley needle; a 20 gauge thin-walled steel tube was introduced through the needle, thus converting the arterial needle into a double lumen assembly. The lumen of the steel tube, which projected 3 cm beyond the tip of the needle, served for arterial sampling. The infusion was delivered through space between the needle and tube. In seven subjects, solution of 0.155 M NaCl (50 ml), Evans blue due (7.5 ml), and [1-^{14}C]oleic acid (0.4 ml), containing 2.5 μCi according to manufacturer, was infused at a constant rate of about 0.1 ml/min (= 0.0043 μCi/min) into the brachial artery. The arterial volume injection rate was measured in each experiment, as was the concentration of [1-^{14}C]oleic acid delivered from the syringe. Total dose did not exceed 1.3 μCi per subject. The [1-^{14}C]oleic acid was stated to have a sp act of 40 μCi/μmol, a concentration of 6.25 μCi/ml, and was bound to human albumin. Less than 4% of impurities of [1-^{14}C]oleic acid could be documented by radiogas-chromatographic analysis. Sodium bicarbonate-^{14}C (1.25 μCi/μmol) was diluted in 35 ml 0.155 M NaCl and 7.5 ml Evans blue dye, and infused at a constant rate (0.1 ml/min) in two other subjects. When the isotopic infusion was discontinued, Evans blue dye in 0.155 M NaCl solution was injected from another syringe at the same rate and concentration to continue the measurement of blood flow. Venous catheters and the arterial sampling lumen were kept patent with a slow infusion of 0.155 M NaCl solution without anticoagulant.

Circulation to the wrist and hand was excluded by a pediatric sphygmomanometer cuff applied to the wrist and inflated to 200 mm Hg during each experimental period, which lasted 20–30 min. Simultaneous samples were obtained from the artery, deep vein, and superficial vein, 10 min and 20 or 30 min after the wrist cuff inflation, and then the cuff was deflated. There were seven or eight such experiments in preheparinized syringes for determinations of blood flow, arterial and venous $^{14}CO_2$, [1-^{14}C]oleic acid, and hematocrit. In four different periods samples were also collected for measurements of endogenous oleic acid and for total oxygen and carbon dioxide contents.

Analytical methods. Immediately after each collection portions of each blood sample were removed for the following analyses:

Blood flow. The hematocrit was determined immediately. A correction factor of 6% was used for trapped plasma. The optical density of Evans blue dye in the plasma was then measured in a Beckman spectrophotometer at 620 nm.

Total oxygen and carbon dioxide. Arterial and deep venous blood samples were analyzed according to the method of Van Slyke and Neill (18).

Labeled carbon dioxide. 4-ml portions of deep venous and arterial blood were transferred immediately to 50 ml Erlenmeyer side arm flasks for $^{14}CO_2$ analyses as described by Saba and DiLuzio (19). This technique was modified by using methyl benzethonium hydroxide instead of 10% potassium hydroxide as trapping agent on filter paper inserted into the attached scintillation vial. Recovery of $^{14}CO_2$ with this procedure, assessed in whole blood with $NaH^{14}CO_3$ was 87±3.3% (SEM, 10 determinations). Each vial, containing filter paper and 20 ml of scintillation solution (5 g/liter 2,5-diphenyloxazole and 0.1 g/liter 1,4-bis-2-(4-methyl-5-phenyloxazoly)-benzene in toluene) was counted to 10,000 counts or for 100 min, whichever came first, in a 3375 Packard TriCarb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Radioactivity of blood was corrected for (*a*) quenching, by channels ratio technique, (*b*) average recovery of standard $NaH^{14}CO_3$, (c) heparin dilution of the dead space in the blood collection syringe, and (*d*) counting efficiency.

[1-^{14}C]oleic acid. Deep venous and arterial plasma lipids were extracted by the method of Dole (20) and separated by thin-layer chromatography (TLC) as described by Brown and Johnston (21) and Goldrick and Hirsch (22). The FFA spot was recovered and eluted from silica gel by repeated extraction by chloroform. The eluate was collected in a glass counting vial and evaporated to dryness with nitrogen. FFA were then dissolved in scintillation solutions, as described above for use with $^{14}CO_2$, and were counted to 10,000 counts or for 100 min, whichever occurred first. Concentration was expressed in disintegrations per minute per milliliter of plasma after correction for quenching, for percent recovery from TLC and lipid extraction, and for counting efficiency. Recovery of known additions of [^{14}C]-oleic acid from TLC ranged from 92 to 98%.

In two subjects radioactivity in triglyceride fractions from TLC were analyzed similarly.

Endogenous oleic acid. Individual FFA from deep venous and arterial plasma were measured by gas chromatography with heptadecanoic acid as an internal standard added to each sample during the Dole extraction process. Lipids were extracted from plasma by the Dole method, and the FFA fraction was separated by TLC as described above for labeled oleic acid. The FFA were eluted with chloroform which was subsequently evaporated and the residue taken up in 5 ml of methanol. FFA were then esterified by addition of 0.2 ml of dimethyl acetal and 0.25 ml of 6.26 M H_2SO_4 and refluxing for 2 h at 70°C. Distilled water was added to break the phases of the system. Methyl esters were extracted in hexane which was subsequently evaporated under nitrogen. A sample of the residue dissolved in heptane was injected into an isothermal gas chromato-

graph with an argon ionization detector. Two columns maintained at a temperature of 170°C, were packed with 80/100 mesh Chromosorb W. Output of the chromatograph was displayed on a recorder and was simultaneously digitalized by a shaft encoder coupled to an IBM 526 key punch (IBM Corp., Armonk, N. Y.). From the output cards, areas under the curve for each fatty acid, corrected for baseline, were determined, and concentrations and percentage distributions were calculated on a CDC-3300 computer. (Control Data Corp., Minneapolis, Minn.)

Calculations. All analytical measurements were corrected for the presence of heparin in the dead spaces of the precalibrated collection syringes (17). Forearm volume was measured by water displacement; 60% of this volume was assumed equivalent to the muscle mass (17). Blood or plasma flow was calculated by constant injection indicator dilution (17) and was expressed in milliliter per minute/100 g of muscle on the assumption that half the brachial arterial flow supplied forearm muscles.

Arterial [1-^{14}C]oleic acid concentration was calculated as the sum of the measured concentration of [^{14}C]oleic acid in arterial blood proximal to the site of injection (recirculating [1-^{14}C]oleic acid) and the added concentration of [1-^{14}C]oleic acid contributed by the continuous infusion. The latter was determined from the known calibrated volume rate of injection (milliliter per minute) multiplied by the measured concentration of [1-^{14}C]oleic acid in a sample collected from the injecting system (disintegrations per minute per milliliter) divided by blood plasma flow through the brachial artery (milliliter per minute). Thus, as an example, an injectate concentration of 0.0432 μCi/ml = 95,000 dpm/ml, multiplied by 0.1 ml/min volume infusion rate = 9,500 dpm/min, divided by 30 ml/min brachial arterial blood flow = 317 dpm/ml. To this is added the recirculating concentration of, say, 5 dpm/ml, giving an arterial [1-^{14}C]oleic acid concentration of 322 dpm/ml. Actual arterial concentrations depended on minor variations in setting the rate of volume infusion and in preparing the solution infused, and, to the greatest extent, on variations in blood flow.

Uptake of oxygen and [1-^{14}C]oleic acid and release of total or labeled carbon dioxide were calculated from the product of the arterio-venous concentration difference and blood (for O_2) or plasma (for oleic acid) flow.

There were at least 10 determinations of arterial and of venous $^{14}CO_2$ and 4 determinations of arterial and of venous total CO_2 during each experiment. Blood and plasma flow and [1-^{14}C]oleic acid were determined on every sample of blood on which $^{14}CO_2$ was determined. Arterial and venous FFA concentrations were measured three or four times during the course of the study. The following formulae were applied:

fractional extraction of [^{14}C] oleic acid from plasma

$$E^* = ([C^*_{18:1}]_A - [C^*_{18:1}]_{DV})/[C^*_{18:1}]_A, \quad (1)$$

where terms on the right-hand side represent concentrations of [^{14}C] oleic acid in arterial (A) and deep venous (DV) plasma, dpm/ml.

Uptake of endogenous oleic acid by muscle, μmole/min per 100 g,

$$\dot{M} = [F_P]_m \times [C_{18:1}]_A \times E^*, \quad (2)$$

where $[F_P]_m$ is plasma flow to muscle (ml/min per 100 g), and $[C_{18:1}]_A$ in concentration of endogenous oleic acid in arterial plasma.

Uptake of [^{14}C]oleic acid by muscle, dpm/min per 100 g,

$$\dot{M}^* = [F_P]_m \ ([C^*_{18:1}]_A - [C^*_{18:1}]_{DV}). \quad (3)$$

Fraction of $\dot{M}^*$ accounted for by simultaneous production of $^{14}CO_2$,

$$Ox^* = [F_B]_m([CO_2^*]_{DV} - [CO_2^*]_A/\dot{M}^* \quad (4)$$

where $[F_B]_m$ is blood flow to muscle.

Rate of oxidation of (or of CO_2 production from) oleic acid taken up by muscle, μmol/min per 100 g,

$$\dot{M}Ox = \dot{M} \times Ox^*. \quad (5)$$

Percent of CO_2 derived from immediate oxidation of oleic acid taken up by muscle,

$$= 100 \times 18 \times \dot{M}Ox/[F_B]_m([O_2]_A - [O_2]_{DV}). \quad (6)$$

Percent of O_2 uptake spent in oxidation of oleic acid,

$$= 100 \times 25.5 \times \dot{M}Ox/[F_B]_m([O_2]_A - [O_2]_{DV}. \quad (7)$$

To estimate blood or plasma flow per 100 g of forearm muscle it was necessary first to estimate the fraction, α, of brachial arterial flow distributed to muscle. The desired fraction, α, is reported to vary uncertainly with total forearm blood flow (23–26). In general, but not in all reports (25), the higher forearm flow, the smaller α. Blood flow to forearm muscle tends to remain constant, while flow to skin and subcutaneous tissues varies. We assumed α was 0.5.

Of the seven formulae, four are independent of the choice of α, the fraction of brachial arterial blood distributed to muscle. Only $\dot{M}^*$ and M and, hence, $\dot{M}Ox$, depended on the choice of α, and the uncertainty contributed thereby to their calculated value is proportional to the uncertainty in α.

RESULTS

Evidence that subjects were stable during the period of infusion. Before we consider those results that pertain uniquely to the main objective, we ask if there were any trends in the state of subjects during these experimental periods which were prolonged compared to many of our previous studies of forearm metabolism. For the group as a whole, forearm plasma flow, oxygen uptake, arterial oleic and [^{14}C]oleic acid concentrations, and percent extraction of [^{14}C]oleic acid were constant throughout the 4–4½ h period (Table I). The apparent increase in the mean value of [^{14}C]oleic acid concentration during the last period is due to the fact that average concentrations happened to be higher among those three subjects in whom infusion continued through the final period.

Muscle respiratory quotient (RQ) and potential O_2 uptake due to oleic acid uptake (Table II). Muscle RQ was 0.76±0.06 during the period of infusion of oleic acid in the seven subjects, in agreement with our previous experience (2). The weighted average RQ for 100% dependence upon FFA, based on observed distribution of FFA in arterial blood, is 0.706. The observed RQ is consistent with the hypothesis that 82% of O_2 uptake was spent in oxidation of lipid and the remainder in oxidation of carbohydrate.

In previous studies on other groups of subjects it was determined that [^{14}C]palmitic acid uptake (27)

TABLE I

Stability During [1-^{14}C] Oleic Infusion

Infusion time, *min*	0–120	120–150	150–180	180–210	210–240	240–270
Plasma flow, *ml/min per 100 g muscle*	2.4±0.4 (7)	2.2±0.4 (7)	2.1±0.3 (5)	2.3±0.4 (6)	2.8±0.7 (7)	2.4±0.6 (6)
Arterial [1-^{14}C] oleic acid, *dpm/ml*	378±52 (7)	382±52 (7)	379±78 (7)	341±49 (7)	344±93 (5)	436±104 (3)
Extraction, oleic acid, %	46±3.5 (7)	46±4.0 (7)	44±3.5 (7)	41±3.6 (7)	30±3.4 (5)	42±2.9 (3)
O_2 uptake, *μmol/min per 100 g*	10.4±1.7 (6)	9.8±1.3 (4)		9.1±0.8 (6)		
Arterial oleic acid, *μmol/ml*	0.44±0.09 (7)	0.40±0.08 (5)		0.48±0.04 (7)		

Numbers in parentheses are numbers of subjects. Datum is mean±SEM.
Infusion time refers to minutes after onset of continuous intra-arterial infusion of [^{14}C]oleic acid.

and [^{14}C]oleic acid uptake from plasma by forearm muscle were stable within 10–15 min after onset of constant infusion, possibly earlier. Blood flow by our indicator-dilution method cannot be measured during the first 5 min of infusion because indicator concentrations in venous blood are still transient.

In the present series, the earliest observation was at 20 min after onset of infusion, and in this subject, as in all those in this series, [^{14}C]oleic acid uptake was stable from the first observation through about 200 min. Among the first four subjects, in whom infusion continued for about 4 h, [^{14}C]oleic acid uptake was stable throughout in one, but decreased after 200 min in the other three, only to return to or toward plateau at the time of the final observation (Fig. 1).

The extraction ratio for oleic acid and oleic acid uptake, 43% and 0.38 μmol/min per 100 g, respectively, agreed with those we observed in an earlier study of a larger number of subjects.

Oxygen uptake by muscle was within the range reported previously from this laboratory.

If all the oleic acid uptake were oxidized directly it could have accounted for 117±23% of the actual simultaneous O_2 uptake. This is greater than in our previous

TABLE II

Percent of Forearm Muscle O_2 Uptake Accounted for by Lipid Oxidation

Subject	Muscle RQ	% lipid, from RQ	E*	$\dot{M}$	$\dot{Q}O_2$	$\dot{M}/O_2$
		%	%	*μmol/min per 100 g*		%
M. F.	0.74	88	49.0	0.30	12.9	70
M. R.	0.63	126	45.0	0.20	6.7	76
L. G.	1.11	−37	43.8	0.30	7.8	94
D. A.	0.62	130	46.1	0.47	12.6	97
W. C.	0.83	57	29.4	0.60	8.9	159
A. S.	0.70	102	54.5	0.40	7.6	237
J. W.	0.72	95	35.0	0.38	11.7	87
Mean	0.76	80 (82)	43.3	0.38	9.8	117
±SEM	0.06	22 (21)	3.2	0.05	1.0	23

Percent lipid, from RQ is percent of oxidized substrate accounted for by oxidation of lipid, based on observed RQ, RQ for plasma FFA mix of 0.706, and RQ for glucose; mean±SEM in parenthesis calculated from mean RQ±its SEM. E* is extraction ratio for plasma [^{14}C]oleic acid. $\dot{M}$ is uptake of endogenous plasma oleic acid by forearm muscle. $\dot{Q}O_2$ is oxygen uptake by forearm muscle. $\dot{M}/O_2$ is percent of $\dot{Q}O_2$ accounted for if all $\dot{M}$ were oxidized completely.

experience because total FFA concentration in arterial blood in these subjects was unexplainedly high, 1.02 ±0.15 μmol/ml, compared to 0.8±0.06 in a large number of subjects. Such high values are not unreported. In their study which included three normal subjects at rest and fasted 16–19 h, Havel et al. (13) found plasma FFA concentrations of 1.07, 1.06, and 0.61 μmol/ml.

$^{14}CO_2$ production by forearm muscle during infusion of [^{14}C]oleic acid. Although uptake of [^{14}C]oleic acid by muscle was stable within 10 or 15 min after onset of constant infusion, no $^{14}CO_2$ was released into forearm venous blood until about 30 min of infusion. $^{14}CO_2$ production increased thereafter relatively rapidly until about 150 min, when it either reached plateau or rose only at a greatly reduced rate (Fig. 1).

Among the first four subjects, the mean value of the percent of [^{14}C]oleic acid uptake accounted for by simultaneous release of $^{14}CO_2$ during the plateau of $^{14}CO_2$ release, Ox*, arbitrarily taken to include all values on or after 150 min of the infusion period, was 15±2% (Table III). This mean includes five to seven determinations for each subject. Subsequently, three other subjects received [^{14}C]oleic acid infusions for a shorter period to allow more time for postinfusion follow-up. These subjects had only three determinations of Ox* during the infusion period from 150 min on. If results from these subjects are included, mean Ox* for all seven subjects was 19±6%. The increase in mean and variance is due to contribution from subject J. W., for whom Ox* was 52±3.0%.

Thus, during a period of about 90 min, among the first four subjects, or of 60–90 min among all seven, while the subjects were in an apparent steady state with respect to [^{14}C]oleic acid uptake and $^{14}CO_2$ production, less than one ^{14}C atom out of every five taken up could be accounted for by $^{14}CO_2$ production.

The rate of oxidation of endogenous oleic acid extracted from plasma by forearm muscle, $\dot{M}Ox$, was therefore only 0.07 μmol/min per 100 g (Table III), contrasted with the potential rate achieved only if all oleic uptake were oxidized directly, 0.38 (cf. Table II). Thus oxidation of oleic acid extracted from plasma accounted for only 17% of observed O_2 uptake (Table III), not 117% (Table II). Since oleic acid was 43% of plasma FFA, oxidation of all FFA extracted from plasma by muscle accounted for about 39% of observed O_2 uptake, although this is a small overestimate since oleic acid is extracted to a slightly greater extent than are other fatty acids.

CO_2 produced by muscle by oxidation of oleic acid extracted from plasma accounted for 17% of total CO_2 production (Table III).

^{14}C content of perchloric acid soluble fraction of plasma during [^{14}C]oleic acid infusion. Hagenfeldt and Wahren (28) reported that after mild exercise 96 ±12% of ^{14}C-FFA uptake by forearm was recovered as $^{14}CO_2$ in forearm venous blood, but after heavy exercise only 67±27% was so recovered. The remainder of the ^{14}C was recovered in forearm venous blood in a perchloric acid extract, tentatively identified as incomplete oxidation products of FFA. On the basis of their reports we analyzed perchloric acid extracts of arterial and deep venous blood from subject D. A., taken at 230 and 255 min after onset of the continuing infusion of [^{14}C]oleic acid.

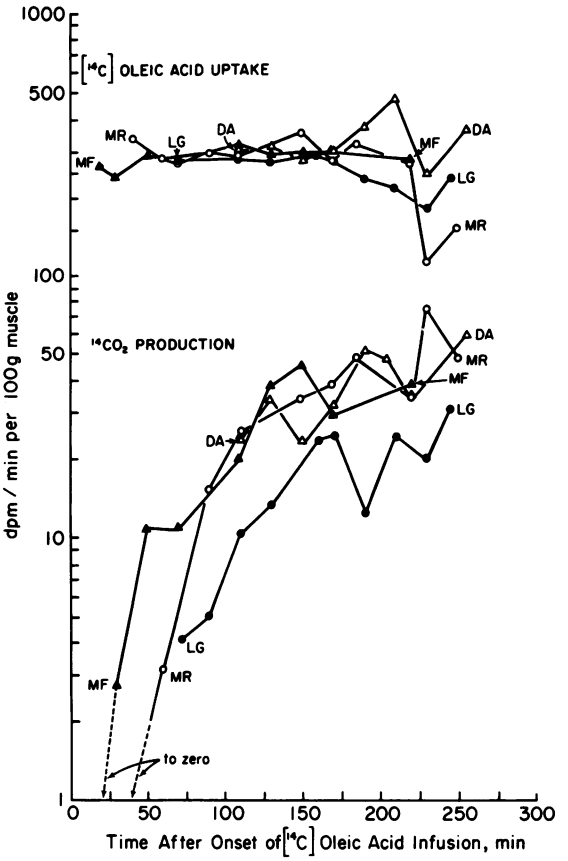

FIGURE 1 Time course of $^{14}CO_2$ production and [^{14}C]oleic acid uptake by forearm muscle during constant intra-arterial infusion of [^{14}C]oleic acid in four subjects.

There was no significant net release of perchloric acid-soluble ^{14}C products into forearm venous blood. Radioactivity in the perchloric acid-soluble fraction of arterial blood was not significantly above background and was only 1% (at 230 min) and 2% (at 255 min) of the [^{14}C]oleic acid activity in brachial arterial blood. ^{14}C contents of Somogyi filtrate were also examined. They did not differ significantly from those in the perchloric acid filtrate. Arterio-venous difference at 230 min was −5±6.5 dpm and 1.1±6.5 dpm at 255

TABLE III

$^{14}CO_2$ Production During its Plateau, During [^{14}C]Oleic Acid Infusion, and Oxidation of Oleic Acid Extracted from Plasma

Subject	$\dot{Q}^*_{CO_2}$	Ox*	$\dot{M}Ox$	$\dot{M}Ox/O_2$	$\dot{M}Ox/CO_2$
	dpm/min/100 g	%	*μmol/min/100 g*	%	%
M. F.	38	17±3.3	0.05	10	9
M. R.	45	16±3.4	0.03	11	13
L. G.	23	10±1.3	0.03	10	6
D. A.	47	15±3.4	0.07	14	15
W. C.	26	12±1.2	0.07	20	22
A. S.	21	8±1.7	0.03	10	10
J. W.	284	52±3.0	0.20	44	45
Mean±SEM (n = 7)	69±36	19±6	0.07±0.02	17±5	17±5
Mean±SEM (first four only)	38±5.4	15±2	0.04±0.01	11±1.0	11±2

Values are averages for each subject of all values obtained at and after 150 min of continuous infusion of [^{14}C]oleic acid, during plateau release of $^{14}CO_2$. $\dot{Q}^*_{CO_2}$, rate of production of $^{14}CO_2$ by forearm muscles; Ox*, percent of [^{14}C]oleic acid uptake accounted for by $^{14}CO_2$ production; $\dot{M}Ox$, rate of complete oxidation of the endogenous oleic acid extracted by muscle from plasma; $\dot{M}Ox/O_2$, percent of observed $\dot{Q}_{O_2}$ accounted for by observed oxidation of oleic acid extracted from plasma. $\dot{M}Ox/CO_2$, percent of observed $\dot{Q}_{CO_2}$ accounted for by observed oxidation of oleic acid extracted from plasma. Duration of infusion in last three subjects was about 3 h; in first four subjects, about 4 h. Where indicated, values are per 100 g forearm muscle.

min, where the standard deviation is that of counting statistics.

Effect of exercise on $^{14}CO_2$ production. It was not our purpose to study effects of exercise. Effects of exercise on $^{14}CO_2$ production in relation to ^{14}C-FFA uptake have been well documented (13, 15, 28). However, in view of the very low rate of production of $^{14}CO_2$ in these subjects at rest, it did seem desirable to see if that rate increased with exercise to levels reported by others. Accordingly, subject L. G. exercised his forearm muscles during the last 20 min of a 280-min infusion of [^{14}C]oleic acid. During the exercise period forearm blood flow tripled, [^{14}C]oleic acid uptake doubled, and $^{14}CO_2$ production increased more than 10-fold. Where only about 10% of [^{14}C]oleic acid uptake was accounted for at rest by concurrent $^{14}CO_2$ production, during mild exercise more than 60% was accounted for, in agreement with reports by others.

$^{14}CO_2$ production by forearm muscle after cessation of infusion of [^{14}C]oleic acid. The disparity between the extent of FFA oxidation predicted by the forearm RQ and the extent deduced from observed $^{14}CO_2$ release during infusion of [^{14}C]oleic acid suggested that most of the oleic acid removed from plasma entered some intracellular lipid pool. If so, it was possible that $^{14}CO_2$ production might continue for some hours after infusion was discontinued. To investigate this possibility $^{14}CO_2$ release was followed for 55 min after cessation of [^{14}C]oleic acid infusion in subject D. A. When analysis showed that $^{14}CO_2$ release continued almost unabated, three additional subjects were studied with longer follow-up periods. To not prolong the total experimental time and because a plateau in $^{14}CO_2$ release seemed to have been reached or approached closely by 150 min of infusion, duration of [^{14}C]oleic acid infusion was reduced to about 200 min. Results appear in Fig. 2.

In all four subjects [^{14}C]oleic acid concentration in deep venous blood was reasonably constant during the last 110–160 min of infusion. In two subjects blood samples were taken 5 min after the end of infusion; deep venous [^{14}C]oleic acid concentration had fallen to 23 and 11% of infusion plateau. By about 30 min postinfusion, deep venous [^{14}C]oleic acid concentration was indistinguishable from the low level of recirculating [^{14}C]oleic acid in arterial blood.

In all subjects $^{14}CO_2$ release during at least the first hour postinfusion was indistinguishable from plateau $^{14}CO_2$ release during infusion. Indeed, in the first three subjects, followed for 55, 105, and 120 min postinfusion, it could not be proven that $^{14}CO_2$ release did not continue unabated throughout. The fourth subject, J. W. was followed for 190 min postinfusion; and in him $^{14}CO_2$ release declined during the last 100 min of the follow-up period, falling to about 30% of plateau release. This subject differed from the other six who

received [^{14}C]oleic acid in that his $^{14}CO_2$ production was seven times greater than the average in absolute value and more than three times greater than the average as percent of [^{14}C]oleic acid uptake. For the four subjects, $^{14}CO_2$ release was 96% of plateau during the first hour postinfusion; for the three subjects observed for at least 2 h postinfusion, it was 81% of plateau during the second hour (92% for each of two subjects, 60% for J. W.). In J. W. $^{14}CO_2$ release during the third hour postinfusion averaged 34% of plateau.

J. W. returned to the laboratory 24 h after the end of infusion. Blood was drawn from the deep vein of the experimental arm and from a corresponding vein of the other arm. The difference in $^{14}CO_2$ concentration was 3 dpm/ml, representing continuing $^{14}CO_2$ release at about 13% of his plateau, although the uncertainty is great since levels were close to background.

In view of accelerated $^{14}CO_2$ production imparted by exercise and since subject J. W.'s activity was uncontrolled between the time he left the laboratory and the time the 24-h sample was obtained, it is likely

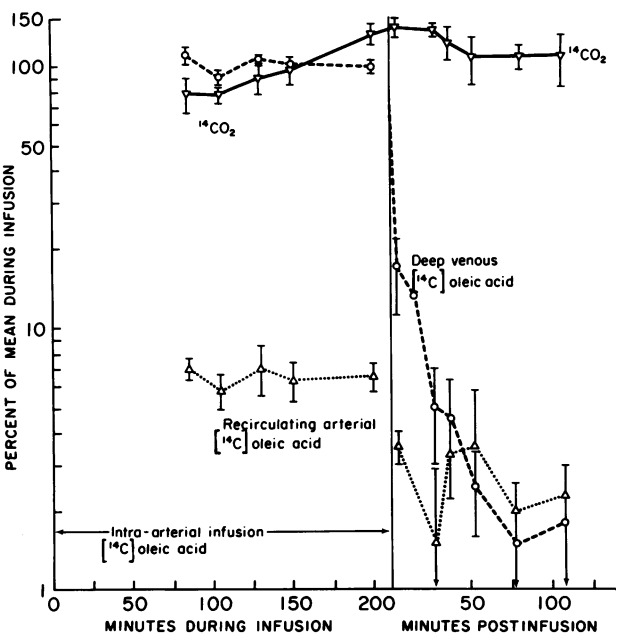

FIGURE 2 $^{14}CO_2$ production after cessation of intra-arterial infusion of [^{14}C]oleic acid. Data are means±SEM for four subjects. $^{14}CO_2$ is displayed as deep venous-arterial $^{14}CO_2$ differences normalized by dividing each v–a difference by the mean v–a difference for that individual determined from data during infusion. Deep venous [^{14}C]oleic acid is displayed as concentration of [^{14}C]oleic acid in deep venous blood normalized by dividing each concentration by the mean concentration for that individual determined from data during infusion. Recirculating arterial [^{14}C]oleic acid is normalized by dividing each recirculating arterial concentration by the mean deep venous concentration for that individual determined from data during infusion.

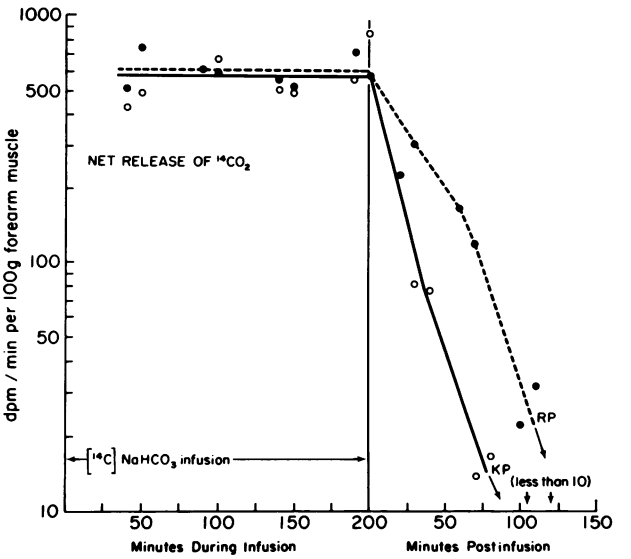

FIGURE 3 Net $^{14}CO_2$ release from forearm during and after intra-arterial infusion of $NaH^{14}CO_3$ in two subjects.

that he had been exhausting intracellular ^{14}C-fatty acids at a rate in excess of that at rest, and that the observed rate of $^{14}CO_2$ production at 24 h was less than it would have been had he been at rest throughout.

By summation of the areas under the curves of $^{14}CO_2$ production and of [^{14}C]oleic acid uptake during the period of infusion (Fig. 2), it can be calculated that cumulative $^{14}CO_2$ production accounted for only 7.5% of cumulative [^{14}C]oleic acid uptake during the period of infusion, approximately 4 h. This left a deficit of $^{14}CO_2$ production of 92.5% to be accounted for during the postinfusion period. Among the three subjects whose $^{14}CO_2$ production was measured during 2 h postinfusion, $^{14}CO_2$ production repaid the deficits at an average rate of 3.5%/h, although that rate was decreasing. If that rate did not decline, it would take 24 h to repay the deficit. Since the rate did decrease, if the subject remained at rest it would take days to produce enough $^{14}CO_2$ to account for all the [^{14}C]oleic acid uptake.

$^{14}CO_2$ release in response to ^{14}C bicarbonate infusion. The delay in appearance of $^{14}CO_2$ and the slow increase in $^{14}CO_2$ release during [^{14}C]oleic acid infusion, and the slow decrease in $^{14}CO_2$ release postinfusion could be due either to distribution of [^{14}C]oleic acid in some large intracellular pools before production of $^{14}CO_2$ or to distribution of $^{14}CO_2$ in a large extravascular CO_2 pool.

To investigate this latter possibility $NaH^{14}CO_3$ was injected intra-arterially at constant rate into two subjects for 200 min (Fig. 3). Plateau of $^{14}CO_2$ release occurred within 40 min. In 30 min after infusion ceased $^{14}CO_2$ release was less than 50% of plateau in one subject, and less than 20% in the other, and by 120 min postinfusion $^{14}CO_2$ had nearly disappeared from venous

blood. $^{14}CO_2$ release fell to half plateau value in about 20 min after HCO_3 infusion ended for the group of two subjects. During the interval from 15 to 30 min post oleic acid infusion, $^{14}CO_2$ release averaged 101% of plateau. During the interval from 90 to 120 min post oleic acid infusion, $^{14}CO_2$ release averaged 85% of plateau, compared to 2% for HCO_3 infusion over the same time interval. These results eliminate the possibility that continued $^{14}CO_2$ release at high intervals after [^{14}C]oleic acid infusion was due to delays within CO_2 pools.

DISCUSSION

In 1967 Havel et al. (16), on the basis of studies in man during prolonged heavy exercise, proposed that there were two possible immediate fates for a molecule of FFA newly entering cells, presumably mainly muscle. There was an even chance that it would be oxidized directly or that it would enter large intracellular lipid compartments. During heavy exercise, according to the analysis of Havel et al. (16), most of stored fraction of FFA reenters the direct oxidative pathway rapidly. Malmendier et al. (29) in 1974 carried out a closely-related study on four normal subjects at rest in whom [^{14}C]palmitic acid was delivered as a slug intravenously and respiratory $^{14}CO_2$ was measured. They accounted for 45% of the labeled FFA leaving plasma by $^{14}CO_2$ in expired air. Both groups performed compartmental analysis of their data. The number of compartments was the same; the arrangement only slightly different. In both cases there was a direct route for FFA oxidation, and a route to temporary storage with return to oxidation.

In contrast to these interpretations are those by Hagenfeldt and Wahren (28). They found that all ^{14}C-FFA uptake was recovered as $^{14}CO_2$ in forearm venous blood during mild exercise, and 67±27% of injected ^{14}C was accounted for as $^{14}CO_2$ during heavy exercise. The remainder was recovered as perchloric acid-soluble ^{14}C substances, tentatively identified as incomplete oxidation products of FFA. Thus, Hagenfeldt and Wahren had no need to invoke an intramuscular lipid pool. However, their observations are not incompatible with models in which there is an intracellular lipid pool. It can be argued that the intracellular pool turns over much more rapidly during exercise.

Hagenfeldt and Wahren correlated the presence in blood of incomplete oxidation products of FFA with pronounced lactate production, occurring only during heavy exercise. If this view is correct, one would not expect such oxidation products to appear in effluent from resting muscle, and, in the one subject in whom we sought these substances, none was found.

Despite the observations by Hagenfeldt and Wahren, there is a body of evidence making it difficult to deny that skeletal muscle contains lipid pools upon which it draws for substrate for oxidation. In a series of reports, beginning in 1958, George and Naik (30) examined cross-striated muscle from several species by histochemical staining for fat, and demonstrated lipid deposits in muscle at rest, with a decrease in lipid in muscles obtained after exercise. Volk et al. (31) and Neptune et al. (32), on the basis of studies of metabolism in excised muscle, suggested that fatty acids were made available for oxidation by lipolysis of intramuscular glycerides. Compatible with storage of FFA inside muscle are the following observations. Total lipid oxidation by muscle is calculated from the observed muscle RQ and O_2 uptake. Based on the observed distribution of plasma FFA, the weighted average RQ for oxidation of FFA is 0.706. From this and the observed RQ 0.76, the fraction of O_2 uptake, total lipid oxidation is calculated. From the observed fractional extraction of [^{14}C]oleic acid and the rate of delivery of FFA in arterial blood, FFA uptake by muscle is calculated. From these calculations, FFA uptake exceeded total lipid oxidation to such an extent that 66% of FFA uptake was not oxidized. It might be argued that this is an unusual event due to the high level of plasma FFA in the sample of the population. However, a similar analysis of data from our laboratory, published in 1961, including measurements of forearm O_2 uptake in 34 subjects and arterial FFA in 35 subjects (2), also demonstrated FFA uptake in excess of lipid oxidation, in this case by 30%.

We therefore analyze our data on the assumption that there is an intracellular lipid pool in muscle into which plasma FFA enters as glycerides and from which lipolysis generates fatty acid substrate for oxidation. In construction of a model the first fact to consider is that there was a 30-min delay before $^{14}CO_2$ was detectable in forearm venous blood, although [^{14}C]oleic acid uptake is measureable within a few minutes after onset of infusion and is constant within 10–15 min. If some [^{14}C]oleic acid were delivered directly from plasma to mitochondrial oxidative sites could there be such a delay? The delay was not due to dilution of $^{14}CO_2$ within the forearm CO_2 pool, as demonstrated by our experiments in which $NaHCO_3$, labeled with ^{14}C, was infused into the brachial artery. Step-by-step tracking of plasma FFA, translocated across the cell membrane and transported through cytosol in association with carnitine to mitochondria, and finally dissimilated to CO_2 and H_2O does not suggest that a delay of 30 min is to be expected. Indeed the large delay argues strongly, in contrast to previous models (16, 29), that none of the FFA is oxidized directly, that all of it is first esterified and deposited among the numerous lipid granules within the muscle cell.

We next consider the slow increase in $^{14}CO_2$ production to a plateau or quasi plateau during steady-state uptake of FFA from plasma. The fact that there is either a plateau or a quasi plateau of $^{14}CO_2$ production at a level competent to account for only about 20% of simultaneous labeled FFA uptake establishes that hydrolysis and subsequent oxidation of the nonlabeled glyceride does not proceed from a well-mixed pool. Nor is it plausible that glyceride stores should be well-stirred. One might expect that there would be varying probabilities of hydrolysis of labeled glyceride depending on the size, composition, and perhaps location of the individual lipid granule. In some cases the rule might be last on, first-off; that is, the probability of hydrolysis might be more favorable for more recent additions to the granule for all but monolayers. In other cases newly-formed glycerides might be inserted ultimately in regions of the granule less accessible to hydrolysis. In still other cases, the newly-formed glycerides might be inserted in lipid granules that turn over much more slowly than the average.

Consistent with this model are the results of those experiments in which $^{14}CO_2$ production continued at a high level for more than 2 h after infusion of [^{14}C]-oleic acid stopped. Observations of this sort, at least qualitatively, have been made previously by others, and indeed in all those experiments by others in which ^{14}C-FFA was injected as a slug it seems likely that the $^{14}CO_2$ detected over several hours arose from oxidation of glyceride stores, not from direct oxidation of plasma FFA. So large are the total lipid stores in muscle, and so well hidden among the lipid granules are some of the labeled fatty acids likely to become with the course of time, that, at the rate observed during resting metabolism, all the ^{14}C would not be recovered as $^{14}CO_2$ in less than a week.

Also consistent with this model are results of experiments during muscle exercise. There is accelerated production of $^{14}CO_2$ during exercise, and $^{14}CO_2$ release accounts for a larger portion of ^{14}C-FFA uptake during light exercise than at rest, facts established by several groups of investigators (13, 33) with which our single observation is in agreement. In our model exercise does not necessarily evoke or accelerate direct oxidation of plasma FFA but rather it is more likely to accelerate lipolysis, as it does in adipose tissues, thus both hydrolyzing sooner the more readily accessible labeled glycerides, and, by reducing the size of the lipid pool, increasing the total proportion of labeled glycerides available for immediate oxidation.

The two previously published models employed compartmental analysis which is not appropriate for inhomogeneous systems, as intramuscular lipid granules are likely to be. We had hoped to treat the system by stochastic analysis; that is, to analyze the system in terms of the probability distribution of residence times of ^{14}C in muscle. Indeed the experiments with $NaH^{14}CO_3$ were carried out with a view to obtaining one of the component distribution functions which, by deconvolution, would give insight into residual distribution of residence times, for events preceding distribution and release of CO_2. However, the data generated cannot be sufficiently well resolved, and the probability seemed high that the system was not stationary (intracellular lipid was accumulating during rest, to be dissimilated later during exercise) so that true distribution functions were unattainable. Violation of stationarity also makes invalid the details of previously reported analyses, since such compartmental analysis is simply a subset of stochastic analysis in which a priori assumptions are made about internal distribution functions of transit times and residence times and about inter-relations among internal systems.

Our experimental design differs in important detail from all previously reported studies. The closest design is that of Hagenfeldt and Wahren (14) who also localized inputs to and outputs from skeletal muscle, thereby eliminating complications due to contributions by or distributions in other organs, and due to systemic effects induced by the experiment. However, they infused ^{14}C-FFA intra-arterially for only 2 min, in contrast to our infusions which were more than 100 times as long. Their studies dealt with periods during or after exercise, not with the resting state as did ours. Despite differences in experimental design of the input-output studies, most of the available reports and our data are consistent with the model we present, that there are intramuscular lipid stores, that the favored, or perhaps only, path for plasma FFA to oxidation in muscle is by way to prior esterification, incorporation into the heterogeneous family of lipid granules, with subsequent hydrolysis at heterogeneous rates, a small portion of which operate under the principle of last-on, first-off, and that exercise, in harmony with its known action on lipolysis in adipopcytes, accelerates turnover from intramuscular lipid granules, and does not evoke or provoke direct oxidation of FFA.

There remains to be explained the reasons for the delay in appearance of $^{14}CO_2$ in our experiments, and the reasons why mitochondria should operate on fatty acids derived from muscle triglycerides but not on fatty acids freshly transported from plasma. The two phenomena are related. We do not know where in muscle acylation of fatty acids occurs, preparatory to formation of triglycerides. By analogy with other tissues, it may occur in association with sarcoplasmic reticulum. The probability that newly transported FFA from plasma will arrive at sarcoplasmic reticulum greatly exceeds the

probability that it will arrive at mitochondria. Since the fat granules in muscle are concentrated in the vicinity of mitochondria, it is required that triglycerides be transported from sarcoplasmic reticulum to fat granules in the vicinity of mitochondria. The two transport processes are likely steps to account for the 30-min delay. It is easy to visualize that hydrolysis of triglycerides in the vicinity of mitochondria floods the mitochondrial region with fatty acids, accounting for preferential oxidation of fatty acids from this source.

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