Kinetic analysis of the oxidation of palmitate-1-^{14}C in man during prolonged heavy muscular exercise

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ABSTRACT Two healthy men with high working capacities were injected intravenously with palmitate-1-^{14}C and $NaH^{14}CO_3$ on two occasions while they were performing strenuous exercise on a bicycle ergometer. From analysis of $^{14}CO_2$ in expired air after injection of $NaH^{14}CO_3$, rate constants and compartment sizes describing a three-compartment system for CO_2 were determined algebraically. These data were combined with those of a separate study in which ^{14}C in free fatty acids of arterial blood plasma and in expired CO_2 were measured after injection of palmitate-1-^{14}C to construct an eight-compartment model with an analogue computer that described precisely the observed data in each subject.

The results indicate that under these conditions almost half of the free fatty acids leaving the blood are oxidized directly (i.e., are transferred to mitochondrial oxidative sites through small intermediate compartments). The remainder enters larger compartments apart from the direct pathway; most of this fraction reenters the direct oxidative pathway within 30 min. These observations suggest that certain esterified fatty acids contained in working muscle cells are rapidly renewed.

Recycling of ^{14}C in plasma free fatty acids and triglyceride fatty acids was substantially reduced during exercise. Values for turnover rate and extent of oxidation of free fatty acids obtained by the method of continuous intravenous infusion of palmitate-1-^{14}C were similar during exercise in these two subjects to those obtained after pulse injection.

KEY WORDS free fatty acids · energy metabolism · fat metabolism · triglyceride metabolism · compartmental analysis

Abbreviations: FFA, free fatty acid(s); TGFA, triglyceride fatty acids.

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It is generally agreed that the free fatty acids (FFA) of blood plasma are the principal form in which fat is transported in the blood from body stores to the sites of oxidative metabolism. The rate and extent of oxidation of FFA have been difficult to measure at rest because the turnover of bicarbonate as expired CO_2 is so slow (2–3% of extracellular bicarbonate is expired per minute) and because only a small fraction of plasma FFA is oxidized rapidly, while the rest is esterified and enters large pools of triglycerides and phospholipids, which are oxidized slowly (1, 2). Earlier studies showed that muscular work greatly increases the amount of FFA that is oxidized rapidly (3–5). During constant intravenous infusion of palmitate-1-^{14}C, the rate of excretion of ^{14}C in expired CO_2 rose rapidly to about 60% of the rate of infusion; it increased very slowly after the first hour of leg exercise at moderate work load (400 kg-m/min), which suggests that delayed oxidation of palmitate stored in tissue esters did not contribute appreciably to its excretion under these conditions (3). In other similar studies, the output of bicarbonate-^{14}C from the exercising leg rose rapidly and then approximately equalled the rate of uptake of palmitate-^{14}C into the leg (R. J. Havel, B. Pernow, and N. L. Jones, unpublished data). It appears, therefore, that most palmitate entering the working muscle cell is transferred rapidly to mitochondrial oxidative sites and that little is stored extramitochondrially in lipid esters.

In the present study, pulse labeling was used for the study of kinetics of CO_2 turnover and conversion of palmitate carbon to expired CO_2 when approximately 90% of oxidative metabolism took place in working

striated muscle. Mathematical and computer analysis permitted construction of a model that precisely describes the data and demonstrates that a fraction of the FFA entering working muscle is stored temporarily in pools apart from those leading directly to sites of oxidation.

METHODS

Subjects and Procedure

Two male athletes were studied in Stockholm three times in 6 months. Subject A, age 24, was a bicycle racer. Subject B, age 35, was a former single-sculls racer (now a fireman). Both were engaged in a continuous program of training which emphasized activities requiring large expenditure of energy over prolonged periods. Their working capacities were 1900 and 1500 kg-m/min (6), respectively, at a heart rate of 170 beats/min. On each occasion, they exercised in the morning for 2 hr at 700–900 kg-m/min on a bicycle ergometer after fasting for 15 hr. Room temperature was 20–22°C. In the first study, palmitate-1-^{14}C was injected intravenously at a constant rate of 0.12 μc/min before, during, and after exercise. In the second study approximately 15 μc of palmitate-1-^{14}C, and in the third, 15 μc of sodium bicarbonate, was injected intravenously within 10 sec, 30 min after exercise began. Expired air was sampled intermittently for 6-min periods at rest, and starting with the instant of injection, for 3-min periods at intervals during the next 90 min of exercise and during the subsequent 2 hr while the subjects rested in a comfortable chair. Samples of brachial arterial blood were taken from a Teflon catheter every minute for 6 min after injection and at longer intervals thereafter.

Materials

Palmitic acid-1-^{14}C, specific activity 5.8 mc/mmole, (New England Nuclear Corp., Boston, Mass.) was complexed with human serum albumin (7), and $NaH^{14}CO_3$, specific activity 5 mc/mmole, was dissolved in 0.15 M sodium chloride solution. In presentation of results, the quantity of ^{14}C injected has been adjusted to 10^6 cpm per kg of body weight.

Analyses

Analyses were performed as described elsewhere (5) with the following additions. During the first 10 min after injection of palmitate-1-^{14}C in the second study, lipids in blood plasma were extracted in ethanol–acetone 1:1, slightly acidified with dilute hydrochloric acid. Portions of the extract were evaporated in counting vials and assayed after the addition of toluene and phosphors (5). ^{14}C in these samples was considered to be solely in FFA (1). Plasma volume was estimated at rest as the volume of distribution of ^{131}I-albumin 10 min after intravenous injection; plasma total protein was determined by the biuret reaction (8). Plasma volume during exercise was corrected for the measured change in concentration of total protein. All analyses were carried out in duplicate. The coefficients of variation of these duplicate analyses were 1.4% for CO_2, 2.5% for $^{14}CO_2$, 2.6% for FFA, 5.6% for ^{14}C-FFA, 3.1% for triglyceride-glycerol, 17% for ^{14}C-triglyceride fatty acids (TGFA), 4.5% for glycerol, and 5.1% for glucose.

Calculations

Values for fractional turnover rate, turnover rate, and oxidation of FFA during study 1 were obtained as described elsewhere (3). Fractional turnover rate of FFA in study 2 was estimated from the slope of the curve relating ^{14}C in FFA and time from the 2nd to the 5th min after injection. Turnover rate was calculated from the product of the plasma FFA concentration at the time of injection, the corrected plasma volume, and the fractional turnover rate. Oxidation of palmitate-1-^{14}C in study 2 was evaluated with the Electronic Associates, Inc., Model TR-48 analogue computer as described in Results.

RESULTS

Selected data obtained during the three studies are presented in Table 1, and serial changes in levels of metabolites in blood plasma and in ventilatory respiratory quotient (RQ) are shown in Fig. 1. Plasma levels of FFA were uniformly higher in subject A at onset of exercise, but increased more in subject B. Glucose levels fell in subject A during the 2nd hr of exercise, but were stable in subject B. RQ was higher during exercise than at rest in both subjects. Heart rates increased gradually during exercise: in subject A from 120 to 138 in the first study, from 117 to 137 in the second, and from 113 to 127 in the third; in subject B from 120 to 152 in the first study, from 116 to 148 in the second, and from 125 to 171 in the third. Rectal temperature, measured with a thermocouple, increased about 1°C during the 1st hr and then remained unchanged. In the second study, the value for cumulative excretion of $^{14}CO_2$ during the 90 min following injection of palmitate-1-^{14}C plus that remaining in the tissue pools of CO_2 was considered to represent rapid oxidation of FFA (see below).

In study 3, cumulative excretion of $^{14}CO_2$ in 90 min was 97% in subject A and 93% in subject B. Values relating specific activity of CO_2 and time for the two subjects are shown in Fig. 2. Curves were first drawn visually through the points and separated into three components by graphical analysis. From the slopes and

TABLE 1 COMPARISON OF CONSTANT-INFUSION AND PULSE-LABELING METHODS FOR EVALUATING METABOLISM OF FFA DURING HEAVY EXERCISE*

Variable	Subject A			Subject B		
	1	2	3	1	2	3
Work load (*kg-m/min*)	900	800	800	700	800	800
Ventilatory RQ	0.79	0.83	0.79	0.79	0.83	0.85
$\dot{V}co_2$ (*mmoles/min*)	84	71	71	65	74	73
Plasma FFA (*μmole/ml*)	0.77	0.85	1.06	1.15	0.35	0.33
Fractional turnover rate (min^{-1})	0.48	0.58	—	0.50	0.58	—
Rapid oxidation of FFA (%)	76	71	—	84	81	—
CO_2 derived from FFA (%)	25	33†	—	39	17†	—

* Studies and methods. Study 1, constant-infusion experimnet with palmitate-1-^{14}C: mean of four values during 2nd hr of exercise; study 2, pulse-labeling experiment with palmitate-1-^{14}C: values after 30 min of exercise; study 3, pulse-labeling experiment with $NaH^{14}CO_3$: values after 30 min of exercise.

† Calculated from: 100 × mmole of FFA-carbon converted to CO_2 per min/$\dot{V}co_2$.

intercepts of these components, we calculated rate constants and compartment sizes for the three-compartment system for CO_2 [as proposed by Shipley, Baker, Incefy, and Clark (9) for the rat] by means of the algebraic method for solution of such a system in the steady state developed by Skinner, Clark, Baker, and Shipley (10). The model and values obtained are given in the figure. The curves shown represent the output of the compartment (y_1) in equilibrium with expired CO_2 from the analogue computer, programmed for this model with rate constants and compartment sizes calculated from the primary data. Independent calculation by another individual gave similar values and an almost identical curve.

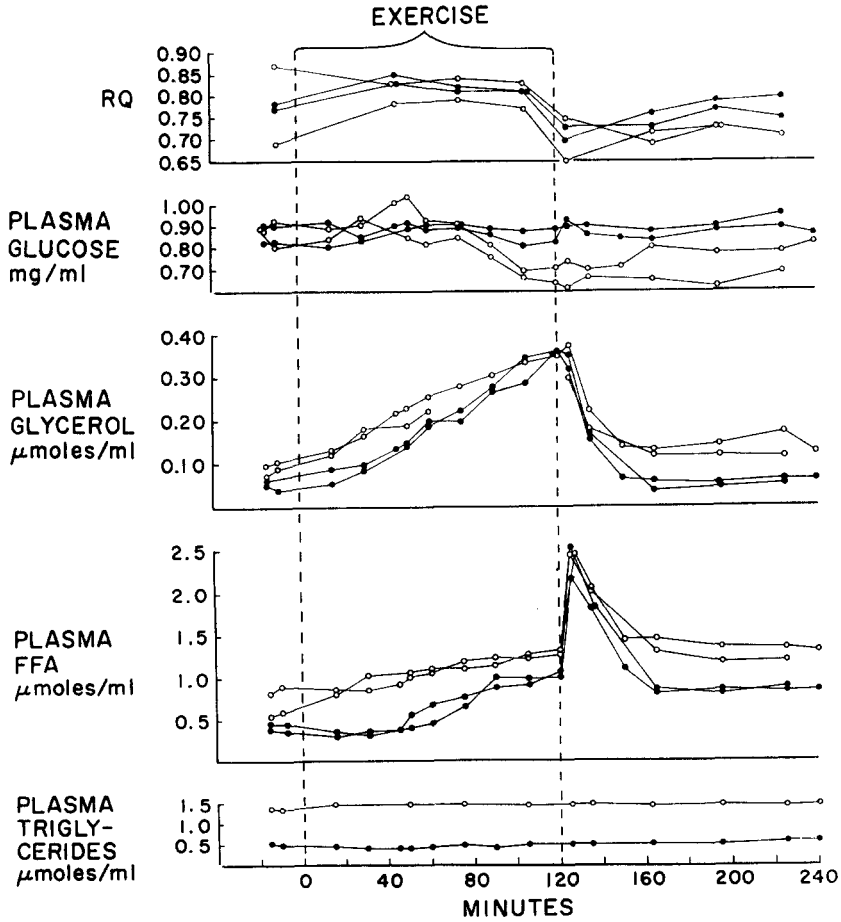

FIG. 1. Ventilatory RQ and metabolite levels during studies 2 and 3. Subject A, open circles; subject B, closed circles. Values for plasma triglycerides are shown only for study 2; they were similar during the other studies. Plasma levels of glucose also fell during study 1 in subject A.

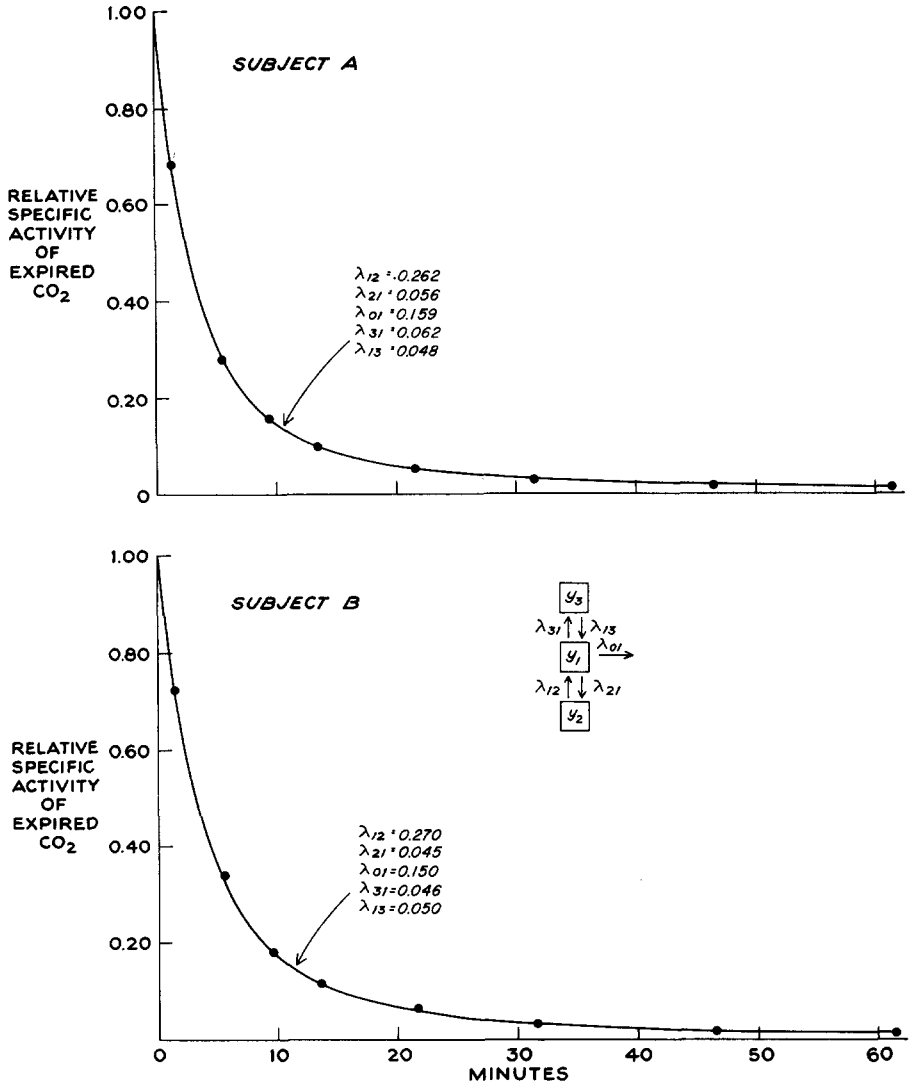

FIG. 2. Specific activity of expired CO_2 during study 3. The points are plotted at the middle of the 3-min collection periods. The curves shown are the outputs from the analogue computer programmed for the three-compartment model shown with the indicated rate constants (see text).

Values relating ^{14}C in FFA and TGFA with time for study 2 are shown in Fig. 3. The fractional turnover rate of FFA was 1.2 min in each subject. The intercept of the slow component of the curve, which is considered to reflect recycling of ^{14}C in FFA, was 0.45% of injected ^{14}C in subject A and 0.68% in subject B. Maximal radioactivity in TGFA was 1.85% and 1.30% of injected ^{14}C.

Values relating specific activity of CO_2 and time for study 2 are shown in Fig. 4. The curves describing the data were obtained with the analogue computer for the eight-compartment model shown, which was the simplest reasonable model and which provided an acceptable fit for the observed values in either subject. Compartment sizes and transfer rates for this model are given in Table 2. Four compartments, y_a, y_1, y_2, and y_3, were fixed from measurements of FFA turnover and from study 3. Early trials indicated that unless two pools were interposed between plasma FFA (pool y_a) and extracellular bicarbonate (pool y_1), the specific activity of y_1 rose too rapidly and reached its maximum too soon. A model which included only the "fixed" compartments and these two compartments (y_b and y_c) did not provide an acceptable fit. Values which provided a maximal specific activity in y_1 at the proper time had a value that was much too great and fell off much too rapidly. Addition of one "side" compartment to y_b or y_c permitted a much better, but not perfect, fit to be obtained. Addition of a second "side" pool to y_b permitted the fit shown in Fig. 4. We found that the two side compartments (y_β and y_γ) could be placed either on the same or on the opposite sides of y_b, and that y_c and y_b could be interchanged with no significant difference in the specific activity–time function in y_1. Additional, more complex models could also be constructed (see Appendix).

TABLE 2 COMPARTMENT SIZES AND TRANSFER RATES OF CARBON*

	Compartment Size			Transfer Rate	
	Subject A	Subject B		Subject A	Subject B
	mmole			*mmole/min*	
y_a	40.6	21.6	0→a		
			a→b	23.7	12.6
y_b	95	35.9	b→c		
			c→1		
y_c	9.5	5.1	b⇄β	32.8	15.0
y_β	258	114	β⇄γ	17.0	4.8
y_γ	4060	520	1⇄2	25.0	22.2
y_1	446	493	1⇄3	27.6	22.7
y_2	95	82	0→1	47.3	61.4
y_3	577	454	1→0	71.0	74.0

* See Fig. 4 for explanation of symbols. "0" refers to compartments out of eight-compartment system.

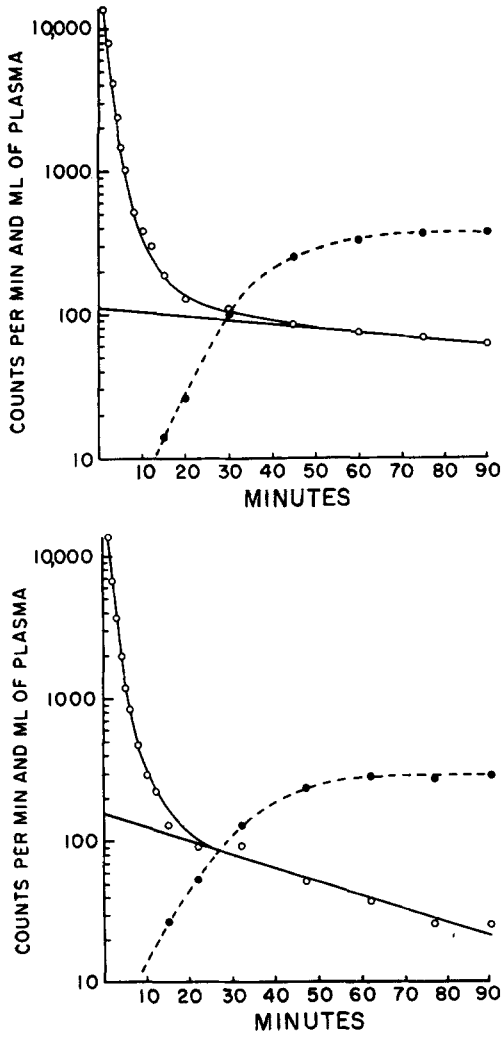

FIG. 3. ^{14}C-content of plasma FFA (open circles) and TGFA (closed circles) during study 2. Top, subject A; bottom, subject B.

DISCUSSION

At an exercise load of 800 kg/min, approximately 75% of the cardiac output enters working muscle and 85–90% of oxidative metabolism occurs there (11). Other studies have shown that the fraction of plasma FFA that enters leg tissues at rest and during exercise is proportional and approximately equal to the fraction of the cardiac output that enters the lower extremities (R. J. Havel, B. Pernow, and N. L. Jones, unpublished data). Thus, in the present studies, about 75% of plasma FFA left the blood in leg tissues and it is reasonable to assume that about 90% entered all working muscles, including the heart. From earlier studies, it was concluded that most FFA that enter working muscles are "rapidly oxidized" (3). The present study allows more precise definition of this term, which clearly includes not only FFA transferred to mitochondrial oxidative sites by the shortest path available, but also those temporarily stored in compartments apart from the "direct" oxidative pathway. In our subjects, the 42 and 45% of FFA oxidized "directly" traversed compartments (y_b and y_c), which together were about twice the size of the plasma FFA compartment. These compartments include extravascular FFA, both without and within cells, the extra- and intramitochondrial pools of fatty acyl-CoA, fatty acyl carnitine, and fatty acyl carbon atoms in intermediates of the fatty acid oxidase system and the Krebs cycle.[1] If working muscle in these experiments is considered to weigh 20 kg (two-thirds of a muscle mass of 40% of body weight), the concentration of these intermediates in μg atoms of carbon is about 5 in subject A and about 2 in subject B. In terms of palmitate (i.e., FFA, fatty acyl-CoA, fatty acyl carnitine), this amounts to about 0.31 and 0.12 μmole/g wet weight. Estimates for rat diaphragm are about 0.15 μmole/g for FFA and 0.05 μmole/g for fatty acyl-CoA (12). Good estimates for fatty acyl carnitine in skeletal muscle are not available. In heart muscle, levels are about

[1] Since palmitate-1-^{14}C was used this study, the results apply strictly only to transfer of the carboxyl carbon through the oxidative pathway. Other studies of fatty acid oxidation, including one in which conversion of palmitate-U-^{14}C to expired CO_2 was measured in exercising men (5), suggest that, once begun, conversion of palmitoyl-CoA to acetyl-CoA proceeds rapidly to completion.

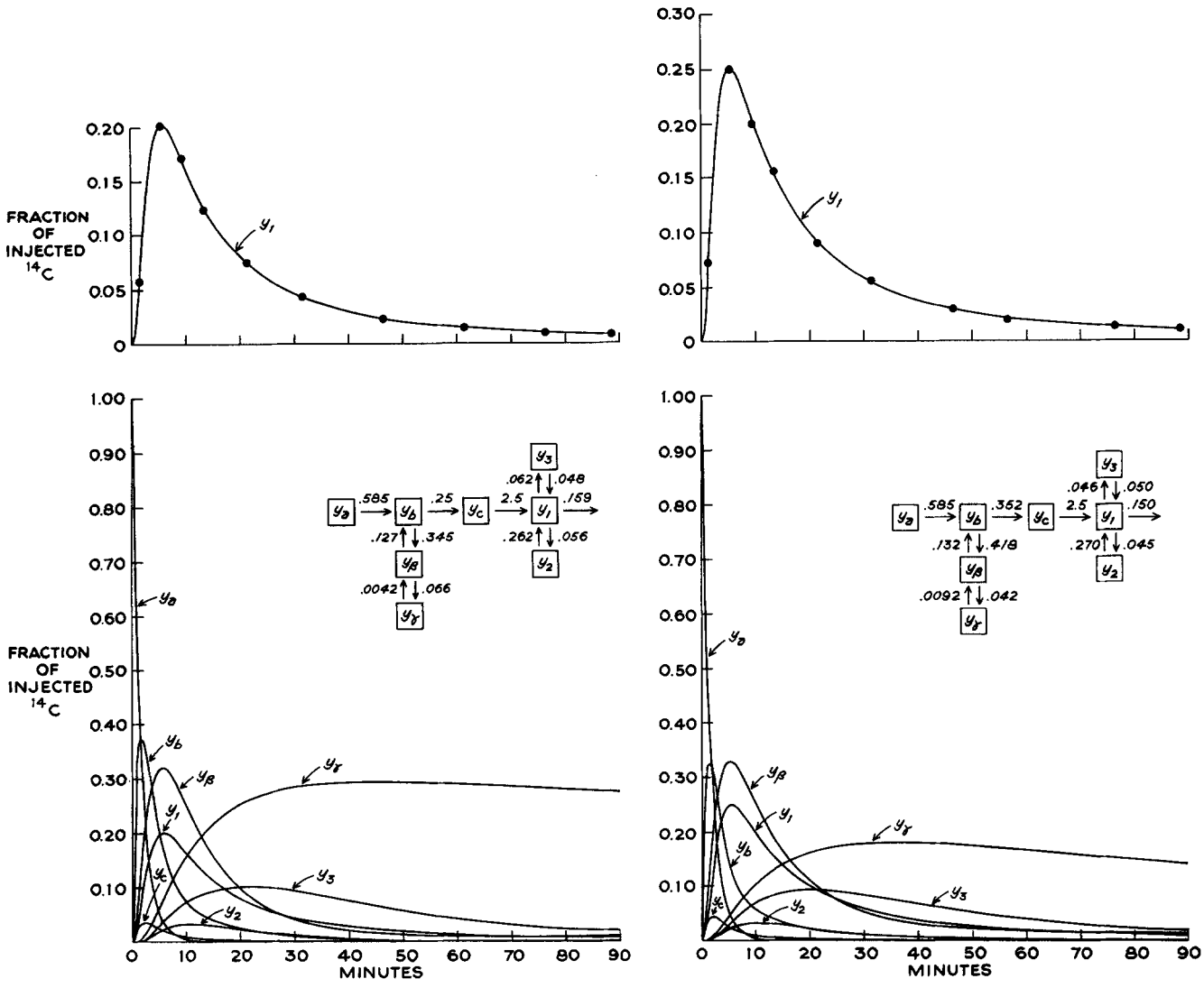

FIG. 4. Left, top: ^{14}C-content of compartment y_1 during study 2 in subject A. The points were calculated from the product: measured specific activity of expired CO_2 × mmole in y_1 (from study 3). The curve is the output from the analogue computer programmed for the eight-compartment model shown with the indicated rate constants. Bottom: computer output for ^{14}C content of each compartment of the eight-compartment model.

Right: data and computer outputs from study 2 in subject B.

0.1 μmole/g (13). Estimates for acetyl-CoA in heart are <0.003 μmole/g (<0.0004 in terms of palmitate) (12). Thus, the estimates for y_b and y_c are reasonable. It is quite possible to construct a series of compartments to replace y_b and y_c which would represent the chemical entities of the direct oxidative pathway for FFA and would fit the data equally well. The eight-compartment model was selected as the simplest which permitted the data to be approximated closely and which was appropriate in terms of known pathways of fatty acid metabolism.

Compartment y_β is most reasonably considered to be a pool of esterified fatty acid that turns over rapidly during exercise and y_γ one which is larger and renewed much more slowly. More than 90% of the FFA oxidized during the 90 min after its injection was excreted in expired air or present in bicarbonate pools after only 30 min. In both subjects, more than half of ^{14}C entering y_β returned rapidly to y_b. The remainder, which entered y_γ, reentered oxidative pathways very slowly. A curve which fitted the data fairly well could be obtained by combining y_β and y_γ into a single compartment (see Appendix), but the precision of the individual values for specific activity of expired CO_2 and the close similarity of the results in the two subjects seem to justify consideration of the more complicated model. In liver, newly synthesized triglycerides are known to be contained in at least two different functional compartments, one of which is related to cytoplasmic organelles and turns over rapidly, while the other is contained in fat droplets and

turns over slowly (1, 14). The relation of these two triglyceride pools to oxidative pathways is not known, and could exist as shown in the preferred model, or in other models (see Appendix). It is probable that triglyceride pools are heterogeneous in red skeletal muscle, since small droplets of fat in close proximity to mitochondria are demonstrable under the electron microscope (15). An unknown fraction of y_β and y_γ may exist in tissues other than working muscle. Recycling of ^{14}C in plasma TGFA indicates that significant quantities of FFA continued to enter liver during exercise. The quantity of FFA which entered these compartments, however, is such that a substantial fraction probably exists in working muscle. If they were contained in 20 kg of muscle, y_β and y_γ together would constitute 2–12 μmole of fatty acid per g (0.05–0.3% of wet weight). That such pools exist is supported by the observation that an appreciable fraction of ^{14}C is present in triglycerides and phospholipids of leg muscles of rats injected intravenously with palmitate-1-^{14}C while running on a treadmill, and killed 2 min later (N. L. Jones and R. J. Havel, unpublished data).

Although FFA continued to recycle through blood plasma during exercise, as indicated from the complex specific activity–time curves for FFA (Fig. 3), the extent was considerably less than that ordinarily observed at rest. This may be the result of a reduced fraction of the cardiac output perfusing adipose tissue, as well as reduced conversion of FFA to plasma TGFA, another source of plasma FFA. The maximal fraction of injected ^{14}C in plasma TFGA was less than 2% during exercise. This can be compared with values of 4.2 to 9.3% obtained in healthy subjects at rest (16). This effect of exercise is the expected result of reduction of the fraction of the cardiac output perfusing the liver from about 25 to 4% (11) and consequent reduction of hepatic influx of FFA as inferred from studies in man (3, 5) and shown directly in rats (N. L. Jones and R. J. Havel, unpublished data).

The present study also provides the first direct comparison of data for turnover rate and oxidation of FFA obtained by the methods of constant infusion and pulse injection (Table 1). Fractional turnover rates were 14–17% lower with the constant-infusion technique. This may be related to neglect of recycling of ^{14}C-FFA in the pulse-injection studies or overestimation of the effective volume of distribution of FFA in the constant-infusion studies. The estimates for "rapid" oxidation of FFA were 4–7% higher in the constant-infusion studies. This difference is probably within limits of biological variation and technical error. The variation in calculated percentage of expired CO_2 derived from rapid oxidation of FFA is mainly the result of differing plasma levels of FFA in studies 1 and 2. In each case, comparison of oxidation of FFA with values for ventilatory RQ indicates that oxidation of FFA accounts for only a fraction (on average, about half) of the fatty acid oxidized during exercise. This agrees with data obtained by the constant-infusion technique at work loads of 300–400 kg-m/min (3, 5).

The specific activity–time curves for expired CO_2 after injection of bicarbonate-^{14}C in our subjects, whose output of CO_2 was about 1.0 mmole/min and kg of body weight, resemble closely those obtained by Shipley et al. (9) in resting rats who excreted about 0.7 mmole of CO_2 per min and kg of body weight. Our values for compartment sizes also agree well with those of Shipley et al. when expressed on the basis of unit weight. The compartment size for y_1 approximates that for extracellular fluid at a concentration of 25 mmole/-liter. Compartment y_2 could be accounted for by a cell mass comprising 50% of body weight and containing 2–3 mmole/kg. Compartment y_3 presumably consists of other pools which turn over relatively slowly and may include organic carbon as well as bone (9). The accuracy of values for compartment sizes and rate constants for these pools is limited by the considerable error in the procedure of graphical subtraction of points on a complex exponential curve (17). The validity of our interpretation of pathways taken by FFA in these studies does not, however, depend upon the validity of the model for bicarbonate, if it is assumed that the behavior of CO_2 derived from oxidation of FFA during exercise does not differ significantly from that of bicarbonate injected intravenously.

APPENDIX

Precision of Estimates of Rate Constants for Bicarbonate Transfers in Three-Compartment System. Effects of varying a single rate constant on the observed function relating ^{14}C in expired CO_2 and time were determined for subject A with the analogue computer. Maximal effect, expressed as vertical deflection in percentage of ordinate value, was least for λ_{21}: variation of $\pm$50% from 0.056 produced a 5% change at 5 min and 8% at 20 min. λ_{12} was moderately sensitive at 10 and 50 min, but rate constants involving compartment 3 were considerably more sensitive.

Precision of Estimates of Rate Constants for Metabolism of FFA in Eight-Compartment System. Effects of varying rate constants were measured as described above. For subject B, varying rate constants $\pm$20% from values shown in Fig. 3, had the following maximal effects: λ_{1c}, +5%, −15% at 1 min; λ_{cb}, +10%, −15% at 3 min; $\lambda_{\beta b}$, −7%, +9% at 4 min; $\lambda_{b\beta}$, +4%, −6% at 15 min; $\lambda_{\gamma\beta}$, −10%, +10% at 40 min; $\lambda_{\beta\gamma}$, +10%, −15% at 90 min. These effects are such as to produce discernibly poorer fits to the observed values in y_1.

Other Models for Metabolism of FFA during Heavy Exercise (Subject A). (*a*) *Six-compartment* model. The best fits that could be achieved showed significant deviations from observed

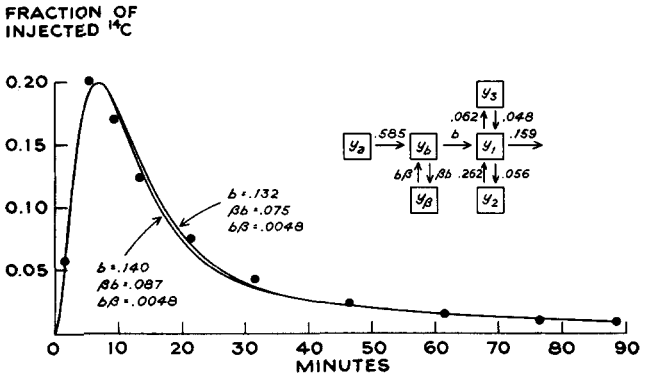

FIG. 5. Data and computer outputs for six-compartment model. Curves for two sets of rate constants are shown.

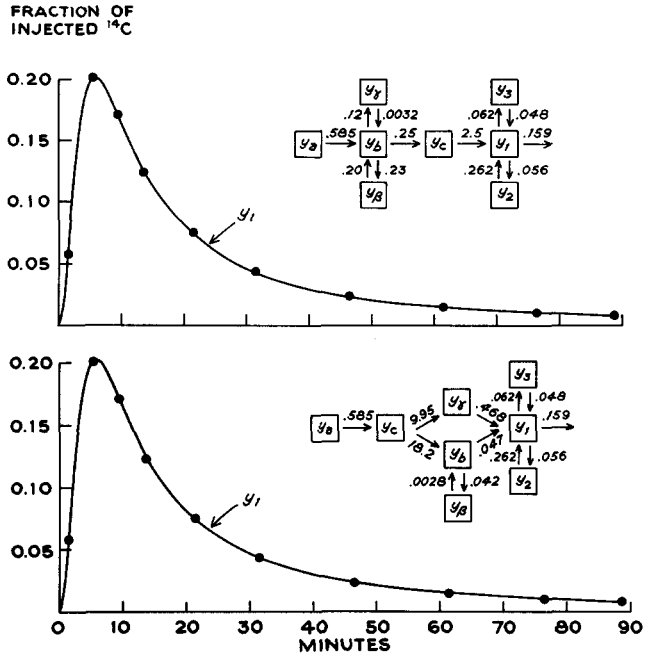

FIG. 6. Alternative eight-compartment models.

data points between 5.5 and 31.5 min (Fig. 5). This was also true for subject B. Addition of compartment y_c produced little improvement in this model. (*b*) Alternative *eight-compartment* models (Fig. 6). Both of these give fits equivalent to that of Fig. 4.

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REFERENCES

1. Havel, R. J., J. M. Felts, and C. M. Van Duyne. 1962. *J. Lipid Res.* **3:** 297.
2. Göransson, G., and T. Olivecrona. 1964. *Acta Physiol. Scand.* **62:** 244.
3. Havel, R. J., A. Naimark, and C. F. Borchgrevink. 1963. *J. Clin. Invest.* **42:** 1054.
4. Carlson, L. A., R. J. Havel, L.-G. Ekelund, and A. Holmgren. 1963. *Metabolism.* **12:** 837.
5. Havel, R. J., L. A. Carlson, L.-G. Ekelund, and A. Holmgren. 1964. *J. Appl. Physiol.* **19:** 613.
6. Sjöstrand, T. 1960. *In* Clinical Cardiopulmonary Physiology. Grune and Stratton, New York.
7. Felts, J. M., and E. J. Masoro. 1959. *Am. J. Physiol.* **197:** 34.
8. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. *J. Biol. Chem.* **177:** 751.
9. Shipley, R. A., N. Baker, G. E. Incefy, and R. E. Clark. 1959. *Am. J. Physiol.* **197:** 41.
10. Skinner, S. M., R. E. Clark, N. Baker, and R. A. Shipley. 1959. *Am. J. Physiol.* **196:** 238.
11. Wade, O. L., and J. M. Bishop. 1962. Cardiac Output and Regional Blood Flow. Blackwell, Oxford. Chapt. 6.
12. Garland, P. B., and P. J. Randle. 1964. *Biochem. J.* **93:** 678.
13. Therriault, D. G., and M. A. Mehlman. 1965. *Can. J. Biochem.* **43:** 1437.
14. Stein, Y., and B. Shapiro. 1959. *Am. J. Physiol.* **196:** 1238.
15. Gauthier, G. F., and H. A. Padykula. 1966. *J. Cell Biol.* **28:** 333.
16. Friedberg, S. J., R. F. Klein, D. L. Trout, M. D. Bogdonoff, and E. H. Estes, Jr. 1961. *J. Clin. Invest,* **40:** 1846.
17. Riggs, D. S. 1963. The Mathematical Approach to Physiological Problems. Williams & Wilkins, Baltimore.