Quantitative aspects of free fatty acid metabolism in the fasted rat

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ABSTRACT Palmitate-1-^{14}C was injected intravenously into unanesthetized, fasted rats. Disappearance of tracer from plasma free fatty acids was studied. A large component of free fatty acid (FFA) recycling was directly demonstrated by reinjection experiments. The latter studies also indicated the existence of an unidentified, rapidly turning over polar lipid in plasma which was synthesized from palmitate-^{14}C. The appearance of ^{14}C in hepatic and extrahepatic triglycerides, in other esters, and in respired CO_2 was also followed. The data were analyzed using a multicompartmental model and a digital computer.

Only a small fraction of the triglycerides formed in liver was derived directly from plasma free fatty acids. The major portion of net triglyceride formation appeared to be by way of an intermediate nontriglyceride ester pool which turned over relatively slowly compared to plasma free fatty acids. Initial approximations are as follows (μmoles of fatty acid per min per 100 g body weight): net free fatty acid mobilization (irreversible disposal) = 2.4; hepatic triglyceride formation directly from plasma free fatty acid = 0.1; total hepatic lipid formation from plasma free fatty acids = 0.5; oxidation of free fatty acids to CO_2 = 0.8; percentage of respired CO_2 from direct oxidation of fatty acids = 12%; extrahepatic triglyceride formation directly from fatty acids = 0.4; total extrahepatic lipid formed directly from fatty acids = 1.2.

IN AN EARLIER STUDY of hepatic TG secretion in fasted rats, we estimated that the TG turnover rate in liver was several times greater than the rate of hepatic TG synthesis from plasma FFA (1). This conclusion was difficult to reconcile with current concepts of FA and TG metabolism in fasted animals, according to which virtually all newly synthesized hepatic TGFA is derived from circulating FFA (2–4). Since hepatic lipogenesis is depressed in the latter condition, newly synthesized hepatic TGFA cannot be synthesized rapidly from non-lipid precursors. Therefore, if TG is turned over in the liver faster than it is synthesized from FFA, the TG must be derived from some other lipid pool such as PL. This conclusion, however, was based upon experiments which were not specifically designed to measure the rate of FFA conversion to liver TG (1).

In the present study, we have reexamined this problem using an experimental approach which focused upon plasma FFA turnover at early times after palmitate-1-^{14}C injection and upon the initial rates of incorporation of plasma FFA into hepatic and extrahepatic TG.

METHODS

Male Sprague-Dawley rats (160–200 g) were deprived of food for 20 hr, but given free access to drinking water. 3 μc of palmitate-1-^{14}C (31 μc/μmole; New England Nuclear Corp., Lot No. 31-238-7) was complexed to 0.30 ml of freshly prepared rat serum (5) and injected into the tail vein of unanesthetized rats. At various time intervals after the injection, a few rats were stunned with a blow and quickly immersed in liquid nitrogen. Other rats were decapitated; the blood was collected in plastic tubes containing solid sodium oxalate and immediately chilled in an ice bath. Simultaneously with the blood withdrawal, the abdominal cavity was ex-

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Abbreviations: FFA, free fatty acid(s); TG, triglyceride(s); TGFA, triglyceride fatty acid; PL, phospholipid(s).

posed and a piece of liver tissue was quickly extirpated, immersed in liquid nitrogen, weighed, and extracted for total lipids with chloroform–methanol 2:1 (6). The remaining liver was also excised and weighed to obtain total liver weight. The carcasses were quickly frozen in liquid nitrogen and either extracted immediately or stored at $-16°C$ for 3 days prior to extraction.

The plasma samples were extracted for lipids as described by Sperry (7). The frozen whole rats and the carcasses were crushed and then extracted with 1800 ml of chloroform–methanol 2:1 in a 1 gallon Waring Blendor. Prior to extraction extreme care was taken to keep the carcasses frozen. The homogenates were diluted to 2000 ml and aliquots of the filtered material washed with $^1/_5$ their volume of water. The water-washed extract was dried under a stream of nitrogen and stored at $-16°C$ in a chloroform solution. The chloroform–methanol extracts of plasma and liver were washed, dried, and stored in a similar manner. The chloroform–methanol aliquots of each of the samples for each time point were pooled, except for the 2-sec and 1-min groups, which were determined individually.

Aliquots of each of the lipid extracts were fractionated by thin-layer chromatography on silica gel (8). The various fractions were detected by exposure to iodine vapors, removed from the plates, and assayed for ^{14}C as previously described (8).

Recycling Experiment

Six unanesthetized rats were fasted and then injected with approximately 15 μc of palmitic acid-1-^{14}C complexed to bovine serum albumin. Exactly 5 min after injection of the labeled material, each rat was decapitated and blood was collected. Serum from each of the rats was pooled and an aliquot was set aside for subsequent assay. 0.50 ml of the labeled serum, which was subsequently found to contain 2920 cpm of polar lipids and 6530 cpm of FFA, was then injected into the tail veins of 14 other fasted, unanesthetized rats. 1, 2, and 5 min after the injection the animals were decapitated and the blood was collected into chilled tubes containing a drop of sodium heparin (10 mg). Plasma lipids and an aliquot of the injected serum were extracted with chloroform–methanol 2:1, washed, dried, and fractionated by thin-layer chromatography (8). Approximate midpoint R_f values were as follows: phospholipids, 0; FFA, 0.65; Tg, 0.8; other lipids, rest of plate. Fractions were scraped from the plates and assayed for ^{14}C (8). Plasma FFA concentrations of six separate rats, which had been injected with saline 5 min before they were decapitated, were determined by titration of unwashed isooctane extracts (9).

Respired CO_2

$^{14}CO_2$ was collected in 50 ml of 1 N NaOH at nine different 4 min intervals, spaced over 6 hr, from each of four rats injected with tracer palmitate-1-^{14}C (3 μc complexed to 0.30 ml of rat serum) and from each of four rats injected with tracer sodium bicarbonate-^{14}C (5 μc in 0.50 ml). The same rats were used for measuring the rate of CO_2 expiration. Aliquots were taken for assay of radioactivity in each sample of alkaline sodium carbonate (10). At seven of the time intervals in each experiment, aliquots obtained from individual rats were pooled thus giving a total of 14 samples from eight rats. The carbonate was precipitated with $BaCl_2$ and analyzed titrimetrically.

RESULTS

Plasma FFA

As we noted previously in mineral oil- and CCl_4-fed fasted rats (11), the disappearance curve of FFA^{14}C from the circulation was a complex exponential function (Fig. 1). 1 min after intravenous injection of palmitate-1-^{14}C 30% of the injected ^{14}C remained in plasma; only 1% remained after 5 min.
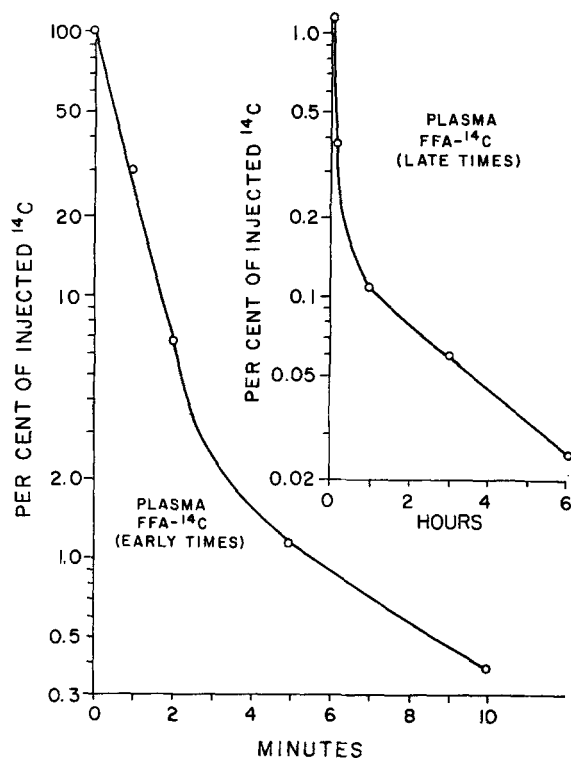

Fig. 1. Percentage of injected palmitate-1-^{14}C in plasma FFA after a single intravenous injection of palmitate-1-^{14}C complexed to rat serum into unanesthetized, fasted rats. Each value represents a pooled aliquot of four rats, except at 1 min when plasmas from four individual rats were analyzed. At 1 min the mean ($\pm$SD) value was 30 $\pm$ 4.8% of the injected ^{14}C as plasma FFA. Data are plotted on a semilogarithmic scale.

The plasma FFA concentration of a separate group of six rats, fasted overnight, was 0.79 ± 0.12 μmole/ml. The plasma volume of these rats of was found to be approximately 4% of body weight (11). From these values, the plasma FFA pool size was estimated to be 3.2 μmoles of FFA per 100 g body weight.

Recycling of FFA

The observations that FFA disappeared from the circulation at an ever-decreasing rate and that a fraction of 1% of the dose persisted in the plasma beyond the 5th min (Fig. 1) suggested that either there was extensive recycling of FFA back into the circulation or that a minor, slowly turning over, labeled substance had been present in the injected dose. The small fraction of ^{14}C present in plasma at 5 min was shown by reinjection into recipient rats to be a rapidly turning over substance (Fig. 2). Therefore, the apparently slow disappearance of FFA-^{14}C from the circulation after the first 5 min of the experiment (Fig. 1) must represent continuous recycling of
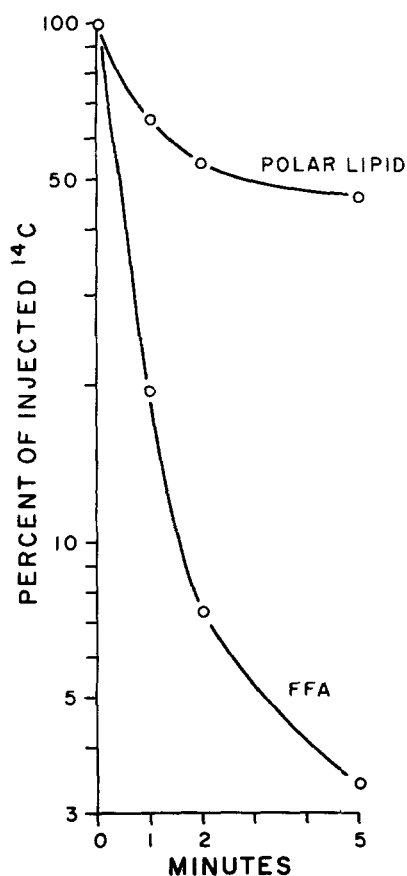
TABLE 1   BALANCE OF INJECTED [14]C

Substance	Per Cent of Injected [14]C						
	0	2 sec	1 min	10 min	1 hr	3 hr	6 hr
FFA (total body)	100	91	44	10	<1	<1	<1
Other lipid (total body)	—	8	32	57	57	42	30
Total lipid	—	99	76	67	57	42	30
Exhaled CO₂	—	0	0.5	8	38	57	65
Missing	—	1	24	25	5	1	5

labeled FFA into plasma. Although no TG-^{14}C is usually found in plasma 5 min after an intravenous injection of palmitate-[14]C (2, 8, 12, 13) a ^{14}C-labeled polar lipid was found in the PL fraction in the serum of donor rats 5 min after injection of labeled palmitate.[1] The PL fraction contained 45% as much radioactivity as the FFA fraction and, as shown in Fig. 2, seemed to disappear at a rapid rate which, together with its early appearance in plasma of the donor rats, suggests that it is a rapidly turning over PL which may be of considerable interest.

Disposition of FFA-[14]C

An attempt was made to measure the chemical form and distribution of ^{14}C that had left the plasma. As shown in Table 1, almost all radioactivity could be accounted for at 2 sec, 1 hr, 3 hr, and 6 hr after the injection. At 2 sec, most of the radioactivity was found to be in the form of FFA and was assumed to be still in the form of albumin-bound palmitate-1-^{14}C in plasma. At 1 hr, 3 hr, and 6 hr, 30–57% of the injected ^{14}C was recovered as TG and PL of hepatic and extrahepatic tissues, and the rest as expired CO₂. However, at 1 and 10 min after injection, a large fraction of ^{14}C was neither present as expired CO₂ nor as chloroform–methanol extractable material (Table 1).

The inability to account for ^{14}C in the lipid extract of the frozen rat 1 min after injection of palmitate-^{14}C had also been observed in separate, preliminary experiments.[2] In one instance 94% of the injected dose was recovered in

FIG. 2.   Disappearance of ^{14}C from plasma FFA and polar lipids after reinjection of plasma obtained 5 min after a single injection of palmitate-1-[14]C. The fractional SD for the values of plasma FFA at the three times studied were ±0.28, ±0.073, and ±0.25 (n = 5, 4, and 5, respectively). Data are plotted on a semilogarithmic scale.

[1] Dr. B. Lombardi, University of Pittsburgh School of Medicine, Pittsburgh, Pa., informed us prior to our experiment (personal communication) that he has found that the specific activity of plasma total phospholipids reached a maximum at 5 min after intravenous injection of palmitate-^{14}C in rats and declined rather rapidly thereafter.

[2] M. C. Schotz, N. Baker, and M. N. Chavez found in a separate study of fasted and glucose-fed rats that 1 min after injection of palmitate-1-^{14}C all of the injected ^{14}C was recovered in a chloroform–methanol extract of frozen animals. Comparison of these experiments with the present data suggested that our missing activity was probably unesterified fatty acid in tissues. We have not established the cause of this discrepancy.

TABLE 2 RECOVERY OF ^{14}C FROM AN ALKALINE ETHANOL EXTRACT OF FROZEN RATS 1 AND 10 MIN AFTER INJECTION OF PALMITATE-1-^{14}C

Expt.	Analysis	2 Sec	1 Min	2 Min	10 Min
				%	
I	Total lipid fatty acid*	— (n = 4)	100 ± 17 (n = 4)	83 ± 10 (n = 4)	46 ± 5 (n = 4)
II	Body bicarbonate†	0 (n = 3)	1.5 ± 0.16 (n = 4)	4.9 ± 0.87 (n = 4)	13.1 ± 3.4 (n = 4)

Values are % of injected ^{14}C, mean ± SD.

* After saponification. In a separate experiment, the recovery of ^{14}C in the whole homogenate (before saponification) at 1 min (n = 3) was not significantly different from that at 2 sec (n = 3) after injection: 4.5 (range, 4.2–4.8) × 10^5 cpm compared with 4.7 (range 4.6–4.7) × 10^5 cpm, respectively.

† Each rat was homogenized in 2 liters of an ethanolic solution of 1 N NaOH. 1.0 ml aliquots of homogenate were added to Erlenmeyer flasks equipped with a center well and a serum stopper. 0.5 ml of 1 N NaOH was present in the center well. 0.2 ml of 5 N H$_2$SO$_4$ was injected into the outer well and the CO$_2$ was allowed to diffuse for 3 hr. The contents of the center well were analyzed for radioactivity. Appropriate bicarbonate-^{14}C controls were run and corrections for recovery made. Each analysis was run in triplicate.

chloroform–methanol at 2 sec, but only 67% was recovered at 1 min (three rats fasted overnight per group). Since about one-fourth of the dose was unaccounted for at both 1 min and 10 min after injection of palmitate-1-^{14}C (Table 1), several experiments were performed in an attempt to account for the missing radioactivity.

At 1 min, all of the injected radioactivity could be recovered as fatty acids if rats were saponified for 1 hr at 100°C in alkaline ethanol (the mixture then being acidified and extracted with petroleum ether) instead of being homogenized in chloroform–methanol. Therefore, all of the missing ^{14}C at 1 min was in the form of lipid. Approximately 13% was recovered at 10 min as "^{14}CO$_2$" after treatment with acid and diffusion (Table 2). No experiments were done to establish (a) whether the ^{14}CO$_2$ was free of other volatile substances and (b) whether substances other than bicarbonate-CO$_2$ had given rise to ^{14}CO$_2$ under the conditions of acid diffusion at ambient temperatures. Nevertheless, these experiments indicated that radioactivity was poorly recovered at 1 min because it was in the form of a lipid which was not readily extractable with chloroform–methanol,^{2} and that ^{14}C recovery was incomplete at 10 min because the palmitate had been converted to nonlipid compounds, a large portion of which behaved like bicarbonate, labile carboxyl groups, or acid-volatile substances.

Conversion of FFA-^{14}C into Hepatic TG and Non-TG Esters

The incorporation of FFA-^{14}C into hepatic lipids is given in Fig. 3. During the 2nd and 10th min of the experiment, when FFA had almost completely disappeared from plasma, ^{14}C incorporation into TG continued at a rate comparable to that found in the first 2 min of the experiment, when 93% of the FFA was being removed from the circulation. On the other hand, the other lipid esters were labeled maximally within the first 2 min of the experi-

ment. The slow incorporation of ^{14}C into TG suggests that most of the FFA-^{14}C that was converted to TG-^{14}C first passed through a relatively large intermediate diluent pool. The pattern of isotopic incorporation into PL (and other esters) was consistent with rapid direct conversion of
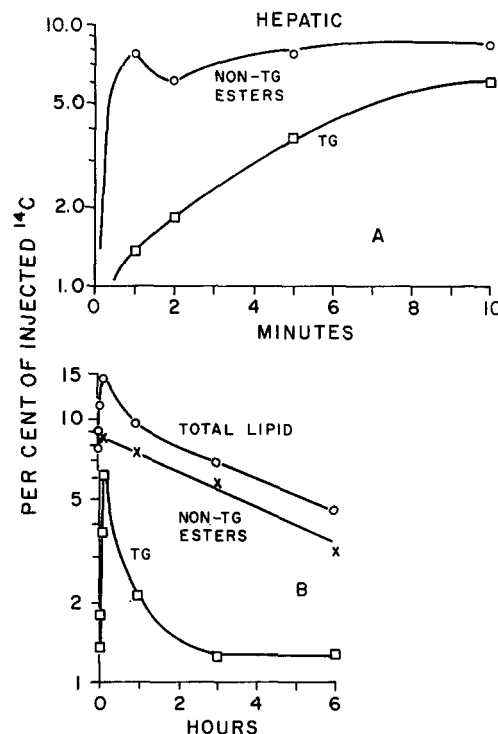

FIG. 3. Incorporation of palmitate-1-^{14}C into hepatic TG and non-TG lipid esters. Each value represents an analysis of four separate extracts, aliquots of which were pooled at each time point except at 1 min. when the individual extracts were analyzed separately. The fractional SD for ^{14}C in TG and non-TG lipids at 1 min were ±0.10 and ±0.30, respectively. Values have been corrected for trapped plasma-^{14}C. Total lipid is the sum of TG + non-TG esters. No FFA-^{14}C was recovered after corrections were made for trapped plasma. Data are plotted on a semilogarithmic scale.

FFA-^{14}C into part of this large lipid pool as an intermediate step in the formation of hepatic TG.

Maximum incorporation of ^{14}C into both TG and non-TG esters occurred within 10 min. As may be seen from Fig. 3B, over $^2/_3$ of the ^{14}C in total hepatic lipids at 10 min turned over in 6 hr. The disappearance was due largely to disappearance of TG-^{14}C in the 1st hr; however, a slow component of TG-^{14}C turnover was also observed. No significant loss of ^{14}C from hepatic TG was observed during the last 3 hr of the experiment. However, non-TG-^{14}C disappearance continued throughout the experiment at a rather constant rate ($t_{1/2}$, approximately 4.5 hr).

Conversion of ^{14}C-FFA into Extrahepatic TG and Non-TG Esters

Data similar to those shown for liver in Fig. 3 are given for extra-hepatic tissue in Fig. 4. As in liver, ^{14}C was incorporated into TG much more slowly than into non-TG lipids during the first 10 min of the experiment. The rapid component of TG turnover that was observed in liver (Fig. 3) was not found in extrahepatic tissue (Fig. 4). The curve of ^{14}C in extrahepatic non-TG esters was complex during the 1st hr; however, all values ranged between 14% and 23% of the injected dose throughout the
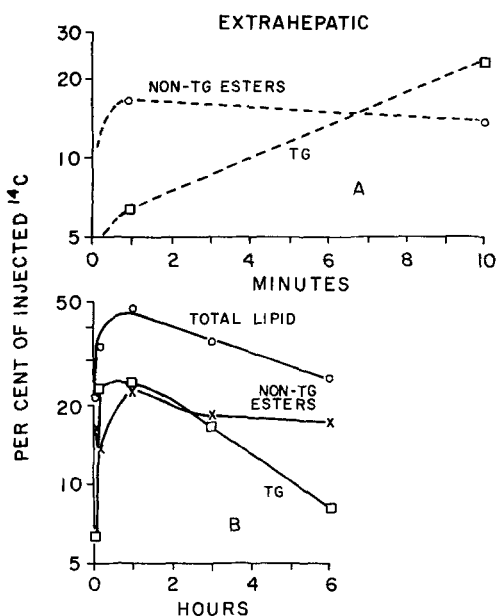
experiment. The fractional rate constant of disappearance of ^{14}C from the TG pool during the last several hours of the experiment ($t_{1/2}$, 2.8 hr) was greater than that of either extrahepatic non-TG-^{14}C or hepatic TG-^{14}C during the same time interval.

Although the total ^{14}C-lipid in extrahepatic tissue at 1 hr was five times greater than that in liver (47% compared to 9.6% of the injected ^{14}C, respectively; cf. Figs. 4 and 3), the fractional rates of disappearance were similar, $t_{1/2}$ about 4.5 hr in liver and about 6 hr in extrahepatic tissues. The fractional disappearance rate of ^{14}C from plasma FFA was similar to that of hepatic TG during t = 10–60 min and to that of extrahepatic TG during the last 5 hr of the experiment.

Turnover of Plasma Bicarbonate-^{14}C

The cumulative appearance of ^{14}CO$_2$ in the breath of fasted rats after intravenous injection of bicarbonate-^{14}C is shown in Fig. 5. Practically all (95%) of the injected ^{14}C was recovered at the end of the experiment. The percentage of injected bicarbonate-^{14}C remaining in the body (100 minus values shown in Fig. 5) is plotted in Fig. 6. The curve is complex and is probably the result of several curves given by different processes including exhalation of CO$_2$ and mixing of extracellular bicarbonate with intracellular inorganic and organic compounds (14). The rate of CO$_2$ exhalation was 106 ± 21 μmoles/min per 100 g of body weight (eight rats).

Oxidation of Palmitate-1-^{14}C to CO$_2$

The cumulative appearance of ^{14}CO$_2$ expired by fasted rats after intravenous injection of palmitate-1-^{14}C is shown in Fig. 5. Approximately $^2/_3$ of the injected ^{14}C was collected as ^{14}CO$_2$ in 6 hr. The amounts of ^{14}CO$_2$ expired after injection of bicarbonate-^{14}C and of palmitate-1-^{14}C during the interval 2–6 hr were 3% and 15% of the injected ^{14}C, respectively. The difference (12% of the dose) corresponds closely to the disappearance of ^{14}C from TG esters in extrahepatic tissues during the corresponding time period.

ANALYSIS AND DISCUSSION

The present study was initiated after several workers had already studied plasma FFA turnover in rats (2, 15). In each case, plasma FFA had been treated as a single homogenous pool which neither exchanged with other FFA pools nor recycled. Initial disappearance of the injected FFA-^{14}C was neither emphasized nor related to measurements of incorporation of ^{14}C into other lipids. Thus, quantitative considerations of the fate of FFA were not adequately emphasized. However, studies of FFA-^{14}C metabolism in man had indicated that FFA-^{14}C in plasma does not behave as though it were part of a single pool,
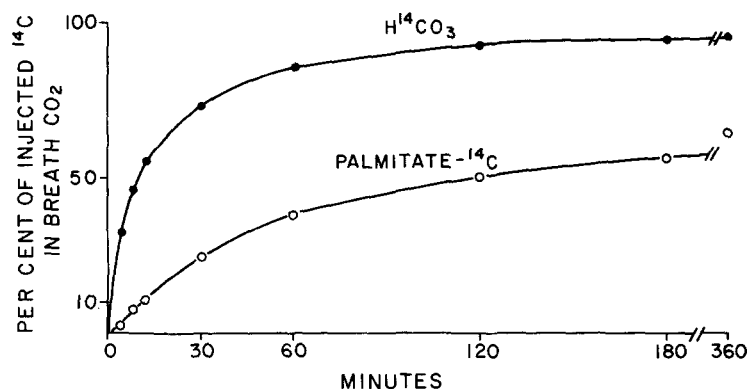

FIG. 4. Incorporation of palmitate-1-^{14}C into extrahepatic TG and non-TG lipid esters. Same numbers of rats as in Fig. 3. One value at 1 min was ignored because all radioactivity was recovered as FFA in contrast to the other samples in this and in other (unpublished) experiments. The range of values at 1 min for TG and non-TG radioactivity, uncorrected for the trapped plasma, were 7.6–10.5 and 17–31% of the injected ^{14}C. The latter range includes FFA-^{14}C which is not included as "total lipid" in the figure. As in Fig. 3, total lipid refers to the sum of TG and non-TG esters. Data are plotted on a semilogarithmic scale.

FIG. 5. Cumulative recovery of $^{14}CO_2$ in breath after intravenous injection of tracer bicarbonate-^{14}C (mean of four rats) or palmitate-1-^{14}C (mean of three rats); ^{14}C data from a fourth rat were ignored because the levels of radioactivity were so low that a technical error seemed likely. The mean fractional SD of the rates of $^{14}CO_2$ exhalation were 0.24 and 0.11 for the bicarbonate-^{14}C and palmitate-^{14}C experiments, respectively.
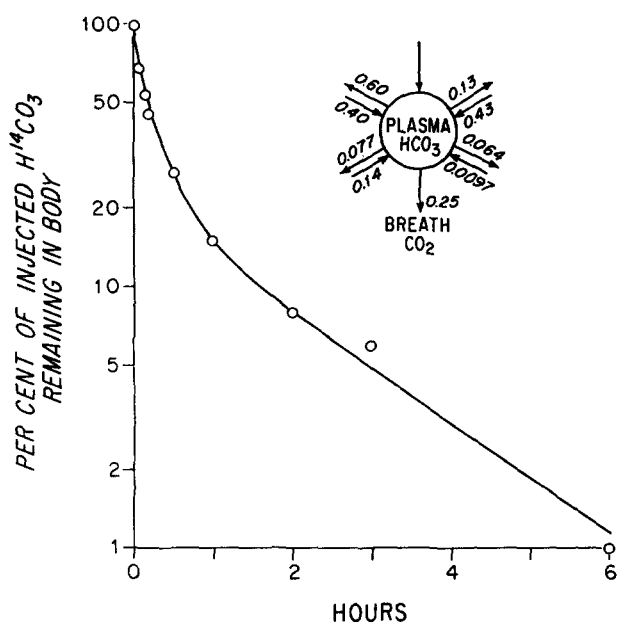

FIG. 6. Disappearance of bicarbonate-^{14}C from the body after intravenous injection of bicarbonate-^{14}C. The semilogarithmic plot of radioactivity was calculated from the data shown in the upper curve of Fig. 5 (amount remaining in body at time $t = 100\%$ $-\%$ of injected ^{14}C excreted in breath at t). These calculated values were used to derive a set of fractional rate constants which would describe operationally the kinetic behavior of bicarbonate-^{14}C as it enters the plasma. The fractional rate constants (min^{-1}) are shown in the upper right portion of the figure. The data may obviously be represented just as accurately by a simpler model; however, the subsequent analysis is independent of the model used to represent the turnover of plasma bicarbonate as long as the turnover of plasma bicarbonate is accurately described by the rate constants.

restricted to the circulation and utilized irreversibly (16). The present data show that the behavior of the FFA pool in fasted rats is also complex and that recycling of FFA is appreciable.

Several papers have appeared which attempted to account for the FFA-^{14}C which disappeared from the circulation (17–19). However, in none of these was the complex nature of the FFA disappearance curve related to the appearance of FA in esters or in respired CO_2. As a result, no integrated, thorough analysis of the quantitative significance of plasma FFA turnover in the rat has been reported. The present data are consistent with most previously published observations. The purpose of this study was to analyze our data to obtain a first approximation of rates of FFA metabolism in a common experimental animal, the rat.

All of the usual shortcomings of multicompartmental analysis apply to the present study: namely, oversimplification; reliance upon many assumptions; arbitrariness with respect to the drawing of smooth curves through scattered points and, therefore, with respect to the solution that is accepted as giving a "best fit" of the data; and compromise between enlarging the model and accepting a poor fit when two or more sets of data are not exactly compatible with a relatively simple model. Although our aim has been to gain some understanding of quantitative aspects of lipid metabolism in the rat, our calculations are only intended to be estimates which apply to rats used under the specific experimental conditions that we employed. These first approximations are a basis for further work, and not absolute values. Analysis and discussion of the observed data have been arranged as follows:

List of assumptions used in calculations
Model of FFA metabolism in fasted rats
Multicompartmental analysis (fractional rate constants of flux)
Specific estimates of selected rates
Discussion of the physiological and biochemical significance of estimated rates as they are presented

Assumptions

The multicompartmental analysis is based upon the following assumptions. Some brief comments have been added parenthetically after each assumption. Compartment numbers are defined in Fig. 10.

1. FFA in compartments *1* and *2* and total body bicarbonate pools did not change in concentration. Similarly, hepatic TG and non-TG pools were in a steady state. [No large changes in plasma FFA concentration were noted during a separate, 10 min study of FFA metabolism.[3] We have never observed increases such as the 240% change reported by Carlson and Nye (20) 15 min after a subcutaneous injection of saline.]

2. Esterification and oxidation of FFA stopped as soon as animals were placed in liquid nitrogen. [Since complete freezing of a rat carcass in liquid nitrogen is known not to be instantaneous, observed values of ^{14}C incorporation into extrahepatic lipid esters will tend to be erroneously high.]

3. No FFA need enter compartments *1, 2,* or *3* during oxidation of lipid esters to CO_2. [Enzymes that can hydrolyze lipid esters and can activate and oxidize fatty acids are located intracellularly; it seems plausible that fatty acids formed from esters can be oxidized to CO_2 without first having to equilibrate with relatively large pools of FFA.]

4. All net input of FFA enters compartment *1* (Fig. 11) without first mixing with extraplasma FFA; newly formed FFA is not appreciably labeled during the experiment. [Stein and Stein have shown that FFA that is converted to TG by adipose tissue is diluted to such an extent that little radioactivity leaves the tissue during brief periods of fasting (21, 22). However, there is still little information regarding the extent to which FFA newly formed from TG mixes with intracellular FFA pools in adipose tissue.]

5. ^{14}C that was not recovered at 1 min in chloroform-methanol (Table 1) was in the form of extraplasmal, bound, but exchangeable, FFA (compartments *2* and *3*; Fig. 11). [The plasma FFA-^{14}C curve indicates that rapid exchange of plasma FFA with some other pool of lipid occurs. One possibility is that plasma FFA exchanges rapidly with extraplasma FFA as discussed below in Specific Estimates of Selected Rates (*b*). More FFA-^{14}C was recovered from whole rats at 1 min than was present in plasma. Therefore significant quantities of ^{14}C must have been present in an unesterified form in tissues at 1 min. The missing ^{14}C was in the form of lipid and could be extracted if animals were homogenized in alkaline ethanol (Table 2). No difficulty was encountered in extracting esterified lipids between $t = 1$ and 6 hr when FFA-^{14}C had virtually disappeared. Moreover, recovery of FFA-^{14}C was quantitative at 2 sec (Table 1) when almost all the labeled FA was probably still in the plasma. We infer from this that the labeled FA was in a unique form at 1 min which made it difficult to extract. Unpublished experiments have indicated that occasionally quantitative recoveries of ^{14}C could be achieved. When this occurred, the "extra" radioactivity was associated with the FFA fraction.[2]]

6. The slowly exchanging component of the plasma FFA-^{14}C curve reflects primarily the slow exchange of plasma FFA with extrahepatic tissue TG by a process which involves no net formation of FFA. [We have obtained no information on recycling other than that it occurs and that it could represent a very rapid process relative to the turnover rate of plasma FFA. All estimates of irreversible disposal, oxidation, and esterification rates must be reduced if recycling of FFA-^{14}C is associated with a net efflux of FFA from nonadipose extrahepatic tissues. Hepatic lipids were assumed not to recycle FFA-^{14}C into plasma; however, this could occur (23).]

7. CO_2 formed during the oxidation of FFA diffuses directly into blood and is expired in the same manner as bicarbonate-^{14}C which is injected directly into plasma. [The basis of this assumption is that CO_2 which forms intracellularly diffuses into red blood cells and into plasma before it mixes with intracellular pools of bicarbonate (14).]

8. All intermediates in the esterification and oxidation of fatty acids that are not shown in Fig. 10 are so small and rapidly turning over that they may be ignored mathematically. [Pool sizes of acyl CoA and of the tricarboxylic acid cycle intermediates are known to be small enough to be consistent with this assumption. However, it is not known to what extent intermediates in the tricarboxylic acid cycle exchange their carbon with large pools of amino acids in the cells that are oxidizing fatty acids to CO_2. Evidence has been presented in humans that acetate is oxidized to CO_2 by way of very rapidly turning over pools (24).]

9. The model itself represents correctly the relationships among FFA, TG, non-TG esters, and CO_2 in the fasted rat. [This assumption is wrong, because of the deletion of additional liver TG pools and of plasma TG and non-TG pools. The model is obviously oversimplified and erroneously treats a highly complex system in a simple manner. The relationships between TG and non-TG esters are not known and the data obtained here do not establish whether or not the model we have chosen is valid.]

10. Palmitate-1-^{14}C added to serum as a potassium salt in aqueous ethanol is complexed to serum albumin like other plasma fatty acids; the fractional rate constant

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[3] N. Baker and H. Rostami. Manuscript in preparation. The formula for irreversible disposal rate (R) is: $R = Q_1/\Sigma(H_i/g_i)$, where Q_1 = pool size of plasma FFA, and H_i and g_i = the intercepts and slopes of the plasma FFA-^{14}C–time curve, respectively.
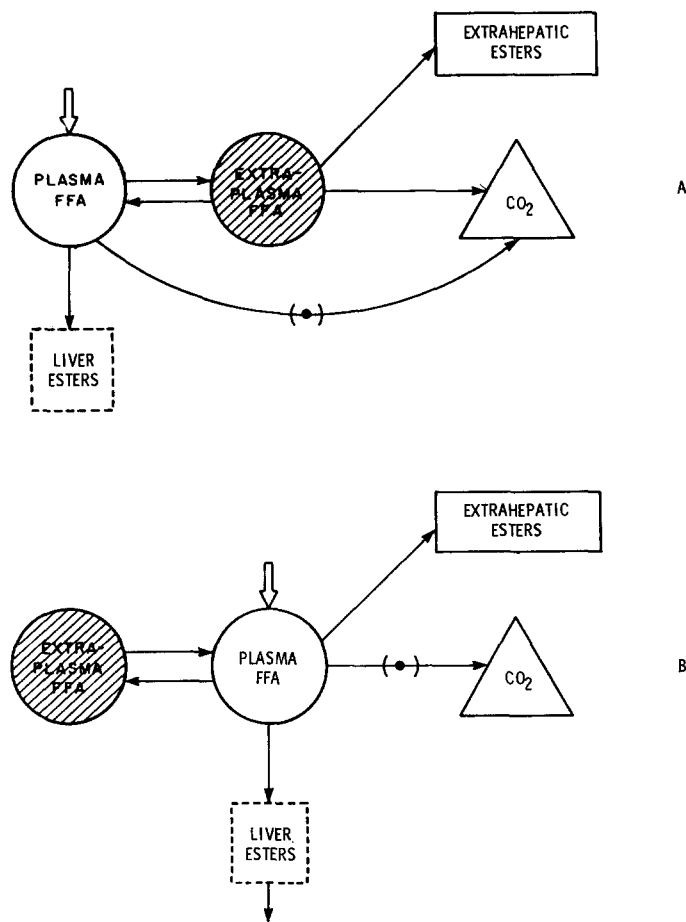
FIG. 7. Two simple models which describe possible relationships of extraplasma FFA and to other products of FFA metabolism. See text.

of turnover of the tracer and the metabolic pathways followed by the labeled fatty acid reflect the behavior of all plasma fatty acids. [We have no information to support this assumption other than the observation that labeled fatty acids in the serum obtained 5 min after injection of the artificial complex (Fig. 2) disappeared from plasma at about the same fractional rate as the initial complex (Fig. 1). The reinjected material was probably labeled in a physiological manner. However, evidence has been presented recently that the rate of palmitate-1-^{14}C disappearance from the circulation of dogs varies according to the method used to complex the tracer with albumin (25).]

11. TG are formed from fatty acids in the same cells as are non-TG esters. [This could be true for native fatty acid albumin and not for an artificial complex. See assumption (10).]

Model of FFA Metabolism

Minimal models of FFA metabolism in fasted rats as defined by other workers are shown in Figs. 7 A and B. Both models A and B take into account experimental evidence that shows that plasma FFA is converted to CO_2 (26, 27) and to lipid esters (2, 19) in both hepatic and extrahepatic tissues. Some evidence suggests that oxidation of FFA to CO_2 occurs primarily in extrahepatic tissues (17, 26); however, conversion of FFA within the liver to intermediate compounds such as ketone bodies which are subsequently oxidized to CO_2 in extrahepatic tissues is also known to occur (27) and is indicated by the symbol ($-\cdot\rightarrow$). Plasma FFA is shown to be exchanging with extraplasma FFA. In Fig. 7A, but not in 7B, the exchangeable FFA pool outside of plasma is considered to act as a diluent through which FFA derived from plasma must pass before it is metabolized by extrahepatic tissues. As Olivecrona pointed out, both types (Fig. 7A and 7B) of exchange probably occur. A large, slowly exchanging pool may exist in adipose tissue (15); a small, rapidly exchanging FFA compartment exists in interstitial fluid and, perhaps, also in the capillary endothelium and extrahepatic cells (28, 29). This is depicted in the simple model shown in Fig. 8. An important feature of this model is the net inflow of FFA, which is shown entering the plasma FFA compartment without first undergoing dilu-
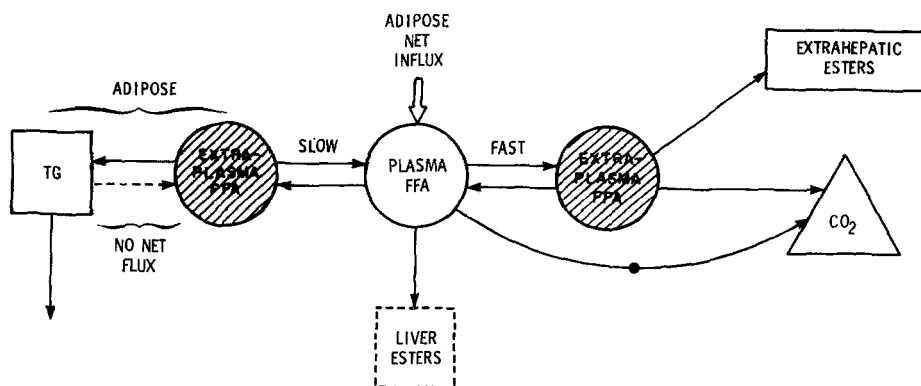
FIG. 8. Simplified model of FFA metabolism. The figure represents a composite of the two models shown in Fig. 7. Two arrows depicting the movement of FA from adipose tissue into plasma FFA are shown. One of these, to the left, represents a hypothetical exchange or mixing phenomenon with no net flow (see assumptions). The broken line from adipose TG to adipose FFA is also assumed to represent an exchange process. The net influx from adipose TG to plasma FFA is assumed to contain negligible radioactivity as depicted by the arrow at the top of the circle representing the plasma FFA compartment. The interchange of plasma with two extraplasma FFA compartments is shown. The exchange was assumed to be relatively fast in nonadipose tissue and slower in adipose tissue.
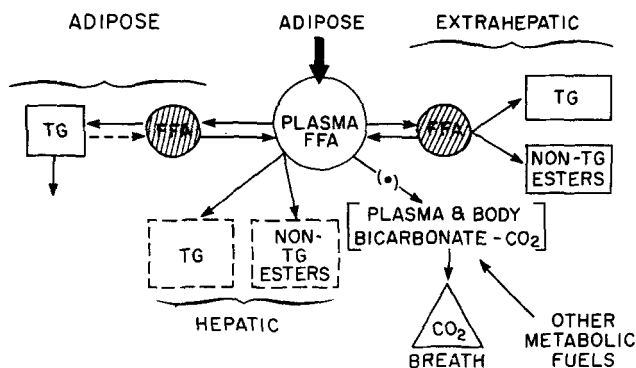

FIG. 9. Expansion of model shown in Fig. 8 to illustrate the final model which served as a basis for the experimental design. Note the simple branching of plasma or extraplasma FFA into TG and non-TG esters in hepatic and extrahepatic (nonadipose) tissues, respectively. The plasma and body bicarbonate pools were shown in Fig. 6 and have been deleted here for simplicity. Also, the exits from hepatic and extrahepatic lipids indicating turnover of these lipids are not shown here but were considered in the subsequent analysis.

tion in the relatively large adipose tissue FFA pool (see assumption 4, above). Some exchange of ^{14}C is considered possible between adipose TG and plasma FFA via the intracellular adipose tissue FFA pool (15) as indicated in Fig. 8.

In order to take into consideration the conversion of plasma FFA to its major initial products, TG and PL, in hepatic and extrahepatic tissues we expanded the model of Fig. 8 to that of Fig. 9. Although FFA must pass through intermediate pools before being incorporated into fatty acyl esters, these pools are not shown because they are thought to be turning over so rapidly that they may be considered negligible from a mathematical standpoint. Any CO_2 formed in the body has been assumed to

enter plasma (14, 30) and to be excreted into breath in the same manner as plasma bicarbonate. A five pool model, not shown, was employed to represent the plasma and body bicarbonate system and the mathematical function was defined experimentally in a separate experiment (Fig. 6).

The model of Fig. 9 was found to be incompatible with the data. Most of the palmitate-^{14}C was apparently incorporated into a relatively large, slowly turning over, non-TG ester pool as an intermediate step during formation of TG in both hepatic and extrahepatic tissues (cf assumption 11 above). It was then necessary to employ a model such as that shown in Fig. 10 in order to find a set of rate constants that would describe the flow of plasma FFA to and from hepatic and extrahepatic lipids and to CO_2. According to the model of Fig. 10, FFA may enter TG by two possible pathways: (a) direct esterification via rapidly turning over intermediates (not shown); (b) indirect esterification, via compartments 5 and 9, which exchange FA with both TG and non-TG esters, but which convert incoming FA to non-TG esters much more rapidly than to TG esters. This model provides for a mechanism that would account for the observed rapid incorporation of palmitate-^{14}C into non-TG esters followed by a relatively slow incorporation of ^{14}C into TG esters. In addition, the possibility of recycling of extrahepatic TGFA into plasma FFA has been included in the model of Fig. 10, which served as the basis of subsequent calculations.

Multicompartmental Analysis of the Data
(Fractional Rate Constants of Flux)

The data of Figs. 1–6 and Tables 1 and 2 are in excess of the minimum required to calculate, uniquely, a set of
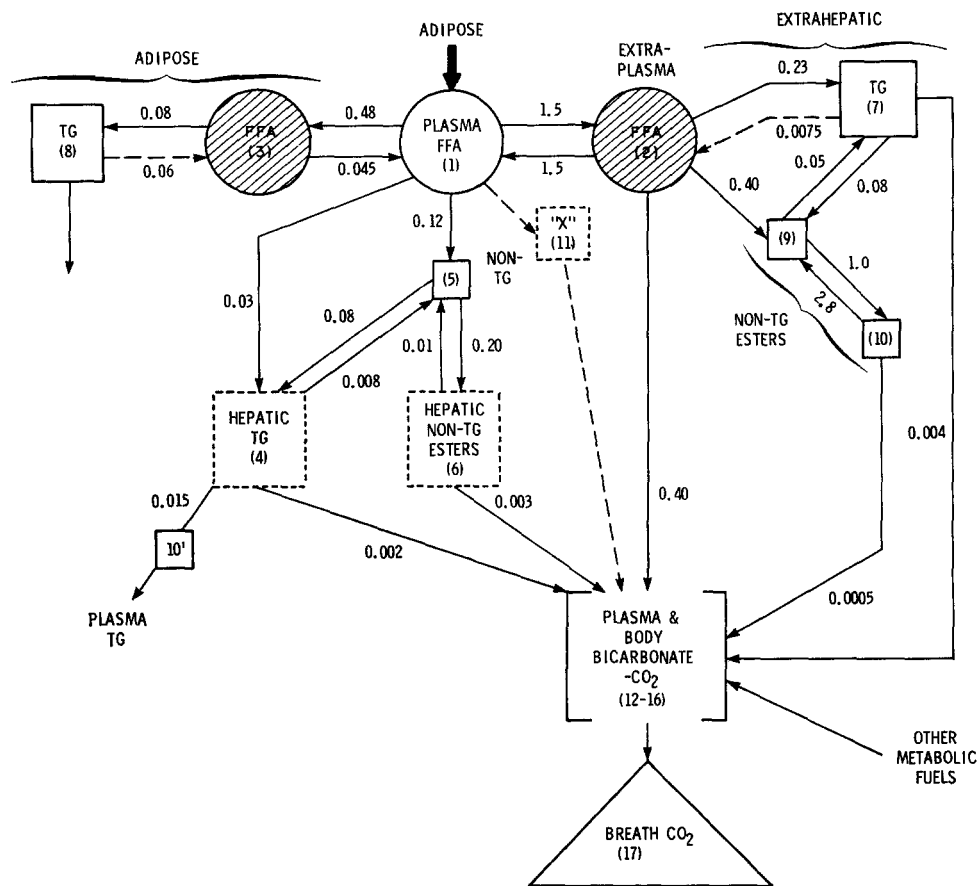
FIG. 10. Final model used to analyze the data. Fractional rate constants (min^{-1}) which were derived by the program of Berman et al. (33) are shown for all arrows except the broken arrows into and out of compartment *11* (see text). However, not all fractional rate constants were uniquely defined by the data even if the model is assumed to be correct. In particular, the interflow among compartments *4, 5,* and *6* and among *7, 9,* and *10* could not be established by the present data. Some of these compartments and arrows were required to depict the unexpected, slow accumulation of ^{14}C into TG as compared to non-TG lipid esters. A broken arrow is also shown from extrahepatic TG (compartment *7*) to extraplasma FFA (compartment *2*). This arrow was inserted to indicate a slow recycling of FA from TG. Although the present data demonstrate clearly that recycling of plasma FFA occurs, neither our experimental results nor those published by others establish the compartments (hepatic or extrahepatic, adipose or nonadipose, TG or non-TG) which are involved in the recycling phenomenon. Havel and Goldfien showed that some recycling occurs both in hepatectomized and in eviscerated dogs (31). It is also not clear from the data whether or not recycling of FFA-^{14}C is associated with a net influx of FFA into plasma.

fractional rate constants of FFA exchange, esterification, and oxidation using a model such as that shown in Fig. 7. Theoretically (32), the only measurements required would be radioactivity against time in plasma FFA (Fig. 1) and one early measurement of radioactivity in liver esters and in exhaled CO_2. Instead, data have been obtained at five times for liver esters and at four times for CO_2. Moreover, there are data available at five times for extrahepatic esters.

Successive independent calculation of all the fractional rate constants shown in Fig. 10 could be achieved, theoretically, using the data of Fig. 1 plus two data points, then with two others, and so on until separate independent values were obtained for each rate constant. An average value and standard deviation of each rate constant could then be calculated. The digital computer program of Berman, Weiss, and Shahn (33), which we have

used, gives an equivalent average value and standard deviations for each rate constant by treating all of the data simultaneously. The reader may see this more readily if he considers the following simple, two pool model

$$\xleftarrow{\lambda_{01}} \boxed{A} \xrightarrow{\lambda_{21}} \boxed{B}$$
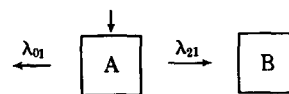
in which B turns over either (*a*) very slowly relative to A, (*b*) not at all (as in the case of an end-product), or (*c*) at a fractional rate which is rapid, but known. In each case, measurement of radioactivity in A [$A^*(t)$], after injecting tracer [$A^*(0)$] at zero time into A, plus one measurement of radioactivity at any time in B [$B^*(t)$] is sufficient to allow estimation of λ_{01} and λ_{21}. If, instead, a complete curve (many values), which defines $B^*(t)$, is obtained, then each extra value of $B^*(t)$ together with the data of
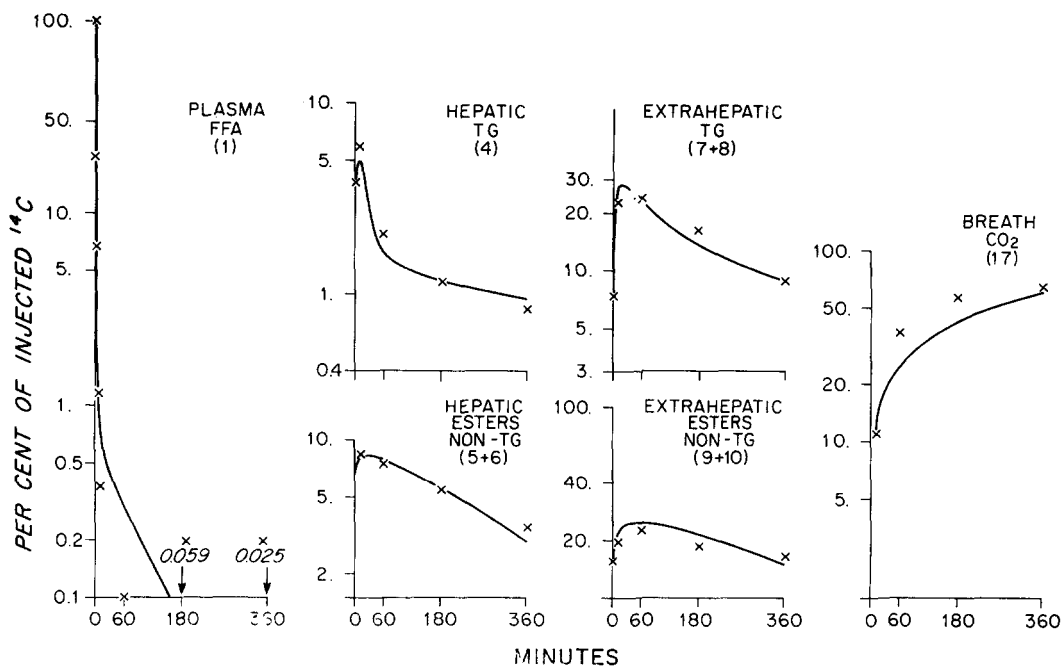
FIG. 11. Comparison of experimental data with ^{14}C–time curves derived by the digital computer on the basis of the model and fractional rate constants shown in Fig. 10. The solid lines represent the calculated curves obtained by the program of Berman et al. (33). Experimental data are indicated by x. The data for extraplasma FFA were not included in the analysis because of the difficulties encountered in extracting the FFA at the early times, especially at 1 min.

$A^*(t)$ may be used to calculate, independently, both the fractional rate constants and standard deviations for each. Similarly, when a model such as that shown in Fig. 9 is used, rates of TG and of non-TG ester formation may be calculated uniquely given only a single early value of hepatic and extrahepatic TG and non-TG esters. Special care was taken to insure that data were in excess of the minimal requirements in order to obtain a valid estimate of these rates. However, as noted above, the data were incompatible with this model. Using the more complex model of Fig. 10 we obtained an acceptable solution, which is also given in Fig. 10. The data do not define uniquely the fractional rate constants of movement of fatty acids among the several hepatic and extrahepatic lipid ester compartments. Some of these values (see legend to Fig. 10) are presented only so that the reader may check the analysis and as a basis for further investigation. The quality of fit is illustrated in Fig. 11 in which some of the observed data are compared with values calculated by a digital computer (33) using the fractional rate constants shown in Fig. 10. Although there are some minor discrepancies between observed and calculated values, no effort has been made to make additional fine adjustments of the estimated rate constants and of the model, since these either would have little effect upon the final values or else the adjustments would be arbitrary and meaningless without additional data. One compartment, which we have not considered in our calculations, but which

should probably be taken into account, is compartment 11 (Fig. 10). The data of Table 2 and for exhaled CO_2 together with published experiments all indicate that this route of FFA metabolism, which would include oxidation of FFA to CO_2 via ketone bodies formed in the liver, should be included in the model.

Specific Estimates of Selected Rates

(a) *Irreversible Disposal (Turnover) Rate of FFA.*   The concept of irreversible disposal rate was first introduced in order to distinguish between the net rate of formation (or utilization) of a substance and the total flux of the substance (34). This is illustrated in the following example, which shows substance x having a total flux of 3 μmoles/min, a recycling rate of 1 μmole/min, and an irreversible disposal rate of 2 μmoles/min. The irreversible disposal
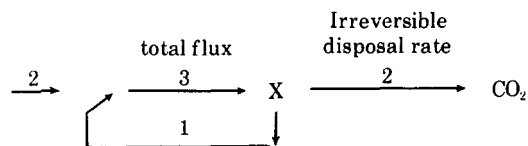
rate in this case is only $^2/_3$ of the total flux. In a constant infusion experiment, which usually does not take recycling into account, the turnover rate corresponds to the irreversible disposal rate of a single injection experiment.

The irreversible disposal rate of FFA was estimated to be 2.9 μmoles of FFA/min per 100 g body weight, from
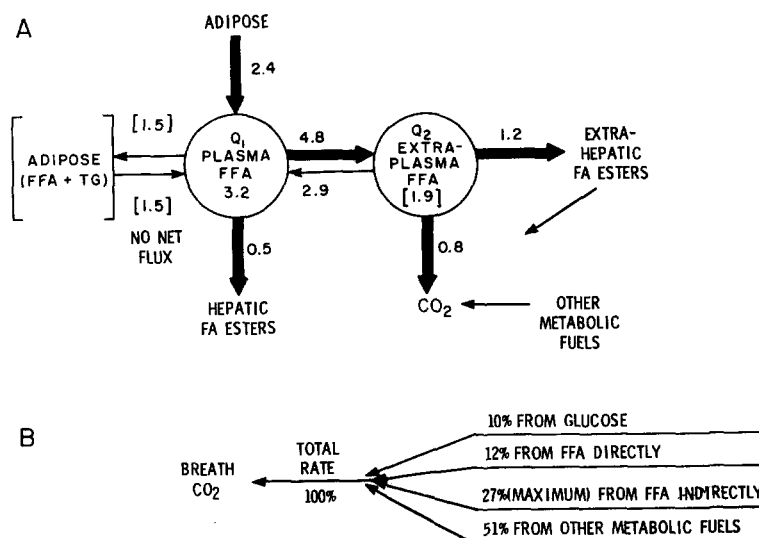
FIG. 12. Rates of plasma FFA exchange, esterification, and oxidation. The complex model of Fig. 10 was simplified to the model shown in A, above, and the estimated rates of flux were calculated on the basis of the appropriate fractional rate constants (Fig. 11) and the plasma FFA pool size. Rates are expressed as μmoles of fatty acid/min per 100 g body weight. Pool sizes are given as μmoles of fatty acid/100 g body weight. Pool size Q_2 is shown in brackets to indicate that the calculated value is a minimum estimate (see text). The lower part of the figure, B, relates the estimated rates of FFA oxidation by direct and indirect pathways to the total rate of CO_2 oxidation.

the data of Fig. 1 and the measured plasma FFA pool size.[3] It should be noted that the calculation does not require construction of a model provided that all newly formed FFA with which plasma FFA mixes enters the plasma before mixing with other FFA pools. As will be seen later, a complex multicompartmental analysis of the data resulted in a somewhat lower estimate (2.4 μmoles/min per 100 g body wt).

(b) *Rates of Plasma FFA Exchange with Extraplasma FA, and Net Rate of FFA Mobilization Based upon a Multicompartmental Analysis.* In Fig. 12 a summary of estimated rates of FFA exchange and net turnover is presented. In the absence of a complete balance of activity at early times, there was about $\pm 25\%$ uncertainty with respect to estimated rates of exchange and extraplasma FFA pool size.[4] However, it is clear from the experimental data that a major fraction of the FFA that left the plasma in the 1st min did not appear as easily extractable FA ester, but rather recycled into the circulation almost as fast as it had left. The uncertainty would be far less if all the activity recovered at 1 min (Table 2) and not accounted for in plasma FFA or in hepatic and extrahepatic esters were known to be in the form of extraplasma FFA (as we have assumed). Nevertheless, the total disposal of fatty acid is

[4] This is a crude estimate of error based upon computer analyses in which fractional rate constants which determine the early disappearance and return of FFA-^{14}C from compartment *1* were systematically varied and the resulting curve for compartment *1* compared with the experimental data. When these rate constants were increased or decreased by 25%, the resulting curves were clearly incompatible with our data.

probably depicted accurately $(\pm 25\%)$[4] by the values (per 100 g body wt) of 1.2 μmoles of FFA esterified per min by extrahepatic tissues, 0.8 μmole of FFA oxidized to CO_2 per min, and 0.5 μmole of FFA esterified in liver per min. The total rate of net FFA mobilization (irreversible disposal) was 2.4 μmoles/min per 100 g body wt. If another 0.5 μmole/min were converted by liver to water-soluble compounds such as glucose and ketone bodies which were subsequently oxidized to CO_2, the irreversible disposal rate would agree with the value calculated from the plasma FFA curve alone (see "Irreversible Disposal Rate of FFA," above) and would improve the correspondence between observed and calculated values for [14]CO_2 shown in Fig. 11.

The size of the most rapidly exchanging extraplasma FFA pool (Q_2, Fig. 12A) was estimated to be slightly more than half that of the plasma FFA pool (Q_1). This is consistent with reports that both albumin and fatty acids exist in interstitial fluid and at a lower concentration than in plasma (28, 29). The calculation of Q_2 was based upon steady state considerations, simplification of a portion of the model in Fig. 10 to that shown in Fig. 12A, the fractional rate constants given in Fig. 10, and the measured plasma FFA pool size: $Q_2 = Q_1\lambda_{21}/\lambda_{22}$, where $\lambda_{21} =$ fraction per min into compartment 2 from *1*, and $\lambda_{22} =$ fraction per min out of compartment 2 by all pathways. If our assumption is incorrect, and if a net inflow, I_2, from extrahepatic FA esters into Q_2 were known and taken into account, a larger estimate of Q_2 would be obtained: $Q_2 = (Q_1 \lambda_{21} + I_2)/\lambda_{22}$. In Fig. 12A the plasma
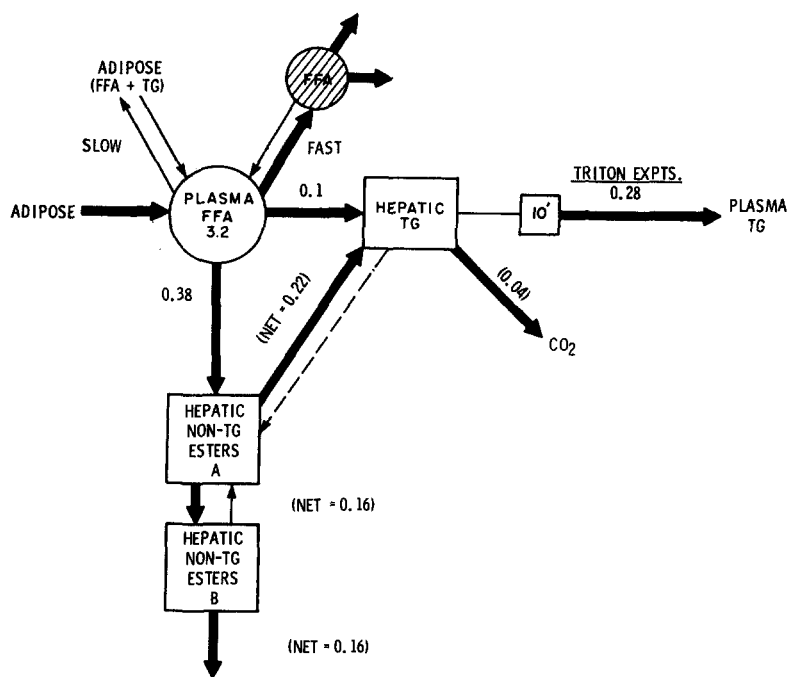
FIG. 13. Rates of hepatic TG formation by direct and relatively indirect pathways. The rates and pool sizes are expressed in the units given in Fig. 12. The rate of TG secretion is taken from separate experiments (see text) and is a minimum estimate. The rate of direct hepatic TG formation from plasma FFA was found to be only a fraction of the total rate of TG formation. The remainder of the required TGFA (0.22 μmoles/-min of TGFA per 100 g body wt) was assumed to come from non-TG esters which were formed from plasma FFA at a rate sufficient to supply the required net flow of FA.

FFA is shown to exchange relatively slowly with a large pool of extraplasma FFA and TG, which we have assumed to be in adipose tissue. The exchange rates are highly uncertain (values in brackets). We emphasize that, although FFA being produced by adipose tissue is shown to be undiluted by the large adipose tissue FFA pool before it enters the circulation, we have presented no experimental data to support this assumption. In the absence of other data, this treatment simplifies calculations.

(c) *Oxidation of FFA to CO_2.* The value of direct FFA oxidation to CO_2, 0.8 μmole/min per 100 g body wt (Fig. 12), represents approximately 33% of the total irreversible disposal of plasma FFA. It also accounts for approximately 13 μmoles of CO_2/min per 100 g body wt (assuming 17 atoms C per molecule of FFA, average) which amounts to only 12% of the total CO_2 actually formed each minute. If the maximal FFA irreversible disposal rate (based upon the FFA-^{14}C curve and plasma FFA concentration[3]) is considered to represent the rate of oxidation of fatty acids to CO_2 by all direct and indirect pathways (including FFA $\rightarrow$ hepatic PL $\rightarrow$ hepatic TG $\rightarrow$ plasma TG $\rightarrow$ extrahepatic lipid $\rightarrow$ CO_2), then approximately 39% of the observed CO_2 expiration may be accounted for. Since only about 10% of the CO_2 formed by fasted rats is derived from glucose by relatively direct oxidative pathways (35), about 50% of exhaled CO_2

must be derived from other metabolic fuels such as protein, amino acids, and lipids which were being oxidized faster than they were replaced. These relationships are summarized in Fig. 12 B.

All estimates of rates of oxidation are dependent upon the assumption that CO_2 formed intracellularly enters the plasma bicarbonate immediately and that the subsequent mixing and exhalation kinetics are identical with those exhibited by intravenously injected bicarbonate (14, 30). This assumption has never been adequately tested; furthermore, the amount of bicarbonate recovered in the whole animal at 10 min (13% of the injected dose, Table 2) was an order of magnitude greater than would be predicted if the model and rate constants shown in Fig. 10 were used. Further comparisons of predicted and measured total body bicarbonate-^{14}C after intravenous injection of bicarbonate-^{14}C and of other labeled metabolic fuels should prove enlightening.

(d) *Rates of Hepatic TG Formation from Plasma FFA by Direct and Indirect Pathways.* The maximum incorporation of FFA-^{14}C into hepatic TG was less than half the values previously reported (1). This observation was the basis of a subsequent study, which showed that the incorporation of FFA-^{14}C into liver TG was markedly stimulated by ether anesthesia (36). However, the most striking observation in the present study was the slow rate of for-

mation of hepatic and extrahepatic TG relative to the rapid disappearance of FFA-^{14}C and to the rapid appearance of radioactivity in other esters, mainly PL.

From the present data the net rates of TG and non-TG ester synthesis from plasma FFA by direct pathways could be estimated. Furthermore, by including data from a separate study of TG secretion in unanesthetized, fasted rats[5] we could get approximate estimates for the quantitative relationships between hepatic TG synthesis and secretion. These calculations are summarized in Fig. 13. The poorly defined fractional rate constants of interchange between compartments *4, 5,* and *6* in Fig. 10 were not necessary for the calculation of these rates. More than enough FFA was taken up by the liver each minute to account for a net TG secretory rate of 0.28 μmole of TGFA per min per 100 g body wt, which was the average value found in a recent study of post-Triton hypertriglyceridemia in fasted rats.[5] However, only 36% of the TGFA secreted was synthesized directly from plasma FFA. The remaining 64% apparently was formed by a route that entailed prior synthesis of a non-TG ester. The calculated percentage of newly synthesized hepatic TGFA formed directly from plasma FFA may be taken as a maximum estimate since this value is based upon an assumed rate of hepatic TG secretion that is considerably below values which have been reported in the literature. This lends support to an earlier suggestion (1) that a major fraction of the net TG synthesized in liver may be derived from relatively large pools of plasma fatty acid, which are replaced by net synthesis from plasma FFA in fasted rats. However, the reader should bear in mind that all calculations are dependent upon many assumptions.

(*e*) *Conversion of Plasma FFA to Extrahepatic TG and Non-TG Ester.* Our experimental design was based upon the hypothesis that FFA would enter extrahepatic TG and non-TG by a branching pathway such as that shown in Fig. 14A. The branching point was assumed to be a small, rapidly turning over pool (Fig. 14A, ●) that could be ignored mathematically, with consequent reduction of Model A to Model B (Fig. 14). The observed data were not compatible with Model B. A more consistent model is shown in Fig. 14C. Even this is a simplification of the more complex model which gave the best fit (Fig. 10). Without an independent measurement of the net rate of extrahepatic TG turnover rate (μmoles/min of TGFA per 100 g of body wt) it was not possible to calculate the net rate of TG formation by indirect pathways. If it is assumed that no net FA formation occurs from extrahepatic TG, one may calculate from the values shown in Figs. 11 and 12 that a minimum of 1.2 μmoles of FFA per min were esterified in extrahepatic tissue per 100 g body

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[5] N. Baker, A. S. Garfinkel, and M. C. Schotz. Manuscript submitted for publication. The rate of triglyceride secretion shown in Fig. 13 is taken from this study.
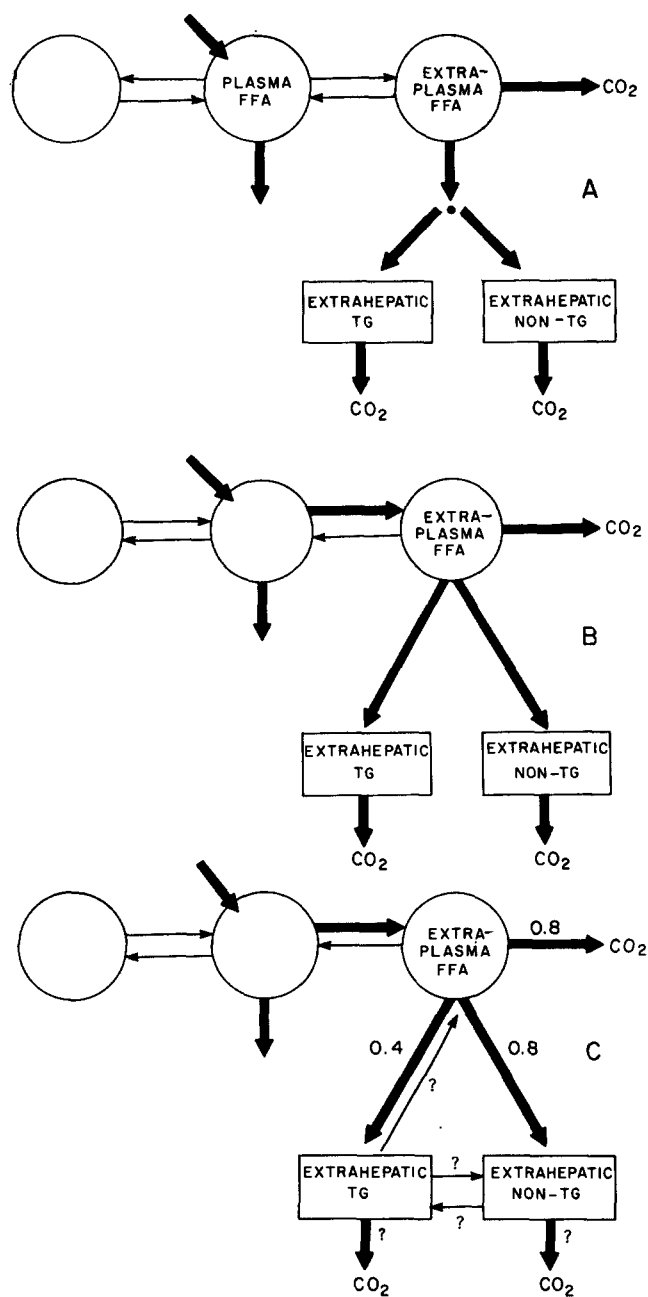

FIG. 14. Estimation of rates of TG and non-TG formation from fatty acids in extrahepatic tissues. A shows that part of the initial model which was to have served as a basis for calculating net rates of esterification after simplification to model B (see text). However, the data indicated that a more complex model such as that shown in C may be required to describe the interrelations between extraplasma FFA and extrahepatic TG and non-TG esters. The data obtained could not define rates of flow shown by question marks. However, if it is assumed that no net extraplasma FFA is formed from TG or non-TG esters in nonadipose, extrahepatic tissues, then the rates of direct esterification shown in C may be calculated from the fractional rate constants shown in Fig. 11 and the pool sizes shown in Fig. 12. Units are as given in Fig. 12.

weight. One-third entered TG directly and the other two-thirds was incorporated into other lipids. Extensive exchange of FFA-^{14}C appeared to take place between TG and non-TG lipids. It was not possible to estimate the actual rates of exchange, oxidation to CO_2, and recycling to FFA in terms of μmoles of esterified FA per min per 100 g body weight. If we assume that no net FA was deposited as lipid esters in extrahepatic tissues of fasted rats, then at least 1.2 μmoles of FA ester per min per 100 g body wt were either recycled to FFA or were oxidized to CO_2 or both. The maximum fractional rate of oxidation to CO_2, defined by the measured CO_2 curve and the decay of the extrahepatic lipid-^{14}C curves, could not have been more than 0.4% and 0.05% per min in the case of TG and non-TG, respectively. If the 0.4 and 0.8 μmole FA per min per 100 g which entered TG and PL, respectively, were not recycled but rather were oxidized to CO_2, the pool sizes of TG and PL would be approximately 100 μmoles of (TG)FA per 100 g body wt and 1600 μmoles of non-TG esterified FA per 100 g body wt. These estimates assume no net flow of FA between TG and non-TG. Further work in the direction of defining rates of FA recycling, exchange, and metabolism in extrahepatic tissues will require more refined methods of sampling and isolation of interstitial fluid FFA, and of halting postmortem esterification during early periods of the experiment. Individual tissues should be sampled and further knowledge of the actual compounds and mechanisms of (TG)FA and of (non-TG)FA must be understood so that the appropriate metabolites may be isolated and assayed.

The present analysis is a limited and very elementary attempt towards ultimately defining the rates of FFA oxidation and esterification under various physiological conditions. We have not considered important points such as ketone body formation and gluconeogenesis from fatty acids in liver. However, the model has already proved useful as a framework for further work in our own laboratory and is presented in the hope that it will stimulate others to formulate new hypotheses and to design future experiments that will yield information regarding quantitative aspects of in vivo fat metabolism.

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