Biochem. J. (1978) **172**, 205–218
Printed in Great Britain

The Contribution of Serum Triacylglycerol to Hepatic Triacylglycerol Turnover in the Starved Rat

By EDWARD W. LIPKIN,* CECIL COOPER*† and REGINALD A. SHIPLEY‡
**Department of Biochemistry, Case Western Reserve University, and*
‡Veterans Administration Hospital, Cleveland, OH 44106, *U.S.A.*

(*Received* 11 *July* 1977)

The present study was undertaken to evaluate quantitatively the turnover of serum triacylglycerol (triglyceride) in the starved rat and to determine whether serum triacylglycerol recycled to liver contributes a significant fraction of the total hepatic triacylglycerol turnover. Serum was labelled *in vitro* with [^{3}H]trioleoylglycerol (glycerol [^{3}H]trioleate) to provide uniform labelling of all lipoprotein species. By using the curves describing disappearance of isotope from serum and its appearance in liver, rate constants for movement of triacylglycerol out of serum ($0.29\,min^{-1}$) and the uptake of serum triacylglycerol by liver ($0.22\,min^{-1}$) were calculated. The total rate of movement (flux) of triacylglycerol in these processes, the product of rate constant and serum pool size, was calculated to be 0.39 and 0.29 mg/min per 100 g body wt. respectively. A model is postulated for whole-body triacylglycerol metabolism consistent with the present data as well as most observations in the literature. From the model it can be predicted that: (1) the entire turnover of liver triacylglycerol in the starved rat can be accounted for on the basis of contributions from serum non-esterified fatty acid and serum triacylglycerol; (2) the entire turnover of the serum triacylglycerol pool can be accounted for quantitatively on the basis of contributions from intestine and liver; (3) the release rate for triacylglycerol from liver should be 0.34 to 0.35 mg/min per 100 g body wt.; (4) triacylglycerol synthesized by liver from non-esterified fatty acid of serum and by intestine can account quantitatively for the irreversible disposal rate of triacylglycerol from serum.

Abrams & Cooper (1976*a*) were able to quantify the contribution of non-esterified fatty acid of serum to liver triacylglycerol synthesis by studying the rate of incorporation of a pulse of [U-^{14}C]palmitate into liver triacylglycerol. They concluded that this source of fatty acid accounted for 0.06 mg of the estimated total hepatic triacylglycerol turnover rate of 0.15 mg/min per 100 g body wt. in a starved rat. The present study was undertaken to determine whether serum triacylglycerol is the source of the unaccounted-for fatty acid contributing 0.09 mg/min per 100 g body wt. to hepatic triacylglycerol formation.

Triacylglycerol turnover has been evaluated *in vivo* in several ways. One of the most common is to administer labelled palmitate or glycerol either by constant infusion or by pulse-labelling and to establish the rate of loss of isotope from serum triacylglycerol after attainment of maximal specific activity (Havel *et al.*, 1962; Farquhar *et al.*, 1965; Kissebah *et al.*, 1974; Havel & Kane, 1975; Canedella & Crouthamel, 1976). Another approach is to infuse prelabelled chylomicrons until serum triacylglycerol approaches constant specific radioactivity, discontinue the infusion, and then establish the rate of loss of label from plasma (Harris & Felts, 1970). The rate constant for turnover is usually estimated from the single exponential decay of radioactivity over relatively short time intervals. The conclusions based on such studies are subject to a variety of qualifications. The approximation of turnover by assigning a single exponential function to observed data is subject to considerable error. Most curves encountered in tracer studies are complex exponential functions in which both early and late components are involved. The labelling of serum triacylglycerol by administration of palmitate or glycerol tends to label very-low-density lipoprotein selectively, and if it accounts for most of the triacylglycerol turnover, this gives a reasonable approximation (Farquhar *et al.*, 1965). However, the point is controversial, since there is some indication that

† To whom requests for reprints should be addressed.

low-density and high-density lipoproteins contribute significantly to whole-body triacylglycerol turnover during starvation (Levy *et al.*, 1971). If very-low-density lipoprotein is used to approximate whole-body triacylglycerol turnover, a conservative estimate would be obtained. This applies also to decay curves of radioactivity in plasma after administration of labelled very-low-density lipoprotein itself (Havel *et al.*, 1962; Farquhar *et al.*, 1965; Kissebah *et al.*, 1974; Havel & Kane, 1975). These experiments are also subject to the limitations outlined above, because data were fitted to single exponential curves.

Triacylglycerol kinetics have also been studied by compartmental analysis and computer simulation of various models of triacylglycerol metabolism (Eaton *et al.*, 1969; Quarfordt *et al.*, 1970; Shames *et al.*, 1970). This technique is usually applied to experiments in which the serum triacylglycerol pool is labelled with either fatty acid or glycerol and is dependent on a complete description of the serum-triacylglycerol specific-radioactivity curve and assumption of models for triacylglycerol metabolism.

In the present study, serum was labelled *in vitro* with [9,10-^{3}H]trioleoylglycerol to provide uniform labelling of all lipoprotein species. From the curve describing the disappearance of isotope from serum, the rate constants for total movement of triacylglycerol out of serum and its irreversible disposal rate from serum were calculated. An estimate of the rate constant for transport to liver was derived from the curve of uptake of label in hepatic triacylglycerol. The rate of movement (flux) of triacylglycerol in these processes, i.e. the product of rate constant and serum pool size, was also calculated. These values, along with some related results from the literature, were assigned to a model showing movement of triacylglycerol to and from serum.

Experimental

Animal procedures

Male Sprague–Dawley rats (Carworth Farms, Portage, MI, U.S.A.) were housed in wire-bottomed cages, and were allowed free access to food (Purina Rat Chow) and water for 10–14 days before use. The animals were maintained in a constant-temperature environment (22°C) with a uniform light cycle (07:00h–19:00h daily). All animals were deprived of food 48h before use and each weighed 200–220g on the day of the experiment. Intravenous injections were made between 08:00 and 13:00h via the lateral tail vein. The rat was restrained in a cage and the protruding tail warmed for 10min with a heat lamp to facilitate intravenous injection.

Preparation of injection solution

The injection solution was 1.0ml of rat serum labelled by exchange *in vitro* (Avigan, 1959; Eaton *et al.*, 1969; Brenneman & Spector, 1974; Brenneman *et al.*, 1974; Faergeman & Havel, 1974) with [9,10-^{3}H]trioleoylglycerol (glycerol [9,10-^{3}H]trioleate; New England Nuclear Corp., Boston, MA, U.S.A.). The injection solution was prepared the day before the experiment. All operations were performed under sterile conditions. All glassware, solutions and dissection instruments used in the collection of blood and the preparation of the injection solution were sterilized for 2h in an autoclave. Fresh rat serum was obtained from 48h-starved animals by aortic puncture. The rats were lightly anaesthetized with diethyl ether. A transverse abdominal incision was then made and the abdominal aorta exposed by blunt dissection. The aorta was pierced at the bifurcation by a 22G needle, and the blood was drawn into a 10ml syringe containing 1ml of 0.15M-NaCl/0.1% EDTA, pH7.4. The blood was transferred to a sterile glass tube and allowed to clot for a minimum of 1h at 0°C. The serum was then obtained by centrifuging the blood for 20min at 500*g* (20°C). An incubation flask was prepared by placing an appropriate amount of HCl and hexane-washed Celite 545 (Fisher Scientific Co., Pittsburgh, PA, U.S.A.) into a 30ml round-bottomed flask fitted with a stopcock (110mg of Celite/ml of serum). Freshly distilled chloroform was added to the flask to form a slurry, and then 0.5mCi of [9,10-^{3}H]-trioleoylglycerol/10ml of serum, or 0.5mCi of trioleoyl[2(*n*)-^{3}H]glycerol ([2(*n*)-^{3}H]glycerol trioleate) plus 250μCi of [1-^{14}C]trioleoylglycerol (glycerol [1-^{14}C]trioleate) (Amersham–Searle, Chicago, IL, U.S.A.)/10ml of serum was introduced into the flask. The flask was shaken to ensure wetting of the Celite and uniform distribution of label. The solvent was removed at room temperature (20°C) under reduced pressure by attaching the flask directly to a vacuum pump fitted with solid-CO_2 trap. Serum was then added, the flask evacuated, and the stopcock closed. The flask was incubated for 4h at 37°C in a rocking-arm shaker, cooled to 0°C, and the contents transferred to a 30ml polycarbonate centrifuge tube. The slurry was centrifuged for 10min at 30000*g* (4°C) in a Sorvall RC2B centrifuge and the supernatant was decanted into a second 30ml polycarbonate centrifuge tube. The solution was centrifuged a second time under the same conditions. The resulting Celite-free supernatant was decanted and stored for 10h at 4°C before use. On the day of the experiment the injection solution was diluted with 0.15M-NaCl if the total volume of injection solution was less than that needed for the injection of 1ml per rat. The purity of lipids in the injection solution was determined by t.l.c. The serum was extracted for total lipids by a modified Bligh & Dyer (1959) procedure outlined below. The injection solution contained 87±2% of the radioactivity in triacylglycerol,

10±2% in non-esterified fatty acid and 3±0.3% in mono- and di-acylglycerol.

Treatment of tissues

After injection of the tail vein, the rats were removed from the restraining cage and allowed free mobility. At specified times thereafter, the rats were decapitated without use of anaesthetic and the blood was collected in a glass centrifuge tube. The blood was allowed to clot for 60min at 0°C and the serum obtained by centrifuging 20min at 500*g* (20°C).

The abdomen was opened after decapitation and collection of blood, and a PE 60 catheter placed in the inferior vena cava. The portal vein was severed, and retrograde perfusion of the liver carried out over a 1min period with 30ml of ice-cold 0.15M-NaCl/0.1% EDTA, pH7.4. The time from decapitation to beginning of perfusion was approximately 60s. After perfusion, the liver was excised, blotted dry, weighed and frozen in liquid N_2. A small lobe (about 1g) located on the animal's right side, dorsal to the large bifurcated lobe, was removed and frozen separately.

Lipid analysis

All glassware used in lipid analyses were washed in dichromate cleaning solution. Serum lipids were extracted by a modified Bligh & Dyer (1959) procedure. Fresh serum (1ml), freshly distilled methanol (2.5ml) and chloroform (1.25ml) were emulsified with a vortex mixer and left for 5min. An additional 1.25ml of chloroform and 1.25ml of triple-distilled water were then added, the emulsion mixed again, and centrifuged at 500*g* for 15min to separate the organic (chloroform) and aqueous (methanol/water) phases. The lower chloroform phase (2.0 out of 2.5ml total) was aspirated with a 20G needle attached to a 10ml glass syringe and transferred to a 12ml glass tube. The chloroform was removed at 55°C with N_2, the lipid residue redissolved in 500μl of chloroform, and 50μl portions taken for radioactivity counting and t.l.c. The remainder of the chloroform extract was dried at 55°C with N_2 and assayed for triacylglycerol as described by Soloni (1971).

The frozen 1g of liver lobe was extracted for total lipids by the method of Folch *et al.* (1957). The tissue was homogenized in 20ml of chloroform/methanol (2:1, v/v) for 2min in a Sorval Omni-Mixer. After filtration of the homogenate on a medium-porosity sintered-glass filter, the filtrate was transferred to a 40ml Folch tube, and the total volume adjusted to 40ml with triple-distilled water. The tube was inverted twice and left overnight. A portion (10ml) of the lower chloroform phase was removed by aspiration with a 10ml glass syringe fitted with a 15cm-long 15G needle and transferred to a 12-ml glass test tube. The chloroform extract was then dried and redissolved in 500μl of chloroform as described above for the serum lipid extract. The lipid extracts were counted for radioactivity directly by transferring 50μl of the chloroform extract to a 5ml scintillation vial and allowing the solvent to evaporate at room temperature.

Serum lipids were separated by t.l.c. on glass plates (20cm×20cm) of silica gel G (0.25mm thick; E. Merck, Darmstadt, Germany). Liver lipids were separated in an identical manner by using glass plates (20cm×20cm) of silica gel G (1.00mm thick; New England Nuclear). The plates used for separation of serum lipids were scored into 2cm-wide lanes, whereas the plates used in the separation of liver lipids were scored into 2.5cm-wide lanes. All t.l.c. plates were activated at 110°C for 30min before use. Chromatography was done in all-glass tanks (20cm×9cm×26cm high) lined with Whatman no. 1 filter paper. The developing solvent was hexane/diethyl ether/acetic acid (70:30:1, by vol.). The tanks were prepared the day before use with 200ml of the developing solvent and allowed to equilibrate overnight.

The lipid extracts in chloroform were chromatographed by spotting 50μl of the sample per lane at the origin and developing the plate for 60min at room temperature. The plates were then air-dried and lipids made visible by exposure to iodine vapour for 1min. The lipid spots were marked, and the iodine was allowed to subliminate at room temperature until the spots were no longer visible (about 1h).

Liquid-scintillation counting of radioactivity

Chloroform extracts were counted for radioactivity after solvent evaporation and addition of 4.0ml of toluene-based scintillant (Lowenstein, 1971). Lipids isolated by t.l.c. were counted for radioactivity after scraping each marked area of the plate into a 20ml scintillation vial, adding 15ml of Aquasol (New England Nuclear), and shaking vigorously. For phospholipids, 5ml of triple-distilled water was also added to the Aquasol/silica gel and the gel shaken to form a homogeneous viscous solution. Background radioactivity was determined with chloroform extracts of tissue devoid of isotope. All samples were kept at 4°C in the dark for at least 1h before counting of radioactivity, and a minimum of two reproducible counts of 10min each (less than 2% counting error) were obtained on each sample. Counting was done on a Packard Tri-Carb liquid-scintillation counter, with an external standard to monitor efficiencies. Quench curves for the external standard were always determined, with [*methyl*-^{3}H]toluene (New England Nuclear) as the standard, and under conditions identical with those used in counting for radioactivity of the chloroform or silica-gel

samples. All counting for radioactivity was normalized to 60% efficiency, which is the maximum efficiency for ^3H with our instrument. Counting efficiencies were 40% for chloroform extracts counted directly, 30% for silica-gel samples counted directly in Aquasol, and 20% for phospholipid samples suspended in Aquasol/water.

Correction of lipid data for trailing of [9, 10-^3H]*trioleoylglycerol on t.l.c.*

All lipid radioactivity counts were corrected for trailing of [9,10-^{3}H]trioleoylglycerol radioactivity. Trailing could be eliminated by underspotting with unlabelled trioleoylglycerol in toluene and by ensuring that the sample was not allowed to dry out. However, the amount of lipid spotted from our biological extracts was the maximum that could be resolved, and thus underspotting did not lend itself readily to our system and was not used. Moreover, in running multiple lanes on a single t.l.c. plate it was impossible to prevent drying-out of the samples. The extent of trailing was accurately determined by chromatography of [9,10-^{3}H]trioleoylglycerol (98% pure) by t.l.c. and counting for radioactivity the entire plate in 0.5cm strips. The results were plotted on linear graph paper, and the extent of trailing was determined by comparing the area under the tail to the area under the trioleoylglycerol peak. It was determined that 7.1% of trioleoylglycerol radioactivity trailed from the trioleoylglycerol spot to the base of the t.l.c. plate. Similar observations were made by the supplier (New England Nuclear, personal communication). Purity was determined at New England Nuclear by using a radioactivity scanner adjusted to baseline on an area of the t.l.c. plate above the point of sample application. All triacylglycerol data were therefore corrected for trailing. It was also determined that an amount of radioactivity equal to 1% of radioactivity counts in the triacylglycerol peak trailed into other discrete lipid spots that co-migrated with purified standards of phospholipids, cholesterol, non-esterified fatty acid and cholesteryl ester. The remaining lipid data were therefore also corrected for this trailing of the trioleoylglycerol peak.

Modifications of procedures for double-label experiments

The liver and injection solution were extracted for total lipid as described above. For the double-label experiments, however, 200μl samples of concentrated chloroform extracts of liver were used for t.l.c. The extract was streaked over a 10cm area at the base of the plate with a mechanical applicator. The plates were then developed and lipids made visible as described above. The triacylglycerol spot was scraped off the plate and into a 30ml glass test tube. After addition of 10ml of chloroform/methanol/diethyl ether (1:1:1, by vol.) (Kates, 1972), the slurry was vortex-mixed for 1min and centrifuged at 2000g for 5min (20°C) in a Sorval GLC-1 centrifuge. The supernatant solution was transferred to a 40ml glass test tube with a Pasteur pipette. The silica-gel pellet was re-extracted in the same manner with two additional 10ml washes of the eluting solvent. The combined eluates were evaporated under N_2 at 55°C and the residue was redissolved in 500μl chloroform.

Lipid extracts with both ^3H and ^{14}C were oxidized to ^3H_2O and $^{14}CO_2$ with a Packard Tri-Carb sample oxidizer (model 306). A portion (200μl) of the concentrated chloroform extract was transferred to a Combusto-Pad (Packard Instrument Co., La Grange, IL, U.S.A.) and the solvent allowed to evaporate at room temperature. Water (100μl) and 200μl of Combust-Aid (Packard) were added to the pad and the sample was oxidized for 1.5min. ^3H was collected as ^3H_2O and counted for radioactivity in 5.0ml of Monophase 40 (Packard), and ^{14}C was collected as $^{14}CO_2$ by Carbo-Sorb (Packard) and counted for radioactivity in 10ml of Permafluor V (Packard). All samples were counted for radioactivity on a Packard liquid-scintillation counter, with an external standard to monitor efficiencies. Counting efficiencies were 30% for ^3H and 50% for ^{14}C. All data were normalized to 60% efficiency.

Gel electrophoresis

Serum was separated into different lipoprotein species as described by Naito *et al.* (1973). On the day of the experiment, fresh serum was obtained from each experimental animal. Electrophoresis (Naito *et al.*, 1973) was performed in duplicate on samples of serum pooled from all recipient animals and on the original injection solution (50μl/gel). One set of gels was stained with Sudan Black and the other was not; both sets were run simultaneously. Electrophoresis was discontinued when the α-band had come within 0.5cm of the bottom of the gel. The gels were removed from the electrophoresis tubes. Gels without stain were cut into 1mm slices and each piece was transferred to a 20ml scintillation vial containing 1.0ml of NCS (Amersham–Searle, Arlington Heights, IL, U.S.A.). After solubilization of the gel slice at 50°C for 2h, 15ml of toluene scintillant (Lowenstein, 1971) was added, and the samples were counted for radioactivity as previously described. Serum samples stained with Sudan Black were stored at 0°C in reservoir buffer, and within 2h from the time of discontinuing the electrophoresis the banding pattern on the gel was recorded photographically on Polaroid F 105 P/N film at f/11, with a 0.25s exposure and a CC 40Y yellow filter. The negatives were immediately treated with 12% (w/v) sodium sulphite, Photo-Flo (Eastman–Kodak Rochester, NY, U.S.A.), and allowed to dry in air.

Densitometric scans of the negatives were made with a Joyce–Loebl double-beam recording microdensitometer with a B-wedge. The identity of the various lipoprotein bands on the gels was determined by electrophoresis of lipoprotein standards obtained by ultracentrifugation of serum in a discontinuous salt gradient (Redgrave *et al.*, 1975). It was found that very-low-density lipoprotein ($\rho < 1.006$ g/ml) and intermediate-density lipoprotein ($\rho = 1.006$–1.019 g/ml) from 48h-starved-rat serum could not be resolved completely from each other by gel electrophoresis. These two lipoprotein classes were therefore treated as a single species. The reproducibility of the gel pattern was assessed by comparing the densitometric scans and radioactivity profiles of multiple gels done on the same serum samples. The quantity of Sudan Black bound to each lipoprotein species was estimated from the area under each peak that could be resolved on the densitometric scan. According to Naito *et al.* (1973), the quantity of Sudan Black bound to each lipoprotein species is proportional to total lipid content. The S.D. for densitometric scans of the same serum sample was 10%. The radioactivity bound to each lipoprotein was assessed by integrating the appropriate areas. The identity of radioactivity peaks was determined by comparison with the densitometric scan of the serum sample stained with Sudan Black. The S.D. for radioactivity in specific areas of replicate gels was less than 5%.

The majority of lipoprotein-bound [9,10-^{3}H]trioleoylglycerol [72±2% (mean±S.E.M.)] in the injection solution, as determined from gels, was in very-low-density and intermediate-density lipoprotein; lesser amounts of [9,10-^{3}H]trioleoylglycerol were incorporated into low-density lipoprotein (9±2%) and high-density lipoprotein (19±2%).

Determination of the chemical distribution of triacylglycerol in the injection solution

Each labelled serum sample was fractionated ($\rho < 1.019$, $\rho < 1.063$, $\rho > 1.063$ g/ml) by ultracentrifugation after adjustment of density with various NaCl/KBr solutions (Havel *et al.*, 1955). The samples were centrifuged at 105000*g* for 22h at 15°C in a no. 40 rotor of a Spinco preparative ultracentrifuge (model L). The lipoproteins of less than solvent density, concentrated at the top of the tube, were aspirated and assayed immediately for triacylglycerol by the method of Soloni (1971).

The majority of lipoprotein-bound triacylglycerol (70±7%) in the injection solution was in very-low-density lipoprotein and intermediate-density lipoprotein, with lesser amounts distributed between low-density lipoprotein (8±6%) and high-density lipoprotein (21±8%). There were no significant differences ($P < 0.03$) in distribution of triacylglycerol versus distribution of [9,10-^{3}H]trioleoylglycerol between different lipoproteins in the injection solution.

Correction of liver data for trapped blood

The percentage of the injected dose of radioactivity in total liver lipids was corrected for trapped blood by using the data of Abrams & Cooper (1976*b*). They used ^{125}I-labelled albumin as a serum marker and found 10% of the liver weight in a 48h-starved rat was attributable to trapped serum, and 80% of this trapped serum could be removed by perfusion. These results were reproduced in the present investigation. Since we routinely perfused the liver before analysis, the radioactivity counts in liver were therefore corrected for the presence of 0.02ml of serum/g of liver.

Results

Movement of isotope through serum and liver

After injection of labelled serum, about 95% of the labelled triacylglycerol was cleared from serum within 60min (Fig. 1). The isotope was taken up rapidly by liver (Fig. 2), with maximal incorporation occurring at 5–10min. Label was subsequently lost from liver, the loss following a time course similar to serum clearance. About 90% of the radioactivity cleared from serum by liver was in triacylglycerol soon after injection (5min).

Intactness of triacylglycerol taken up by liver. Studies were carried out to determine whether the triacylglycerol taken up by liver was intact or had been hydrolysed. In these experiments the serum was prelabelled with trioleoylglycerol containing ^{14}C in the fatty acid moiety and ^{3}H in the glycerol. The ratio of ^{14}C to ^{3}H incorporation into liver was approx. 1.0 up to 10min after injection, but began to increase gradually thereafter (Fig. 2). The fact that the ratio remained unchanged during the time of rapid radioisotope accumulation shows that the triacylglycerol was taken up intact for the first 10min after injection. The increase in the ^{14}C/^{3}H ratio after 10min was concomitant with a decrease in the amount of radioisotope in total liver lipid and liver triacylglycerol, and indicates that hydrolysis occurred at that time.

Calculations

Working model. The estimation of rate constants and flux rates from the type of data described above requires only a simplified model consistent with pertinent physiological events. The information necessary to completely define the situation *in vivo* is not available at present, but a minimal model for movement of triacylglycerol into and out of serum is shown in Fig. 3. A pertinent feature of the model is the reversible movement of triacylglycerol between liver and serum. No attempt is made to specify which

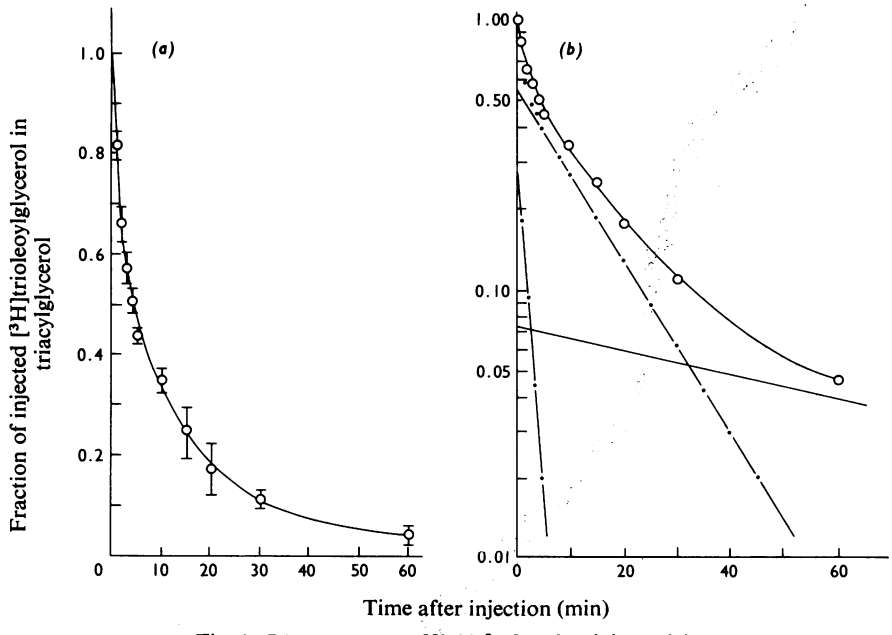

Fig. 1. *Disappearance of* [9,10-^3H]*trioleoylglycerol from serum*

The curve in (*a*) is a plot of fraction of injected [^{3}H]trioleoylglycerol remaining in serum triacylglycerol. Each data point (○) is the mean±S.E.M. obtained with 6–25 rats. Unit dose at zero time represents label proved by analysis to reside in triacylglycerol (87% of total radioactivity in injected preparation). The solid curve (○) in (*b*) is the same as in (*a*), but is displayed in a semi-logarithmic plot. —— represents the curve calculated from the equation given below. The three straight lines are three exponential components obtained by graphic curve analysis. Values shown as 'dots' in (*b*) were obtained by subtraction from the curve. The resulting function describing the curve is:

$$y = 0.36^{-0.66t} + 0.56e^{-0.074t} + 0.072e^{-0.010t}$$

where y is the fraction of injected [9,10-^{3}H]trioleoylglycerol remaining in serum triacylglycerol and t is the time in minutes after injection.

of the triacylglycerol pools in liver and serum are involved in this reversible movement. The symbol k_{ba} represents the rate constant for movement to pool (*b*) from pool (*a*). It is the fraction of total serum triacylglycerol delivered to liver per minute. When multiplied by the triacylglycerol content of serum the product is F_{ba}, the flux rate from serum to liver. All of the values shown in Fig. 3 are flux rates. The rate constant k_{oa} is the fraction of serum triacylglycerol moved from serum to extrahepatic sites and F_{oa} is the flux rate for this process. The sum of k_{ba} and k_{oa} will be designated k_{aa}, the overall rate constant of turnover of serum triacylglycerol. There are certain minimal assumptions necessary to make the following calculation. These are: (1) there are no changes in rate constants or pool sizes during the experimental period; (2) the movement of triacylglycerol radioactivity between pools is equivalent to the movement of triacylglycerol molecules; and (3) the change in tracer content within sampled compartments of the system reflects a kinetic behaviour that may be represented by complex exponential functions.

Calculation of rate constants. (1) Graphic analysis of the serum curve. Rate constants were estimated by stochastic methods, i.e. via integrals and derivatives of the curves rather than by compartmental analysis. This avoids the need to describe the overall model in complete detail. The curve plotted in both linear and semilogarithmic forms in Fig. 1 reflects the reversible movement of triacylglycerol out of serum. It may be analysed to give the three-component function included in the legend of Fig. 1. The simplified two-compartment model in Fig. 3 would yield only two compartments, but the overall physiological system is undoubtedly much more complex. The potential number of compartments and associated exponential components is likely to be even more than three. Regardless of the actual total, the rate constant of overall turnover of labelled species in serum, i.e. k_{aa}, may be calculated from a fitted complex exponential curve having a limited number of components. With a curve from a biological system such as this, the practical upper limit for the number of components is usually three.

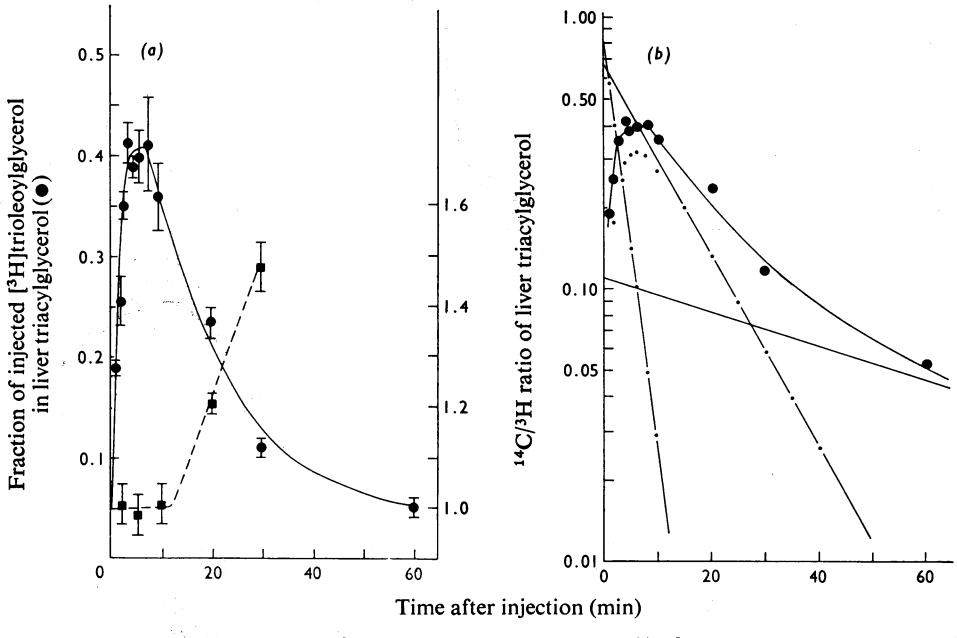

Fig. 2. *Appearance of* [9,10-^{3}H]*trioleoylglycerol in liver and* $^{14}C/^3H$ *ratio of liver triacylglycerol*

The fraction of injected [9,10-^{3}H]trioleoylglycerol in liver triacylglycerol was determined from experiments with serum single labelled with [9,10-^{3}H]trioleoylglycerol serum. Each point (●) in (*a*) is the mean ± S.E.M. obtained with 8–24 rats. The data have been corrected for the contribution of labelled serum non-esterified fatty acid to liver triacylglycerol, the exit of labelled triacylglycerol from liver, and the presence of trapped serum in liver. The solid curves in (*a*) and (*b*) were obtained by graphical analysis and the straight lines in (*b*) derived in such an analysis represent the function:

$$y = -0.79e^{-0.34t} + 0.68e^{-0.082t} + 0.11e^{-0.014t}$$

where y is the fraction of injected [9,10-^{3}H]trioleoylglycerol in liver triacylglycerol and t is the time in minutes after injection. The $^{14}C/^3H$ ratio of liver triacylglycerol (■, in *a*) was determined after injection of double-labelled serum trioleoyl[2(*n*)-^{3}H]glycerol plus [1-^{14}C]trioleoylglycerol. Each point is the mean ± S.E.M. obtained with eight rats. Values shown as 'dots' in (*b*) were obtained by subtraction from the curve.

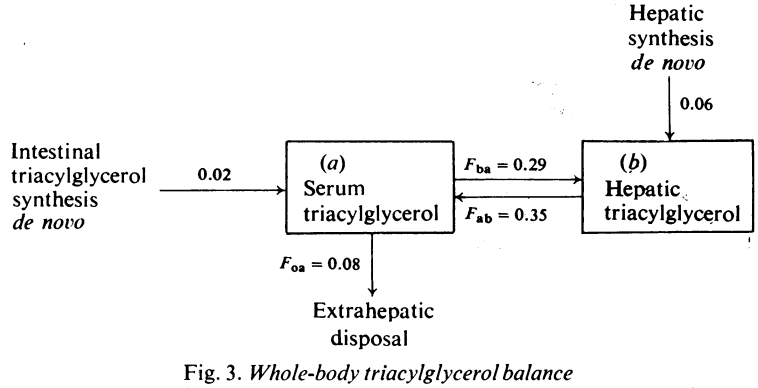

Fig. 3. *Whole-body triacylglycerol balance*

Illustrated are the flux rates (mg/min per 100g body wt.) for triacylglycerol into various whole-body triacylglycerol compartments in rats starved for 48h. The values of F_{ba}, F_{ab}, and F_{oa} were determined in these studies. The values for triacylglycerol synthesis *de novo* were obtained from the literature sources described in the text.

The gradual attenuation of the serum curve (Fig. 1) reflects the return of labelled triacylglycerol to serum. If no such reflux existed, the curve would be a simple exponential function, give a straight line when plotted semilogarithmically, and its slope would be k_{aa}. In the presence of reflux, the value of k_{aa} is

the slope of the complex curve for only an instant after zero time before tracer has time to return in significant amounts. This instant slope may be calculated by mathematical treatment of the curve as a whole if activity is expressed as fraction of administered dose. It is the derivative of the equation for the curve evaluated at zero time (Shipley & Clark, 1972). The equation is given in the legend of Fig. 1. The zero-time derivative is the sum of the products of each coefficient and its exponential constant:

$$k_{aa} = (0.36)(-0.66) + (0.56)(-0.074) + (0.072)(-0.010) = -0.28$$

The sign is negative because it represents a downslope.

The curve for serum activity may also be used to determine a rate constant for irreversible disposal of serum triacylglycerol. With dose normalized to unity, it is the reciprocal of the area under the disappearance curve from zero to infinity. The area may be determined by planimetry, weighing or by calculation from the integral of the equation for the curve (Shipley & Clark, 1972). Thus the rate constant is:

$$\frac{1}{\left(\frac{0.36}{0.66} + \frac{0.56}{0.074} + \frac{0.072}{0.010}\right)} = 0.065$$

Because little if any such irreversible disposal involves liver, this constant should be an estimate of k_{oa} if minimal tracer recycles from the periphery during the period of observation. Since k_{aa} is the sum of k_{ba} and k_{oa}, then:

$$k_{ba} = k_{aa} - k_{oa} = 0.28 - 0.065 = 0.22$$

(2) Graphic analysis of liver curve. An independent estimate of k_{ba} can be made from the curve describing movement of isotope into and out of liver. As expected for a secondary pool in a reversible system, the radioactivity in hepatic triacylglycerol will first rise then fall (Fig. 2). Again, it is possible to use the derivative of the curve evaluated at zero time. In this instance it represents k_{ba}, the rate constant for entry into hepatic triacylglycerol from serum (Shipley & Clark, 1972). From the respective coefficients and exponential constants for the function given in the legend of Fig. 2:

$$k_{ba} = (0.79)(0.34) - (0.68)(0.082) - (0.11)(0.014) = 0.21$$

This value corresponds to the upslope near zero time before appreciable tracer is lost from the hepatic pool. This estimate of k_{ba} requires only that there be direct access of triacylglycerol to liver from serum. It is not influenced by the existence of various hepatic subcompartments or other details omitted from the simplified model in Fig. 3. The agreement between these two measurements of k_{ba} provides some reassurance of the accuracy of this value.

(3) Errors of estimates. Errors in the instant slope as estimated by the zero-time derivative can be quite large. A prime requirement is that the early portion of the curve should be well-defined by numerous closely spaced and accurately timed data points. Figs. 1 and 2 show no gross ambiguity of alignment of early points. The coefficients-of-error of mean values represented by points from 1 to 10min on the serum and liver curves ranged from 2 to 7%. Timing errors were kept within approx. 2s by use of a stopwatch to measure the interval between the rapid intravenous injection (1s duration) and the time of decapitation.

Delay in mixing and transport of tracer to tissues is a potential source of error. This may be appreciated by noting that the initial direction of the curve is affected not only by the placement of the first few data points, but also by the point in time the curve is considered to begin. Onset is actually when mixed blood encounters tissues, rather than when tracer is injected. The delay between injection and such meeting with tissue can be assessed roughly in the graphic analysis of the curves. The lines that represent the peeled components in Fig. 1 should give values at the zero-time (injection-time) intercept that add to 1.0 if delay is not great. For Fig. 2 the sum should be zero. These conditions were fulfilled within limitations of visual fitting. To evaluate the delay directly, the time of transit of ^{99m}Tc-labelled albumin from tail vein to tissues of the head was made with the aid of a scintillation camera. The curve began to rise at 2–3s after injection and reached a stable plateau value at 6s. There was no fluctuation at the plateau such as would result from recirculation of an unmixed bolus. The 6s delay in reaching tissue distal to the central circulation means that the timing of all data points may be corrected by subtracting 0.1min from the interval between injection and sampling. This correction is too small to affect graphic analysis, but it may be introduced in least-squares fitting, as will be discussed below.

If the molecular size of the labelled species is small, the zero-time derivative of the plasma curve bears no relationship to any metabolic process. For example, the very first loss with labelled glucose is by diffusion into erythrocytes and interstitial fluid. In such a case the curve before 1min is not susceptible to meaningful analysis and intercepts derived from points beyond this time add to less than 100% of dose at injection time because during the first min much tracer has 'leaked' from serum. This should not be a problem in the present study because triacylglycerol, as a component of lipoprotein, is relatively non-diffusible.

(4) Least-squares fitting of curves. Because graphic curve-fitting can be flawed by subjectivity, several

Table 1. *Comparison of various fitted functions*

Line A is the graphic fit shown in Fig. 1; B is a three-component Newton–Raphson least-squares fit with points weighted by $1/y^2$, C is a two-component unweighted Newton–Raphson least-squares approximation with a program that gives the S.E. of parameters (Dixon, 1976). Line D is the graphic fit of Fig. 2; E is a three component Newton–Raphson least-squares fit with points weighted by $1/y^2$, F is a three-component unweighted least-squares fit by a generalized decreased-gradient program (Lasdon *et al.*, 1973), G is a two-component unweighted least-squares fit with S.E. of parameters (Dixon, 1976).

Type of fit	Parameters* ...	I_1	g_1	I_2	g_2	I_3	g_3	Residual deviation†		k_{aa}	k_{0a}	k_{ba}
								Numerical	Percentage			
(i) Serum												
A Three-component graphic		0.36	−0.66	0.56	−0.074	0.072	−0.010	0.018	3.5	0.28	0.065	
B Three-component least squares		0.374	−0.677	0.565	−0.074	0.062	−0.0074	0.015	3.3	0.30	0.060	
C Two-component least squares		0.466	−0.494	0.534	−0.051			0.015	15.2	0.26	0.088	
		±0.046‡	±0.073	±0.046	±0.0057							
(ii) Liver												
D Three-component graphic		−0.79	−0.34	0.68	−0.082	0.11	−0.014	0.017	9.3			0.21
E Three-component least squares		−0.769	−0.358	0.688	−0.079	0.081	−0.014	0.018	7.2			0.22
F Three-component least squares		−1.088	−0.310	0.724	−0.153	0.364	−0.035	0.018	9.3			0.21
G Two-component least squares		−0.623	−0.419	0.623	−0.051			0.018	12.6			0.23
		±0.064	±0.060	±0.064	±0.0070							

* The function is $I_1e^{-g_1t}+I_2e^{-g_2t}+I_3e^{-g_3t}$.

† The numerical value is root-mean-square deviation of data points from the curve. The percentage value is root mean square of the percentage deviation of points on the curve from data points.

‡ S.E. of estimate of the parameter.

least-squares estimates of a fitted function were obtained for the data from both serum and liver. The results are shown in Table 1. Sampling time was corrected for the aforementioned delay by subtracting 0.1 min from the interval between injection and sampling. With such a correction having been made, the computer programs were constricted so that the coefficients of the equations added to one for serum and to zero for liver. In other words, each curve was considered to begin when tracer reached tissues at 0.1 min after injection.

Results in lines B and E of Table 1 were obtained by the least-squares method when data points were weighted by $1/y^2$. This is a conventional weighting when the mean values have a constant coefficient of error, i.e. when the percentage deviation from the mean tends to be constant from high to low values and absolute numerical deviation is less for lower values. Except for the 15 and 20 min values in Fig. 1, this was true for all the data in Figs. 1 and 2. A satisfactory unweighted three-component fit could not be obtained for the serum data with any computer program tested. The problem was either failure to converge or what amounted to a two-component adjustment to points up to 30 min and neglect of the final point until far down on the curve, when the response of the computer was to generate either a zero slope or a positive upslope so as to pass through the 60 min point. Such solutions are not physiological and are not subject to meaningful integration.

The two-component unweighted solutions giving the functions in lines C and G of Table 1 disregarded the last point altogether. This is reflected in the relatively high percentage residual deviation. The value of k_{oa} (line C) was overestimated because of the abbreviated area resulting from the premature terminal downslope. Line F is a successful unweighted three-component fit for the liver data and was determined with a generalized decreased-gradient program. The last data-point was approached closely, and the residual error was comparable with that with the other three-component functions.

Choice of fitted functions and calculation of flux rates

Although the various fitting procedures listed in Table 1 do not give seriously divergent values for k_{aa} and k_{ba}, the derived functions with a three-component least-squares fit on lines B and E are the best choice as judged jointly by the absolute and percentage residual error-of-fit. Thus a final choice is 0.30 for k_{aa} and 0.22 for k_{ba}. The function on line B also is preferred for k_{oa} which becomes 0.060.

Flux rates are calculated by introducing the pool size for serum triacylglycerol. The mean $\pm$ S.E. for serum triacylglycerol concentration was found to be $0.31+0.02$ mg/ml ($n = 205$), and a value of 4.2 ± 0.2 ml of serum/100 g body wt. confirmed the serum volume found by Abrams & Cooper (1976*a*). Thus the serum triacylglycerol pool size is 1.3 ± 0.1 mg/100 g body wt. This value multiplied by rate constants gives respective rates of flux:

$F_{oa} = (0.060)\ (1.3) = 0.08$ mg/min per 100 g body wt.

$F_{ba} = (0.22)\ (1.3) = 0.29$ mg/min per 100 g body wt.

$F_{aa} = (0.30)\ (1.3) = 0.39$ mg/min per 100 g body wt.

The model of Fig. 3 would require that $F_{aa} = F_{oa} + F_{ba}$, i.e. 0.37 mg/min. This is quite close to 0.39 calculated directly by obtaining k_{aa} via the derivative of the serum curve.

Discussion

Provided the present data reflect reliably events occurring *in vivo*, certain noteworthy conclusions may be made (see Fig. 3). The first is that the entire turnover of serum triacylglycerol can be accounted for on the basis of contributions from liver and intestine. Abrams & Cooper (1976*a*) estimated that non-esterified fatty acid of serum is converted into 0.06 mg of hepatic triacylglycerol/min per 100 g body wt. The results above show that serum triacylglycerol contributes 0.29 mg/min per 100 g body wt. to liver triacylglycerol. There is no increase in the triacylglycerol content of liver during the exponential period (Abrams & Cooper, 1976*a*) and presumably none in adipose tissue in a starved animal. Therefore the net input of triacylglycerol into liver or serum must equal the net output. The rate of hepatic triacylglycerol input should be the sum of the contributions of non-esterified fatty acid of serum and serum triacylglycerol, or $0.29+0.06 = 0.35$ mg/min per 100 g body wt. The total serum triacylglycerol turnover should be the sum of the intestinal and liver inputs. Studies in the literature indicate that intestine contributes about 0.02 mg of triacylglycerol/min per 100 g body wt. to serum in the starved rat (Baxter, 1966; Windmueller & Levy, 1968; Ockner *et al.*, 1969; Mistilis & Ockner, 1972). The total turnover is then $0.35+0.02$ or 0.37 mg/min per 100 g body wt. This is close to the value of 0.39 for F_{aa} measured directly from the disappearance of [9,10-^{3}H]trioleoylglycerol from serum (Fig. 1).

These experiments were carried out under circumstances in which the concentration of serum triacylglycerol remained constant. There is, however, a continual irreversible loss of serum triacylglycerol to extrahepatic tissues at a rate that we have determined to be 0.08 mg/min per 100 g body wt. For this to occur, new triacylglycerol must be produced at the same rate, otherwise the serum concentration would fall. It is noteworthy that we can account quantitatively for the required production *de novo* on the basis of hepatic triacylglycerol synthesis from non-esterified fatty acid of serum (0.06) and from precursors in intestine (0.02).

Two surprising findings are that the estimated release rate of triacylglycerol from liver (0.35 mg/min per 100 g body wt.) and the total turnover rate of serum triacylglycerol (0.39 mg/min per 100 g body wt.) are much larger than would have been predicted on the basis of available information. Current estimates of liver triacylglycerol-release rate, as estimated by the intravenous administration of Triton WR-1339, are approximately 0.15 mg/min per 100 g body wt. (Schotz *et al.*, 1964; Recknagel, 1967; Abrams & Cooper, 1976*a*). By using this value the estimated total turnover of serum triacylglycerol would be $0.15+0.02 = 0.17$ mg/min per 100 g body wt. Although Triton has been shown to inhibit esterification of cholesterol *in vitro* (Klauda & Zilversmit, 1974), and to enhance the activity of hydroxymethylglutaryl-CoA reductase (Goldfarb, 1975), it is difficult to predict its effects on whole-body triacylglycerol metabolism. However, it is apparent that the use of Triton probably gives erroneously low estimates of the hepatic triacylglycerol release rate.

One of the more intriguing questions to arise from the above-mentioned studies is the physiological significance of the recycling of serum triacylglycerol to liver. A possible explanation of our findings is that these serum lipoproteins return to liver to pick up a missing component, e.g. peptide, after which they re-enter the circulation without being degraded. An alternative explanation is that lipoprotein particles still containing significant triacylglycerol may be involved in returning specific peptides to liver to facilitate very-low-density-lipoprotein release (Roheim *et al.*, 1965, 1976).

It is not clear whether this recycling phenomenon reflects intracellular or extracellular processes within liver. Radioautographic studies of uptake of triacylglycerol, cholesteryl ester, and apoprotein suggest the lipoprotein returning to liver may be bound to the hepatocyte plasma membrane, but substantial amounts of labelled material are also found intracellularly (Stein & Stein, 1967; Stein *et al.*, 1969, 1974). The quantitative significance of such data is difficult to assess. Another question is raised by the experiments with double-labelled serum triacylglycerol (Fig. 2). The results suggest a delayed metabolism of this triacylglycerol, indicating that the model shown in Fig. 3 may contain other less obvious components.

It has been reported that liver has the capacity to bind and metabolize lipoprotein-bound triacylglycerol. This has been shown with isolated hepatocytes (Green & Webb, 1964; Higgins & Green, 1966; Higgins, 1967; Ontko, 1967), liver homogenates (Ontko, 1967), liver slices and perfused liver (Ontko & Zilversmit, 1967) and *in vivo* by using single- and double-labelled chylomicron triacylglycerol (French & Morris, 1958; Nestel *et al.*, 1962; Olivecrona, 1962; Belfrage *et al.*, 1965; Elovson *et al.*, 1965; Olivecrona & Belfrage, 1965; Belfrage, 1966; Schotz *et al.*, 1966). Similar findings were made by Schotz *et al.* (1966) with plasma triacylglycerol single-labelled *in vivo* and by Stein & Shapiro (1960) with double-labelled serum triacylglycerol prepared *in vitro* by exchange. Similar conclusions were made in studies using lipoprotein-containing labelled cholesteryl ester (Quarfordt & Goodman, 1967; Faergeman *et al.*, 1975). On the other hand, Felts (1965) and Mayes & Felts (1967) concluded that lipoprotein-bound triacylglycerol was not taken up by liver. When tests were done *in vivo*, the labelled chylomicron triacylglycerol could be removed by a brief liver perfusion (Felts, 1965). This is in contrast with our results in which labelled triacylglycerol, derived from chylomicron-free serum lipoproteins, remained associated with liver even after perfusion. The reason for the different results is not clear. Our conclusion that liver can bind or take up intact serum triacylglycerol is diametrically opposed to theirs, that uptake by liver only occurs after extrahepatic hydrolysis and recirculation of the non-esterified fatty acid back to liver (Mayes & Felts, 1967). However, none of these studies provided a quantitative estimate of the importance of this phenomenon to turnover of hepatic triacylglycerol.

Most of the studies of kinetics of whole-body triacylglycerol metabolism cannot be compared directly with ours, because the very early stages of clearance were defined inadequately, and no companion results for liver were presented. The studies of Havel *et al.* (1962), Farquhar *et al.* (1965), Eaton *et al.* (1969), Harris & Felts (1970), Kissebah *et al.* (1974) and Havel & Kane (1975) did not include measurements of the very early rapid processes and approximated clearance from a single exponential decay of prelabelled or endogenously labelled triacylglycerol.

Some potential sources of error that might influence our above results are: (1) serum present in isolated liver; (2) hepatic esterification of radioactive fatty acid, mono- or di-acylglycerol present as contaminants in the labelled serum injected; and (3) secondary labelling of serum triacylglycerol via labelled fatty acid produced by lipolysis at extrahepatic sites. Because each of these can be measured, the data in Figs. 1 and 2 have been corrected to account for these contributions. The retained serum was estimated as described in the Experimental section. Since serum triacylglycerol is cleared rapidly (Fig. 1), this correction is of significance only during the first 2–3 min. The corrections to be applied to the radioactivity in hepatic triacylglycerol range from 1.1% at 1 min after injection to 0.8% at 3 min.

The error from contaminating fatty acid, mono- or di-acylglycerol in injected material is very small. From the data of Abrams & Cooper (1976*a*) and

Palmer *et al.* (1978) an estimate may be made of the percentage of contaminating labelled non-esterified fatty acid appearing in hepatic or serum triacylglycerol at various times after injection. Their data show that after injection of labelled palmitate the amount of radioactivity appearing in liver triacylglycerol between 1 and 10min does not exceed 4.3% of the dose, and it declines to about 2% at 60min. The labelled serum used in these experiments contained 10% of the total radioactivity in non-esterified fatty acid. Assuming that oleate behaves like palmitate, the contribution of this contaminant to observed hepatic triacylglycerol radioactivity would add 0.2% to an observed value of 19% at 1min, 0.4% to observed 40% at 5–10min, and 0.2% to the observed 5% at 60min. Similar calculations may be made for the effect of this contaminant on triacylglycerol in serum by using the data of Palmer *et al.* (1978). The first significant appearance would be near 10min. The following list gives (respectively) time, observed activity, and expected content of contaminant for presently observed values: 15min, 25%, 0.07%; 20min, 20%, 0.1%; 30min, 11%, 0.1%; 60min, 4%, 0.05%.

The results in Fig. 4 show that the third potential source of error exists. After injection of labelled serum with a 10% contaminant radioactivity in non-esterified fatty acid, the isotope in non-esterified fatty acid of serum (upper curve) falls less rapidly than predicted by experiments (Abrams & Cooper, 1976*a*) in which labelled palmitate was injected (----). The difference is attributable to labelled fatty acid released from extrahepatic sites. The upper curve in Fig. 4 may be used to estimate the total contribution of labelled non-esterified fatty acid reaching hepatic triacylglycerol from the contaminating radioactive fatty acid plus that derived from lipolysis at extrahepatic sites. The labelled non-esterified fatty acid in serum at a given time on this curve multiplied by the rate constant of transport to hepatic triacylglycerol gives an estimate of the amount of triacylglycerol radioactivity derived from this source. The rate constant of conversion of non-esterified fatty acid of serum into hepatic triacylglycerol is $0.076\,min^{-1}$ (Abrams & Cooper, 1976*a*). For example, in the 0–1 min interval, there is an average of 6.7% of the injected radioactivity in non-esterified fatty acid of serum (Fig. 4) and the conversion into hepatic triacylglycerol will be (6.7) (0.76) = 0.5% of dose. During the second minute the amount added to this will be (3.0)(0.076) = 0.2%. The correction is therefore 0.5% at 1min, 0.7% at 2min and so on until 10min, at which time the total accumulation adds up to 1.8% of injected dose. After 10min this incremental input will be offset by loss to serum from the hepatic triacylglycerol pool. The channel of such loss is assumed to be via the microsomal pool, where 56% of total hepatic triacylglycerol label is located at 10min after an injection of [U-^{14}C]palmitate (Palmer *et al.*, 1978). The rate constant of movement to serum from this pool was estimated by Palmer *et al.* (1978) to be $0.13\,min^{-1}$. Therefore the loss between 10 and 11min is (1.8%) (0.56)(0.13) = 0.13% of label originally injected. During this same time the input is (1%)(0.076) = 0.076%, giving a net loss of 0.06% of dose, leaving 1.7% of injected activity in hepatic triacylglycerol arising from non-esterified fatty acid in both injected material and that coming indirectly from lipolysis at peripheral sites. Thereafter the calculations become more complex, because of the interchange of triacylglycerol between serum and liver, but the hepatic pool will not contain more than 1% of dose from these sources during the next hour. The interpretation of the disappearance curve for labelled serum triacylglycerol will therefore not be complicated by recirculation of label from non-esterified fatty acid of serum. Similar calculations for the mono- and di-acylglycerol contaminants were not attempted because they constitute only 3% of the injected dose and because of uncertainties about the metabolic fate of these moieties. Another compelling argument against fatty acid recycling as a source of significant hepatic triacylglycerol soon after injection is apparent from the broken line (----) in Fig. 2(*a*).

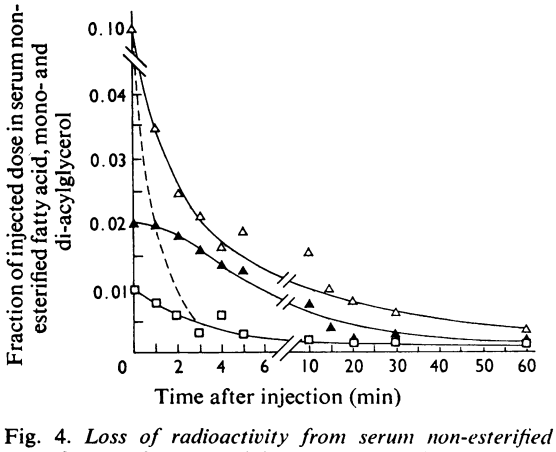

Fig. 4. *Loss of radioactivity from serum non-esterified fatty acid, monoacylglycerol and diacylglycerol*

[^{3}H]Trioleoylglycerol-labelled serum was injected intravenously into rats starved for 48h. At the specified times the animals were decapitated, and the fraction of total injected ^{3}H remaining in non-esterified fatty acid (△), monoacylglycerol (▲) and diacylglycerol (□) was determined. Each point is the mean of results obtained with 6–25 rats. The broken curve (----) represents values for non-esterified fatty acid predicted from the data of Abrams & Cooper (1976*a*) assuming there is no lipolysis of labelled serum triacylglycerol.

A series of experiments were performed with serum containing double-labelled triacylglycerol. During the first 10min the ratio $^{14}C/^{3}H$ in hepatic triacylglycerol remained the same as that of the injected material, indicating that no hydrolysis occurred during the period of maximum triacylglycerol uptake by liver. It does change after 10min, but by this time the radioactivity in hepatic triacylglycerol passed its peak and was decreasing. Therefore the principal source of labelled triacylglycerol in liver cannot be ascribed to recycling to liver of labelled fatty acid from extrahepatic sites.

The calculations made from the observed curves for determining the overall turnover rate constant of serum triacylglycerol (k_{aa}) or the rate constant of direct entry of triacylglycerol to liver from serum (k_{ba}) are unaffected by recycling. The only uncertainty is in the value of F_{oa}, the disposal rate of serum triacylglycerol to non-hepatic sites. Although considered to represent irreversible disposal, if recipient sites are imperfect sinks for tracer and appreciable labelled fatty acid returns via liver to serum triacylglycerol during the latter portion of the period of observation, the area under the serum triacylglycerol curve will be increased, leading to an underestimate of F_{oa}.

One of the major points to be considered in evaluating the above data is the validity of the experimental technique used to obtain it. The questions of turnover of serum lipoproteins and hepatic uptake of serum lipoprotein and/or their individual components has been studied frequently. Two types of approaches have been used. The first involves labelling serum lipoproteins *in vivo* by injection of an appropriate precursor, e.g. glycerol, fatty acid, cholesterol or amino acid, and trying to monitor clearance of labelled serum lipoproteins in the same animal. The second technique is to inject a labelled lipoprotein subjected previously to varying extents of processing *in vitro*. The processing *in vitro* might include prolonged centrifugation, exposure to high ionic strength, labelling procedures *in vitro* and storage for varying periods of time before reinjection.

There are two experimental protocols that may be used with lipoproteins prepared *in vitro*. The first is to study the clearance of a 'purified and characterized' lipoprotein species and the second is to treat serum lipoproteins as a 'black box' and study turnover of the group.

During starvation there are no chylomicrons to contend with, and the bulk of serum triacylglycerol is in lipoprotein fractions designated 'very low density' and 'intermediate density'. It is the consensus at present that very-low-density lipoprotein is first acted on by extrahepatic tissues until some of its triacylglycerol component is removed, at which time the 'remnant' may be taken up and metabolized by liver. If this is true, it means that a given serum-lipoprotein class represents a temporal spectrum of all metabolic stages of the species and that its site of metabolism varies according to, presumably, the status of the peptide–phospholipid–cholesterol membrane surrounding the lipoprotein complex.

We elected to use a method involving processing *in vitro* in which there is no serum fractionation or exposure to non-physiological ionic strength. We have also studied some of the same phenomenon by labelling *in vivo* with radioactively labelled palmitate. These results are presented in the following paper (Palmer *et al.*, 1978). Because processing *in vitro* may introduce structural alterations, thereby rendering such complexes unsuitable for tracer studies, it is important to try to characterize the product used. To this end, labelled serum samples were subjected to discontinuous gradient flotation, polyacrylamide-gel electrophoresis and peptide analysis. In each case the labelled sample appeared identical with unprocessed serum. However, such tests are relatively crude, and it seems likely that any alterations induced by the labelling procedure will be much more subtle and will be detected clearly only by a test involving its metabolic utilization. One such test is to compare the irreversible disposal rate of serum triacylglycerol by using triacylglycerol labelled by procedures both *in vivo* and *in vitro*. We have done this and have obtained similar rates (see above and Palmer *et al.*, 1978). We therefore consider that the results obtained in the present study are as valid as those obtained with any of the alternative experimental approaches used in the past.

We greatly appreciate the technical assistance of Ksenija Dimitrov and Rebecca Coapman. This work was supported by grants from the Northeast Ohio Chapter American Heart Association, the Department of Health of Ohio, and a training grant (GM-00035) from the National Institutes of Health. Some of this work forms part of a thesis presented by E.W.L. in partial fulfilment of the requirements for the Ph.D. degree at Case Western Reserve University. We are particularly grateful to Dr. Barry Lindley for his assistance with curve-fitting.

References

Abrams, M. A. & Cooper, C. (1976*a*) *Biochem. J.* **156**, 33–46

Abrams, M. A. & Cooper, C. (1976*b*) *Biochem. J.* **156**, 47–54

Avigan, J. (1959) *J. Biol. Chem.* **234**, 787–790

Baxter, J. H. (1966) *J. Lipid Res.* **7**, 158–166

Belfrage, P. (1966) *Biochim. Biophys. Acta* **125**, 474–484

Belfrage, P., Elovson, J. & Olivecrona, T. (1965) *Biochim. Biophys. Acta* **106**, 45–55

Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

Brenneman, D. E. & Spector, A. A. (1974) *J. Lipid Res.* **15**, 309–316

Brenneman, D. E., McGee, R. & Spector, A. (1974) *Cancer Res.* **34**, 2605–2611
Canedella, R. J. & Crouthamel, W. G. (1976) *J. Lipid Res.* **17**, 156–166
Dixon, W. J. (1976) in *BMD: Biomedical Computer Programs; BMD 07R-Non Linear Least Squares* (Dixon, W. J., ed.), pp. 387–396, University of California Press, Berkeley
Eaton, R. P., Berman, M. & Steinberg, D. (1969) *J. Clin. Invest.* **48**, 1560–1579
Elovson, J., Olivecrona, T. & Belfrage, P. (1965) *Biochim. Biophys. Acta* **106**, 34–44
Faergeman, O. & Havel, R. J. (1974) *J. Clin. Invest.* **55**, 1210–1218
Faergeman, O., Sata, T., Kane, J. P. & Havel, R. J. (1975) *J. Clin. Invest.* **56**, 1396–1403
Farquhar, J. W., Gross, R. C., Wagner, R. M. & Reaven, G. M. (1965) *J. Lipid Res.* **6**, 119–134
Felts, J. M. (1965) *Ann. N.Y. Acad. Sci.* **131**, 24–42
Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509
French, J. E. & Morris, B. (1958) *J. Physiol. (London)* **140**, 262–271
Goldfarb, S. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 662
Green, C. & Webb, J. A. (1964) *Biochim. Biophys. Acta* **84**, 404–411
Harris, K. L. & Felts, J. M. (1970) *J. Lipid Res.* **11**, 75–81
Havel, R. J. & Kane, J. P. (1975) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **34**, 2250–2257
Havel, R. J., Eder, H. A. & Bragdon, J. H. (1955) *J. Clin. Invest.* **34**, 1345–1353
Havel, R. J., Felts, J. M. & Van Duyne, C. M. (1962) *J. Lipid Res.* **3**, 297–308
Higgins, J. A. (1967) *J. Lipid Res.* **8**, 636–641
Higgins, J. A. & Green, C. (1966) *Biochem. J.* **99**, 631–639
Kates, M. K. (1972) *Techniques of Lipidology*, pp. 444–445, American Elsevier, New York
Kissebah, A. H., Adams, P. W. & Wynn, V. (1974) *Clin. Sci. Mol. Med.* **47**, 259–278
Klauda, H. C. & Zilversmit, D. B. (1974) *J. Lipid Res.* **15**, 593–601
Lasdon, L. S., Fox, R. L. & Ratner, M. W. (1973) *Technical Memorandum No. 325*, Department of Operations Research, Case Western Reserve University
Levy, R. I., Bilheimer, D. W. & Eisenberg, S. (1971) in *Plasma Lipoproteins* (Smellie, R. M. S., ed.), pp. 3–17, Academic Press, New York
Lowenstein, J. M. (1971) *J. Biol. Chem.* **246**, 629–632
Mayes, P. A. & Felts, J. M. (1967) *Biochem. J.* **105**, 18c
Mistilis, S. P. & Ockner, R. K. (1972) *J. Lab. Clin. Med.* **80**, 34–46
Naito, H. K., Wada, M., Ehrhart, L. A. & Lewis, L. A. (1973) *Clin. Chem.* **19**, 228–234
Nestel, P. J., Havel, R. J. & Bezman, A. (1962) *J. Clin. Invest.* **41**, 1915–1921
Ockner, R. K., Hughes, F. B. & Isselbacher, K. J. (1969) *J. Clin. Invest.* **48**, 2079–2088
Olivecrona, T. (1962) *J. Lipid Res.* **3**, 439–444
Olivecrona, T. & Belfrage, P. (1965) *Biochim. Biophys. Acta* **98**, 81–93
Ontko, J. A. (1967) *Biochim. Biophys. Acta* **137**, 13–22
Ontko, J. A. & Zilversmit, D. B. (1967) *J. Lipid Res.* **8**, 90–96
Palmer, J. F., Cooper, C. & Shipley, R. A. (1978) *Biochem. J.* **172**, 219–226
Quarfordt, S. H. & Goodman, D. S. (1967) *J. Lipid Res.* **8**, 264–273
Quarfordt, S. H., Frank, A., Shames, D. M., Berman, M. & Steinberg, D. (1970) *J. Clin. Invest.* **49**, 2281–2297
Recknagel, R. O. (1967) *Physiol. Rev.* **19**, 145–208
Redgrave, T. G., Roberts, D. C. K. & West, C. E. (1975) *Anal. Biochem.* **65**, 42–49
Roheim, P. S., Miller, L. & Eder, H. A. (1965) *J. Biol. Chem.* **240**, 2994–3001
Roheim, P. S., Edelstein, D. & Pinter, G. G. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1757–1760
Schotz, M. C., Baker, N. & Chavez, M. N. (1964) *J. Lipid Res.* **5**, 569–577
Schotz, M. C., Arnesjo, B. & Olivecrona, T. (1966) *Biochim. Biophys. Acta* **125**, 485–495
Shames, D. M., Frank, A., Steinberg, D. & Berman, M. (1970) *J. Clin. Invest.* **49**, 2298–2314
Shipley, R. A. & Clark, R. E. (1972) *Tracer Methods for in vivo Kinetics*, pp. 1–44, 65–76, 193–194, Academic Press, New York
Soloni, F. G. (1971) *Clin. Chem.* **17**, 529–534
Stein, O. & Stein, Y. (1967) *Lab. Invest.* **17**, 436–446
Stein, O., Stein, Y., Goodman, D. S. & Fidge, N. H. (1969) *J. Cell Biol.* **43**, 410–431
Stein, O., Rachmilewitz, D., Sanger, L., Eisenberg, E. & Stein, Y. (1974) *Biochim. Biophys. Acta* **360**, 205–216
Stein, Y. & Shapiro, B. (1960) *J. Lipid Res.* **1**, 326–331
Windmueller, H. G. & Levy, R. I. (1968) *J. Biol. Chem.* **243**, 4878–4884