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THE METABOLISM OF FREE FATTY ACIDS AND CHYLOMICRON TRIGLYCERIDES BY THE ISOLATED PERFUSED LIVER OF THE RAT

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When triglycerides or free fatty acids labelled with ^{14}C are injected into the blood stream, a large proportion of the radioactivity can be recovered from the liver (French & Morris, 1957; Bragdon & Gordon, 1958). It appears, however, that these two forms of lipid are distributed differently between the tissues following their removal from the blood. This is due largely to differences in the amounts of free fatty acids and triglycerides taken up by the liver and adipose tissue and appears to be related to the nutritional state of the animal (Bragdon & Gordon, 1958).

The injection of heparin intravenously increases the rate at which chylomicron triglycerides are removed from the blood stream and the rate at which they are oxidized to CO_2 by the tissues (French & Morris, 1958). It is thought that this effect of heparin is due to the release of a lipolytic enzyme (clearing factor) into the blood stream which causes intravascular hydrolysis of triglycerides (Robinson & French, 1957). The fatty acids released by hydrolysis leave the circulation very rapidly (Havel & Fredrickson, 1956).

Morris & French (1958) studied the uptake and metabolism of chylomicron fat by the isolated perfused liver of the rat. They found that the liver took up labelled chylomicron triglycerides and oxidized them to CO_2 when there was no apparent hydrolysis occurring in the perfusate. When the triglycerides were hydrolysed by pre-incubating the chylomicron fat with perfusate which contained clearing factor, the subsequent rate of uptake and oxidation of the fat by the liver was increased. This finding suggested that free fatty acids were taken up by the liver and oxidized to CO_2 more readily than triglyceride fatty acids. Experiments by Hillyard, Cornelius & Chaikoff (1959) have also shown that free fatty acids were taken up by the perfused liver more rapidly than triglycerides.

The form in which long-chain fatty acids are carried in the blood may play an important part in determining the mechanism by which the fatty

acids are removed from the circulation and also in determining their subsequent distribution and fate in the tissues. Experiments designed to compare the uptake and oxidation of fat in the form of chylomicron triglycerides and free fatty acids by the isolated perfused rat's liver are described in this paper. Some observations have also been made on the intermediary metabolism of free fatty acids and triglycerides in the liver and an attempt has been made to assess the extent to which these substances are retransported in the blood following their uptake by the liver.

METHODS

Animals. Rats of both sexes were used for all the experiments. They were an albino strain bred and housed at the laboratory. The livers for perfusion were obtained from two groups of animals with body weights between 207 and 242 g; those designated 'fed livers' were obtained from rats fed on a diet of rat nuts *ad libitum* up to the time of the experiments, while those designated 'starved livers' were obtained from rats which were given no food for 24 hr before the experiments. An analysis of variance of the body weights of the rats used showed no significant difference between the starved and fed groups or between the males and the females. There were, however, significant differences between the weights of the livers taken from the fed and starved groups (variance ratio 18:9, $P < 0{\cdot}001$). The livers from the fed rats had a mean weight of 6·79 g, while the mean weight of the livers taken from the starved rats was 5·44 g.

Liver perfusions. The apparatus and the perfusion technique have been described previously (Morris, 1960). All perfusions were carried out at 38° C and a pressure of 15 cm H_2O. The perfusion fluid was made up of 50 ml. rats' blood, 25 ml. electrolyte solution, 25 ml. bovine albumin solution (Armour), 5 ml. amino acid supplement and 5 ml. phosphate buffer. The composition of these solutions has been described (Morris, 1960). The rat's blood was collected on to 30 mg dry powdered heparin (Evans) and filtered through gauze to remove any clots. The amino acid supplement was added to the perfusion to give an amino acid content in the perfusate similar to that normally found in rat's plasma (Henderson, Schurr & Elvehjem, 1948). The phosphate buffer was included to minimize changes in the pH of the perfusate when equilibrated against the O_2 gas phase (Morris, 1960). The haematocrit value of the final perfusion fluid varied between 19 and 25 %.

Labelled substrates. The ^{14}C-labelled fatty acids were used in the experiments either in the form of chylomicron triglycerides or as free fatty acids bound to albumin. ^{14}C-labelled palmitic acid and ^{14}C-labelled glyceryl tripalmitate were obtained from the Radiochemical Centre, Amersham. The palmitic-acid–albumin complex was prepared by evaporating a solution of the fatty acid in benzene to dryness and then neutralizing the fatty acid with 0·01 N-NaOH solution. The solution was heated and rat serum added rapidly to the mixture. Electrophoretic analysis of the fatty-acid–serum mixture showed that at least 92 % of the total radioactivity was bound to the albumin.

Labelled chylomicron triglycerides were obtained by feeding 20 μc ^{14}C-labelled glyceryl tripalmitate dissolved in 1 ml. olive oil to rats in which thoracic duct fistulae had been established previously (French & Morris, 1957). The distribution of radioactivity in samples of chyle collected in this way showed that 90–94 % of the total activity was present as triglycerides. Between 3 and 6 % of the activity was present as phospholipids and between 2 and 4 % as free fatty acids.

The fatty acids of the ^{14}C-tripalmitin–olive-oil mixture and of samples of radioactive chyle were converted to their methyl esters with boron trifluoride as a catalyst. The methyl esters were separated on a diethylene glycol succinate column at 188° C. A hydrogen flame ioniza-

tion detector and an automatic printing integrator were used to record and measure the various peaks. The fractions were identified against quantitative mixtures of samples of pure methyl esters of fatty acids (Applied Science Laboratories, Pa.). The relative amounts of the main fatty acids present in the fed olive oil and in the chylomicron fat are given in Table 1.

Labelled palmitic acid was added to the perfusate in trace amounts to give an initial activity of about 0·1 μc/ml. perfusate. The chylomicron fat was added to give an initial concentration of 1 mg labelled esterified fatty acids/ml. perfusate.

TABLE 1. The relative amounts of the principal fatty acids present in the fed olive oil and in samples of chylomicron fat. The chain length of the various fatty acids and their degree of unsaturation are indicated by the figures heading each column; i.e. 18:1 represents fatty acids with a chain length of 18 C atoms and one double bond

	Percentage of total fatty acids					
	14:0	16:0	16:1	18:0	18:1	18:2
Olive oil sample	—	15·99	2·62	2·26	57·77	21·45
Chylomicron fat						
Sample 1	1·21	18·35	3·65	3·81	51·87	20·54
Sample 2	0·33	16·32	2·83	2·98	55·38	22·17

Sampling procedure. At intervals throughout the experiments samples of perfusate were taken from the portal vein inflow, the hepatic vein outflow and the perfusate reservoir. Each hepatic vein sample was taken immediately after the portal vein sample. The difference in the concentration of radioactivity in the portal vein and hepatic vein samples gave a measure of the movement of the ^{14}C-labelled carboxyl C. The amount of radioactivity removed from the perfusate in a single transit through the liver has been calculated from these measurements.

Measurement of radioactivity. The activity in the samples of perfusate was measured by plating 0·2 ml. amounts directly on polythene planchets and counting with a thin end-window G–M tube. The activity in the other fractions was measured both by direct plating and by plating as $BaCO_3$ after converting the samples to CO_2 by the method of Van Slyke & Folch (1940). The activity in each sample was corrected for self-absorption to zero mass.

Collection and analysis of CO_2. The CO_2 produced by the liver was collected in scrubbing towers containing N-NaOH solution which was initially free of carbonate. The completeness of absorption of CO_2 was checked with a tower containing lime water. The NaOH towers were changed at half-hourly intervals and the CO_2 precipitated with an excess of $BaCl_2$ in stoppered tubes. The tubes were centrifuged at 1500 rev/min for 10 min and the alkali decanted. The $BaCO_3$ precipitate was washed three times in CO_2-free distilled water and a measured excess of N-HCl solution was added and the CO_2 liberated. The residual acid was back-titrated with N-NaOH solution, screened methyl orange being used as an indicator. The amount of CO_2 was calculated from the amount of acid used.

Analytical methods. The samples of perfusate were chilled immediately they were collected and centrifuged. The plasma was separated from the cells and kept at 0° C. At the end of the experiment the liver was removed from the apparatus, blotted dry and weighed quickly. The liver was then dropped into liquid air and after freezing it was broken up, transferred to a flask and freeze-dried. The freeze-dried powder was weighed and stored at −20° C under N_2.

Analysis of the perfusate. The following analyses were made on the perfusate plasma:
Total esterified fatty acids by the method of Stern & Shapiro (1953);
Free fatty acids by the method of Dole (1956);
Glucose by the method of Nelson (1944);
Total protein by the method of Gornall, Bardawill & David (1949);
Ketone bodies by the method of Werk, McPherson, Hamrick, Myers & Engel (1955).

Analysis of the liver. Glycogen. Samples of freeze-dried liver weighing about 50 mg were taken into tared centrifuge tubes. One ml. KOH solution 30 g/100 ml. was added and the tubes heated in a boiling water-bath for 30 min. Glycogen was precipitated by adding 1·2 ml. 95 % ethanol and after heating again the tubes were cooled to room temperature and centrifuged. The tubes were drained and the residual alcohol removed by heating for 1–2 min. The glycogen was hydrolysed by heating for 2½ hr with 2 ml. N-H_2SO_4. The solution was neutralized with N-NaOH, phenol red being used as an indicator, and after centrifuging the supernatant was made up to a volume of 10 ml. and a portion taken for sugar analysis according to the method of Nelson (1944).

Isolation of fractions for analysis of radioactivity. Glucose. Samples of glucose were isolated from the perfusate as their phenylosazones according to the method of Feller, Strisower & Chaikoff (1950). 3 ml. samples of perfusate were used and deproteinized with 6 ml. 0·3 N-$Ba(OH)_2$ solution and 6 ml. $ZnSO_4$ solution 5 g/100 ml. 20 mg of carrier glucose and 300 mg sodium acetate were added before heating with 200 mg phenylhydrazine hydrochloride. The phenylosazones were purified by recrystallizing 4 times from hot 50 % ethanol.

Proteins. 0·5 ml. samples of plasma were pipetted into 50 ml. 3:1 ethanol–ether mixture and 0·2 ml. N-sodium acetoacetate, 0·2 ml. N-sodium β-hydroxybutyrate and 0·5 ml. sodium acetate solution 50 g/100 ml. were added as carriers. The mixtures were heated to boiling on a steam-bath and then centrifuged. The supernatants were discarded and the residues re-extracted once with boiling 1:1 chloroform–methanol mixture and three times with trichloracetic acid solution 6 g/100 ml. at room temperature.

Ketone bodies. The ketone bodies were separated from the perfusate by the method of Van Slyke (1917). Denigès precipitates were prepared from 1 ml. samples of plasma after the addition of sodium β-hydroxybutyrate, sodium acetoacetate and sodium acetate as carriers. The precipitates were washed three times with ice-cold distilled water and dried for 1 hr at 110° C.

Plasma lipids. Total lipids were extracted with 3:1 ethanol–ether mixture. The extracts were taken to dryness under N_2 and the residue was extracted with petroleum–ether. The petroleum–ether extract was taken to dryness, and the lipids dissolved in 5 ml. chloroform. The phospholipids were separated from the free fatty acids and triglycerides on silicic acid columns. The free fatty acids and triglycerides were separated by partitioning between alkaline ethanol and petroleum–ether according to the method of Borgström (1952).

Liver glycogen. Approximately 1 g freeze-dried liver was added to 10 ml. KOH solution 30 g/100 ml. and heated in a steam-bath for 1 hr. The digest was cooled and centrifuged and carrier β-hydroxybutyrate, sodium acetoacetate, and sodium acetate were added to the supernatant solution. The glycogen was precipitated by adding 15 ml. 95 % ethanol and heating in a water-bath at 80° C for 5 min. The mixture was allowed to cool for 1 hr and was then centrifuged for 10 min and the supernatant discarded. 15 ml. trichloracetic acid solution 10 g/100 ml. was added to the precipitate and the mixture shaken. The protein precipitate was removed by centrifuging, and the glycogen dissolved in the supernatant was decanted. 25 ml. 95 % ethanol was added to the supernatant and the glycogen precipitated. The glycogen was dissolved in water, heated on a steam-bath and precipitated again with 95 % ethanol. The precipitate was washed successively with 60 % ethanol and a mixture of 95 % ethanol and acetone in equal parts.

Liver lipids. The total lipids present in 1 g samples of freeze-dried liver were extracted with three portions of 25 ml. boiling 2:1 chloroform–methanol mixture. The volume of the extract was reduced and purified according to the method of Folch, Lees & Sloane Stanley (1957). The purified extract was dried under N_2 and the lipids dissolved in 20 ml. petroleum–ether. The lipids in petroleum–ether were then separated into their various classes by gradient elution chromatography on silicic acid columns, according to the method of Hirsch & Ahrens (1958). The weight of the lipids added to the silicic acid columns varied from 155

to 189 mg. The weights of the various fractions were measured by the technique described by Craig, Hausmann, Ahrens & Harfenist (1951). The various lipid fractions were analysed as described by Hirsch & Ahrens (1958).

Sources of error in the experiments. In some experiments the concentration of radioactivity in the perfusate was measured at various times throughout the 4 hr period of perfusion. Samples were taken from the perfusate reservoir, the portal vein inflow and the hepatic vein outflow. In these experiments a total number of thirty-six samples were taken. The volume of perfusate removed by this sampling procedure was approximately 10 ml., about 9 % of the original volume of the perfusate. No corrections were made for this sampling error. Negligible losses of gas occurred from the perfusion system and the amounts of total CO_2 and $^{14}CO_2$ measured were accurate to within ± 5 %. Other analytical techniques were accurate to within ± 5 %.

The performance of the perfused liver over a period of 4 hr depends in some measure on the time taken to remove the liver from the donor rat and to establish the perfusion. It was found that in a series of thirty perfusions, the mean time taken to remove the livers from the donor rats was significantly less for the second 15 experiments than for the first 15 experiments. To eliminate any systematic errors of this nature, all the experiments were randomized.

RESULTS

Changes in the glucose content of the perfusate

No glucose was added to the perfusion fluid at the start of the experiments. The initial concentration of glucose in the perfusate varied between 60 and 80 mg/100 ml. In all the experiments with fed livers the glucose concentration in the perfusate increased by about 400 % by the end of the first hour. There were only small increases when livers from starved rats were used. There was no significant difference between the amount of glucose added to the perfusate by the livers in experiments with chylomicron triglycerides or free fatty acids. An average rate of glycolysis in the perfusate of 11 mg/100 ml./hr occurred when the perfusion fluid was incubated at 38° C (Haft & Miller, 1958).

The hydrolysis of chylomicron triglycerides in the perfusate

Experiments were carried out to test whether the incubation of fatty chyle with the perfusion fluid produced any release of free fatty acids from chylomicron triglycerides. Samples of perfusate were taken before and at various intervals during a perfusion experiment and two 1·0 ml. portions of each sample were mixed with 0·1 ml. fatty chyle and 1 ml. bovine albumin solution 4 g/100 ml. The free fatty acids of one of each pair of mixtures were extracted immediately and their concentration measured. The other samples were incubated for 1 hr at 37° C before the free fatty acids were extracted and measured. The results of this experiment are given in Table 2. It appeared that some free fatty acids were liberated during the incubation period but the amount was small. In other experiments, in which changes in optical density were used as an index of

hydrolysis, no clearing occurred when chyle was added to samples of perfusate and incubated for 1 hr at 37° C.

No increase occurred in the amount of radioactivity in the free fatty acid fraction of the perfusate when radioactive chyle was added to the perfusate and circulated through the apparatus for 3 hr in the absence of the liver.

TABLE 2. The release of free fatty acids *in vitro* when samples of perfusate were incubated with fatty chyle for 1 hr. The reaction mixtures contained 1 ml. perfusate plasma, 1 ml. bovine albumin solution 4 g/100 ml. and 0·1 ml. fatty chyle. The perfusate samples were taken before and during a 3 hr perfusion

Time of sampling after start of perfusion (min)	Period of incubation (min)	F.F.A. concn. (mg/100 ml.)	Change in F.F.A. concn. (mg/100 ml.)
0	0	35·05	−1·45
	60	33·60	
15	0	35·78	+2·36
	60	38·14	
30	0	35·78	+1·82
	60	37·60	
60	0	35·42	+1·45
	60	36·87	
120	0	34·60	+2·81
	60	37·41	
180	0	35·48	+0·73
	60	36·21	

The uptake and oxidation of chylomicron triglycerides by the perfused liver in the absence of added heparin

Some experiments were carried out initially to test the metabolism of chylomicron fat by the perfused liver in the absence of any added heparin. The ^{14}C-labelled chylomicrons were obtained from radioactive chyle that was collected and allowed to clot. The perfusion fluid for these experiments was made up with defibrinated rat's blood and no heparin was injected into the rats from which the livers were taken. In 3 experiments with fed livers carried out in this way, 70, 60 and 61 % of the labelled chylomicron fat was taken up in 4 hr. Radioactive CO_2 was excreted from the first half hour and 1·60, 2·02 and 1·73 % of the chylomicron fat was oxidized to CO_2 in 4 hr.

These results showed that the perfused liver extracted chylomicron fat from the perfusate and oxidized it to CO_2 in the absence of added heparin. However, the injection of heparin into the rat immediately before removing the liver gave more satisfactory preparations. All the subsequent experiments were carried out by using powdered heparin as an anticoagulant and injecting heparin into the liver donor.

The uptake of labelled triglycerides and free fatty acids from the perfusate

When ^{14}C-labelled free fatty acids were added to the perfusate, it was found that about 80 % of them were removed in the first hour. By the end of 4 hr only about 5 % of the initial activity was left in the free fatty acid fraction of the perfusate. In experiments with fed livers the total radioactivity in the perfusate rose after the first 60–90 min indicating that the ^{14}C-label was accumulating in the perfusate. By this time labelled esterified fatty acids appeared and by the end of the 4th hr a large proportion of the residual activity in the perfusate was in the form of neutral

TABLE 3. The distribution (%) of the residual radioactivity in the lipid fraction of the perfusate at various intervals throughout the perfusion. Experiments were carried out with livers from fed and starved rats perfused with (^{14}C)-palmitic acid or ^{14}C-labelled chylomicron triglycerides. The results given are the means and standard errors for three experiments in each group

Experiment		Time after start of perfusion (min) 0	60	120	180	240
Fed livers perfused with free fatty acids	In F.F.A.	100	85·4 ± 3·3	56·3 ± 1·6	32·3 ± 1·4	23·2 ± 1·5
	In triglycerides and phospholipids	0	14·6 ± 3·3	43·7 ± 1·6	66·6 ± 1·4	76·8 ± 1·5
Starved livers perfused with free fatty acids	In F.F.A.	100	83·4 ± 1·5	63·2 ± 3·9	54·5 ± 10·0	58·9 ± 12·3
	In triglycerides and phospholipids	0	16·6 ± 1·5	36·4 ± 3·9	45·2 ± 10·0	41·2 ± 12·3
Fed livers perfused with chylomicrons	In F.F.A.	2·5 ± 0·9	24·7 ± 6·7	33·5 ± 4·7	27·5 ± 3·7	18·2 ± 4·6
	In triglycerides and phospholipids	97·5 ± 0·9	75·3 ± 6·7	66·5 ± 4·7	72·5 ± 3·7	81·8 ± 4·6
Starved livers perfused with chylomicrons	In F.F.A.	3·2 ± 0·6	19·0 ± 4·8	31·3 ± 5·5	29·9 ± 5·4	29·8 ± 6·9
	In triglycerides and phospholipids	96·7 ± 0·6	81·0 ± 4·8	68·7 ± 5·5	70·1 ± 5·4	70·2 ± 6·9

glycerides. In the experiments with fed livers the concentration of total esterified fatty acids in the perfusate had doubled by the end of the perfusion. It was assumed that these fatty acids had been retransported from the liver. In experiments with starved livers the concentration of esterified fatty acids in the perfusate changed only slightly. The amount of ^{14}C-activity in the perfusate in the form of esterified fatty acids was higher in fed livers than in starved livers at the end of the 4 hr perfusion (Table 3).

In the experiments with chylomicron triglycerides the labelled fat was removed from the perfusate more slowly. A mean of 75 % was removed in 4 hr by starved livers and a mean of 62 % by fed livers. The concentration of total esterified fatty acids in the perfusate did not change significantly in the experiments with fed livers, even though the ^{14}C-labelled triglycerides

were removed effectively. In the experiments with starved livers the concentration of esterified fat in the perfusate fell by about 65 mg/100 ml. The distribution of radioactivity between the free and esterified fatty acid fractions at various times throughout these experiments is shown in Table 3.

The changes which occurred in the concentration of free fatty acids in the perfusate are shown in Fig. 1. The most striking changes were seen in the perfusions with chylomicron triglycerides. In these experiments,

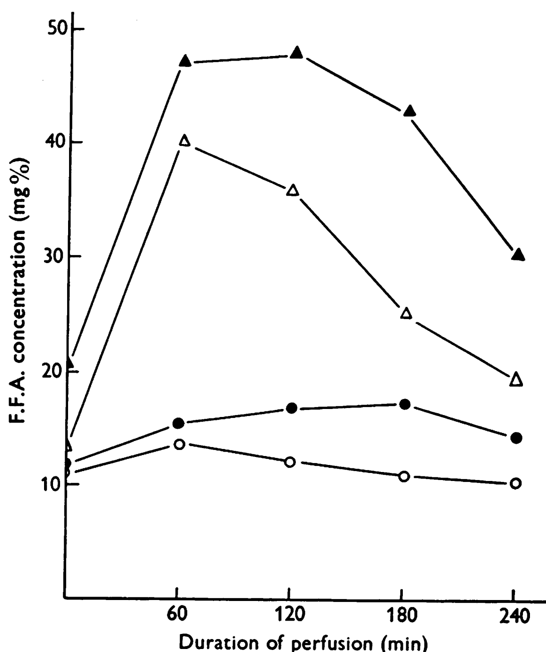

Fig. 1. The changes in the concentration of free fatty acids in the perfusate. The results are means of paired experiments. ● Fed livers perfused with [^{14}C]-palmitic acid. ○, Starved livers perfused with [^{14}C]-palmitic acid. ▲ Fed livers perfused with chylomicron triglyceride. △ Starved livers perfused with chylomicron triglyceride.

the concentration of free fatty acids in the perfusate increased rapidly to a maximum between the 1st and 2nd hours. In livers perfused with free fatty acids, although it has been seen that labelled free fatty acids were removed from the perfusate very rapidly, there was little change in their concentration in the perfusate. This suggested that a rapid exchange occurred between the free fatty acids in the perfusate and fatty acids in the liver.

The increase in the concentration of free fatty aids in the perfusate which occurred in experiments with labelled chylomicrons was accom-

panied by a rapid increase in radioactivity in this fraction. In view of the slow rate at which fatty acids were released from chylomicron triglycerides incubated with perfusate *in vitro* it seemed likely that some of the free fatty acids were added to the perfusate during the passage of the chylomicron fat through the liver. If, however, the behaviour of the labelled palmitic acid is taken as an index of the behaviour of the other long-chain fatty acids in the chylomicron triglycerides, it appeared that a part of the increase in free fatty acids in the perfusate came from sources other than the labelled chylomicron fat. The specific activity of the increment in free fatty acids appearing in the perfusate compared with the specific activity of the fatty acids in the chylomicron triglycerides was always less than 1 (Table 4). This suggested that the labelled free fatty acids in the perfusate were diluted with unlabelled fatty acids coming from the liver.

TABLE 4. The specific activity of the increment of free fatty acids in the perfusate relative to the specific activity of the fatty acids of the added chylomicron triglycerides. The relative specific activity was calculated as (counts/m-mole/increment of F.F.A. in perfusate)/(counts/m-mole added chylomicron fatty acids). The mean results are given for 4 experiments in each group, together with their standard errors

Time sample was taken (min)	Relative specific activity	
	Fed livers	Starved livers
60	0·57 ± 0·06	0·43 ± 0·02
120	0·62 ± 0·07	0·68 ± 0·04
180	0·57 ± 0·02	0·68 ± 0·13
240	0·45 ± 0·05	0·75 ± 0·12

The uptake and oxidation of free fatty acids in the presence of chylomicron triglycerides

Some experiments were carried out to study the rate of uptake and oxidation of ^{14}C-labelled palmitic acid in the presence of chylomicron triglycerides. Unlabelled chylomicron triglycerides were added to the perfusate at the rate of 4·0 mg/ml., together with trace amounts of ^{14}C-labelled palmitic acid. The uptake and oxidation of the labelled palmitic acid by perfused livers was then followed. The results of these experiments are given in Table 5. The presence of high concentrations of chylomicron triglycerides in the perfusate did not appear to alter either the rate of uptake or the rate of oxidation of the labelled free palmitic acid. There was no evidence from these results that free fatty acids and triglycerides were competing for a common mechanism of removal in the liver.

The efficiency of extraction of labelled triglycerides and free fatty acids by the perfused liver

A measure of the efficiency with which the liver removed chylomicron triglycerides and free fatty acids from the perfusate was obtained from

the differences in the radioactivity of the lipids in the blood taken from the portal and hepatic veins. The efficiency of extraction was measured during the first 20 min of the perfusion before the re-cycling of radioactivity was significant. In perfusions with chylomicron triglycerides, about 8 % of the labelled fat was extracted in a single passage through the liver. A much greater proportion of labelled palmitic acid was extracted as it passed through the liver. In the first 20 min of the perfusions the extraction efficiency for the labelled palmitate varied between 30 and 50 %. There

TABLE 5. The uptake and oxidation of free ^{14}C-labelled palmitic acid in the presence of unlabelled chylomicron triglycerides. The experiments were carried out on fed livers perfused at 15 cm pressure and 38° C for 4 hr. The control perfusion had no chylomicron triglycerides added. The specific activities of the CO_2 are given as the mean of 8 measurements taken at half-hourly intervals throughout the perfusion. The same amount of radioactivity was added to the perfusate in each experiment

Experiment no.	Remaining in perfusate (%)	Recovered in liver (%)	Expired as CO_2 (%)	Mean specific activity of CO_2 (counts/min/m-mole)
1	27	64·7	4·8	9,411
2	30	54·8	9·8	17,344
3	35	56·0	7·3	16,212
Control	27	63·9	5·2	9,569

was no significant difference in the efficiency with which starved or fed livers took up either chylomicron triglycerides or free fatty acids (variance ratio 1·1) but in these series of experiments livers from female rats extracted fat more efficiently than livers from male rats (variance ratio 21·5, $P < 0{\cdot}001$). This was true for both triglycerides and free fatty acids.

The distribution of ^{14}C activity in the liver

Liver lipids. At the end of the experiments the livers were separated into their various lobes. The lipids in each lobe were extracted separately and the radioactivity in the extracts was measured. It was found that there was no significant difference between the amount of radioactivity recovered in the right lateral + caudate, central or left lateral lobes when comparisons were made on a basis of their wet weights. The amount of radioactivity recovered in the papillary lobe, however, was significantly higher than in the other lobes on a wet weight basis (Table 6).

The distribution of the radioactivity between the various lipid fractions in the liver was determined by silicic acid chromatography. The results of 8 experiments in which these separations were made are given in Table 7. A typical elution diagram for an experiment is shown in Fig. 2. In all experiments with free fatty acids or chylomicron triglycerides, more than 90 % of the total activity recovered in the liver lipids was present in the triglyceride and phospholipid fractions. The percentage of the total activity

in the liver lipids recovered as free fatty acids varied between 0·4 and 1·7 % and there was no significant difference between experiments with palmitic acid or chylomicron triglycerides. Significantly more radioactivity was recovered in the triglyceride fraction of the liver lipids in experiments with chylomicrons. The mean percentage recovered as triglycerides was 37·6 for the chylomicron experiments and 12·6 for the palmitic acid experiments.

Most of the radioactivity was recovered in the liver phospholipids. The lecithin fraction contained 41–56 % of the total activity while the cephalin fraction contained 10–33 %. The cholesterol, cholesterol ester and sphingomyelin fractions each contained a very small amount of activity. The

TABLE 6. The weights of the various lobes of the liver after 4 hr perfusion and the amount of radioactivity recovered in the lipids extracted from each lobe. Mean results together with their standard errors are given for 9 experiments. The livers were taken from fed rats and were perfused at 38° C and a pressure of 15 cm H_2O. Radioactive chylomicron fat was used in each experiment

Liver lobe	Final wt. (g)	Radioactivity/ g wet wt.
Right lateral + caudate	2·17 ± 0·07	9,492 ± 1200
Centrals	3·55 ± 0·11	11,143 ± 628
Left lateral	2·96 ± 0·08	10,203 ± 517
Papillary	0·80 ± 0·04	12,896 ± 597

TABLE 7. The distribution of ^{14}C-activity between the various lipid fractions isolated from livers perfused for 4 hr with (^{14}C)-palmitic acid and (^{14}C)-chylomicron triglycerides. Results are given for separate experiments

	Activity recovered in the liver lipids (%)							
Experiment	Triglycerides	F.F.A.	Cholesterol	Cholesterol esters	Monoglycerides	Diglycerides	Cephalin	Lecithin
Starved liver perfused with F.F.A.	8·69	0·51	0·31	0·30	0·48	0·44	33·15	54·46
Starved liver perfused with F.F.A.	12·01	0·62	0·24	0·26	0·50	0·94	27·72	56·20
Fed liver perfused with F.F.A.	15·76	1·70	0·10	0·27	0·43	1·71	24·31	51·72
Fed liver perfused with F.F.A.	14·02	0·64	0·21	0·34	0·32	1·54	23·68	56·17
Starved liver perfused with chylomicron triglyceride	34·98	0·38	0·0	0·40	0·41	0·93	12·38	42·64
Starved liver perfused with chylomicron triglyceride	31·20	0·40	0·34	0·21	0·28	1·26	13·84	54·09
Fed liver perfused with chylomicron triglyceride	45·69	0·61	0·30	0·35	0·39	2·09	9·71	41·33
Fed liver perfused with chylomicron triglyceride	38·34	0·42	0·21	0·42	0·50	1·32	12·71	43·77

monoglyceride and diglyceride fractions were labelled; the activity of the diglycerides was significantly greater than that of the monoglycerides.

Liver glycogen and protein. The activity recovered from the liver as glycogen and protein was less than 0·5 % of the total activity in the liver. The mean amount of glycogen in livers obtained from fed rats after 4 hr perfusion was 107 mg/g dry liver; in livers from starved rats it was 6·6 mg/g dry liver. There was no significant difference in the amount of glycogen in livers perfused with free fatty acids or chylomicron triglycerides. In all experiments the amount of radioactivity in the perfusate glucose and in the perfusate plasma proteins was less than 0·2 % of the original activity.

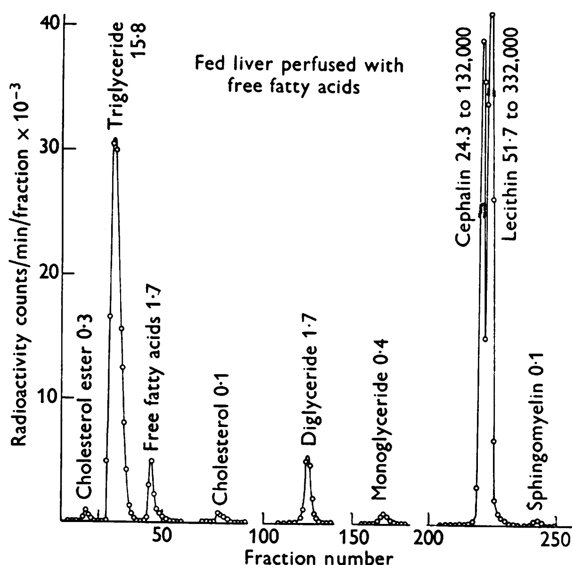

Fig. 2. The distribution of ^{14}C-activity between the various lipid fractions isolated from the liver of a fed rat after 4 hr perfusion with free [^{14}C]-palmitic acid.

The oxidation of labelled triglycerides and free fatty acids to CO_2 and ketone bodies

The livers from fed rats produced significantly more total CO_2 than did the livers from starved rats (fed, 6·01 m-mole/4 hr; starved, 4·66 m-mole/4 hr; variance ratio, 28·3, $P < 0{\cdot}001$). There was no significant difference in total CO_2 production between livers from male and female rats (5·44 and 5·23 m-mole/4 hr). The mean amounts of labelled fat oxidized to CO_2 are given in Table 8, together with the summary of the analysis of variance.

Livers from starved rats oxidized significantly more free fatty acids and triglycerides to CO_2 than did livers from fed rats and this was true for

livers from both males and females. A significantly greater proportion of the free [^{14}C]-palmitic acid was oxidized than the labelled triglycerides. There was a highly significant interaction between starvation and the type of fat presented to the liver for oxidation ($P < 0.001$); starvation increased the amount of free fatty acids oxidized to CO_2 by about 300 %, whereas the oxidation of triglycerides was increased by only about 30 %.

TABLE 8. Summary of the analysis of variance of the percentage of (^{14}C)-free palmitic acid and chylomicron triglycerides oxidized to CO_2 in 4 hr. Livers were taken from starved and fed rats of both sexes. Mean results are given for groups of 6 experiments

		Starved	Fed	Male	Female
Mean percentage oxidized	Livers perfused with chylomicron triglycerides	4·19	3·04	3·41	3·82
	Livers perfused with free fatty acids	18·00	6·27	12·49	11·79

Source of variation	D.F.	M.S.	F	Signif. (P)
Starved *v.* fed (X)	1	249·0	56·5	< 0·001
Male *v.* female (Y)	1	0·1	< 1	N.S.
F.F.A. *v.* triglyceride (Z)	1	436·1	99·1	< 0·001
Interactions				
XY	1	12·4	2·8	N.S.
XZ	1	168·1	38·2	< 0·001
YZ	1	1·9	< 1	N.S.
XYZ	1	3·1	< 1	N.S.
Error. res. S.S.	16	4·4	—	—

Figure 3 shows the percentage of the labelled fat oxidized to CO_2 in each half hour during the perfusion for experiments with [^{14}C]-palmitic acid and chylomicron triglycerides. The livers from starved rats oxidized increasing amounts of triglycerides as the perfusion proceeded and maximum rates of oxidation occurred in the last half hour. Livers from fed rats oxidized most labelled triglyceride during the third hour. In experiments with [^{14}C]-palmitic acid the maximum rate of oxidation occurred at about 1 hr with livers from fed rats and at about 2 hr with livers from starved rats.

There was a significant increase in the concentration of ketone bodies in the perfusate in all experiments. The smallest changes occurred in experiments with fed livers. Concentrations of ketones in the perfusate up to 50·6 mg/100 ml. were reached in experiments with starved livers perfused with chylomicron triglycerides. It was found that fed livers perfused with [^{14}C]-palmitic acid oxidized 2–3 times more fat to CO_2 than to ketones. Starved livers on the other hand oxidized approximately the same amount of palmitic acid to CO_2 as to ketones. In experiments with triglycerides the starved livers oxidized 3–5 times more fat to ketones than to CO_2 (Table 9).

In order to compare the specific activities of the ketone bodies with the

specific activities of the free fatty acids and triglyceride fatty acids in the perfusate, a theoretical maximum yield of 4 moles acetoacetic acid was assumed to come from each mole fatty acid. The specific activity of the ketone bodies derived from a fatty-acid molecule labelled in the carboxyl carbon was therefore diluted fourfold. It has been demonstrated by Chaikoff, Goldman, Brown, Dauben & Gee (1951) that the ratio ^{14}CO to $^{14}COOH$ in acetoacetic acid formed from the oxidation of palmitic acid-1-^{14}C is 1.

As only the activity in the carbonyl carbon of acetoacetate was measured in the Denigès precipitates, the specific activities of the ketone bodies were

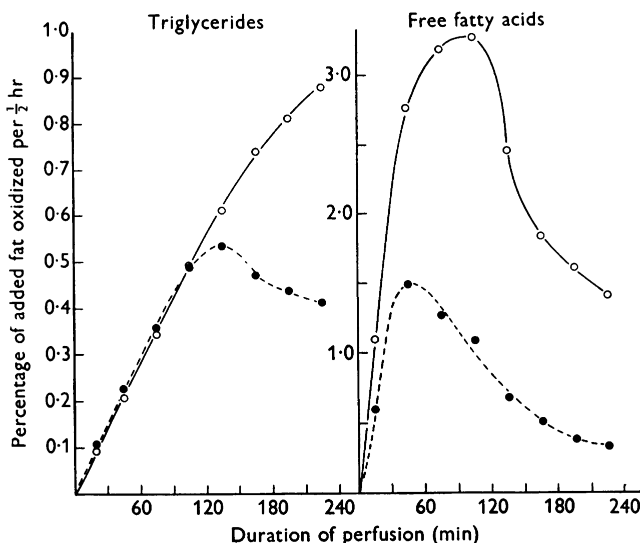

Fig. 3. The percentage of the fatty acids present initially in the perfusate which was oxidized to CO_2 during the perfusion. The results are the means of 6 experiments in each group. ● Livers from fed rats. ○ Livers from starved rats.

TABLE 9. The oxidation of free (^{14}C)-palmitic acid and (^{14}C)- chylomicron triglycerides by the perfused liver. The mean percentages oxidized to CO_2 and to ketones are given for 4 experiments in each group together with their standard errors

Experiment	Oxidized (%)		
	To CO_2	To ketones	Total
Fed livers perfused with [^{14}C]-palmitic acid	5·05 ± 0·34	2·04 ± 0·42	7·09 ± 0·44
Starved livers perfused with [^{14}C]-palmitic acid	21·41 ± 5·58	20·81 ± 2·11	42·22 ± 8·72
Fed livers perfused with chylomicron triglycerides	2·48 ± 0·40	0·89 ± 0·24	3·37 ± 0·37
Starved liver perfused with chylomicron triglycerides	4·27 ± 0·96	16·41 ± 3·17	20·68 ± 3·83

multiplied by 2 to correct for the loss of labelled carboxyl carbon. Calculations of the relative specific activity of the ketone bodies in the perfusate to the specific activity of the free fatty acids and esterified fatty acids in the perfusate at the start of the experiments are given in Table 10 together with the concentrations of ketones.

TABLE 10. The specific activity (counts/m-mole fatty acid equivalent) and concentration of ketone bodies produced by the liver compared with the specific activities of the free fatty acids and chylomicron fatty acids (counts/m-mole) in the perfusate at the start of the experiment. Mean results are given for 4 experiments together with their standard errors

	Starved		Fed	
Time (min)	Relative specific activity	Concentration (mg/100 ml.)	Relative specific activity	Concentration (mg/100 ml.)
		[^{14}C]-Palmitic acid experiments		
0	0	$2{\cdot}13 \pm 0{\cdot}17$	0	$3{\cdot}21 \pm 0{\cdot}12$
60	$0{\cdot}30 \pm 0{\cdot}07$	$10{\cdot}08 \pm 1{\cdot}49$	$0{\cdot}12 \pm 0{\cdot}01$	$4{\cdot}82 \pm 0{\cdot}13$
120	$0{\cdot}27 \pm 0{\cdot}05$	$12{\cdot}50 \pm 2{\cdot}40$	$0{\cdot}04 \pm 0{\cdot}01$	$9{\cdot}77 \pm 2{\cdot}50$
180	$0{\cdot}09 \pm 0{\cdot}01$	$15{\cdot}67 \pm 0{\cdot}38$	$0{\cdot}01 \pm 0{\cdot}01$	$15{\cdot}79 \pm 1{\cdot}52$
240	$0{\cdot}06 \pm 0{\cdot}01$	$22{\cdot}29 \pm 1{\cdot}61$	$0{\cdot}01 \pm 0{\cdot}01$	$18{\cdot}66 \pm 2{\cdot}01$
		Chylomicron triglyceride experiments		
0	0	$1{\cdot}95 \pm 0{\cdot}73$	0	$2{\cdot}64 \pm 0{\cdot}41$
60	$0{\cdot}57 \pm 0{\cdot}07$	$8{\cdot}30 \pm 1{\cdot}80$	$0{\cdot}01 \pm 0{\cdot}01$	$2{\cdot}94 \pm 0{\cdot}32$
120	$0{\cdot}98 \pm 0{\cdot}05$	$13{\cdot}00 \pm 0{\cdot}50$	$0{\cdot}26 \pm 0{\cdot}02$	$5{\cdot}44 \pm 0{\cdot}31$
180	$0{\cdot}67 \pm 0{\cdot}01$	$36{\cdot}61 \pm 2{\cdot}21$	$0{\cdot}37 \pm 0{\cdot}03$	$9{\cdot}03 \pm 1{\cdot}01$
240	$0{\cdot}60 \pm 0{\cdot}05$	$44{\cdot}31 \pm 6{\cdot}32$	$0{\cdot}16 \pm 0{\cdot}01$	$16{\cdot}64 \pm 1{\cdot}27$

An estimate of the proportion of the ketone bodies derived from the oxidation of lipid taken up from the perfusate was made by assuming that the oxidation of palmitic acid was an index of the oxidation of most of the other fatty acids present. In experiments with free [^{14}C]-palmitic acid, the maximum specific activity of the ketone bodies occurred by the end of the first hour and the level reached in experiments with starved livers was about three times higher than in the experiments with fed livers. At the end of the first hour about 30 % of the ketone bodies had come from the oxidation of free fatty acids taken up from the perfusate. The early peak specific activity of the ketone bodies coincided with a similar early peak specific activity of the expired CO_2 found when livers were perfused with labelled free fatty acids. In the experiments in which starved livers were perfused with chylomicron triglycerides the specific activity of the ketones in the perfusate had almost reached the initial specific activity of the perfusate esterified fatty acids by the end of the second hour; after this the specific activity of the ketones decreased and at the end of the perfusion was about 60 % of its peak value. The specific activities of the ketones were significantly higher in experiments with starved livers than in experiments with fed livers, and this finding was consistent with a higher rate of oxidation of fat to CO_2 by starved livers.

DISCUSSION

In the interpretation of some of the results concerning the metabolism of free fatty acids and chylomicron triglycerides the behaviour of the labelled palmitic acid has been equated with the behaviour of other long-chain fatty acids. Although there are certain differences in the metabolism of various fatty acids (cf. Dittmer & Hanahan, 1959; Simpson-Morgan & Morris, 1962), it appears that palmitic, oleic and linoleic acids incorporated into chylomicron triglycerides are taken up and oxidized by the perfused rat liver at essentially the same rate: this also appears to be true for the intact rat (Simpson-Morgan & Morris, 1962). In the experiments reported in this paper about 20% of the fatty acids present in the chylomicron triglycerides were palmitic acid.

The differences in the rate of removal and subsequent metabolism of free fatty acids and chylomicron triglycerides suggest that the fate of long-chain fatty acids in the body will depend in a large measure on the form in which they are transported in the blood. Bragdon & Gordon (1958) have shown that the nutritional state of the animal also plays a part in determining the fate of injected fat. When chylomicron triglycerides or free fatty acids were injected intravenously into rats, their subsequent distribution between tissues varied. It was found that most of the radioactivity was recovered from the livers of fasting rats injected intravenously with labelled chylomicrons, whereas in rats fed on carbohydrate most of the radioactivity was recovered in the fat depots. Heimberg, Weinstein, Klausner & Watkins (1962) have shown differences in the rate of uptake and release of triglycerides by perfused livers from fed and starved rats and they related these differences to the effects of hormones on the liver. The results of the present paper also show that the nutritional state of the animal from which the liver is taken influences the subsequent retransport and oxidation of fat. In the case of livers from fed rats more retransport and less oxidation of both triglycerides and free fatty acids occurred.

It seems that the level of fat metabolism and the availability of alternative oxidative substrates determine, at least in part, the extent to which retransport occurs. The retransport of intravenously injected palmitic acid into the blood stream has been studied in the intact rat (Laurell, 1959) and in the rat's liver perfused *in situ* (Stein & Shapiro, 1959). These experiments showed that following the uptake of [^{14}C]-palmitic acid the liver retransported labelled triglycerides into the blood stream. It has also been shown by Kay & Entenman (1960) that the isolated perfused liver of the rat is able to add particulate fat to the perfusate, and Heimberg *et al.* (1962) have demonstrated the simultaneous uptake and retransport of triglycerides into the perfusate by the liver. These results have been

confirmed in the present paper where the extent of this retransport by fed livers perfused with chylomicron fat was of the order of 150 mg/4 hr.

The appearance of labelled free fatty acids in the perfusate in experiments with chylomicron fat indicated that retransport of fat from the liver occurred in this form as well. It was not possible, however, in these experiments to be certain that some of the labelled free fatty acids did not originate in the perfusate itself.

The efficiency with which the liver extracts free fatty acids and triglycerides gives a measure of the part this organ may play in removing fat from the blood stream. It can be calculated that extraction efficiencies of the order of those measured in the perfused liver experiments could account for the removal of practically all the chylomicron fat which enters the circulation following a fat meal (Borgström & Jordan, 1959; Morris, 1963). However, chylomicrons entering the blood from the thoracic duct pass through the pulmonary and the systemic circulations before entering the liver and other tissues are known to take up chylomicron fat from the circulating blood (French & Morris, 1958).

The removal of free fatty acids from the perfusate appears not to be related to the removal of chylomicron triglycerides. Under physiological conditions both forms of fat will be present in the circulation. It has been shown (Morris, 1963) that the efficiency with which the liver extracts triglycerides falls off as the concentration of fat in the circulation rises. High levels of triglycerides in the blood, however, appear to have little effect on the mechanisms concerned with the extraction of free fatty acids from the circulation, and this suggests that the uptake of free fatty acids and triglycerides may occur in different ways.

Triglyceride fatty acids and free fatty acids were rapidly incorporated into phospholipids and triglycerides once they were taken up by the perfused liver. Only a small amount of activity was recovered as free fatty acids. Whereas it is probable that the size of the free fatty acid pool in the liver is restricted within narrow limits, the pool of esterified fatty acids represents a distensible compartment into which fatty acids are rapidly incorporated for storage or subsequent mobilization. The extent of incorporation of fat into liver triglycerides and phospholipids is related to the amount of fat being transported through the liver and to the metabolic requirements of the liver itself.

It appeared likely that some of the differences in metabolism between starved and fed livers were due to differences in the glycogen content of the livers. In livers replete with glycogen much more substrate was available for metabolism and the rate and extent of oxidation of both free fatty acids and triglycerides were significantly lower than with starved livers. There was also a smaller production of ketone bodies by these

livers. When the livers contained little glycogen, however, oxidative metabolism was confined largely to the break-down of fat. This interpretation is consistent with the finding that the respiratory quotient of perfused livers taken from fed rats is close to 1, whereas livers from starved rats have a respiratory quotient of 0·76 (Mishkel & Morris, 1963). In the chylomicron triglyceride experiments, where large amounts of substrate were being taken up by the liver, a significant proportion of the liver's total metabolism was provided by the oxidation of labelled triglycerides as they were extracted from the perfusate.

SUMMARY

1. 1-^{14}C-palmitic acid, both in the free form and incorporated into chylomicron triglycerides, was extracted from the perfusate and oxidized to CO_2 and ketone bodies by the rat's isolated perfused liver. Free fatty acids were taken up by the liver and oxidized to CO_2 more rapidly than chylomicron triglycerides.

2. A significant amount of retransport of the ^{14}C label occurred as esterified fatty acids. Some radioactivity was also retransported from the liver as free fatty acids. Retransport of fat was significantly greater in experiments with livers from fed rats than in experiments with livers from starved rats.

3. The maximum rate of oxidation of free fatty acids occurred in the first 60–90 min of perfusion, whereas the maximum rate of oxidation of chylomicron triglycerides occurred between 180 and 240 min. Livers from starved rats oxidized significantly more free fatty acids and triglycerides than did livers from fed rats.

4. There was an increase in the concentration of ketone bodies in the perfusate in all experiments, the maximum levels occurring in experiments with livers from starved rats perfused with chylomicron triglycerides. Livers from starved rats oxidized significantly more fat to ketones than to CO_2.

5. At the end of the 4 hr perfusion more than 90 % of the ^{14}C activity in the liver was recovered as esterified fatty acids. More activity was present in phospholipids than in triglycerides. The amount of activity recovered as triglycerides was significantly lower in the free fatty acid experiments than in the chylomicron experiments.

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