J. Lipid Research, October 1963
Volume 4, Number 4

Fate of palmitate and of linoleate perfused through the isolated rat liver at high concentrations

PAUL J. NESTEL* and DANIEL STEINBERG

Laboratory of Metabolism,
National Heart Institute,
National Institutes of Health,
Bethesda 14, Maryland

[Manuscript received February 25, 1963; accepted June 19, 1963.]

SUMMARY

When rat livers were perfused with red cell-albumin solutions containing high concentrations of free fatty acids (FFA), the FFA were very rapidly taken up. Concomitantly, the glyceride content of the liver rose by 25–60% over control values, the absolute increment corresponding roughly to the amount of FFA taken up. The net increase in liver glyceride content was independent of whether palmitate, linoleate, or a combination of the two was used to raise the FFA concentration of the perfusate. The results support the conclusion drawn from earlier in vivo studies that high serum FFA levels can directly contribute to development of fatty liver. When the initial FFA concentration of perfusate was less than 1 μEq/ml, there was no significant increment in the glyceride content of the perfusate. When the initial FFA concentration of the perfusate was high (2–3 μEq/ml), the glyceride content of the perfusate rose significantly during a 90-min perfusion. The changes in the fatty acid pattern of the glycerides in the liver and in the perfusate indicated that the fatty acid added to the perfusate to raise the FFA concentration to high levels was being preferentially utilized for formation of the new glycerides, but the quantitative effects when perfusing with palmitate alone and linoleate alone differed. Perfusion with palmitate markedly increased the percentage of palmitate in liver glycerides but increased only slightly the percentage of palmitate in the perfusate glycerides. Linoleate had less effect on the fatty acid pattern of the liver glycerides and a greater effect on that of the perfusate glycerides. The differences between the metabolic fates of the two fatty acids are consistent with results of in vivo studies reported previously. Studies of utilization of labeled FFA by liver slices showed that relatively more labeled palmitate was incorporated into glycerides and relatively more linoleate into phospholipids. Incorporation into other lipids and conversion to CO_2 were also compared. Livers were perfused with palmitate at low and at high concentration adding palmitate-1-C^{14} as a tracer. It was shown that high FFA concentrations strongly stimulated conversion to $C^{14}O_2$ and incorporation into glycerides but had a less marked effect on incorporation into phospholipids.

It is clear that the metabolic fates of different classes of long-chain fatty acids differ both qualitatively and quantitatively. This is implicit in the rather specific positional distribution of saturated and unsaturated fatty acids in phospholipid and triglyceride molecules, the special fatty acid composition of cholesterol esters, and the well-established difference between saturated and polyunsaturated fatty acids in the diet with regard to their effects on serum lipid levels. Recent studies have demonstrated significant differences in the metabolism of linoleate and palmitate when administered intravenously (to dogs) as albumin complexes of the free fatty acids (FFA) (1, 2). Linoleate was shown to leave the circulation more rapidly than palmitate and to reappear more rapidly and to a greater extent in the triglycerides of circulating low-density lipoproteins (2). A relatively higher turnover rate for linoleate in hepatic triglycerides was observed again when labeled linoleate and palmitate were adminstered to intact dogs in the form of chylomicron triglycerides rich in the respective fatty acids (2). The precise mechanisms responsible for these differences are not understood, and the complexities of tracer studies in intact animals leave a measure of interpretational

* Work done during tenure as a Fellow of Life Insurance Medical Research Fund of Australia and New Zealand. Present address: Department of Medicine, University of Melbourne, Australia.

uncertainty due to the unknown degree of dilution of the tracer and the possible contributions of exchange reactions. One of the purposes of the present studies was to compare the fate of palmitate and linoleate in the isolated perfused liver and thus establish any differences in their metabolism attributable to the liver. By "loading" the perfused liver with high concentrations of these two fatty acids, separately and together, it has been possible to show differences in their metabolism using nonisotopic methods. The fate of labeled palmitate and linoleate in liver slices was also studied, and certain quantitative differences in their metabolism have been observed.

Previous studies have shown that when serum FFA levels in intact dogs are maintained at high levels by constant infusion of norepinephrine into a femoral vein, there is a progressive and marked increase in liver triglyceride concentration (3). It was concluded that the increase in liver triglycerides was probably attributable to a high level of FFA perfusing the liver. In other studies,[1] it has been shown that the rate of synthesis of triglycerides by liver slices incubated with labeled FFA is a function of the medium concentration of FFA, again consistent with a direct effect of FFA concentration on the rate of triglyceride formation in the liver. A second purpose of the present studies was to demonstrate a similar relationship in perfused liver.

MATERIALS AND METHODS

Liver Perfusion Studies. Livers of male Sprague-Dawley nonfasted rats weighing 150 g were perfused by the method of Mortimore (4). The perfusing fluid was prepared from defibrinated blood obtained from nonfasted rats anesthetized with ether. The red blood cells were spun down and, in most studies, washed twice with 0.15 M NaCl to remove adsorbed serum and thus reduce the initial concentration of triglycerides and FFA in the perfusing fluid. The red cells were then diluted to a final packed-cell volume of 20% by the addition of 0.15 M NaCl and a bovine albumin–fatty acid mixture, pH 7.4, containing the FFA under investigation. The final concentration of albumin was 5%. The basic perfusion fluid, prior to the addition of the albumin–FFA mixture, contained FFA at a concentration of approximately 0.15 μEq/ml, representing plasma trapped in the washed red cells. When the red cells were not washed, the initial FFA content was higher, 0.36 μEq/ml. Chromatographically purified palmitic acid, or linoleic acid (Hormel Institute, Austin, Minn.), or both were added in the form of sodium salts bound to bovine serum albumin to raise the FFA concentration of the perfusate as indicated. The perfusing fluid was oxygenated with humidified 95% O_2–5% CO_2 in a rotating spherical flask. The total volume of perfusing fluid was 30–40 ml.

The portal vein of rats under ether anesthesia was cannulated, and delivery of oxygenated perfusing fluid was started at once. The inferior vena cava was then quickly cannulated above the diaphragm and ligated below the liver. The venous return from the vena cava was delivered to the oxygenating reservoir. Since the liver was perfused with blood throughout the brief procedure of cannulation, the experiments were considered to have commenced immediately after cannulation. The livers were perfused at a constant rate of 6 ml/min by means of a Bowman pump. Some slight degree of hemolysis occurred unavoidably. The liver was left in the carcass and the preparation was kept in a 37° incubator during perfusion. The oxygenating flask was operated as a closed system to permit the collection of CO_2. Effluent gas from it was then dispersed through a sintered glass column into 30 ml of 10% KOH.

At the end of each study, the liver was briefly perfused with iced saline. Portions of the liver were homogenized at once and the lipids extracted into chloroform–methanol 2:1 (v/v). The perfusate was centrifuged at 800 × g for 15 min, and aliquots of the plasma were extracted with chloroform–methanol.

The chloroform–methanol was converted into a two-phase system by the addition of 1/5 volume of 0.02 N HCl, acidification being used to insure quantitative extraction of FFA. The lipids in the chloroform phase were separated into three fractions on 1-g columns of 325-mesh silicic acid (Bio-Rad Laboratories, Richmond, Calif.). It was found that cholesterol esters uncontaminated with triglycerides could be eluted with 25 ml 1% diethyl ether in hexane. Triglycerides, partial glycerides, free cholesterol, and FFA were eluted together with 50 ml chloroform; phospholipids were eluted with 20 ml methanol (5). Free fatty acids were extracted from the chloroform fraction with alkaline ethanol and then reextracted into isooctane after acidification (6). Free fatty acids were titrated by Dole's method (7). In the case of the perfusate lipids, hydroxamate-forming ester bonds were determined in aliquots of the glyceride fraction (8). Glyceride concentrations in the liver lipids were determined by first adsorbing phospholipids onto silicic acid, then saponifying and determining glycerol as described by Jover (9).

Methyl esters of fatty acids were prepared by refluxing with 2% H_2SO_4 in methanol for 1 hr at 70° and

[1] Rose, H., M. Vaughan, and D. Steinberg. Unpublished results.

were analyzed by gas-liquid chromatography using a 15% ethylene glycol–adipate chromosorb W column at 180° and an argon ionization detector. Results are presented as uncorrected area percentages determined by triangulation. The linearity of the response of the apparatus was checked periodically using a standard mixture of fatty acid esters of known composition.

Liver Slice Studies. Livers were taken from male rats allowed free access to food. Slices 0.5 mm thick were prepared using a Stadie-Riggs microtome. The slices (approximately 500 mg in each flask) were incubated for 1 hr under O_2 at 37° in 3 ml Krebs-Ringer phosphate buffer, pH 7.4, containing 3% bovine albumin (Armour Pharmaceutical Co., Kankakee, Ill.). The titratable FFA content and the fatty acid composition of the bovine albumin were determined. An appropriate amount of purified linoleate was added to make the final concentrations of palmitate and linoleate in the incubation medium equal (0.11 μEq/ml for each). The final total concentration of FFA in the medium was 0.33 μEq/ml. Either palmitic acid-1-C^{14} (Nuclear Chicago Corp., Chicago, Ill.) or linoleic acid-1-C^{14} (Applied Sciences Laboratories, State College, Pa.) of similar specific activity was added to each of a pair of flasks that contained slices from the liver of the same rat. CO_2 was collected in KOH in a glass center well.

At the end of 60 min, the incubation was stopped by the addition of 0.3 cc of 4 N H_2SO_4 to the medium, and the flasks were shaken for an additional 20 min to ensure complete CO_2 collection. The slices of liver were homogenized and the lipids extracted into chloroform–methanol. The KOH from the center well was quantitatively transferred with several washes of water to a graduated cylinder and diluted to 10 ml. Aliquots of 3 ml were added to 1 g of anthracene crystals (Distillation Products Industries, Rochester, N. Y.), and the $C^{14}O_2$ was measured in a Tricarb liquid scintillation spectrometer (10).

The lipids of the liver were fractionated on silicic acid as described above. Free cholesterol was precipitated with digitonin from an aliquot of the lipids of the chloroform effluent (taken to dryness and redissolved in acetone–ethanol), and the digitonide was dissolved in methanol. Radioactivity was measured in a liquid scintillation spectrometer, using 0.3% diphenyloxazole in toluene as scintillator-solvent.

Fate of Palmitate-1-C^{14} During Perfusions at Low and at High FFA Concentrations. A further set of experiments was performed in which four livers were perfused, two with fluid containing 0.36 μEq/ml of FFA and two with fluid to which palmitate had been added to raise the total FFA concentration to 2.06 μEq/ml. During each perfusion, 20 μc of palmitate-1-C^{14} was added. CO_2 was collected. The liver was then flushed with iced saline, and portions of the liver and aliquots of the perfusion fluid were extracted with chloroform–methanol.

The lipids were separated on silicic acid columns into "neutral lipids" and phospholipids by eluting first with chloroform and then with methanol. Free fatty acids were removed from the "neutral lipid" fraction by Borgström's method (6). Phospholipids were freed from any residual contaminating FFA by adsorption onto silicic acid, which was then washed twice with chloroform and twice with methanol. The chloroform washes were added to the "neutral lipid" fraction prior to extraction of FFA. The methanol washes, containing the phospholipids, were combined.

Radioactivity was measured in CO_2; in aliquots of the FFA, neutral lipids and phospholipids of the perfusate; and in neutral lipids and phospholipids of the liver.

RESULTS

Liver Perfusion Studies

Net Changes of FFA and Glyceride in Perfusion Fluid and in Liver. When livers were perfused with the red cell–albumin solution without additional FFA, somewhat less than one-half of the FFA initially present (0.36 μEq/ml) was taken up in 90 min (Table 1). The concentrations of free palmitate and linoleate in the basic perfusing fluid were similar, each accounting for about 25% of the total. The net uptake was 4 μEq. The glyceride content of the perfusate changed very little. The small increments noted cannot be given much importance since they represent a small percentage increase over the initial glyceride content (18 μEq glyceride fatty acids).

When the initial FFA concentration of the perfusion fluid was increased to high levels (1.79–3.97 μEq/ml) by addition of palmitate, linoleate, or both, it was found that over 80% of the FFA was removed in the course of the infusion. When both fatty acids were added, they were added in equal concentrations, maintaining the relationship found in the basic perfusing fluid. At the highest initial concentration studied (3.97 μEq/ml), the total amount of FFA removed was 115-130 μEq, over 15 μEq/g of liver perfused.

Aliquots of the livers to be perfused were not taken for determination of zero time values to avoid the possibility of leakage during the perfusion. Glyceride levels were measured in the livers of two groups of three control rats killed at the same time as those used in Experiments B and C, respectively. These control

TABLE 1. UPTAKE OF FFA FROM PERFUSING FLUID AND CHANGES IN GLYCERIDE CONTENT OF LIVER AND PERFUSATE AFTER 90-MIN PERFUSIONS OF RAT LIVERS AT DIFFERENT INITIAL FFA CONCENTRATIONS

Expt. No.	Fatty Acid Added to Perfusion Fluid	Total FFA Conc. of Perfusion Fluid		Total FFA Uptake	Net Increase in Total Liver Glyceride Fatty acids*	Net Increase in Total Perfusate Glyceride Fatty Acids
		Initial	Final			
		μEq/ml	*μEq/ml*	*μEq*	*μEq*	*μEq*
Control	None	0.36	0.20	4	—	2
Control	None	0.36	0.20	4	—	1
A-1	Linoleate	1.82†	0.27	47	—	4
A-2	Linoleate	1.82	0.27	47	—	10
A-3	Palmitate	1.79	0.31	45	—	9
A-4	Palmitate	1.79	0.31	45	—	6
B-5	Linoleate	2.10	0.32	54	40	4
B-6	Linoleate	2.10	0.25	56	40	3
B-7	Palmitate	2.35	0.29	60	50	3
B-8	Palmitate	2.35	0.25	63	49	3
C-9	Equal amounts	3.97	0.32	130	108	9
C-10	of linoleate	3.97	0.65	115	80	5
C-11	and palmitate	3.97	0.32	130	92	8
C-12		3.97	0.32	130	110	8

* The weights of the livers perfused varied from 7.10 to 9.28 g. The increments in liver glyceride fatty acids are based on control values determined in the livers of control groups of animals which averaged 23 μEq/g (range 19.5 to 25.9).

† In Experiments 1–12, the red cells were carefully washed after harvesting to reduce the initial level of FFA. In these experiments, the level of FFA in the basic perfusion fluid was 0.15 μEq/ml, and, thus, the added FFA accounted for over 90% of the total.

values were, on the average, 23 μEq glyceride fatty acid/g liver (range 19.5–25.9). As seen in Table 1, there was a striking increase in liver glyceride content when the initial FFA concentration of the perfusing fluid was elevated, averaging about 25% in the group B experiments and 50% in the group C experiments. The increment in liver glyceride fatty acids was equivalent to 70% or more of the FFA disappearing from the perfusate during the 90 min of perfusion.

When the initial FFA concentration of the perfusing fluid was high, there was a significant rise in the glyceride content of the perfusate. At the highest concentration of FFA studied (3.97 μEq/ml, Expt. C), the increment averaged 7.5 μEq of glyceride fatty acids—a 40% increase over zero time values (0.6 μEq/ml; 18 μEq total). It should be noted, however, that in this limited series of perfusions, there was no proportionality between the absolute initial FFA level and the increment in perfusate glyceride content.

With regard to the net changes discussed above, there did not appear to be any difference whether the FFA added to the perfusate was linoleate, palmitate, or a combination of the two (Table 1).

Changes in Fatty Acid Composition of Liver and Perfusate Glycerides. When livers were perfused with high concentrations of linoleate, the liver glycerides showed a slightly higher linoleate content than that found in the glycerides of control livers, 37% instead of 32% (Table 2, Expt. 5 and 6). There was a slight decrease in the proportion of oleate found. By contrast, when the liver was perfused with high concentrations of palmitate, there was a marked distortion of the fatty acid pattern of the liver glycerides. The palmitate content rose from 23 to 50% and 44% in the two experiments shown (Expts. 7 and 8, Table 2), and the oleate and linoleate content fell.

The absolute changes in linoleate and palmitate in the glyceride fraction of liver can be estimated using the data of fatty acid composition (Table 2) and the

TABLE 2. COMPOSITION OF FATTY ACIDS IN THE GLYCERIDE FRACTION OF THE LIVER BEFORE AND AFTER PERFUSION*

Fatty Acid	Con-trols	Expt. B-5 (Linoleate)	Expt. B-6 (Linoleate)	Expt. B-7 (Palmitate)	Expt. B-8 (Palmitate)	Expt. C-9 (Linoleate plus Palmitate)	Expt. C-10 (Linoleate plus Palmitate)	Expt. C-11 (Linoleate plus Palmitate)	Expt. C-12 (Linoleate plus Palmitate)
14:0	1	1	1	1	1	1	1	1	1
16:0	23	22	22	50	44	30	33	27	33
16:1	3	3	3	2	2	4	2	3	3
18:0	4	3	3	4	5	4	3	3	3
18:1	36	33	34	20	27	27	26	30	26
18:2	32	37	37	19	20	33	34	35	33
20:4	1	1	1	1	1	1	1	1	1

* Results for the same liver perfusions shown in Table 1 according to the same code numbers. Values shown represent uncorrected area percentages determined by GLC.

TABLE 3. CALCULATED CHANGES IN TOTAL GLYCERIDE, GLYCERIDE PALMITATE, AND GLYCERIDE LINOLEATE IN LIVERS PERFUSED WITH HIGH CONCENTRATIONS OF FFA

Expt. No.	Fatty Acid Added to Perfusion Fluid*	Net change in Total Liver Glyceride Fatty Acids	Calculated Net Change† in Total Liver Glyceride Content of	
			Palmitate	Linoleate
		μEq	*μEq*	*μEq*
B-5	Linoleate	+40	+6	+25
B-6	Linoleate	+40	+6	+25
B-7	Palmitate	+50	+64	+2
B-8	Palmitate	+49	+54	−8
C-9	Linoleate plus palmitate, 1:1	+108	+44	+39
C-10		+80	+44	+31
C-11		+92	+36	+37
C-12		+110	+54	+40

* Initial and final FFA concentrations of perfusing fluid shown in Table 1 for corresponding experiments.

† Net increments calculated from percentage composition (Table 2) and estimated increments in total triglyceride fatty acid (Table 1).

calculated net increments in glycerides. The results are shown in Table 3. It can be seen that in livers perfused with linoleate, only a little over 60% of the total increment in glycerides can be attributed to the linoleate accumulating in this fraction. On the other hand, the increase in liver glycerides when palmitate was perfused appeared to be completely accounted for by the net increase in palmitate in this fraction. Under the unusual circumstances studied here of exposure to an essentially monotonous serum source of FFA, the liver drastically altered the pattern of glycerides laid down by increasing the palmitate content but did not to a similar extent distort the pattern by radically increasing the linoleate content. Because zero time values were not obtained for each liver perfused, these calculated results are not considered to be quantitatively exact, but the qualitative difference between the results using the two different fatty acids seems clear.

When livers were perfused with equal concentrations of linoleate and palmitate simultaneously, the relative palmitate content of the liver glycerides increased very significantly (from 23% to as much as 33% of the total). The relative linoleate content again changed only very slightly, as when linoleate was used alone. The calculated absolute increments in linoleate and palmitate in the liver glycerides (Table 3) were quite comparable.

The changes in fatty acid composition of the glycerides in the perfusate are shown in Table 4. When high concentrations of linoleate were used in the perfusion, the linoleate content of the perfusate glycerides rose from control values of 24–25% to 36–45%. The corresponding changes when palmitate was used were from control values of 25–26% to 31–33%. When both fatty acids were added to the perfusate, the shifts in glyceride fatty acid pattern were smaller but in a similar direction to those observed in the previous experiments. When lower initial concentrations of FFA were used (0.5 μEq/ml each of palmitate and linoleate) in a series of preliminary studies lasting 60–100 min, there were no significant changes in the fatty acid composition of the glycerides in the perfusate despite a total uptake of up to 30 μEq FFA and small but probably significant net increments of up to 3 μEq of glyceride fatty acid in the perfusate.

Changes in Fatty Acid Composition of Liver and Perfusate Phospholipids. As shown in Table 5, perfusing with linoleate led to a significant increase in the linoleate content of the liver phospholipid fraction—from initial levels of 32% up to 39 and 40% in two studies. The change was accompanied by a nearly equal decrease in oleate content. When palmitate was used in the perfusion, only a very slight increment in the palmitate content of the liver phospholipid was seen. The changes

TABLE 4. FATTY ACID COMPOSITION OF GLYCERIDES OF PERFUSING FLUID BEFORE AND AFTER PERFUSION*

Fatty Acid	Initial Perfusing Fluid A	Expt. A-1 Linoleate	Expt. A-2 Linoleate	Expt. A-3 Palmitate	Expt. A-4 Palmitate	Initial Perfusing Fluid B	Expt. B-5 Linoleate	Expt. B-6 Linoleate	Expt. B-7 Palmitate	Expt. B-8 Palmitate	Initial Perfusing Fluid C	Expt. C-9 Linoleate plus Palmitate	Expt. C-10 Linoleate plus Palmitate	Expt. C-11 Linoleate plus Palmitate	Expt. C-12 Linoleate plus Palmitate
<16:0	3	1	1	1	2	7	6	5	5	4	9	7	5	5	4
16:0	26	21	21	33	32	25	18	20	31	31	25	27	29	30	28
16:1	5	2	3	3	4	3	3	3	3	2	6	5	4	4	6
18:0	10	6	2	5	7	15	13	11	13	14	7	5	4	7	4
18:1	30	30	26	30	30	27	22	24	25	25	30	24	24	23	23
18:2	25	39	45	25	24	24	37	36	22	22	22	30	31	30	34
20:4	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

* Basic data and net changes for these perfusions shown in Table 1, and changes within livers shown in Tables 2 and 3. Results shown as uncorrected area percentages determined by GLC.

in the fatty acid pattern of the phospholipids in the perfusate were similar to those in the liver (Table 5).

Liver Slice Studies

Two groups of experiments were carried out at different times; since the results, with regard to relative incorporation into glyceride and phospholipid, differed systematically, the results are separately tabulated (Table 6). Liver slices from each rat were divided between two flasks, one containing palmitate-1-C^{14} and the other linoleate-1-C^{14}. The total concentrations of linoleate and palmitate in the medium were made equal (0.11 μEq/ml) by addition of the appropriate amount of unlabeled linoleate (see Methods). As shown in Table 6, at least 80–90% of the radioactivity recovered in the five fractions analyzed was found in phospholipids and glycerides. In the Group A experiments, over two-thirds of the recovered radioactivity was found in glycerides and less than one-quarter in phospholipids; in the Group B experiments, the incorporation into glycerides and into phospholipids was of a similar magnitude. The reason for the difference between the two groups of experiments is not known. In both sets of experiments, the fraction of labeled linoleate recovered in glycerides was consistently below the fraction of palmitate recovered in glycerides. Conversely, relatively more linoleate-C^{14} than palmitate-C^{14} was found in the phospholipid fraction.

TABLE 5. FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION IN LIVER AND IN PERFUSING FLUID BEFORE AND AFTER PERFUSION

Fatty Acid	Liver					Perfusing Fluid				
	Control	B-5 (Linoleate)	B-6 (Linoleate)	B-7 (Palmitate)	B-8 (Palmitate)	Control	B-5 (Linoleate)	B-6 (Linoleate)	B-7 (Palmitate)	B-8 (Palmitate)
14:0	1	2	2	2	2	1	1	1	1	1
16:0	14	12	12	16	15	33	30	29	34	35
16:1	7	6	6	6	7	1	1	1	2	1
18	15	12	13	14	13	22	19	21	21	21
18:1	27	21	20	20	20	20	18	18	16	16
18:2	32	39	40	32	33	24	32	31	24	26
20:4	5	4	3	4	5	Not determined				

Only a few per cent of the recovered radioactivity appeared as $C^{14}O_2$. In every experiment except one (Group A, Rat 3), relatively more linoleate-C^{14} than palmitate-C^{14} was oxidized. Similarly, relatively more linoleate-C^{14} than palmitate-C^{14} was incorporated into free cholesterol.

On the average, the relative recovery of palmitate-C^{14} in cholesterol esters was twice that of linoleate-C^{14}. The distribution of label between fatty acid and sterol was not determined, but since the reverse relationship was seen in free cholesterol fraction, the label was probably predominantly in the fatty acid moiety. The true difference in utilization of the two fatty acids for sterol ester formation, then, would undoubtedly be even greater than that observed.

Fate of Palmitate-1-C^{14} During Perfusions at Low and at High FFA Concentration

In order to gain better insight into the effects of FFA concentration on the initial fate of the fatty acid being perfused into the liver, studies were done in which palmitate-1-C^{14} was added to the perfusate, and the perfusions were carried out for a shorter time (30 min). Two studies were carried out in which only a tracer amount of palmitate-1-C^{14} was added and two in which unlabeled palmitate was added to the basic perfusing fluid to raise the total initial FFA concentration to 2.06 μEq/ml. The total amount of palmitate-1-C^{14} added was the same in each case so that the initial specific radioactivity of palmitate was much higher in the perfusing fluid with low initial concentration of FFA (Table 7).

The total amounts of radioactivity recovered in $C^{14}O_2$, in the glycerides and phospholipids of the liver, and in the glycerides and phospholipids of the perfusing fluid were determined. Approximately one-quarter of the radioactivity initially added remained in the FFA fraction of the perfusate. The total recovery of radioactivity ranged from 75 to 85%.

The total radioactivity recovered in each of the fractions analyzed was divided by the specific radioactivity of the palmitate-1-C^{14} in the perfusing fluid to give the *minimum* number of micromoles of fatty acid metabolized along the several pathways. This, of course, does not take account of dilution of the radioactivity occurring within the liver, and the values represent, therefore, *minimum* estimates. As shown in Table 7, the conversion of labeled palmitate to CO_2 was increased more than 15-fold when the initial FFA concentration of the perfusing fluid was raised, and the incorporation into liver triglycerides was increased more than 30-fold. Incorporation into the liver phospholipids was also increased, but to a lesser extent. When the FFA concentration was low, approximately equal amounts of the label, about 25% of the total, were incorporated into phospholipids and glycerides. However, at the higher initial concentration of FFA, the amount of label incorporated into glycerides was four times that incorporated into phospholipids.

The incorporation of labeled palmitate into glycerides in the perfusate was again stimulated to a much greater extent by the high concentration of FFA in the perfusing fluid than was incorporation into perfusate phospholipids.

TABLE 6. TOTAL UPTAKE AND PERCENTAGE DISTRIBUTION OF RADIOACTIVITY AMONG LIPID FRACTIONS AND IN $C^{14}O_2$ AFTER INCUBATION OF LIVER SLICES WITH LINOLEATE-1-C^{14} OR PALMITATE-1-C^{14}

Group	Rat	Total Radioactivity Taken Up*		Percentage Distribution of Recovered Radioactivity									
				CO_2		Free Cholesterol		Cholesterol Esters		Triglycerides		Phospholipids	
		LA	PA	LA	PA	LA	PA	LA	PA	LA	PA	LA	PA
		cpm	*cpm*										
A	1	85,000	97,000	3.7	3.4	4.3	2.9	2.0	2.8	70	83	20	8
	2	76,000	97,000	4.1	2.6	5.2	2.7	2.4	2.5	70	80	18	11
	3	132,000	120,000	1.7	2.3	4.0	2.3	1.0	2.7	70	76	24	14
	4	67,000	79,000	5.7	3.4	7.1	4.0	2.2	2.9	60	78	24	14
	5	76,000	84,000	3.5	2.3	2.9	2.7	1.3	3.3	68	75	23	16
Mean		87,000	95,000	3.7	2.8	4.7	2.7	1.8	2.8	68	78	24	13
B	1	115,000	129,000	3.1	1.4	5.1	1.5	1.1	3.9	48	62	43	31
	2	159,000	165,000	4.9	0.8	1.8	1.5	1.2	3.5	43	61	49	33
	3	140,000	165,000	3.2	1.3	5.4	1.3	1.3	6.4	35	49	55	40
	4	124,000	157,000	4.3	1.4	3.4	1.1	1.9	4.1	42	58	48	36
Mean		134,000	154,000	3.9	1.2	3.9	1.3	1.4	4.5	42	57	49	35

* Initial concentration of linoleate and palmitate equal at 0.11 μEq/ml; total initial FFA concentration 0.33 μEq/ml. Total radioactivity added 420,000 cpm in experiments of Group A; 500,000 cpm in experiments of Group B. LA = linoleate, PA = palmitate added to perfusing fluid.

DISCUSSION

The observed increments in liver glyceride fatty acids when the liver was perfused with FFA at high concentrations were as much as 25–60% above control values. These increments corresponded to only slightly less than the total amount of FFA taken up by the liver (Table 1). The net changes were quantitatively similar whether palmitate or linoleate was used as the predominant fatty acid in the perfusing fluid, but the ultimate distribution of the two fatty acids in the liver lipids differed significantly (Table 2). Perfusion with palmitate caused a marked increase in the percentage of palmitate in liver glycerides. Perfusion with linoleate increased the percentage of linoleate in the liver glycerides, but not to the same extent. Because the initial pool of palmitate in the liver glycerides was smaller than that of linoleate, equal absolute increments in the two fatty acids would be reflected in a relatively larger increment in the percentage of palmitate. A truer comparison is given by calculation of the absolute increments in the amounts of the two fatty acids in the liver glycerides (Table 3). These calculations show that the absolute as well as the relative increment in palmitate exceeded that of linoleate. Since perfusing with linoleate or palmitate produced quantitatively similar increments in liver glyceride, and since the absolute increment in linoleate accounted for only about 60% of the increment, it is probable that, during perfusion with linoleate, significant amounts of other fatty acids were incorporated at the same time. Such fatty

TABLE 7. FATE OF PALMITATE-C^{14} DURING 30-MIN PERFUSIONS OF LIVERS AT LOW AND AT HIGH FFA CONCENTRATIONS

Expt. No.	Total Initial FFA Conc.	Total Initial Palmitate Conc.	Total FFA Taken Up	Initial Palmitate Specific Radioactivity	Calculated Minimum Number of Microequivalents of Palmitate Converted to Fractions Shown*				
					CO_2	Liver Glycerides	Liver Phospholipids	Perfusate Glycerides	Perfusate Phospholipids
	μEq/ml	*μEq/ml*	*μEq*	*cpm/μEq*					
1	0.36	0.09	5.7	6.7×10^6	0.04	0.72	0.63	0.018	0.004
2	0.36	0.09	4.5	6.7×10^6	0.04	0.75	0.68	0.016	0.004
3	2.06	1.79	44.5	3.4×10^5	0.74	26.1	4.6	0.76	0.008
4	2.06	1.79	40.2	3.4×10^5	0.74	23.2	7.5	0.97	0.013

* Observed total radioactivity in fraction (cpm) divided by specific radioactivity of palmitate in perfusing fluid (cpm/μEq).

acids might have been derived from de novo synthesis, or transfer from preformed fatty acids in other stores within the liver, perhaps from phospholipids, or both. This suggests that a relatively greater quantity of linoleate than palmitate must have become incorporated into other lipid fractions, phospholipids being the most likely. This is supported by the finding of a greater percentage increment in linoleate than palmitate in liver phospholipids when the corresponding fatty acids were used in the perfusate (Table 5). It should be noted that the fatty acid composition of the liver glycerides was determined after 90 min of perfusion whereas most of the FFA were probably removed in the course of the first hour. Thus the fatty acids taken up may have undergone redistribution due to a metabolic turnover within the liver. If this were extensive, the fatty acid distribution need not reflect initial rates of incorporation. However, the apparently greater incorporation of palmitate into glycerides and the relatively greater incorporation of linoleate into phospholipids are consonant with the results of the liver slice studies in which the rate of incorporation of the labeled fatty acids is measured (Table 6). In vivo studies in dogs (1), and in vitro studies with liver microsomes (11) have also shown preferential incorporation of labeled linoleate into liver phospholipids.

When the initial FFA concentration of perfusing fluid was low (0.36 μEq/ml), there was only a barely detectable increment in the glyceride content of the perfusate. Increasing the FFA concentration to very high levels markedly stimulated the output of glycerides. Separation into various lipoprotein classes was not attempted in the present experiments, but Kay and Entenman have shown in similar studies that the newly secreted glycerides in the perfusate are found predominantly in lipoproteins of density less than 1.006 (12). Although the use of perfusion fluids with high FFA concentration gave a highly significant threefold increase in the amount of glyceride put out into the perfusate, the absolute increments were still rather small, equivalent to 10% or less of the amount of FFA taken up by the liver (Table 1). When the liver was perfused with high concentrations of labeled palmitate (Table 7), again only a small percentage of the label taken up reappeared in the perfusate as glycerides. The marked change in the fatty acid composition of the glycerides in the perfusate indicates that these were newly synthesized, utilizing the predominant fatty acid in the perfusing fluid.

These studies are in general agreement with the previous results of Gidez, Roheim, and Eder (13). Using longer periods of perfusion, these workers observed larger increments in perfusate glyceride levels induced by perfusing with high concentrations of FFA. The present results are in contrast to those of Heimberg et al. (14), who reported much greater increments in perfusate glycerides even when no extra FFA were added to the perfusing fluid. The reasons for the differences in results are not known.

From the net increments and the changes in fatty acid composition of the perfusate glycerides, it can be calculated that a larger amount of linoleate than of palmitate appeared in the perfusate glycerides when these fatty acids were used separately to raise the initial FFA concentration (Tables 1 and 4). This is consonant with previous results in intact dogs showing that labeled linoleate injected as the free fatty acid tends to appear more rapidly in serum very-low-density lipoproteins secreted by the liver (2). The fatty acid composition of the phospholipids of the perfusate was also altered by perfusing with high concentrations of linoleate or palmitate, a greater amount of the former appearing in this fraction at the end of 90 min (Table 5).

Liver slices incubated in the presence of equal concentrations of linoleate and palmitate incorporated relatively more palmitate into glycerides and relatively more linoleate into phospholipids, although the differences were not large. In the absence of specific information regarding the diluting pools of intermediates, interpretation of these isotopic results must be tentative. Getz et al. (15) found that the FFA fraction of liver contained about 30% palmitate and only less than 20% linoleate. Similar results have been obtained in this laboratory,[1] and the total FFA content has been found to be, on the average, 0.56 μEq/g wet weight. If all of the labeled FFA entering the tissue were diluted by this entire pool of tissue FFA (which is by no means certain), the incorporation of palmitate would tend to be decreased relative to that of linoleate. However, the incorporation of palmitate into glycerides and into cholesterol esters was consistently greater than that of linoleate. These results, then, probably reflect true differences in the rates measured.

It was also found that liver slices oxidized significantly more linoleate than palmitate (Table 6). The difference, especially in the second set of experiments, appears to exceed that which might have been produced by relative dilution of the precursors in the FFA pool of the liver. The greater incorporation of radioactivity from linoleate into the free cholesterol fraction may merely reflect a greater availability of labeled 2-carbon precursors derived from degradation of the fatty acid.

The results of perfusions with labeled palmitate (Table 7) confirm and extend previous reports of similar studies and support the conclusions derived from the

present studies of net changes discussed above. The distribution of radioactivity among the lipid classes of the perfusate and liver when the initial FFA concentration in perfusate was low are similar to those reported by Hillyard, Cornelius, and Chaikoff (16). The much larger FFA uptake at high perfusate FFA concentrations is in agreement with the results of in vivo studies reported by Fine and Williams (17), McElroy, Siefert, and Spitzer (18), and Armstrong et al. (19). At higher perfusate FFA concentrations, the conversion of labeled palmitate to CO_2 was also increased. An effect of FFA concentration on oxidation has been observed both with liver slices[1] and with isolated skeletal muscle preparations (20, 21).

It has been reported previously that when the serum FFA concentration in dogs is maintained at high levels for several hours by constant intravenous infusion of norepinephrine, there are marked increases in liver glyceride content but only small increases in phospholipid content (3). The present findings support the conclusion that the observed deposition of glycerides under the conditions of the previous studies was, in fact, a direct consequence of the sustained high levels of serum FFA. In the present perfusion studies utilizing high concentrations of palmitate, glyceride synthesis was stimulated to a much greater extent than was phospholipid synthesis (Table 7). Rose et al.,[1] using the rate of incorporation of labeled glycerol as a measure of the rate of synthesis, have shown in a liver-slice system that high concentrations of medium FFA stimulate the rate of glyceride synthesis markedly with little or no effect on the rate of phospholipid synthesis. All of these in vivo and in vitro studies, then, show that high concentrations of FFA exert an important influence on the rate of glyceride synthesis in the liver. These findings help to clarify the mechanism by which the rate of fat mobilization from the depots in the form of FFA plays a key role in the development of certain types of fatty liver (3).

The present studies, and those of Gidez et al. (13), also support the hypothesis presented previously (3, 22) to explain the rises in serum lipoproteins induced by administration of epinephrine to rats and dogs. On the basis of the correlation between FFA responses and lipoprotein responses after epinephrine administrations, it was proposed that an increased rate of delivery of FFA to the liver could somehow stimulate production and secretions of lipoproteins. The present results with an isolated liver system are consonant with such a mechanism. The quantitative importance of FFA uptake by the liver as a possible determinant of serum lipoprotein levels deserves further exploration.

REFERENCES

1. Dustin, J.-P., D. S. Fredrickson, Ph. Laudat, and K. Ono. *Federation Proc.* **20**: 270, 1961.
2. Nestel, P. J., A. Bezman, and R. J. Havel. *Am. J. Physiol.* **203**: 914, 1962.
3. Feigelson, E. B., W. W. Pfaff, A. Karmen, and D. Steinberg. *J. Clin. Invest.* **40**: 2171, 1961.
4. Mortimore, G. E. *Am. J. Physiol.* **200**: 1315, 1961.
5. Borgström, B. *Acta Physiol. Scand.* **25**: 101, 1952.
6. Borgström, B. *Acta Physiol. Scand.* **25**: 111, 1952.
7. Dole, V. P. *J. Clin. Invest.* **35**: 150, 1956.
8. Snyder, F., and N. Stephens. *Biochim. Biophys. Acta* **34**: 244, 1959.
9. Jover, A. *J. Lipid Res.* **4**: 228, 1963.
10. Steinberg, D. *Anal. Biochem.* **1**: 23, 1960.
11. Stein, Y., and B. Shapiro. *Biochim. Biophys. Acta* **34**: 79, 1959.
12. Kay, R. E., and C. Entenman. *J. Biol. Chem.* **236**: 1006, 1961.
13. Gidez, L. I., P. S. Roheim, and H. A. Eder. *Federation Proc.* **21**: 289, 1962.
14. Heimberg, M., I. Weinstein, H. Klausner, and M. L. Watkins. *Am. J. Physiol.* **202**: 353. 1962.
15. Getz, G. S., W. Bartley, F. Stirpe, B. M. Notton, and A. Renshaw. *Biochem. J.* **80**: 176, 1961.
16. Hillyard, L. A., C. E. Cornelius, and I. L. Chaikoff. *J. Biol. Chem.* **234**: 2240, 1959.
17. Fine, M. B., and R. H. Williams. *Am. J. Physiol.* **199**: 403, 1960.
18. McElroy, W. T., Jr., W. L. Siefert, and J. J. Spitzer, *Proc. Soc.* Exptl. Biol. Med. **104**: 20, 1960.
19. Armstrong, D. T., R. Steele, N. Altzuler, A. Dunn, J. S. Bishop, and R. C. DeBodo. *Am. J. Physiol.* **201**: 9, 1961.
20. Eaton, P., and D. Steinberg. *J. Lipid Res.* **2**: 376, 1961.
21. Fritz, I. B. *Physiol. Rev.* **41**: 52, 1961.
22. Shafrir, E. and D. Steinberg. *J. Clin. Invest.* **39**: 310, 1960.