Interactions between fatty acid synthesis, oxidation, and esterification in the production of triglyceride-rich lipoproteins by the liver

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Abstract In a series of experiments with male rat livers perfused with or without 5-tetradecyloxy-2-furoic acid (TOFA) in the presence and absence of oleate, the relationships between fatty acid synthesis, oxidation, and esterification from newly synthesized and exogenous fatty acid substrates have been examined. When livers from fed rats were perfused without exogenous fatty acid substrate, 20% of the triglyceride secreted was derived from de novo fatty acid synthesis. Addition of TOFA caused immediate and nearly complete inhibition of fatty acid synthesis, measured by incorporation of ^3H_2O into fatty acids. Concurrently, ketone body production increased 140% and triglyceride secretion decreased 84%. These marked reciprocal alterations in fatty acid synthesis and oxidation in the liver almost completely abolished the production of very low density lipoproteins (VLDL). Cholesterol synthesis was also depressed by TOFA, suggesting that this drug also inhibited lipid synthesis at a site other than acetyl-CoA carboxylase. When livers from fed rats were supplied with a continuous infusion of [1-^{14}C]oleate as exogenous substrate, similar proportions, about 45–47%, of both ketone bodies and triglyceride in the perfusate were derived from the infused [1-^{14}C]oleate. The production of ketone bodies was markedly increased by TOFA; the secretion of triglyceride and cholesterol were decreased. Altered conversion of [1-^{14}C]oleate into these products occurred in parallel. While TOFA decreased esterification of oleate into triglyceride, incorporation of [1-^{14}C]oleate into liver phospholipid was increased, indicating that TOFA also affected glycerolipid synthesis at the stage of diglyceride processing. The decreased secretion of triglyceride and cholesterol following TOFA treatment was localized almost exclusively in VLDL. The specific activities of ^{3}H and of ^{14}C fatty acids in triglyceride of the perfusate were greater than those of liver triglyceride, indicating preferential secretion of triglyceride produced from both de novo fatty acid synthesis and from infused free fatty acid substrate. These observations suggest the following chain of events in the liver following TOFA treatment: *1*) inhibition of fatty acid and cholesterol synthesis; *2*) increased fatty acid oxidation and ketogenesis; *3*) decreased triglyceride synthesis as a result of *a*) inhibition of fatty acid synthesis, *b*) stimulation of fatty acid oxidation, and *c*) altered partition of diglyceride between triglyceride and phospholipid synthesis; and *4*) decreased production of VLDL. These comparative rat liver perfusion experiments indicate that free fatty acids provide the major source of substrate for the hepatic production of triglyceride-rich lipoproteins. Fatty acid synthesis, while contributing a substantial quantity of fatty acid substrate for this process, is of somewhat less significance in quantitative terms. These studies demonstrate the effectiveness of TOFA in the inhibition of VLDL production from both sources.—**Fukuda, N., and J. A. Ontko.** Interactions between fatty acid synthesis, oxidation, and esterification in the production of triglyceride-rich lipoproteins by the liver. *J. Lipid Res.* 1984. **25:** 831–842.

Supplementary key words cholesterol • ketone bodies • lipogenesis • metabolism • oleic acid • phospholipids • secretion • 5-(tetradecyloxy)-2-furoic acid • TOFA • VLDL

Fatty acids utilized for the alternative pathways of oxidation and esterification in the liver are mainly derived from plasma free fatty acids, from de novo fatty acid synthesis, and from intrahepatic lipolytic processes. The relative contributions of these fatty acid sources for utilization in the liver are variable and are under hormonal and nutritional control (1–6).

Fatty acids which enter the esterification pathway are either retained within the liver cell for the formation of membrane phospholipids and for storage in triglyceride droplets or they are secreted in the form of triglyceride-rich lipoproteins (1–11). The metabolism of fatty acids from different sources, i.e., de novo synthesis and plasma free fatty acids, for these processes likewise proceeds under homeostatic regulation.

From isolated liver perfusion experiments the rate of fatty acid synthesis (12, 13) and the concentration of circulating free fatty acids (14, 15) are clearly determinants in hepatic triglyceride synthesis and VLDL production. However, these determinants have not been compared in detail under the same experimental conditions, i.e.,

Abbreviations: TOFA, 5-(tetradecyloxy)-2-furoic acid; VLDL, very low density lipoproteins.

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with the same animals fed the same diet and with identical liver perfusion procedures. It has, therefore, been difficult to assess the relative quantitative importance of these two fatty acid sources for VLDL production. It has also been difficult to determine the similarities and differences in the intracellular processing of fatty acids synthesized de novo and fatty acids derived from the plasma free fatty acid pool. Such evaluations have been made in the present study. In addition, interactions between these processes were investigated by examination of the consequences of treatment with 5-(tetradecyloxy)-2-furoic acid (TOFA), a potent inhibitor of fatty acid synthesis (16–18). The effects of this drug, although complex, provide a degree of integration of the metabolic activities underlying the secretion of triglyceride-rich lipoproteins.

EXPERIMENTAL PROCEDURES

Animals

Male Holtzman rats (Cr1:CD H(SD)BR) were obtained from Charles River Breeding Laboratories. All animals (330 g–370 g) were maintained on Purina Laboratory Chow and water ad libitum, and were housed at constant temperature (22°C, with lights on at 6:00 AM and off at 6:00 PM).

Liver perfusion

Liver perfusions were performed by essentially the same technique and apparatus as described in detail previously (19, 20). On the day of perfusion, the animals were anesthetized with an intraperitoneal injection of Nembutal (5 mg/100 g body weight) between 9:00 AM and 9:30 AM. The perfusion was started between 9:30 AM and 10:00 AM. The perfusion medium (recirculating volume of 120 ml) consisted of Krebs-Henseleit buffer (pH 7.4) containing 25 mM glucose, 1.5% fatty acid-free bovine serum albumin (fraction V fatty acid free, Miles Laboratories), and 25% aged human erythrocytes.

In experiments designed to measure the effect of TOFA on fatty acid synthesis, 10 mCi of ^3H_2O in 1.0 ml of 0.9% NaCl was added to the perfusion medium and the livers were perfused with the medium described above.

In experiments designed to measure the effect of TOFA on the metabolism of [1-^{14}C]oleate substrate, 5 ml of 20 mM potassium [1-^{14}C]oleate solution (100 μmol) was added at the beginning of perfusion and the same solution was infused continuously (4.5 ml, 90 μmol/hr) with a mechanical infusion pump. The perfusions were continued for 225 min after recirculation was established. Every 45 min, 18 ml of perfusion medium was removed for analyses of lipids and ketone bodies, and the same amount of fresh medium was added to the mixing chamber to maintain 120 ml of recirculating volume.

5-Tetradecyloxy-2-furoic acid (TOFA, RM1 14,514) was complexed with 3.0% fatty acid-free bovine serum albumin in 0.9% NaCl. Four ml of 1 mM TOFA was added at 0 min or 90 min, and the same amount was added after each 45-min interval thereafter, as indicated in Fig. 1.

All assays of the perfusate were carried out after removing erythrocytes by centrifugation. After the termination of perfusion, the livers were removed from the perfusion apparatus, rinsed with cold saline, weighed after removal of non-hepatic tissues, and then homogenized with 4 volumes of cold saline. Very low density lipoproteins ($d < 1.006$ g/ml) were isolated from the cell-free perfusate at each 45-min period by ultracentrifugation after layering with aqueous NaCl ($d = 1.006$ g/ml) containing 0.2% EDTA and 0.01% thimerosol as described previously (19, 20).

Lipid analyses

The lipids in the perfusate, liver homogenates and lipoprotein fractions were extracted and purified according to the procedure of Folch, Lees, and Sloane Stanley (21). The extracts were then evaporated to dryness in a rotary evaporator, and the lipids were then dissolved in a known volume of chloroform. Triglyceride and cholesterol were measured by the methods described elsewhere (19, 20). Phospholipids were determined colorimetrically (22).

Perfusate, lipoprotein, and liver lipid radioactivities

The radioactivity in lipid fractions derived from [1-^{14}C]oleate was measured after separation by thin-layer chromatography on Silica Gel 60G (Brinkman Instrument, Inc., Westbury, NY) containing the fluorescent agent Ultraphor (23) with a solvent mixture of n-hexane–diethylether–glacial acetic acid 80:20:1 (v/v/v) (23). The bands corresponding to cholesteryl ester, triglyceride, free fatty acid, partial glyceride, and phospholipid were identified with ultraviolet light and scraped into counting vials containing 10 ml of Insta-Gel (Packard Instrument Co., Downers Grove, IL).

The total rates of fatty acid and cholesterol synthesis in perfusate and liver lipids were measured from the incorporation of tritium from ^3H_2O into total fatty acids and cholesterol (13, 24, 25). The radioactivities in lipids of sequential samples of perfusate, and of liver at the end of the perfusion period, were measured as previously described (19, 20). The incorporation of label into triglyceride fatty acids and phospholipid fatty acids was determined in washed chloroform–methanol 2:1 (v/v) extracts, processed and separated by thin-layer chromatography as described (19, 20). The bands corresponding to

triglyceride and phospholipid were scraped into tubes containing chloroform–methanol 2:1 (v/v) and chloroform–methanol–glacial acetic acid–water 50:39:1:10 (v/v/v/v), respectively (26). These extracted lipids were saponified and fatty acids were then extracted with n-hexane three times after acidification. The combined extracts were counted in Insta-Gel. The digitonin-precipitable sterol was purified according to Sperry and Webb (27) and counted in 1 ml of methanol and 10 ml of Insta-Gel. Radioactivities were counted by liquid scintillation and corrected for quenching by external standard.

The total rates of fatty acid and cholesterol synthesis were calculated from the following quotient: (^{3}H in lipid in dpm)/(sp act of ^3H_2O) times 1.15 or 1.31 (13, 28) and expressed as μmol acetyl incorporated into fatty acid or digitonin-precipitable sterol, respectively.

Other analyses

Acetoacetate (20) and β-hydroxybutyrate (20) in the perfusate were measured in deproteinized samples (29). Conversion of [1-^{14}C]oleate to [^{14}C]acetoacetate was measured by the aniline citrate decarboxylation procedure described previously (30). Total radioactivity in ketone bodies was calculated from the β-hydroxybutyrate/acetoacetate ratio.

RESULTS

Effects of TOFA on ketogenesis, fatty acid and cholesterol synthesis, and triglyceride secretion by livers perfused in the absence of fatty acid substrate

Ketone body production by the control livers, in the absence of exogenous fatty acid, proceeded at a rather constant rate throughout the 225 min of perfusion (**Fig. 1**). Addition of TOFA complexed with bovine serum albumin at the beginning of perfusion caused an immediate increase in the production of ketone bodies as did the administration of TOFA at 90 min. These results provide the net rate of endogenous ketogenesis, presumably arising from the oxidation of fatty acid substrates derived from lipolytic events in the liver. TOFA promoted the accumulation of both β-hydroxybutyrate and acetoacetate. The average β-hydroxybutyrate:acetoacetate ratios ranged between 0.5 and 0.8 throughout the perfusion period in all groups, indicative of a satisfactory mitochondrial pyridine nucleotide redox state and, accordingly, a normal adenine nucleotide phosphorylation state. The ratio was not appreciably affected by TOFA.

The net secretion of triglyceride by these livers is shown in **Fig. 2.** The administration of TOFA at the beginning of perfusion and at 90 min decreased triglyceride accumulation 72.2% and 54.2%, respectively, at the end of the perfusion period. Thus, following a lag period, probably due to the secretion of preformed VLDL particles (20), net triglyceride secretion was almost completely abolished by TOFA.

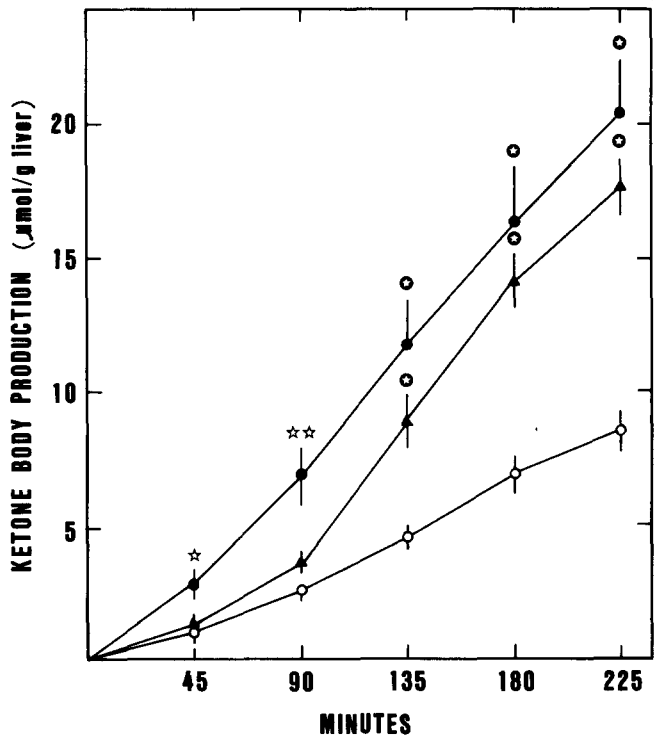

Fig. 1. Effect of TOFA on the production of ketone bodies by the perfused liver. Livers from fed rats were perfused with Krebs-Henseleit buffer (pH 7.4) containing 25 mM glucose, 1.5% bovine serum albumin (fatty acid-free), and 25% human aged erythrocytes, at a rate of 20 ml/min. A solution of TOFA (4 μmol) complexed with bovine serum albumin was added at 0 or 90 min and at each 45 min thereafter, as described in Experimental Procedures. The mean body weights of the donor animals in the control, TOFA at 90 min, and TOFA at zero time groups were: 372 ± 17, 375 ± 8, and 370 ± 13 g ± SEM, respectively. The corresponding liver weights were: 15.9 ± 0.4, 16.0 ± 0.7, and 15.3 ± 0.4 g ± SEM, respectively. Each group had four animals. The values indicated by the single star, double star, and circled star are significantly different from the control values at $P < 0.025$, 0.01, and 0.005, respectively. ○, Control; ●, TOFA at 0 min; △, TOFA at 90 min. The vertical bars indicate SEM.

Fatty acid synthesis was measured by incorporation of tritium from ^3H_2O into fatty acids (**Fig. 3**). The lag period in the incorporation of label into fatty acids of the perfusate indicates the periods required for the synthesis, assembly, and secretion of triglyceride-rich lipoprotein particles by the liver. In control livers, newly synthesized fatty acid secreted into the perfusate at 225 min was 10.2 μmol of acetyl units (**Table 1**). This was 23% of the amount found in the liver, in agreement with the results of Windmueller and Spaeth (12) and Kirk, Verrinder, and Hems (31). The accumulations of newly synthesized fatty acid in the perfusate following the addition of TOFA at the beginning and at 90 min were decreased 96% and 88%, respectively. These decreased rates of secretion of newly synthesized fatty acids by the liver may be explained by

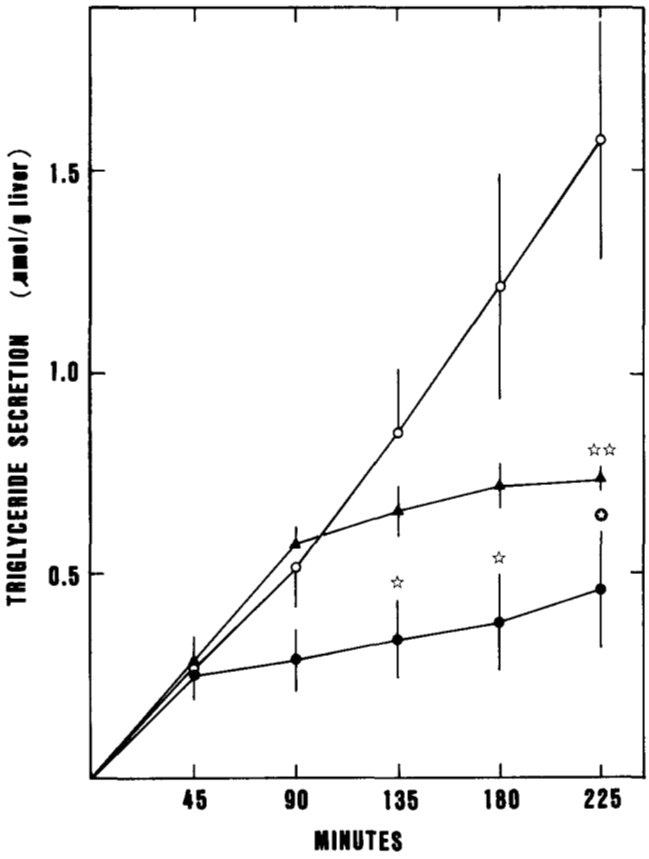

Fig. 2. Effect of TOFA on the net secretion of triglyceride by the perfused liver. The experiments are the same as those described in the legend to Fig. 1. The values indicated by the single star, double star, and circled star are significantly different from the control values at $P < 0.05$, 0.025, and 0.02, respectively. ○, Control; ●, TOFA at 0 min; △, TOFA at 90 min. The vertical bars indicate SEM.

inhibition of fatty acid synthesis by TOFA. Division of 10.2 μmol acetyl units by 8.5 yields 1.2 μmol of long chain fatty acid (0.6 μmol palmitate and 0.6 μmol stearate) synthesized. We have observed approximately 85% of the fatty acids synthesized and secreted by the isolated perfused liver in the triglyceride fraction; the remainder was found in the phospholipid fraction. Based on this distribution, 85% of 1.2 μmol equals 1.02 μmol of newly synthesized fatty acids in triglyceride secreted during 225 min of perfusion. Moreover, when divided by 3, this yields 0.34 μmol of triglyceride synthesized. These and related calculations are summarized in Table 1.

TOFA is reportedly a potent inhibitor of acetyl-CoA carboxylase (18, 32), a key enzyme in the biosynthesis of fatty acids (33). Fatty acid synthesis by the livers treated with TOFA was completely inhibited (Fig. 3, Table 1). Concurrently, cholesterol synthesis was decreased 42%. The extent of inhibition of fatty acid synthesis was much greater. Inhibition of cholesterol synthesis by TOFA cannot be explained by inhibition of acetyl-CoA carboxylase.

As described above, newly synthesized triglyceride secreted into the perfusate was 0.34 μmol. When fatty acid synthesis was inhibited by TOFA, net secretion of triglyceride decreased to a much greater extent, by 1.1 μmol (Table 1). This suggests that TOFA also decreased the secretion of triglyceride by another mechanism.

In the liver, newly synthesized fatty acid is primarily incorporated into phospholipid and triglyceride. The ratio of the newly formed fatty acid in phospholipid:triglyceride was about 2:1. In TOFA-treated livers, the incorporation into triglyceride was predominantly depressed, and the ratio was approximately 8:1.

Specific radioactivities of triglyceride fatty acids were much higher in the perfusate than in the liver in both control and TOFA-treated systems. Therefore, newly synthesized fatty acids were preferentially secreted.

Effects of TOFA on fatty acid oxidation and esterification, triglyceride and cholesterol secretion, and VLDL production by livers perfused with oleic acid substrate

In the foregoing experiments, livers were perfused in the absence of exogenous fatty acid substrate. TOFA

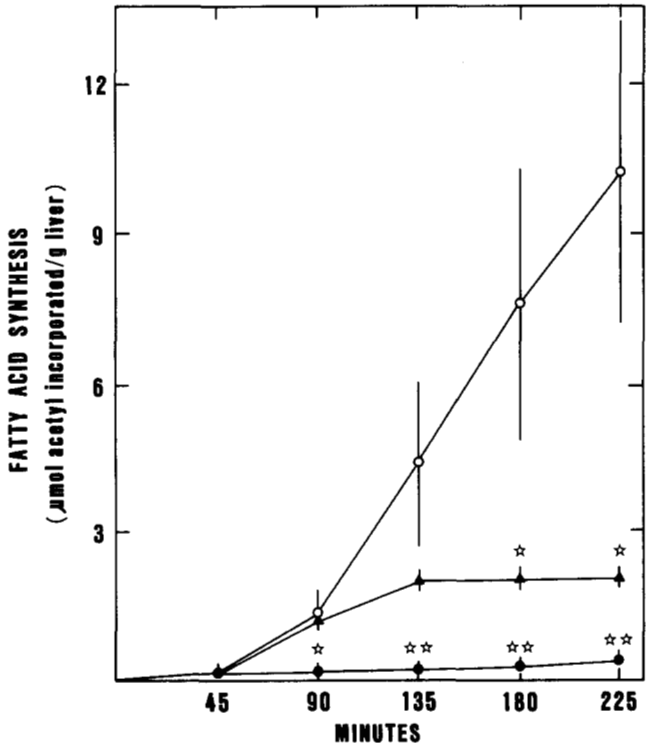

Fig. 3. Effect of TOFA on the secretion of newly synthesized fatty acids by the perfused liver. The experiments are the same as those described in the legend to Fig. 1. Newly synthesized fatty acids in total lipids of the perfusate were calculated from the incorporation of ^3H from ^3H_2O into total fatty acids extracted after saponification. The values indicated by the single and double stars are significantly different from the control values at $P < 0.05$ and 0.025, respectively. ○, Control; ●, TOFA at 0 min; △, TOFA at 90 min. The vertical bars indicate SEM.

TABLE 1. Effects of TOFA on fatty acid synthesis by perfused livers from fed rats

	Control (4)	TOFA at 0 min (4)	P
Perfusate			
TG secretion (μmol/g per 225 min)	1.56 ± 0.27	0.46 ± 0.14	<0.02
Total FA synthesis (μmol C_2/g per 225 min)[a]	10.15 ± 3.58	0.33 ± 0.08	<0.05
Total FA synthesis (μmol FA/g per 225 min)	1.20 ± 0.42	0.04 ± 0.01	<0.05
Newly synthesized FA in TG (μmol FA/g per 225 min)	1.02 ± 0.36	0.03 ± 0.01	<0.05
Newly synthesized TG (μmol TG/g per 225 min)	0.34 ± 0.12	0.01 ± 0.00	<0.05
Contribution of newly synthesized TG to TG (%)	19.61 ± 4.49	2.54 ± 0.53	<0.01
Specific activity (C_2/μmol TG)	5.01 ± 1.16	0.65 ± 0.13	<0.01
Liver			
TG content (μmol/g)	5.56 ± 1.10	5.94 ± 0.36	NS
Total FA synthesis (μmol C_2/g)	43.63 ± 10.49	8.34 ± 3.00	<0.02
FA synthesis in TG (μmol C_2/g)	13.77 ± 3.95	0.83 ± 0.47	<0.02
FA synthesis in phospholipid (μmol C_2/g)	23.32 ± 6.43	6.90 ± 2.32	<0.05
TG-FA/phospholipid-FA	0.59 ± 0.02	0.15 ± 0.09	<0.05
Newly synthesized FA in TG (μmol FA/g)	1.62 ± 0.47	0.10 ± 0.06	<0.02
Newly synthesized TG (μmol TG/g)	0.54 ± 0.16	0.03 ± 0.02	<0.02
Contribution of newly synthesized TG to TG (%)	9.59 ± 1.68	0.59 ± 0.33	<0.005
Specific activity (C_2/μmol TG)	2.44 ± 0.43*	0.15 ± 0.08**	<0.005
Cholesterol synthesis (μmol C_2/g)[a]	6.44 ± 0.64	3.65 ± 1.11	<0.05

The experiments are described in the legend to Fig. 1. Values represent the mean ± SEM. The number of perfusions in each group is shown in parentheses. Fatty acid and cholesterol synthesis are expressed as μmol acetyl incorporated into fatty acid or digitonin-precipitable sterol per g liver per 225 min of perfusion.

*,**, Indicate values significantly different from the corresponding specific activities of the perfusate at $P < 0.05$ and 0.01, respectively.

[a] Based on the incorporation of ^3H_2O as described in Experimental Procedures.

concurrently decreased fatty acid synthesis, increased ketogenesis, and decreased triglyceride secretion by the perfused liver. To gain further insight on the interactions between these processes, effects of TOFA on the metabolism of [1-^{14}C]oleic acid were investigated. Livers received a continuous infusion of [1-^{14}C]oleate (90 μmol/hr) after an initial priming dose of 100 μmol. TOFA was added at the beginning of the perfusion and at each 45-min interval. There was no difference between the uptake of [1-^{14}C]oleate by the control and TOFA-treated livers (**Table 2**). Thus, we could directly compare the utilization of infused oleate by the livers in each group. Effects of TOFA could, therefore, be attributed to actions of the drug on the fatty acid substrate after its entry into the liver cells.

TABLE 2. Uptake of ^{14}C-oleic acid substrate by the perfused liver

Time	Control (4)	TOFA at 0 min (4)	P
min	*μmol/g liver*		
45	9.3 ± 0.7	9.0 ± 0.6	NS
90	14.1 ± 1.0	13.6 ± 0.7	NS
135	18.4 ± 1.2	17.7 ± 1.0	NS
180	22.7 ± 1.5	22.0 ± 1.2	NS
225	26.1 ± 1.9	25.3 ± 1.4	NS

Livers were perfused with constant infusion of [1-^{14}C]oleate (90 μmol/hr) after an initial priming dose (100 μmol). The uptake of oleate was calculated from radioactivities remaining in the free fatty acid fraction of the perfusate. The average body weights ± SEM of the rats in the control and TOFA-treated groups were 352 ± 12 g and 352 ± 12 g, respectively. The corresponding liver weights were 15.3 ± 1.0 g and 15.5 ± 1.0 g, respectively. All values represent the mean ± SEM. Figures in parentheses refer to the number of perfusions. TOFA was added at 0 min and at 45-min intervals, as described in Experimental Procedures.

The rates of ketone body production by the control livers were 4.6-fold higher in the presence of oleate (**Fig. 4**) than in its absence (Fig. 1). Addition of TOFA caused a further stimulation of ketogenesis. The concurrent incorporation of [1-^{14}C]oleate into perfusate ketone bodies was elevated to a similar extent. The β-hydroxybutyrate:acetoacetate ratio in the liver perfusate was increased by TOFA (Fig. 4, legend) as observed in hepatocytes (17, 18). The proportions of ketone body carbon derived from infused oleate were calculated as described previously (19). At 45, 90, 135, 180, and 225 min these values were: 55.9, 53.5, 46.4, 46.6, and 44.9% (control) and 59.8, 56.7, 52.5, 52.8, and 48.8% (TOFA), respectively. In both groups, 45–49% of ketone bodies (at 225 min) were derived from infused oleate, indicating that the relative contributions of endogenous and infused substrate to ketone body formation were similar. TOFA accordingly increased ketogenesis from both sources.

Triglyceride secretion by the control livers was increased 50% by oleate infusion (Fig. 2 and **Fig. 5**). Triglyceride secretion was substantially decreased by TOFA. The percentage decrease in the presence of oleic acid substrate (Fig. 5) was 45%, somewhat less than the 72% decrease observed in the absence of infused fatty acid (Fig. 2). However, in absolute terms the decreases were

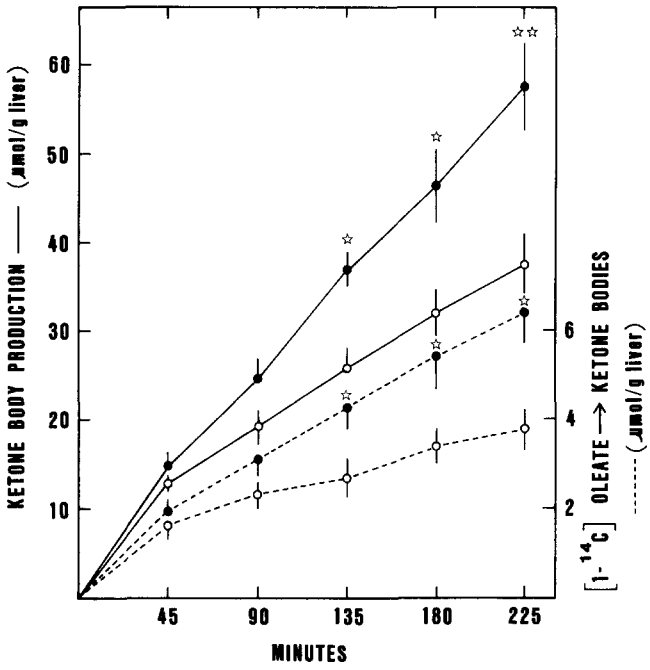

Fig. 4. Effect of TOFA on ketone body production by the perfused liver and incorporation of [1-^{14}C]oleate into perfusate ketone bodies. Livers from fed rats were perfused with a continuous infusion of oleate (90 μmol/hr) after the initial priming dose (100 μmol). A solution of TOFA (4 μmol) complexed with bovine serum albumin was added at 0 min and at each 45 min thereafter. The experimental animals and livers are described in Table 2. The values indicated by the single and double stars are significantly different from the control values at $P < 0.05$ and 0.02, respectively. The β-hydroxybutyrate:acetoacetate ratios in the perfusates at the consecutive time intervals, starting at 45 min, averaged: 0.94, 0.83, 0.76, 0.84, and 0.97 (control) and 1.22, 1.17, 1.09, 1.08, and 1.11 (TOFA), with the TOFA group significantly elevated ($P < 0.001$). Four livers were perfused in each group: O, Control; ●, TOFA at 0 min. The vertical bars indicate SEM.

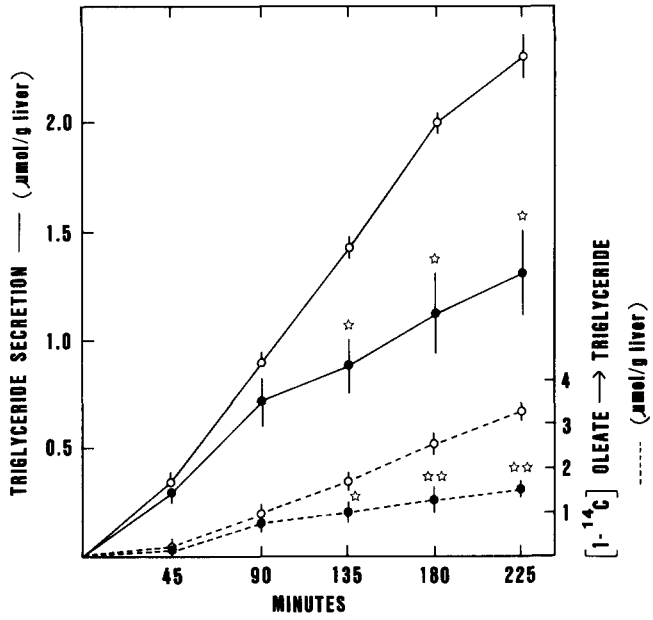

Fig. 5. Effect of TOFA on the net secretion of triglyceride by the perfused liver and incorporation of [1-^{14}C]oleate into perfusate triglyceride. The experiments are the same as those described in Table 2 and Fig. 4. The values indicated by the single and double stars are significantly different from the control values at $P < 0.01$ and 0.005, respectively. O, Control; ●, TOFA at 0 min. The vertical bars indicate SEM.

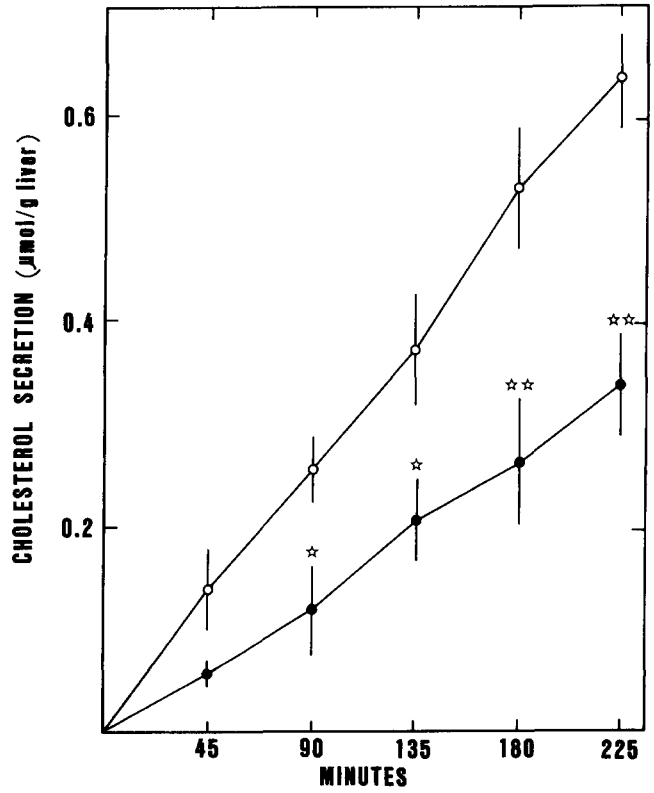

Fig. 6. Effect of TOFA on the net secretion of total cholesterol by the perfused liver. The experiments are the same as those described in Table 2 and Figs. 4 and 5. The values indicated by the single and double stars are significantly different from the control values at $P < 0.05$ and 0.02, respectively. O, Control; ●, TOFA at 0 min.

very similar, namely 1.1 μmol/g (Fig. 5) and 1.0 μmol/g (Fig. 2). Incorporation of [1-^{14}C]oleate into secretory triglyceride was likewise depressed (Fig. 5). TOFA also decreased cholesterol secretion (**Fig. 6**). The contributions of infused oleate to perfusate triglyceride were 47.1 and 38.3% at the end of perfusion for control and TOFA-treated livers, respectively (**Table 3**). This was significantly different. The contribution of [1-^{14}C]oleate to total liver triglyceride, however, was not significantly altered (Table 3). Accordingly, esterification of both [1-^{14}C]oleate and endogenous fatty acids into liver triglyceride decreased in parallel following TOFA administration. If TOFA inhibited only fatty acid synthesis, a higher contribution of infused fatty acid to liver triglyceride would be anticipated.

The lipid content and radioactivities of hepatic lipid fractions at the end of perfusion are shown in **Table 4.** Significantly less cholesterol, but similar concentrations of triglyceride and phospholipid, were found after TOFA treatment. Whereas the incorporation of [1-^{14}C]oleate into liver triglyceride was considerably decreased, the

TABLE 3. Contributions of perfusate oleic acid substrate to the perfusate and liver triglyceride

Time	Control (4)	TOFA at 0 min (4)	P
min	%	%	
Perfusate			
45	19.1 ± 3.2[a]	23.4 ± 2.4	NS
90	37.4 ± 1.4	34.2 ± 3.9	NS
135	39.9 ± 2.2	37.3 ± 3.0	NS
180	42.2 ± 0.9	37.7 ± 2.3	NS
225	47.1 ± 1.8	38.2 ± 2.3	<0.025
Liver			
225	25.4 ± 3.2	21.1 ± 2.6	NS

The experiments are the same as described in Table 2. These values were calculated from the data in Fig. 5. Values represent the mean ± SEM. Subtraction of the percentage in this table from 100 accordingly represents the contribution of endogenous fatty acids to the perfusate triglyceride, which accumulated during each period, and to the liver triglyceride at 225 min.

[a] [Amount (μmol) of [1-^{14}C]oleic acid substrate converted to triglyceride ÷ μmol of triglyceride × 3] × 100.

conversion of infused oleate into phopholipid conversely increased, suggesting that TOFA affected glycerolipid synthesis.

The disposition of infused [1-^{14}C]oleate by the livers is summarized in **Table 5.** In control livers, about half of the [1-^{14}C]oleate substrate utilized by the liver was esterified and a similar quantity entered the pathway of oxidation. TOFA decreased esterification and increased oxidation, affecting the disposition of label into triglyceride and ketone bodies in a reciprocal manner. Oxidation to CO_2 was unaffected.

Since the incorporation of [1-^{14}C]oleate into total liver phospholipids was increased by TOFA (Table 4), individual phospholipid subclasses were separated by thin-layer chromatography and analyzed. The relative percentages (±SEM) of phospholipid phosphorus in phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, phosphatidic acid, and lysophosphatidylcholine at 225 min were, respectively, 50.0 ± 0.8, 28.6 ± 0.7, 12.1 ± 1.0, 7.6 ± 0.9, and 2.2 ± 0.5% in the control, and 51.3 ± 1.4, 28.0 ± 0.4, 10.0 ± 1.4, 7.4 ± 1.1 and 3.4 ± 1.4% in the TOFA-treated livers. The distributions of radioactivity in these fractions were, respectively, 68.0 ± 1.0, 14.7 ± 2.1, 6.4 ± 1.7, 6.3 ± 1.5 and 4.6 ± 1.5% in the control group and 69.1 ± 1.6, 17.0 ± 1.7, 4.7 ± 1.0, 5.1 ± 1.8 and 4.0 ± 0.9% in the livers exposed to TOFA. Phosphatidylcholine and lysophosphatidylcholine were of higher specific radioactivity, indicating preferential incorporation of [1-^{14}C]oleate into these phospholipids. No specific effects of TOFA on the content of total phospholipids (Table 4) and on the relative abundance of, and distribution of radioactivity in, phospholipid subclasses were observed.

In another series of experiments, perfusates from livers infused with [1-^{14}C]oleate were removed at 45-min intervals and fractionated at a solvent density of 1.006 g/ml by ultracentrifugation. Triglyceride and cholesterol in the d< and d > 1.006 g/ml fractions were measured. In this experiment, ketone body production by the control and TOFA-treated livers responded in the same manner as observed in the foregoing experiment, shown in Fig. 4. In all perfusates triglyceride was predominantly recovered in VLDL and similar percentages of the total triglyceride radioactivity, derived from [1-^{14}C]oleate substrate, were likewise found in VLDL except at the earliest (45 min) interval, during which preformed (unlabeled) VLDL were secreted. These results are summarized in **Table 6.** The same proportions of radioactivity were recovered in the perfusate VLDL (d < 1.006 g/ml) (data not shown). These results demonstrated that the TOFA-induced decreases in both net secretion of, and incorporation of label into, triglyceride (Fig. 5) were primarily

TABLE 4. Effects of TOFA on the content of hepatic lipids and incorporation of [1-^{14}C]oleate into hepatic lipid fractions

	Control (4)	TOFA at 0 min (4)	P
	μmol/g liver		
Lipid content			
Triglyceride	7.94 ± 0.52	6.22 ± 0.56	NS
Cholesterol	6.65 ± 0.27	5.36 ± 0.04	<0.01
Phospholipid	38.26 ± 0.74	41.18 ± 1.55	NS
Incorporation of [1-^{14}C]oleate in			
Cholesteryl ester	0.22 ± 0.02	0.14 ± 0.02	<0.05
Triglyceride	5.95 ± 0.62	3.87 ± 0.24	<0.02
Free fatty acid	0.13 ± 0.03	0.16 ± 0.03	NS
Diglyceride	0.31 ± 0.03	0.12 ± 0.03	<0.01
Phospholipid	1.91 ± 0.19	2.45 ± 0.14	<0.05

The experiments are the same as described in Table 2. At 225 min, the livers were removed from the perfusion apparatus and assayed for triglyceride, cholesterol, phospholipid, and radioactivities in lipid fractions. Values represent the mean ± SEM. Figures in parentheses refer to the number of livers perfused.

TABLE 5. Conversions of infused oleic acid substrate by the perfused liver

Products of esterification	Metabolism of Oleate (% of Total Uptake) Control (4)	TOFA at 0 min (4)	P
In perfusate			
Triglyceride	12.9 ± 1.7	5.8 ± 0.8	<0.02
Diglyceride	0.3 ± 0.1	0.1 ± 0.0	NS
Phospholipid	0.4 ± 0.0	0.4 ± 0.0	NS
Cholesteryl ester	0.2 ± 0.0	0.2 ± 0.1	NS
In liver			
Triglyceride	22.9 ± 1.9	15.0 ± 0.4	<0.01
Diglyceride	1.2 ± 0.1	0.5 ± 0.1	<0.005
Phospholipid	7.3 ± 0.2	9.7 ± 0.6	<0.01
Cholesteryl ester	0.9 ± 0.0	0.6 ± 0.1	NS
Products of oxidation			
Ketone bodies	14.2 ± 0.7	24.4 ± 1.8	<0.005
Carbon dioxide[a]	39.8 ± 2.0	43.5 ± 1.9	NS

These data were calculated from the values in Fig. 4, Fig. 5, and Table 6 and thin-layer chromatographic analysis of the perfusate and liver lipids. Results are expressed as percentages of the total [1-^{14}C]oleic acid utilized. Figures in parentheses refer to the number of perfusions.

[a] These values represent the total radioactivity utilized minus the amounts present in esterified fatty acids and ketone bodies. This is primarily CO_2 but also contains some water-soluble intermediates of oxidation such as acetyl-CoA and Krebs cycle intermediates.

associated with diminished secretion of VLDL. The decreased net secretion of cholesterol (Fig. 6) was also found to be a result of decreased cholesterol secreted in the form of VLDL (Table 6).

Mean bile production ± SEM by the 12 control and 12 TOFA at zero time livers in all experiments was 1520 ± 140 μl and 1412 ± 124 μl, respectively. Bile volume was not affected by TOFA under the conditions employed.

DISCUSSION

The production of VLDL by the liver is a complex function of the synthesis of various chemical constituents, their assembly into particulate structures, maturation by remodeling including chemical modifications during intracellular transport within membrane-bound compartments, and secretion of the nascent particles. We have investigated the metabolic contributions of circulating

TABLE 6. Lipid composition of perfusate very low density lipoproteins

Lipid Composition	Fraction Analyzed Perfusate	d < 1.006	d > 1.006
		nmol/ml	
Triglyceride			
Control (4)	292.6 ± 30.7	233.0 ± 52.3	28.9 ± 1.4
TOFA at 0 min (4)	117.2 ± 4.7	80.2 ± 4.8	14.9 ± 3.2
P	<0.005	<0.05	<0.01
Total cholesterol			
Control (4)	63.1 ± 6.6	34.7 ± 7.0	22.7 ± 4.0
TOFA at 0 min (4)	41.7 ± 2.9	13.8 ± 2.9	20.3 ± 4.2
P	<0.025	<0.05	NS

The lipoproteins of density < 1.006 g/ml and >1.006 g/ml were isolated by ultracentrifugation of the liver perfusates after 225 min of perfusion. The mean body weights ± SEM of the rats in the control and TOFA-treated groups were 343 ± 19 g and 334 ± 17 g, respectively. The corresponding liver weights were 14.8 ± 0.5 g and 15.2 ± 0.7 g, respectively. There were four perfusions in each group. All values are the mean ± SEM.

free fatty acids, fatty acid synthesis, and intrahepatocellular lipolysis in the provision of fatty acid substrate for the formation of lipid ester constituents of hepatic VLDL, and we have examined effects of TOFA thereon.

In the first series of experiments (Table 1, Figs. 1–3), rat livers were perfused in the absence of free fatty acid substrate to provide near maximum rates of fatty acid synthesis from glucose under the experimental conditions employed, namely in livers from rats fed chow ad libitum. Ketogenesis (Fig. 1), triglyceride secretion (Fig. 2), and the secretion of newly synthesized triglyceride after 90 min of perfusion (Fig. 3) were constant, demonstrating the metabolic steady state in this perfused organ system.

The net triglyceride secretion rate by the control livers was 0.42 μmol of triglyceride/g per hr (Fig. 2). At 225 min the calculated contributions of newly synthesized fatty acids to the triglyceride secreted was 19.6%. The liver contained 5.56 μmol of triglyceride/g (Table 1). The calculated contribution of fatty acid synthesis to this total liver triglyceride was 9.6%. Kuksis et al. (34) found that about 9% of the fatty acids and glyceride-glycerol in total liver lipids was replaced by newly synthesized fatty acids and glycerol during 3 hr of perfusion, measured by deuterated water incorporation. Approximately 6% of the triglyceride fatty acids were replaced in this period (34). The present results on ^3H_2O incorporation resemble their observations.

The ketone bodies produced and 80% of the triglyceride secreted by the control livers were derived from endogenous sources. This corresponds to the processing of about 1.9 and 3.6 μmol of fatty acids/g per 225 min, respectively. These fatty acid substrates are apparently derived from endogenous lipolytic processes (35–40). It is also possible that a portion of the endogenous triglyceride secreted was a consequence of en bloc transfer of triglyceride from the droplet pool to the secretory pool (41). These two hepatic triglyceride pools are metabolically and morphologically distinct (7–11, 42, 43).

TOFA appears to inhibit fatty acid synthesis at the site of acetyl-CoA carboxylase (18, 44–46). It has been suggested (18) that the active inhibitor is 5-(tetradecyloxy)-2-furoyl-CoA. Since 20% of the triglyceride secreted by the control livers was derived from fatty acid synthesis (Table 1), the marked 80–90% inhibition of fatty acid synthesis by TOFA might be expected to decrease triglyceride secretion about 20%. However, TOFA decreased triglyceride secretion by 70% (Table 1, Fig. 2). Therefore, TOFA clearly also inhibited the secretion of triglyceride derived from other, endogenous sources, causing an additional 50% decrease (0.8 μmol/g) in the accumulation of triglyceride in the perfusate. The marked increase in ketogenesis following TOFA treatment (Fig. 1) provides an explanation. Thus, TOFA increased ketogenesis about 12 μmol/g, equivalent to the oxidation of about 2.8 μmol of long chain fatty acid (with an estimated average chain length 17 carbons), corresponding to about 0.9 μmol of triglyceride. This is very close to the deficit observed. Therefore, TOFA exerted dual and additive effects; *1*) decreased fatty acid synthesis and *2*) increased oxidation of endogenous fatty acids, which consequently decreased triglyceride secretion 20% and 50%, respectively. The subsequent studies provided an integrated mechanism for these effects of TOFA, as discussed later.

The perfusion of oleic acid substrate enhanced ketogenesis (Figs. 1, 4) and triglyceride secretion (Figs. 2, 5). These processes reached a metabolic steady state after 90 min of perfusion. The high rate of ketogenesis in the first 45 min (Fig. 4) was probably a consequence of the priming dose of oleic acid at zero time. The initial delay in attaining a constant rate of triglyceride secretion (Fig. 5) was caused by the time required for the assembly and secretion of VLDL particles. The appearance of radioactivity from the infused fatty acid substrate in ketone bodies and secretory triglyceride paralleled the accumulation of ketone bodies and triglyceride in the perfusate (Figs. 4, 5). It was found that 45–47% of both ketone bodies and secretory triglyceride were derived from the oleic acid substrate. Accordingly, the same blend of exogenous and endogenous fatty acids was processed in the pathways of oxidation and VLDL production. This suggests that a common intracellular pool of free fatty acids was utilized for oxidation by mitochondria and for esterification by the endoplasmic reticulum.

The liver utilized newly synthesized fatty acids and infused free fatty acids for esterification and lipid secretion in a similar manner in the following processes: *a*) of the total conversion of these fatty acids to esterified products, 20–30% were secreted and 70–80% were utilized for the synthesis of liver lipids; *b*) of the newly synthesized and infused fatty acids which were secreted, 85–95% were in the form of secretory triglyceride; *c*) of the fatty acids utilized for the synthesis of liver lipid esters, although most of the de novo synthesized fatty acids were utilized for phospholipid formation and most of the infused oleate was converted to triglyceride, the actual quantities of phospholipid produced from these two fatty acid sources were similar. Therefore, phospholipid synthesis from either source proceeded preferentially in the esterification process and the surplus was converted to triglyceride, and *d*) triglycerides produced from both fatty acids synthesized de novo and free fatty acids from the perfusate were preferentially secreted. It is concluded that fatty acids derived from both sources enter a common precursor pool in the formation of lipid esters for intracellular use and for the construction of secretory lipoproteins.

TOFA exerted marked and reciprocal effects on oleic acid oxidation to ketone bodies and esterification to tri-

glyceride (Table 5), accounting for the decreased synthesis and secretion of triglyceride-rich lipoproteins by the perfused rat liver (Fig. 5, Table 6). However, TOFA treatment did not substantially alter the specific radioactivities of the ketone bodies and triglyceride in the perfusate. Although TOFA caused a small decrease in the specific radioactivity of secretory triglyceride, this change became significant only at 225 min and was quantitatively minor (Table 3). It is therefore clear that TOFA increased ketone bodies and decreased the secretion of triglyceride synthesized from both infused free fatty acid substrate and from fatty acids arising from endogenous sources.

While TOFA inhibited the synthesis of triglyceride from [1-^{14}C]oleate, conversion of this substrate to phospholipids was actually elevated (Table 5). Total glycerolipid synthesis remained considerably less. The decrease in labeled diglyceride may be attributed to decreased flux in the acylation of glycerophosphate. These observations suggest that TOFA concurrently inhibited acylation of diglyceride and stimulated phospholipid synthesis, the latter by lack of competition for diglyceride or by some type of direct stimulatory action. Phospholipid synthesis was preferentially maintained, in support of the earlier observation that when the long chain fatty acid supply is limiting, the fatty acid is preferentially esterified to phospholipid (see Fig. 6 in Ref. 4).

The decrease in net secretion of cholesterol caused by TOFA (Fig. 6) appears to be a combined result of depressed cholesterol synthesis (Table 1) and decreased secretion of triglyceride-rich lipoproteins (Table 6), which contain substantial amounts of cholesterol. TOFA decreased the secretion of VLDL triglyceride and VLDL cholesterol to a similar extent (66% and 60%, respectively, Table 6). Accordingly, with regard to these major core components, the lipid composition of the VLDL particles was not appreciably affected.

It is clear from the studies of McGarry and Foster (46–48) that fatty acid oxidation is inhibited by malonyl-CoA, via inhibition of carnitine acyltransferase. Depression of the hepatic malonyl-CoA concentration (45–46), therefore, appears to be the mechanism by which TOFA stimulates ketogenesis. In the present study livers were derived from fed rats. In this nutritional state fatty acid oxidation by liver mitochondria is most sensitive to malonyl-CoA (49–51). Carnitine acyltransferase has also been implicated in this control process (52–54).

In rat hepatocytes (18, 45, 55) TOFA stimulated ketogenesis in the absence of fatty acid substrate, or at low fatty acid concentrations (0.5 mM), but had little or no effect at high concentrations of oleate (1–2 mM). McCune and Harris (18) explained this on the basis of inhibition of acetyl-CoA carboxylase by CoA esters of both oleate and TOFA. When acetyl-CoA carboxylase of liver cells was almost completely inhibited by high concentrations of oleate, TOFA exerted no further inhibition and had little effect on the concentration of malonyl-CoA. In the present study the concentration of oleic acid in the perfusion medium was maintained at approximately 0.3 mM and TOFA stimulated ketogenesis (Fig. 4). This indicates that the concentration of oleate provided to the perfused liver did not fully inhibit acetyl-CoA carboxylase activity.

Although TOFA increased oleate oxidation to ketone bodies, it did not affect the complete oxidation of oleate to CO_2 (Table 5). Since TOFA did not alter the ketone body specific radioactivity, it presumably did not change the intramitochondrial acetyl-CoA specific radioactivity. Therefore, the lack of effect of TOFA on $^{14}CO_2$ production indicates that TOFA did not alter citric acid cycle flux.

In the present experiments, circulating free fatty acids were quantitatively more important substrates than de novo fatty acid synthesis in the formation and packaging of triglyceride for export by the liver in VLDL particles. It is probable that fatty acid synthesis contributes a greater portion than presently observed, following a high carbohydrate, low fat meal, when hepatic fatty acid synthesis is enhanced (1, 13, 33, 56, 57). Conversely, fatty acid synthesis is probably of less significance than currently found, when its activity is suppressed by an elevated level of dietary fat (1, 33, 56, 57). The contribution of fatty acid synthesis to hepatic VLDL production, therefore, varies according to the prevailing nutritional state.

The observed reduction in net secretion of triglyceride and cholesterol by the perfused rat liver following addition of TOFA to the perfusion medium (Figs. 2, 5, 6, Tables 1, 6) demonstrates that the hypolipidemic action of TOFA (58) is caused, at least in part, by decreased hepatic production of triglyceride-rich lipoproteins.

The present studies have described the kinetics of fatty acid metabolism by the perfused rat liver by quantitative analyses of the component processes of fatty acid synthesis, oxidation, and esterification in relation to the production of triglyceride-rich lipoproteins. Investigation of the consequences of TOFA treatment on these conversions has provided determination of basic relationships between these metabolic processes. These studies suggest the importance of inhibition of fatty acid synthesis in initiating a series of metabolic adjustments culminating in decreased secretion of triglyceride and cholesterol in the form of very low density lipoproteins.

The expert technical assistance of Pamela Everett is gratefully acknowledged. We thank Dr. William L. Albrecht of Merrell Research Center, Cincinnati, OH, for the sample of RMI 14,514 (TOFA). This investigation was supported by Grant HL 23181 from the U.S. Public Health Service.

Manuscript received 3 January 1984.

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