# Decreased hepatic production of very low density lipoproteins following activation of fatty acid oxidation by Ro 22-0654

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**Abstract** In fed rat livers perfused with [1-$^{14}$C]oleic acid, Ro 22-0654 (4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester hydrochloride), an inhibitor of fatty acid synthesis, activated ketogenesis and decreased the secretion of triglyceride in very low density lipoproteins (VLDL). Ro 22-0654 was without effect on total oleic acid uptake and utilization by the liver. The liver triglyceride content, urea synthesis, and bile production were also unaffected. Ro 22-0654 increased the conversion of both exogenous and endogenous fatty acid substrates to ketone bodies, while decreasing the secretion of triglyceride synthesized from both of these sources. Depressed fatty acid synthesis accounted for a relatively small portion of the decrease in secretory triglyceride derived from endogenous sources. $^{14}CO_2$ from [1-$^{14}$C]oleic acid was unchanged by Ro 22-0654. This drug decreased the malonyl-CoA content of rat liver freeze-clamped in vivo, providing an explicable mechanism for its activation of fatty acid oxidation. Hepatic citrate was also diminished. The present studies indicate the following sequence of events in the liver of fed rats following the administration of Ro 22-0654: decreased formation of citrate and malonyl-CoA, decreased fatty acid synthesis via decreased carbon supply and increased fatty acid oxidation via stimulation of acylcarnitine formation, decreased synthesis of triglyceride from both endogenous and exogenous fatty acids, resulting in the decreased formation and secretion of VLDL. — **Yamamoto, M., N. Fukuda, J. Triscari, A. C. Sullivan, and J. A. Ontko.** Decreased hepatic production of very low density lipoproteins following activation of fatty acid oxidation by Ro 22-0654. *J. Lipid Res.* 1985. **26:** 1196–1204.

**Supplementary key words** cholesterol • citrate • fatty acid synthesis • ketone bodies • malonyl-CoA • oleic acid • perfused liver • phospholipids • secretion • triglyceride

Very low density lipoproteins (VLDL) are major conveyors of triglyceride and cholesterol from the liver to extrahepatic tissues (1–4). This transport process is characterized by intravascular lipolysis of VLDL triglyceride and the formation of low density lipoproteins (LDL). Accordingly, the rate of VLDL production by the liver is a key determinant of plasma lipid concentrations.

Our previous studies on the regulation of VLDL production have examined metabolic and genetic conditions characterized by the hypersecretion of VLDL particles (5–9). Multiple effects of 5-tetradecyloxy-2-furoic acid (TOFA) in the perfused liver, culminating in decreased hepatic triglyceride and cholesterol secretion, were also investigated (10). We now report depression of hepatic VLDL production by the drug Ro 22-0654 (**Fig. 1**), an inhibitor of fatty acid synthesis (11), and definition of underlying alterations in hepatocellular fatty acid metabolism. This agent decreased hepatic triglyceride secretion without concurrent accumulation of lipids in the liver. Results implicate activation of hepatic ketogenesis as a major event in the sequence of metabolic alterations.

## EXPERIMENTAL PROCEDURES

### Animals

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

Abbreviations: VLDL, very low density lipoproteins; Ro 22-0654, 4-amino-5-ethyl-3-thiophenecarboxylic acid methyl ester hydrochloride; TOFA, 5-tetradecyloxy-2-furoic acid.

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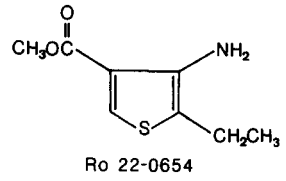

Ro 22-0654

**Fig. 1.** 4-Amino-5-ethyl-3-thiophenecarboxylic acid methyl ester.

### Liver perfusion

Liver perfusions were at 37°C as described previously (5, 6). Total $^{14}CO_2$ was measured in the perfusate (12) and by collecting effluent vapors in 500-ml gas washing bottles fitted with coarse (c) porosity fritted glass discs. These bottles each contained 250–270 ml 5 N KOH. One bottle collected vapors from the silastic tubing lung (13). Two bottles in series collected vapors from the mixing chamber. Gas flow was maintained by application of a partial vacuum at the gas washing bottle exit side arm. $^{14}CO_2$ in 0.5-ml samples of 5 N KOH was counted in 2 ml of methanol and 10 ml of Ready-Solv (Beckman Instruments) with correction for quenching. By this procedure, 96% of standard $NaH^{14}CO_3$ was recovered as $^{14}CO_2$ during 225 min of recirculating perfusion in the absence of a liver.

The recirculating liver perfusion medium (6) contained 90 ml of Krebs-Henseleit buffer (pH 7.4), 25 mM glucose, 1.5% fatty acid-free bovine serum albumin (Fraction V fatty acid free, Miles Laboratories) and 30 ml of aged human erythrocytes. A priming dose of [1-$^{14}$C]oleic acid substrate (5 ml of 20 mM potassium oleate in 0.9% NaCl) was added at the beginning of the perfusion and the same solution was infused continuously (4.5 ml/hr) for 225 min. Under these conditions delivery of oleate to the liver in the portal vein plasma stabilized at 290–320 μM, while the oleate concentration in the effluent perfusate plasma (hepatic venous return via the vena cava) was 180–210 μM. The hematocrit was not significantly altered during the perfusion.

In the single-pass perfusions, after 160 min of recirculation as described above, fresh medium was introduced at the same constant rate of 20 ml/min. This medium was the same as that perfused at the beginning of the perfusion (zero time) except that it contained 0.30 mM [1-$^{14}$C]oleic acid in the plasma (0.22 mM in the total medium containing erythrocytes). This substrate was of the same specific radioactivity as that perfused throughout the recirculating period. The single-pass perfusion proceeded for 20 min. During this period the effluent perfusate was collected at 5-min intervals.

Ro 22-0654 (hydrochloride salt from Hoffmann-La Roche, Nutley, NJ) was added at zero time (20 μmol in 4 ml of 3% fatty acid-free bovine serum albumin at pH 7.4 in 0.9% NaCl) and 5 μmol in the same solution (1 ml) was added at 45-min intervals in recirculating perfusions. In single-pass perfusions, 350 μM Ro 22-0654 was present in the perfusion plasma. These concentrations inhibit hepatic fatty acid synthesis by 70%, as described herein, which corresponds to inhibition observed in vivo following an i.p. dose of 89 mg or 400 μmol/kg body wt.

### Analytical methods

The methods employed for the lipid, lipoprotein, and ketone body analyses in the livers and liver perfusates were described previously (5, 6). Hepatic malonyl-CoA was measured by the method of McGarry, Stark, and Foster (14) and citrate was assayed fluorometrically (15) in the same perchloric acid extracts. Urea was analyzed in perchloric acid extracts of the perfusate (5) by the procedure of Chaney and Marbach (16). Fatty acid synthesis was measured with $^3H_2O$ as previously described (9).

## RESULTS

The present studies on hepatic VLDL production have been focused on alterations in the utilization of infused oleic acid substrate by the perfused liver, since free fatty acids are major precursors of hepatic secretory VLDL triglyceride (1–6, 17–22).

Data for the animals and livers in the first series of liver perfusion experiments are shown in **Table 1**. The average liver weight at the end of the perfusion period and the mean perfusate flow rate were not affected by Ro 22-0654, indicating no significant alteration of hepatic circulation and fluid balance. Likewise, urea synthesis and bile production were unaltered by the presence of Ro 22-0654.

The effects of Ro 22-0654 on ketone body production are shown in **Fig. 2.** Ketogenesis was elevated by Ro 22-0654, as was the incorporation of radioactivity from the infused [1-$^{14}$C]oleic acid substrate into ketone bodies. The specific radioactivity of ketone bodies was not altered

TABLE 1. Characteristics of the animals and perfused livers

| | Control (3)[a] | Ro 22-0654 (3) | *P* |
|---|---|---|---|
| Body weight (g) | 335 ± 13[b] | 347 ± 14 | NS[c] |
| Liver weight (g)[d] | 13.7 ± 0.3 | 14.0 ± 0.2 | NS |
| Stomach weight (g) | 8.9 ± 0.5 | 8.5 ± 0.2 | NS |
| Bile production (μl)[e] | 2210 ± 185 | 2553 ± 168 | NS |
| Urea production (μmol/g)[f] | 12.7 ± 0.4 | 10.8 ± 1.3 | NS |
| Perfusate flow (ml/min)[g] | 17.2 ± 0.1 | 17.0 ± 0.1 | NS |

[a]Indicates the number of animals and perfused livers in each group.
[b]Standard error of the mean.
[c]NS, not significant.
[d]Determined at the end of the perfusion period.
[e]Entire volume produced in 225 min of perfusion.
[f]Amount produced in 225 min of perfusion per g of liver.
[g]The flow rate was measured at 45-min intervals in each perfusion. The average rate in each perfusion was used to calculate the mean value.

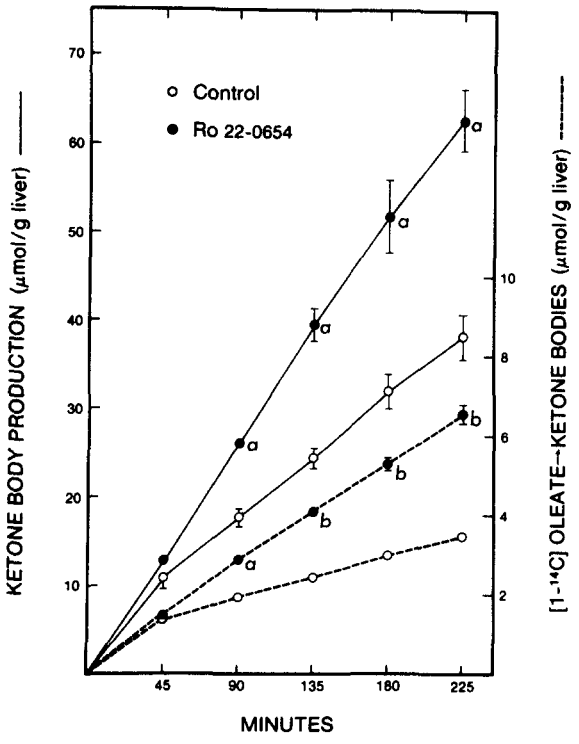


**Fig. 2.** Effect of Ro 22-0654 on the production of ketone bodies by the perfused rat liver. Livers from fed rats were perfused with Krebs-Henseleit buffer (pH 7.4) containing 25 mM glucose, 1.5% bovine serum albumin (fatty acid-free), and 25% human aged erythrocytes, at a rate of 20 ml/min. Ro 22-0654 complexed with bovine serum albumin was added at 0 min and at each 45-min interval, as described in Experimental Procedures. The animals and livers are described in Table 1. Each group had three animals. Solid lines indicate total ketone body production. Dotted lines indicate [1-$^{14}$C]oleate conversion to ketone bodies. The values designated a and b are significantly different from the control values at $P < 0.005$ and 0.001, respectively. ○, Control; ●, Ro 22-0654. The vertical bars indicate SE.

by the drug (data not shown). The contributions of [1-$^{14}$C]oleic acid substrate to perfusate ketone bodies, calculated as previously described (5), in the control system were 58, 49, 46, 42, and 41% at the consecutive time intervals. The corresponding contributions in the Ro 22-0654 group were 52, 48, 47, 46, and 47%, respectively. These values were not appreciably affected by Ro 22-0654. Accordingly, the drug did not alter the proportions of endogenous and exogenous substrates converted to ketone bodies. Ro 22-0654 caused a mild but significant decrease in the β-hydroxybutyrate:acetoacetate ratio. These values in the control perfusates (Fig. 2) at 45, 90, 135, 180, and 225 min were 1.00 ± 0.12, 0.85 ± 0.06, 0.93 ± 0.08, 0.93 ± 0.05, and 1.11 ± 0.13 (mean ± SE), respectively. The corresponding ratios in the experimental group were 0.58 ± 0.03 ($P < 0.05$), 0.57 ± 0.02 ($P < 0.02$), 0.63 ± 0.05 ($P < 0.02$), 0.68 ± 0.03 ($P < 0.05$), and 0.72 ± 0.01 ($P < 0.05$), respectively.

Ro 22-0654 decreased net triglyceride secretion (**Fig. 3**). Although evident at 135 min, this alteration required 180 min for statistical significance. The conversion of infused [1-$^{14}$C]oleic acid into secretory triglyceride paralleled triglyceride accumulation in the perfusate. Triglyceride specific radioactivities were unaffected by Ro 22-0654 (data not shown). The contributions of infused [1-$^{14}$C]oleic acid to total triglyceride fatty acids in the perfusate, calculated as previously described (10), were 33, 43, 46, 49, and 49% at the successive 45-min intervals in the control system. Following drug treatment, these values were 40, 45, 44, 44, and 43%. Ro 22-0654 clearly decreased the secretion of both exogenous and endogenous fatty acid substrates in the form of triglyceride.

The contribution of fatty acid synthesis to liver triglyceride synthesis and secretion was examined in other experiments under identical conditions of perfusion, in which $^3H_2O$ and unlabeled oleic acid were infused. It was found that only 4% of the triglyceride secreted by the control livers was derived from fatty acids synthesized de novo during the perfusion period. Ro 22-0654 inhibited fatty acid synthesis by 70%. In these experiments, 90% of the fatty acids synthesized de novo was recovered in liver lipids at the end of the perfusion period. Thus, a relatively small fraction of the newly synthesized fatty acids was incorporated into the lipids of nascent plasma lipoproteins.

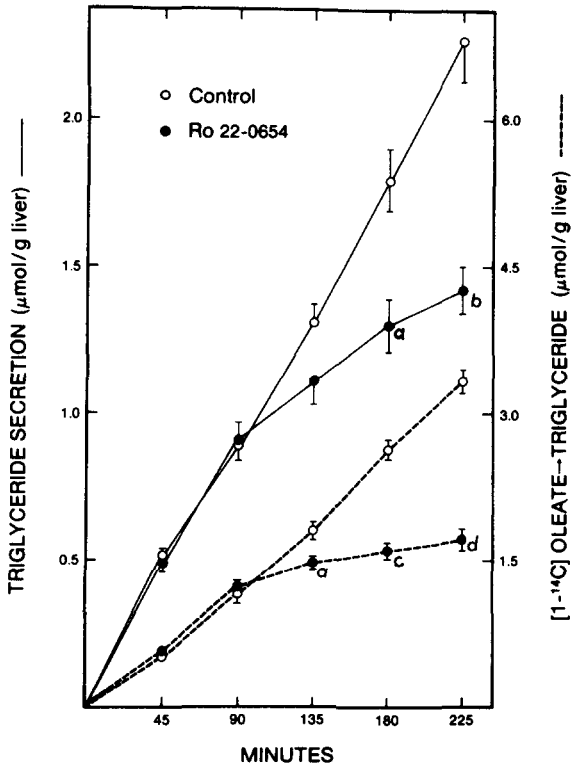


**Fig. 3.** Effect of Ro 22-0654 on the net secretion of triglyceride by the perfused rat liver. The experiments are the same as those described in the legend to Fig. 2. The values designated a, b, c, and d are significantly different from the control values at $P < 0.05$, 0.01, 0.005, and 0.001, respectively. ○, Control; ●, Ro 22-0654. The vertical bars indicate SE.

Cholesterol secretion (**Fig. 4**) was less affected by Ro 22-0654 than triglyceride secretion (Fig. 3). However, when the cholesterol secretion rates between 135 and 225 min were compared, Ro 22-0654 decreased the net rate of cholesterol secretion from the control value of 0.19 ± 0.01 μmol/g to 0.15 ± 0.01 μmol/g ($P < 0.05$).

The amounts of triglyceride and cholesterol in the liver were not altered by 225 min of persusion with Ro 22-0654 (**Table 2**). The uptake of [1-$^{14}$C]oleic acid substrate from the perfusion medium was likewise unaffected. Total utilization of the infused oleic acid substrate was not altered by the drug, since total free fatty acid uptake by the liver and that recovered in the liver at 225 min were unchanged (Table 2). Consistent with the decrease in triglyceride secretion (Fig. 3), the esterification of [1-$^{14}$C]oleic acid into liver triglyceride and diglyceride was depressed by Ro 22-0654 (Table 2). The conversions of infused oleic acid to liver phospholipids and cholesteryl esters were unaltered.

Effects of Ro 22-0654 on the overall metabolism of [1-$^{14}$C]oleic acid, provided by constant infusion in the recirculating liver perfusion system, are summarized in **Table 3.** Although Ro 22-0654 increased the oxidation of [1-$^{14}$C]oleic acid to ketone bodies, it did not alter the production of $^{14}CO_2$. The increase in oxidation products and the decrease in esterification products were balanced. Approximately 18% of the oleic acid utilized in the control system was not recovered in the specific fractions identified. This amount is as expected from earlier studies (11) and may be attributed to the presence of oleic acid radioactivity in water-soluble intermediates of oxidation, such as Krebs cycle intermediates, acetyl-CoA, acetylcarnitine, glutamate, and aspartate. Ro 22-0654 did not alter the amount of radioactivity in this unrecovered fraction.

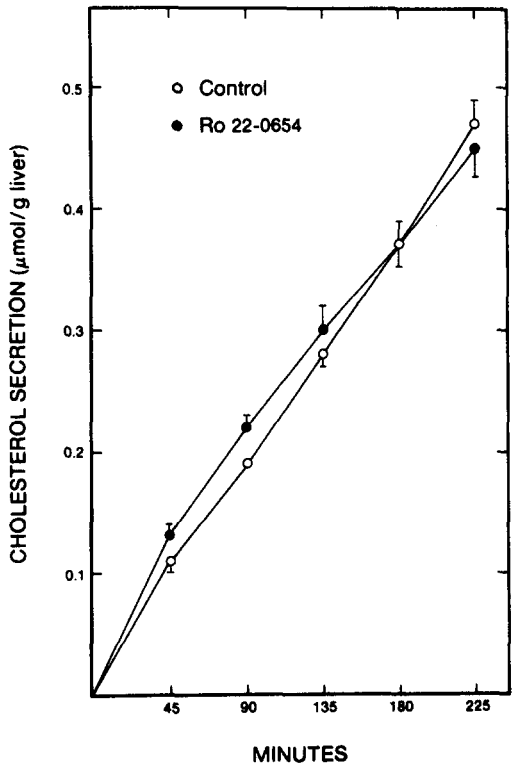


**Fig. 4.** Effects of Ro 22-0654 on net secretion of cholesterol by the perfused rat liver. The experiments are the same as those described in the legend to Fig. 2. ○, Control; ●, Ro 22-0654. The vertical bars indicate SE.

Since Ro 22-0654 caused such a marked reduction in triglyceride secretion (Fig. 3), the quantities of triglyceride and cholesterol in the very low density lipoproteins (d < 1.006 g/ml) of the perfusate were examined. In this experiment (**Table 4**), Ro 22-0654 decreased the perfusate triglyceride concentration 32% and this was found to be

TABLE 2. Effects of Ro 22-0654 on hepatic lipids, uptake of [1-$^{14}$C]oleate, and incorporation of [1-$^{14}$C]oleate into hepatic lipid fractions

| | Control (3) | Ro 22-0654 (3) | *P* |
|---|---|---|---|
| | *μmol/g liver* | | |
| Lipid content | | | |
| Triglyceride | 8.20 ± 0.36 | 7.38 ± 0.25 | NS |
| Cholesterol | 5.13 ± 0.15 | 5.29 ± 0.12 | NS |
| Uptake of [1-$^{14}$C]oleate | 29.7 ± 0.6 | 28.9 ± 0.4 | NS |
| Incorporation of [1-$^{14}$C]oleate in liver | | | |
| Cholesteryl ester | 0.36 ± 0.01 | 0.29 ± 0.04 | NS |
| Triglyceride | 7.46 ± 0.50 | 6.34 ± 0.28 | <0.05 |
| Free fatty acid | 0.15 ± 0.02 | 0.16 ± 0.03 | NS |
| Diglyceride | 0.54 ± 0.06 | 0.27 ± 0.03 | <0.05 |
| Phospholipid | 2.71 ± 0.04 | 2.40 ± 0.13 | NS |

The experiments are the same as described in Table 1. At 225 min livers were removed from the perfusion apparatus and assayed for lipids and radioactivity in lipid fractions. The uptake of oleate was calculated from the total amount infused (100 μmol priming dose plus 90 μmol/hr) less the amount remaining in the perfusate at 225 min. All values represent the mean ± SE. Values in parentheses refer to the number of perfused livers in each group.

TABLE 3. Conversions of infused oleic acid substrate by the perfused liver

| | Oleate Utilization (% of total) | | |
|---|---|---|---|
| | Control (3) | Ro 22-0654 (3) | *P* |
| | % | | |
| Products of esterification | | | |
| *In perfusate* | | | |
| Triglyceride | 12.2 ± 0.5 | 6.3 ± 0.4 | <0.005 |
| Diglyceride | 0.1 ± 0.0[a] | 0.1 ± 0.0 | NS |
| Phospholipid | 0.5 ± 0.0[b] | 0.4 ± 0.0 | NS |
| Cholesteryl ester | 0.4 ± 0.0[c] | 0.2 ± 0.0 | <0.02 |
| *In liver* | | | |
| Triglyceride | 25.1 ± 1.2 | 19.9 ± 1.1 | <0.05 |
| Diglyceride | 1.8 ± 0.5 | 0.9 ± 0.1 | <0.05 |
| Phospholipid | 9.1 ± 0.3 | 8.3 ± 0.3 | NS |
| Cholesteryl ester | 1.2 ± 0.1 | 1.0 ± 0.1 | NS |
| Products of oxidation | | | |
| Ketone bodies | 12.7 ± 0.2 | 24.7 ± 0.8 | <0.001 |
| Carbon dioxide | 19.0 ± 0.6 | 18.7 ± 0.5 | NS |
| Undetermined[d] | 18.4 ± 1.5 | 18.9 ± 1.0 | NS |

These values were calculated from the data in Fig. 2, Fig. 3, Table 3, thin-layer chromatographic analysis of perfusate lipids, and $^{14}CO_2$ collection. Results are expressed as percentages of the total [1-$^{14}$C]oleic acid utilized ± SE. Total utilization represents total [1-$^{14}$C]oleic acid infused less that recovered in the free fatty acid fraction at the end of the perfusion.

[a]The SD were 0.01 (control) and 0.00 (Ro 22-0654).

[b]The SD were 0.05 (control) and 0.02 (Ro 22-0654).

[c]The SD were 0.06 (control) and 0.04 (Ro 22-0654).

[d]These values refer to total utilization less that recovered in the various fractions above. The total recoveries were therefore 81.6% and 81.1% in the control and Ro 22-0654 groups, respectively.

localized in the VLDL density fraction. Less than 10% of the triglyceride was recovered in the d > 1.006 g/ml density fraction (low density plus high density lipoproteins). Slightly more than half of the cholesterol secreted by the liver was found in VLDL and this was not appreciably altered by Ro 22-0654.

In the recirculating liver perfusion system, it is possible that differences in the rates of accumulation of ketone bodies and triglyceride in the perfusate are, at least in part, caused by differential utilization of ketone bodies (23) and removal of triglyceride (24–26) by the liver. To examine these possibilities, ketone body production and triglyceride secretion were measured during the single-passage of perfusion medium through the liver. Livers were perfused for 160 min by recirculation and fresh medium was then continuously perfused for a period of 20 min without recirculation. In this manner total production of ketone bodies and total secretion of triglyceride were measured (**Fig. 5**). Ro 22-0654 significantly increased total hepatic ketogenesis and decreased total liver triglyceride secretion during the single passage of perfusion medium.

This experiment, by providing a comparison between net ketone body production and net triglyceride secretion during the recirculating perfusion and total ketogenesis and triglyceride secretion during the single-pass perfusion, provided an analysis of hepatic ketone body utilization and the degree of recycling (uptake) of triglyceride. These data are shown in **Table 5.** Ketogenesis during the single-pass perfusion was the same as that observed during recirculation. The livers therefore did not utilize ketone bodies to a measurable extent. In contrast, triglyceride secretion during the single-passage of perfusion medium exceeded the rate of triglyceride accumulation during recirculation. The difference was 12 nmol of triglyceride/g per 5 min (control) and 15 nmol of triglyceride/g per 5 min (experimental). Accordingly, the livers removed triglyceride at the rate of 2.4–3.0 nmol/g per min during recirculation.

Since malonyl-CoA is an inhibitor of fatty acid oxidation (27, 28), the mechanism by which Ro 22-0654 increased ketogenesis (Fig. 2) was investigated by measurements of the hepatic malonyl-CoA concentration. Ninety min after the intraperitoneal injection of Ro 22-0654, the malonyl-CoA content of rat liver was decreased by more than 50% (**Table 6**). Hepatic citrate was also depressed (Table 6). The dose of Ro 22-0654 in this experiment (89 mg or 400 μmol/kg body weight) was chosen as we have found that it provides concentrations of the drug that inhibit hepatic fatty acid synthesis in vivo and in the perfused liver, as described above, to a similar extent.

## DISCUSSION

The perfused rat liver, provided with a continuous infusion of [1-$^{14}$C]oleic acid, exhibited constant rates of accumulation of ketone bodies and triglyceride in the recirculating perfusate, the specific radioactivities of

TABLE 4. Accumulation of triglyceride in liver perfusate lipoprotein density fractions

| | Triglyceride | | |
|---|---|---|---|
| Density Fraction | Control (3) | Ro 22-0654 (3) | *P* |
| *g/ml* | *nmol/ml* | | |
| Perfusate | 174.4 ± 8.1[a] | 119.4 ± 3.7[a] | <0.005 |
| d < 1.006 g/ml | 156.2 ± 7.7[b] | 99.1 ± 8.2[b] | <0.01 |
| d > 1.006 g/ml | 12.4 ± 1.6[c] | 9.5 ± 0.7[c] | NS |

VLDL were isolated from liver perfusates, after 160 min of recirculation, by ultracentrifugation at d < 1.006 g/ml, after removal of erythrocytes, as described in Experimental Procedures. The livers are the same as those described in Fig. 5.

[a]The concentrations of total cholesterol were 30.6 ± 2.7 nmol/ml (control) and 30.8 ± 1.0 nmol/ml (Ro 22-0654).

[b]The concentrations of total cholesterol were 17.1 ± 1.3 nmol/ml (control) and 16.9 ± 0.9 nmol/ml (Ro 22-0654).

[c]The concentrations of total cholesterol were 12.2 ± 1.5 nmol/ml (control) and 11.8 ± 0.2 nmol/ml (Ro 22-0654).

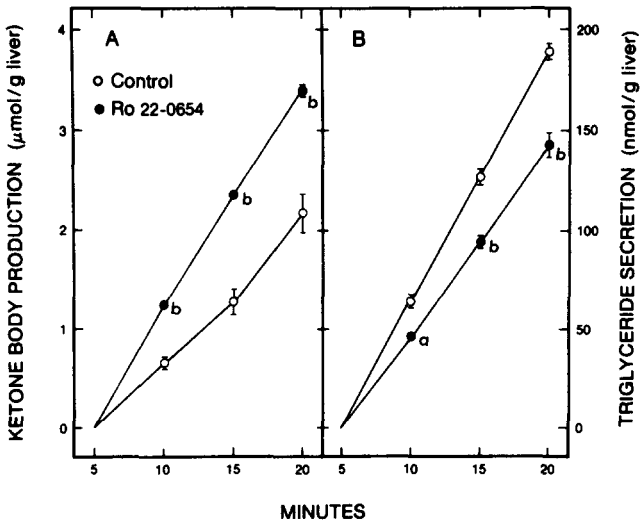


**Fig. 5.** Effects of Ro 22-0654 on ketogenesis and triglyceride secretion by rat livers perfused via single-pass. The livers were perfused for 160 min of recirculation (100 μmol primary dose of [1-$^{14}$C]oleic acid and a constant infusion of 90 μmol of [1-$^{14}$C]oleic acid/hr) and then 20 min of single-passage (0.30 mM [1-$^{14}$C]oleic acid) as described under Experimental Procedures. The three control livers weighed 15.5 ± 0.01 g and the three Ro 22-0654 livers were 15.3 ± 0.3 g. The liver donor animals were 361 ± 4 g (control) and 349 ± 9 g (Ro 22-0654). The perfusates collected during the first 5 min of single-pass perfusion contained residual recirculating perfusate and were therefore not used. The values designated a and b are significantly different from the control values at $P < 0.01$ and 0.005, respectively. O, Control; ●, Ro 22-0654. The vertical bars indicate SE.

which became constant after 90 min of perfusion in both control and treated groups (Figs. 2 and 3). Achievement of these steady metabolic states validates direct kinetic comparison of the two systems. The observed lag period in the development of constant rates represents the time required for *a*) the metabolic adjustments of the liver to the priming dose of [1-$^{14}$C]oleic acid and to the artificial perfusion medium, and *b*) the attainment of constant specific radioactivities in cellular metabolic pools. Secretory triglyceride exhibited a longer lag period than ketone bodies, owing to the greater time required for the construction and secretion of VLDL particles (20–22). Ro 22-0654 exerted effects on fatty acid metabolism rapidly, with differences in ketogenesis reaching significance at 90 min. Sensitive parameters of liver function, namely bile production and urea synthesis (Table 1), were not measurably altered by Ro 22-0654. Although Ro 22-0654 decreased the β-hydroxybutyrate:acetoacetate ratio (see Results), this ratio remained sufficiently high to maintain a normal hepatic adenine nucleotide phosphorylation state (29). In addition, total fatty acid uptake and utilization were unaffected by Ro 22-0654 (Table 2). Alterations in the metabolism of the [1-$^{14}$C]oleic acid substrate following treatment with Ro 22-0654 may therefore be attributed to effects of the drug on intracellular metabolism. Accordingly, in terms of all measured indices, Ro 22-0654 produced no observable toxic effect on the liver.

Fatty acid oxidation was clearly activated by Ro 22-0654. While the conversion of both exogenous (infused) and endogenous fatty acids to ketone bodies was elevated (Fig. 1, text), complete oxidation of the fatty acid to $CO_2$ via the Krebs cycle was unaffected (Table 3). The specific radioactivity of acetyl-CoA was unaffected by Ro 22-0654, as demonstrated by the unaltered ketone body specific radioactivity. Accordingly, Ro 22-0654 increased β-oxidation of long chain fatty acids to acetyl-CoA and its subsequent conversion to ketone bodies but did not alter Krebs cycle carbon flux. The mechanism by which Ro 22-0654 enhanced β-oxidation of oleic acid is suggested by the decreased hepatic malonyl-CoA concentration (Table 6). The studies of McGarry and Foster have demonstrated an inhibitory effect of malonyl-CoA on long chain fatty acid oxidation by inhibition of acylcarnitine formation (27, 28). Ro 22-0654, by decreasing cytosolic malonyl-CoA, may promote acylcarnitine formation and, consequently, acylcarnitine transport across the inner mitochondrial membrane followed by β-oxidation of long chain acyl groups to acetyl-CoA. Ro 22-0654 is also an inhibitor of fatty acid synthesis (9). The observed decrease in liver malonyl-CoA and citrate (Table 6) also provides metabolic localization of this inhibitory action, namely at the level of citrate lyase and/or at some prior reaction. Decreased fatty acid synthesis was not a significant factor in the Ro 22-0654-induced depression of triglyceride secretion, since *a*) only about 4% of the triglyceride secreted by the control livers was derived from de novo synthesis, and *b*) the specific radioactivity of perfusate triglyceride following [1-$^{14}$C]oleate infusion would have been increased by Ro 22-0654 if the total triglyceride synthesized from the unlabeled precursors was preferentially

TABLE 5. Comparative rates of ketogenesis and triglyceride secretion by isolated rat livers during recirculating and single-pass perfusion

| | Rates of Production | | |
|---|---|---|---|
| | Recirculation[a] | Single-Pass[b] | *P* |
| | *μmol/g per 5 min* | | |
| Ketone bodies | | | |
| Control (3) | 0.57 ± 0.05[c] | 0.63 ± 0.02 | NS |
| Ro 22-0654 (3) | 1.32 ± 0.04 | 1.15 ± 0.05 | NS |
| *P* | <0.001 | <0.001 | |
| Triglyceride | | | |
| Control (3) | 0.051 ± 0.001 | 0.063 ± 0.001 | <0.001 |
| Ro 22-0654 (3) | 0.033 ± 0.002 | 0.048 ± 0.001 | <0.005 |
| *P* | <0.001 | <0.001 | |

The liver perfusions were described in Fig. 5. The period from 45–160 min was used to calculate the net rates of ketogenesis and triglyceride secretion during recirculation. The 5–20 min period of single-pass perfusion was employed to determine the total rates of production.

[a] These values are net rates of production.

[b] These values are total rates of production.

[c] The mean value ± SE.

TABLE 6. Effect of Ro 22-0654 on malonyl-CoA and citrate contents of rat liver in vivo

| | Control (4)[a] | Ro 22-0654 (4) | P |
|---|---|---|---|
| Body weight (g) | 353 ± 9[b] | 344 ± 6 | NS |
| Malonyl-CoA (nmol/g liver)[c] | 4.70 ± 0.34 | 2.15 ± 0.19 | <0.001 |
| Citrate (nmol/g liver)[c] | 276 ± 17 | 164 ± 4 | <0.001 |

Rats fed laboratory chow ad libitum were injected intraperitoneally with Ro 22-0654 (89 mg/kg body wt) dissolved in 0.9% NaCl (89 mg/2.0 ml) at 8:30-9:00 AM. Livers were freeze-clamped at −196°C 90 min later and perchloric acid extracts were prepared for malonyl-CoA and citrate assays.

[a] Number of animals in each group.

[b] Mean ± SE.

[c] nmol/g fresh liver weighed in frozen state.

diminished to a significant extent; however, the triglyceride specific radioactivity was unaffected.

Conclusive evidence that Ro 22-0654 enhanced ketogenesis and diminished triglyceride secretion by the liver was provided by the single-pass perfusion experiments (Fig. 5, Table 5), which verified that the absolute rates of ketone body formation and triglyceride secretion were reciprocally altered by the drug. The possibility that enhanced hepatic VLDL catabolism contributed to the decrease in net triglyceride secretion by livers perfused with Ro 22-0654 in the recirculating system has not been excluded.

An equal and opposite effect of Ro 22-0654 on total esterification of infused [1-$^{14}$C]oleic acid (Tables 3 and 4) accompanied the activation of fatty acid oxidation by Ro 22-0654. After 225 min of perfusion, half of this decrease in esterification was found in perfusate VLDL triglyceride and the remainder in liver triglyceride, with a small concurrent depression in liver diglyceride. Considering the undetermined radioactivity (Table 3) to be water-soluble oxidation products, the oleate oxidation:esterification ratio was increased from 1.0 to 1.7 by Ro 22-0654.

The ketone body specific radioactivities were very similar to those of perfusate triglyceride throughout these experiments. Thus, at 225 min 41-49% of the ketone bodies and secretory triglyceride fatty acids were derived from the infused [1-$^{14}$C]oleic acid. It may be concluded that free fatty acids entering the hepatocyte from the plasma and those derived from endogenous turnover (lipolysis) of liver lipid esters enter a common intracellular free fatty acid pool, which provides substrate for both fatty acid oxidation in the mitochondria and synthesis of secretory triglyceride in the endoplasmic reticulum. Liver triglyceride exists in two separate pools, the cytoplasmic droplets and the VLDL particles in the endoplasmic reticulum (20-22, 30-32). The present observations indicate that the "en bloc" transfer of intact triglyceride molecules from the droplet pool to the VLDL particle does not play a major role in the intracellular assembly of nascent triglyceride-rich plasma lipoproteins. If such a process did contribute substantially to the assembly of VLDL, the addition of such unlabeled endogenous triglyceride to the VLDL particles would have decreased the specific radioactivity of secretory triglyceride relative to that of ketone bodies. Such a decrease was only observed at 45 min and this was caused by the initial secretion of unlabeled VLDL present in the livers at zero time. The time required for the synthesis and secretion of a VLDL particle is approximately 25 min (30-33). The observations of Kondrup (34) are also inconsistent with the transfer of intact triglyceride molecules from the droplet pool to the VLDL particle.

The observed effects of Ro 22-0654 are analogous to the actions of clofibrate (35-39) and 5-tetradecyloxy-2-furoic acid (TOFA) (8, 40-42). Thus, these agents also increase fatty acid oxidation and decrease triglyceride synthesis in the liver. Clofibrate appears to promote the enrichment of peroxisomes with a fatty acid oxidizing system (39). However, several days of drug treatment prior to liver perfusion are required for enzyme induction. TOFA may alter hepatic fatty acid metabolism by a mechanism more similar to that of Ro 22-0654. Thus, both TOFA (42-44) and Ro 22-0654 (Table 6) decreased hepatic malonyl-CoA. However, TOFA also exerts a separate effect on diglyceride processing, causing an increase in phospholipid synthesis (8). The effects of TOFA on lipid metabolism therefore appear to be more complex. It is evident that TOFA increased (42) whereas Ro 22-0654 decreased (Table 6) the concentration of citrate, a key intermediate in de novo fatty acid synthesis, in the liver. Also, TOFA increased (8, 40, 42) whereas Ro 22-0654 decreased the $\beta$-hydroxybutyrate:acetoacetate ratio. Further studies with Ro 22-0654 may provide additional insight on the regulation of hepatic metabolism. In addition, this novel compound may be of value in the study of lipid transport processes and in the control of the plasma lipid concentration.

The present observations provide evidence for the following sequence of events in the liver following exposure to Ro 22-0654: *a*) inhibition of citrate and malonyl-CoA formation, *b*) decreased de novo fatty acid synthesis and enhanced acylcarnitine formation, *c*) stimulation of fatty acid oxidation and ketogenesis, *d*) decreased diglyceride and triglyceride synthesis, and *e*) decreased formation and secretion of triglyceride-rich lipoproteins.

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