Halofenate and clofibrate: mechanism of hypotriglyceridemic action in the rat[1]

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Abstract Rats fed a fat-free diet containing no drug, 0.02% or 0.10% halofenate, or 0.25% clofibrate for 14 days were injected intravenously with equivalent amounts of either [2-^{3}H]glycerol or [1(3)-^{3}H]glycerol. Blood samples were collected at times up to 150 min after injection and serum triglycerides were isolated and assayed for radioactivity. Kinetic analysis of the serum appearance and clearance curves of ^{3}H-labeled triglyceride permits estimation of serum total ^{3}H-labeled triglyceride formation and triglyceride fractional turnover rates. The total amounts of ^{3}H-labeled triglyceride formed from [2-^{3}H] or from [1(3)-^{3}H] glycerol in control-fed animals were very similar. Over 95% of the serum ^{3}H-labeled triglyceride formed from either substrate circulated in a rapidly turning-over triglyceride pool ($t_{1/2}$ = 8 min). Treatment with 0.10% halofenate or 0.25% clofibrate decreased labeling of serum triglycerides by 75–80% without increasing serum ^{3}H-labeled triglyceride fractional turnover rates. Furthermore, both drugs decreased incorporation in vivo of ^{14}C from [U-^{14}C]glycerol into hepatic but not intestinal triglycerides without significantly decreasing incorporation of ^{14}C into total phospholipids of either tissue. From these observations we suggest that, in the intact normal rat, sustained reduction of serum triglyceride levels produced by treatment with halofenate or clofibrate is due to inhibition of hepatic triglyceride formation.

Supplementary key words hypotriglyceridemic drugs • serum triglyceride formation • serum clearance kinetics • triglyceride turnover • hepatic versus intestinal triglyceride synthesis • glycerol-3-phosphate • dihydroxyacetone phosphate • [2-^{3}H]-, [1(3)-^{3}H]-, or [U-^{14}C]glycerol

Halofenate, 2-acetoamidoethyl(*p*-chlorophenyl)(*m*-trifluoromethylphenoxy)acetate, first recognized in 1971 (1), is a promising new hypolipidemic drug which has undergone considerable clinical testing (2–9). Like clofibrate (CPIB), halofenate (HFA) appears to have a greater effect on circulating triglyceride levels than on cholesterol levels (2–5, 10, 11). In contrast with CPIB, however, very little information is available on the mechanism by which HFA lowers triglyceride concentrations. The present study examines the ability of HFA, as compared with CPIB, to influence the kinetics of serum triglyceride formation and turnover in the rat subsequent to intravenous injection of either [2-^{3}H]-glycerol or [1(3)-^{3}H]glycerol. Values are presented which describe both the extent of total synthesis and the elimination kinetics of two serum triglyceride fractions. This approach permits estimation of whether drug-induced hypotriglyceridemia is due to decreased formation of serum triglycerides and (or) to changes in the fractional turnover rates of serum triglycerides. The experimental design was initially patterned after the method described by Nikkila and Kekki (12) for measurement of plasma triglyceride kinetics in man from intravenous injection of [2-^{3}H]glycerol. However, a more complicated kinetic approach was ultimately adopted, one which is similar in many respects to that used by Shames et al (13) for studying triglyceride transport in man after pulse injection of [^{14}C]palmitate. In the present study, serum levels of ^{3}H-labeled triglyceride radioactivity were determined for 150 min after pulse intravenous injection of either [2-^{3}H]- or [1(3)-^{3}H]glycerol. Using the kinetic model selected, the computer fit of the experimental data was excellent. Kinetic analysis of the data was accomplished using the SAAM 25 computer program of Berman and Weiss (14). This analysis permitted calculation of rate constants for the entry and removal of labeled serum triglyceride and the estimation of serum total ^{3}H-labeled triglyceride formation in control and treated rats.

Both [2-^{3}H]glycerol and [1(3)-^{3}H]glycerol were separately used as precursors of triglyceride synthesis in an attempt to distinguish between triglyceride formed solely by acylation of glycerol-3-phosphate ([2-^{3}H]glycerol) and that formed by acylation of both glycerol-3-phosphate and dihydroxyacetone phosphate ([1(3)-^{3}H]glycerol). One-half or more of hepatic total triglyceride formation could proceed through acylation of dihydroxyacetone phosphate (15, 16). In vivo incorporation of [U-^{14}C]glycerol into hepatic and intestinal lipids of control and drug-fed rats was measured to further interpret observations made in the studies with the ^{3}H-labeled glycerols.

Abbreviations: HFA, halofenate; CPIB, clofibrate; dpm, disintegrations per minute; TLC, thin-layer chromatography; K, rate constant; $t_{1/2}$, half-life.

[1] Preliminary accounts of these investigations have been presented at the 5th Int. Symp. on Drugs Affecting Lipid Met., Milan, Italy (Sept., 1974) and at the 59th Ann. Meeting of the Fed. Am. Soc. Exp. Biol., Atlantic City, N.J. (April, 1975).

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METHODS

Treatment of animals

Male Wistar rats (200–240 g) were maintained for 14 days on a fat-free diet (17) containing either no drug (controls) or HFA (0.02% or 0.10%) or CPIB (0.25%). At the time of experimental use there was less than 10% difference in the average body weights of the various groups. Rat food consumption was measured on several occasions for 11 different rats fed one of the three diets and was found to average 97 g/kg body wt/day (range 86–109). Therefore, 0.10% HFA or 0.25% CPIB in the diet correspond to an oral drug dose of approximately 100 or 250 mg/kg/day, respectively. These doses are each about seven times higher than the therapeutic oral dosage levels in man. The rats were given free access to the diet until about 1 hr prior to experimental use. The animals were anesthetized with chloralose (60 mg/kg, ip) and the right external jugular vein and the left femoral vein were cannulated as previously described (18). A blood sample, collected prior to injection of the radio-labeled glycerol, was saved for measurement of triglyceride and cholesterol levels by established automated methods (19, 20). In several experiments plasma free glycerol levels were measured (21).

Measurement of serum triglyceride formation and turnover

Control and treated rats were injected via the femoral vein with 75 μCi/kg of [2-^{3}H]glycerol (200 mCi/mmole, New England Nuclear Corp., Boston, Mass.) or 75 μCi/kg of [1(3)-^{3}H]glycerol (200 mCi/mmole, Amersham/Searle Co., Arlington Heights, Ill.) dissolved in sterile saline (0.3–0.4 ml). At specific times from 10–150 min after injection, samples of whole blood (0.2–0.3 ml) were collected from the jugular vein, immediately taken up into microhematocrit tubes (no anticoagulant), and the serum was separated by centrifugation. Hematocrits generally fell only 5–10% over the experimental period. The serum was recovered, total triglycerides isolated, and the ^{3}H content of the triglycerides was determined as described in detail before (18). The radioactivity content of the serum was expressed as the dpm of ^{3}H-labeled triglyceride/ml serum at each of the times investigated. Since all rats were fed a fat-free diet, the measurements of triglyceride formation represent synthesis of endogenous serum triglyceride. After collecting the last blood sample (150 min), the animals were killed with pentobarbital and the livers were immediately removed, weighed, frozen in liquid nitrogen, and stored at −20°C.

Measurement of hepatic and intestinal lipid formation

Control and treated rats, anesthetized and prepared as above, were injected via the femoral vein with 20 μCi/kg of [U-^{14}C]glycerol (7.4 mCi/mmole, New England Nuclear Corp.) dissolved in sterile saline (0.3–0.4 ml). Rats were killed with intravenously injected pentobarbital in groups of four at 10, 15, 25, 40 and 70 min after giving the ^{14}C-labeled glycerol. The whole liver and small intestines (stomach to caecum) were immediately removed, separately transferred to large test tubes which were then immersed in liquid nitrogen and subsequently stored at −20°C.

Individual livers and small intestines were later thawed, weighed, and minced. The contents of the intestinal lumen were removed with gentle peristaltic pressure and adipose tissue was quickly and carefully dissected from this tissue before weighing and mincing it. One g portions of the tissues were homogenized in 2 ml of isotonic saline (Polytrom homogenizer) and the homogenates were then extracted with 20 vol of 2:1 chloroform–methanol. The mixture was extracted overnight at room temperature with gentle agitation. The recovered total lipid was dissolved in chloroform, the chloroform–lipid solution was washed twice with 0.5 vol of chloroform–methanol–0.5% aqueous NaCl 3:47:50 and subsequently dried over anhydrous Na_2SO_4.

Tissue total lipid content was determined gravimetrically and hepatic triglyceride and cholesterol content colorimetrically. One-half of the total extracted lipid (equal to 0.5 g of fresh tissue) was chromatographed on TLC plates (silica gel G, 500 μ thick) and developed in *n*-hexane–diethylether–glacial acetic acid 73:25:2. The silica gel areas corresponding to standards of lecithin, cholesterol, free fatty acid, triglyceride, and cholesterol ester were recovered and the ^{14}C content was determined by scintillation counting. In several cases aliquots of the recovered total tissue lipid were saponified in ethanolic KOH, followed by separate recovery of the nonsaponifiable lipid and the total fatty acids. Only trace radioactivity was measured in the nonsaponifiable lipids and less than 2% of the total lipid-^{14}C was recovered with the total fatty acids. The remaining 98% of the saponified lipid-^{14}C was present as water soluble radioactivity. Thus, essentially all of the ^{14}C incorporated into triglyceride and phospholipid is present in the glycerol backbone of these molecules. The ^{14}C radioactivity contents of the hepatic and intestinal triglycerides and phospholipids were separately expressed as dpm/g tissue (wet wt) at each of the times investigated.

Measurement of hepatic free glycerol and glycerol-3-phosphate

When hepatic free glycerol and glycerol-3-phosphate levels were to be measured the rats were killed immediately prior to injection of the radiolabeled glycerols. The livers were removed, frozen in liquid nitrogen, and stored at −20°C. The livers were later thawed and homogenized, using the Polytrom homogenizer, in ice-cold isotonic saline (1 g liver in 3 ml saline). The homogenates were quickly transferred to screw-capped centrifuge tubes which were then sealed and placed in a boiling water bath for 3 min. The tubes were cooled, centrifuged, and the supernatant was recovered and assayed for glycerol-3-phosphate and free glycerol using the enzymatic system described by Chernick (21).

Kinetic treatment of the data

Kinetic treatment of the data for serum appearance and clearance of the ^{3}H-labeled triglyceride was accomplished using the SAAM25 program of Berman and Weiss (14) in

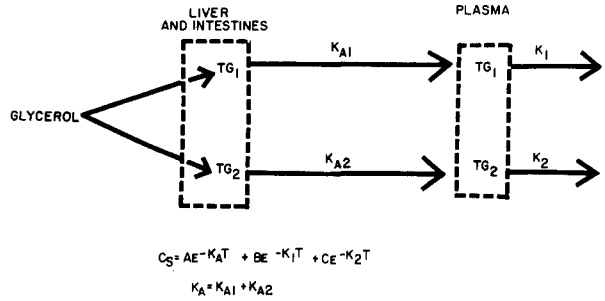

Fig. 1. The physiological model and equation used in fitting the experimental data. C_s is the ^{3}H-labeled triglyceride concentration (dpm/ml) in serum at any given time, K_A (min^{-1}) is the overall rate constant for ^{3}H-labeled triglyceride transport from the liver and intestine to serum, K_1 (min^{-1}) is the rate constant for loss of a rapidly turning-over ^{3}H-labeled triglyceride fraction, K_2 (min^{-1}) is the rate constant for loss of a slowly turning-over ^{3}H-labeled triglyceride fraction (these two fractions are taken to account for all of the ^{3}H-labeled triglyceride which entered the circulation), the coefficient A is the estimated total amount of ^{3}H-labeled triglyceride that entered the circulation (in dpm/ml), the coefficient B is the estimated total amount of the rapidly turning-over ^{3}H-labeled triglyceride fraction, the coefficient C is the estimated total amount of the slowly turning-over ^{3}H-labeled triglyceride, and T is time (min). TG_1 is the fast turning-over triglyceride and TG_2 is the slower turning-over triglyceride.

conjunction with an IBM 360-75 computer. This program uses the equations implied by the model and adjusts the parameters until the best least squares fit to the experimental data is obtained. In this case, computer fitting took place between the theoretically calculated serum concentration of radioactive triglyceride and the experimentally determined ^{3}H-labeled triglyceride serum concentrations. The model and equation used in fitting the data are shown in **Fig. 1**, where C_s is the ^{3}H-labeled triglyceride concentration (dpm/ml) in serum at any given time, K_A is the rate constant for transport of ^{3}H-labeled triglyceride from the liver and intestine to serum, K_1 is the rate constant for loss of a fast turning-over ^{3}H-labeled triglyceride fraction, K_2 is the rate constant for loss of a slow turning-over ^{3}H-labeled triglyceride fraction (these two fractions are taken to account for all of the ^{3}H-labeled triglyceride that entered the circulation), the coefficient A is the estimated total amount (dpm/ml) of ^{3}H-labeled triglyceride that entered the circulation, the coefficient B is the estimated total amount of the rapidly turning-over ^{3}H-labeled triglyceride fraction, the coefficient C is the estimated total amount of the slowly turning-over ^{3}H-labeled triglyceride fraction, and T is time. Our justification for this model is presented in the Discussion.

Initial estimates of the parameters for the computer program were determined by graphical analysis (22). The "lag time", the time required for ^{3}H-labeled triglyceride to first enter the circulation following injection of the ^{3}H-labeled glycerol, was estimated graphically (22) and found to be between 14–15 min in most cases. The coefficients A, B, and C were estimated by extrapolation of the K_A, K_1, and K_2 lines to the "lag time" using the method of residuals to obtain the K_A and K_1 lines as described previously (18).

Each of the 48 individual sets of serum ^{3}H-labeled triglyceride data was fitted separately using iterative SAAM25 program (14). The individual rate constants and coefficients from these fits were then averaged and are reported in Tables 4 and 5. Total triglyceride synthesis (Tables 4 and 5) was estimated from the coefficients B and C in Figure 1. The Student's t test was used to evaluate for statistically significant differences between groups. Because it is difficult to resolve two rate constants whose values are close to one another, the rate constant K_A is probably uncertain. This should not be a factor in resolving K_1 and K_2 however. In the data presented here only two kinetically distinct elimination rate constants were found. It is conceivable that if the serum triglyceride data were followed for a longer period of time, additional minor components could appear.

RESULTS

Effect of drug treatment upon liver size and upon liver and plasma constituents

Treatment of the rat for 14 days with 0.10% HFA or 0.25% CPIB in a fat-free diet resulted in increases in liver size of 20% and 45% respectively (**Table 1**). HFA produced no significant changes in hepatic total lipid or cholesterol concentration (mg/g liver) but it did result in a significant 47% decrease in triglyceride concentration. Some but not all of the HFA effect could be attributed to the 20% increase in liver size. In contrast, treatment with CPIB produced

TABLE 1. Effect of HFA and CPIB on rat liver size and lipid concentrations[a,b]

Parameter	Units[c]	n Rats	Control	n Rats	HFA (0.10%)	n Rats	CPIB (0.25%)
% Body Wt.	%	29	3.78 ± 0.07	26	4.55 ± 0.06[d]	26	5.50 ± 0.06[d]
Total Lipid	mg/g	25	54.0 ± 1.4	27	56.9 ± 1.5	20	42.3 ± 0.7[d]
Cholesterol	mg/g	24	2.16 ± 0.09	25	1.99 ± 0.06	19	1.87 ± 0.03[e]
Triglyceride	mg/g	22	4.52 ± 0.46	25	2.38 ± 0.24[d]	19	3.13 ± 0.28[e]

[a] HFA and CPIB were fed to the rats for 14 days at levels of 0.10 and 0.25%, respectively, of a fat-free diet. Controls received the fat-free diet without drugs.
[b] The values are means ± one standard error.
[c] Measurements are based upon wet weight of liver. Total lipid was determined gravimetrically and cholesterol (total) and triglyceride were determined colorimetrically.
[d] $P(t)$ of difference from control < 0.001 (Student's t test).
[e] $P(t) < 0.02$.

TABLE 2. Effect of HFA and CPIB on rat plasma lipids

Treatment[a]	Triglycerides[b] n Rats	Triglycerides[b] mg/100 ml	Total Cholesterol[b] n Rats	Total Cholesterol[b] mg/100 ml
Controls	32	74.6 ± 4.5	30	96.6 ± 2.6
HFA (0.02%)	7	59.3 ± 14.3	7	70.3 ± 4.9[c]
HFA (0.10%)	21	34.9 ± 3.0[c]	27	44.2 ± 1.1[c]
CPIB (0.25%)	19	43.5 ± 3.4[c,d]	28	41.1 ± 1.9[c]

[a] Rats were fed a fat-free diet for 14 days containing either 0.02 or 0.10% HFA, 0.25% CPIB, or no drug (controls).
[b] Values are means ± one standard error.
[c] $P(t)$ of difference from control <0.001 (Student's t test).
[d] $P(t)$ of difference from halofenate (0.10%) < 0.07.

modest decreases in liver relative content of total lipid, cholesterol, and triglyceride. These changes could be explained solely by the marked hepatomegaly that occurred in the CPIB-treated rats.

Plasma triglyceride and cholesterol levels both decreased by 50–55% in rats given HFA at a level of 0.10% of the diet (**Table 2**). These lipid fractions decreased 20–25% with 0.02% HFA, although the decrease in plasma triglyceride concentration was not statistically significant. The reduction in plasma concentrations of triglycerides and cholesterol produced by 0.25% dietary CPIB were similar to those seen with 0.10% HFA; however, the decrease in triglyceride level was somewhat greater with HFA. The comparable ability of 0.10% HFA and 0.25% CPIB to produce major decreases in plasma lipids, particularly triglycerides, supported focusing on these respective drug doses in the mechanism of action studies.

Treatment with either HFA (0.10%) or CPIB (0.25%) did not significantly change plasma free glycerol concentrations although there was a tendency for HFA to raise and for CPIB to lower the levels (**Table 3**). The effects of drug treatment upon hepatic free glycerol concentrations were not statistically significant. However, CPIB produced a highly significant 35% decrease in concentration of hepatic glycerol-3-phosphate. The high concentration of hepatic glycerol rela-

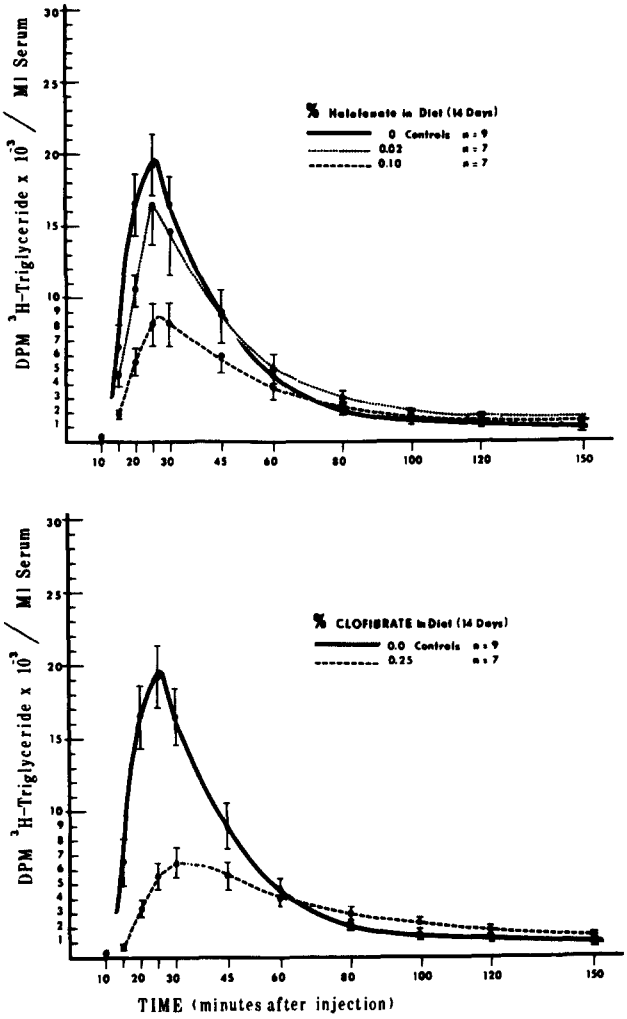

Fig. 2. Arithmetic plot of the serum appearance and disappearance of ^{3}H-labeled triglyceride after intravenous injection of 75 μCi/kg of [2-^{3}H]glycerol (200 mCi/mmole) into control and drug fed rats. Rats were fed for 14 days a fat-free diet containing no drug (control), 0.02 or 0.10% halofenate (HFA), or 0.25% clofibrate (CPIB). Values are means. Bars are standard errors.

TABLE 3. Effect of HFA and CPIB on plasma and liver free glycerol and on liver glycero-3-phosphate concentrations

Treatment[a] Group	n Rats	Plasma[b] Glycerol (nmoles/ml)	Liver[b] Glycerol (μmoles/g)	Liver[b] Glycero-3-phosphate (μmoles/g)
Controls	6	139 ± 15	1.01 ± 0.07	1.13 ± 0.03
HFA (0.10%)	6	180 ± 12[d]	1.03 ± 0.05	1.05 ± 0.04[d]
CPIB (0.25%)	6	120 ± 20	0.88 ± 0.04[d]	0.74 ± 0.03[c]

[a] Rats were fed a fat-free diet for 14 days which contained 0.10% HFA, 0.25% CPIB, or no drug (controls).
[b] Values are means ± one standard error. Values are expressed as nM/ml of plasma or μM/g liver (wet wt).
[c] $P(t)$ of difference from control < 0.001.
[d] $P(t)$ < 0.10.

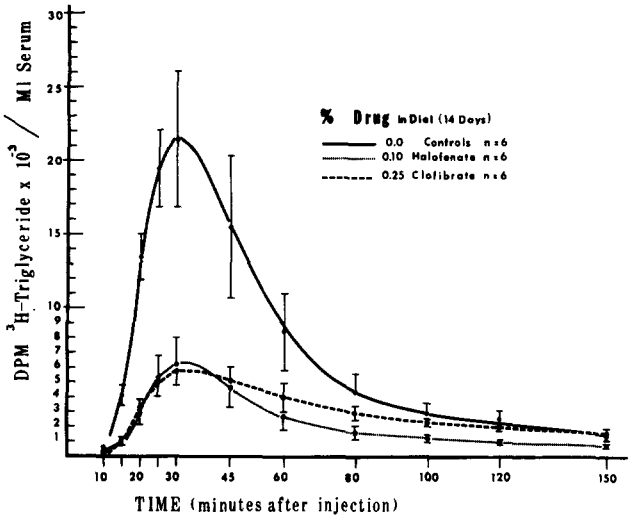

Fig. 3. Arithmetic plot of the serum appearance and disappearance of ^{3}H-labeled triglyceride following intravenous injection of 75 μCi of [1(3)-^{3}H]glycerol per kg (200 mCi/mmole) into control and drug-fed rats. Rats were fed for 14 days a fat-free diet containing no drug (control), 0.10% halofenate (HFA), or 0.25% clofibrate (CPIB). Values are means. Bars are standard errors.

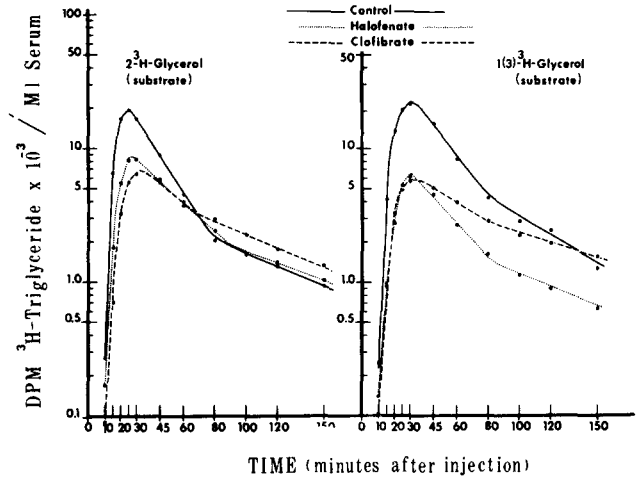

Fig. 4. Semilogarithmic plot of the data presented in Figs. 2 and 3 except that the curves for the 0.02% halofenate (HFA) fed rats are not shown.

tive to plasma glycerol encountered in the present study was unexpected. It is conceivable that the high hepatic level could be an artifact of the isolation procedures used. McGarry and Foster (23) found unusually high levels of certain hepatic metabolic intermediates upon precipitation of liver proteins by heat. They explain that accelerated hepatic enzyme activity could occur before total denaturation of proteins is accomplished.

Formation and clearance of serum triglyceride in control and treated rats

After intravenous injection of either [2-^{3}H]glycerol or [1(3)-^{3}H]glycerol into control rats, peak levels of serum triglyceride radioactivity were observed at 25–30 min and reached about 20,000 dpm/ml (**Figs. 2** and **3**). These directly observed values for serum maximum ^{3}H-labeled triglyceride levels are, however, very poor estimates of the total ^{3}H-labeled triglyceride that actually entered the circulation since the observed values are dependent upon both the rates of entry and clearance of labeled triglyceride to and from the circulation, two very rapid processes. When the curves shown in Figs. 2 and 3 are expressed as semilogarithmic plots (**Fig. 4**) one sees that the elimination of ^{3}H-labeled triglyceride from the serum is biphasic, apparently reflecting two different populations of labeled serum triglycerides, one which turns over rapidly and one with a slow turnover. Alternate interpretations of these data are discussed later. When these data are subjected to computer analysis using the SAAM program

TABLE 4. Effect of HFA and CPIB upon serum triglyceride formation from [2-^{3}H]glycerol

Treatment[a]	n Rats	Rate Constants (min^{-1} × 10^{-2})[b]			Total TG Syn. (dpm/ml × 10^8)[c]	
		K_a	K_1	K_2	K_1-TG-Pool	K_2-TG-Pool
Control	9	13.65 ± 1.80 (5.08)	8.93 ± 0.74 (7.76)	1.20 ± 0.29 (57.8)	205.6 ± 38.8	3.54 ± 0.69
HFA 0.02%	7	10.67 ± 0.94 (6.49)	8.58 ± 0.73 (8.08)	1.35 ± 0.09 (51.3)	224.4 ± 61.4	5.94 ± 1.56
HFA 0.10%	7	14.60 ± 2.64 (4.75)	5.17 ± 0.41[f] (13.40)	0.81 ± 0.22 (85.6)	45.3 ± 31.8[e]	4.15 ± 1.73
CPIB 0.25%	7	11.56 ± 1.98 (5.99)	5.34 ± 0.84[e] (12.98)	0.90 ± 0.09 (77.0)	52.0 ± 31.0[d]	4.29 ± 0.85

[a] Rats fed for 14 days a fat free diet containing no drug (controls), 0.02% or 0.10% HFA, or 0.25% CPIB were injected intravenously with 75 μCi/kg of [2-^{3}H]glycerol (200 mCi/mmole). Serum ^{3}H-triglyceride content (dpm/ml) was determined at times from 10–150 min after injection. Presented values are means ± one standard error or harmonic means ($t_{1/2}$ values) of those determined for individual animals.

[b] K_A is the rate constant describing the serum entry of ^{3}H-labeled triglyceride. K_1 and K_2 are rate constants respectively describing a rapid and slow component of ^{3}H-triglyceride elimination from the serum. The values in parentheses are the half-life values corresponding to the rate constants (in minutes).

[c] Total TG synthesis is the estimated total dpm of ^{3}H-labeled triglyceride (per ml of serum) which entered the rapid (K_1) and slow (K_2) turning-over pools of serum triglyceride. K_1-TG-Pool is the coefficient B and K_2-TG-Pool is the coefficient C in the equation used to fit the data (Fig. 1).

[d] $P(t)$ of difference from control < 0.02.

[e] $P(t) < 0.01$.

[f] $P(t) < 0.005$.

TABLE 5. Effect of HFA and CPIB upon serum triglyceride formation from [1(3)-^{3}H]glycerol

Treatment[a]	n Rats	Rate Constants (min^{-1} × 10^{-2})[b]			Total TG Syn. (DPM/ml × 10^3)[c]	
		K_A	K_1	K_2	K_1-TG-Pool	K_2-TG-Pool
Control	6	10.49 ± 0.35 (6.61)	8.17 ± 0.78 (8.48)	1.59 ± 0.08 (43.6)	210.2 ± 49.7	10.17 ± 2.53
HFA	6	12.25 ± 2.31 (5.66)	7.21 ± 0.72 (9.61)	1.01 ± 0.20[d] (67.9)	40.5 ± 12.6[e]	2.89 ± 1.16[d]
CPIB	6	10.23 ± 1.52 (6.77)	7.24 ± 0.80 (9.57)	0.72 ± 0.14[f] (96.3)	46.0 ± 11.1[e]	3.80 ± 0.18[d]

[a] Rats fed for 14 days a fat-free diet containing no drug (controls), 0.10% HFA, or 0.25% CPIB were injected intravenously with 75 μCi/kg of [1(3)-^{3}H]glycerol (200 mCi/mmole). Serum ^{3}H-triglyceride content (dpm/ml) was determined at times from 10–150 min after injection. Presented values are means ± one standard error or harmonic means ($t_{1/2}$ values) of those determined for individual animals.

[b] K_A is the rate constant describing the serum entry of ^{3}H-triglyceride. K_1 and K_2 are rate constants respectively describing a rapid and slow component of ^{3}H-triglyceride elimination from the serum. The values in parentheses are the half-life values corresponding to the rate constants (in minutes).

[c] Total TG synthesis is the estimated total dpm of ^{3}H-labeled triglyceride (per ml serum) which entered the rapid (K_1) and slow (K_2) turning-over pools of serum triglyceride.

[d] $P(t)$ of difference from controls < 0.05.

[e] $P(t) < 0.02$.

[f] $P(t) < 0.001$.

of Berman and Weiss (14) and the kinetic model in Fig. 1 (see Methods for description of the kinetic analysis), rate constants are obtained for the serum entry (K_A) and clearance (K_1 and K_2) of the ^{3}H-labeled triglyceride. As stated before, Fig. 1 describes the model upon which the computer analysis of the data in Fig. 4 is based. K_A is the overall rate constant for transport of ^{3}H-labeled triglyceride from the liver and intestine into the circulation. K_1 and K_2 are the serum elimination rate constants for the labeled triglyceride. K_1 describes the rapidly turning-over triglyceride pool and K_2 the much slower one. The total amount of ^{3}H-labeled triglyceride that individually entered these two pools can be separately calculated from extrapolation of the respective elimination curves, described by K_1 and K_2, to time zero; that is, the time of first appearance of ^{3}H-labeled triglyceride in the serum. These values represent the maximum concentration (dpm/ml) of ^{3}H-labeled triglyceride that would have appeared in the circulation had the respective removal rates been zero. The numerical values that quantitatively describe these parameters are shown in **Tables 4** and **5**. Because the two serum triglyceride fractions, TG_1 and TG_2, could not be routinely separated, the individual rate constants for transport of TG_1 and TG_2 from the liver and intestines into serum could not be determined. Hence, the rate constant for triglyceride transport from the liver and intestine into the serum is expressed as a single overall rate constant K_A representing the sum of the individual rate constants K_{A1} and K_{A2}.

Serum triglyceride formation in control-fed rats was very similar regardless of whether intravenously injected [2-^{3}H]-glycerol or [1(3)-^{3}H]glycerol served as substrate. After injection of either of these radiolabeled glycerols, radioactive triglycerides rapidly entered the circulation after a delay of about 15 min. The half-life of this entry process was 5.0–6.5 min (Tables 4 and 5). Serum ^{3}H-labeled triglyceride was distributed between two fractions, a rapidly versus a slowly turning-over one. The rapidly turning over triglyceride fraction had a net serum half-life of about 8 min and accounted for over 95% (200,000–220,000 dpm/ml) of the total ^{3}H-labeled triglyceride that entered the circulation. The net serum half-life of the remaining serum radioactive triglyceride, 5% of total, was 45–60 min.

Rats fed a fat-free diet containing 0.10% HFA or 0.25% CPIB for 14 days incorporated 75–80% less total radioactivity into serum triglycerides after intravenous injection of either [2-^{3}H]glycerol or [1(3)-^{3}H]glycerol than rats fed the diet without drug (Tables 4 and 5). Essentially all of this reduction was accounted for by decreased formation of the rapidly turning-over serum triglyceride fraction ($t_{1/2}$ 8 min). When [2-^{3}H]glycerol served as substrate neither HFA nor CPIB significantly changed the amount of radioactivity incorporated into the slowly turning-over ^{3}H-labeled triglyceride fraction. In contrast, the appearance of label in this slowly turning-over fraction was apparently decreased by 60–70% in drug-treated as compared to control rats after injection of [1(3)-^{3}H]glycerol.

The influence of drug treatment upon the kinetics of serum triglyceride formation and clearance is of special interest. Neither drug significantly changed the rate constant for the serum entry of ^{3}H-labeled triglyceride. Even more important, the drugs did not accelerate the removal of the labeled triglycerides from the circulation. In fact, the serum elimination of both the rapidly and the slowly turning-over ^{3}H-labeled triglyceride fractions tended to be slower in the treated rats (Tables 4 and 5). HFA at 0.02% of diet had no significant effects upon any of the measured parameters of serum triglyceride formation or turnover (Table 4). This observation is consistent with the finding that HFA at this dose did not significantly decrease plasma triglyceride levels (Table 2).

Effect of drug treatment upon hepatic and intestinal lipid synthesis

Glycerol labeled with ^{14}C rather than ^{3}H was used for studies of tissue triglyceride and phospholipid synthesis, in part, to minimize possible isotope effects and, in part, to permit evaluation of glycerol incorporation into lipids via de novo fatty acid formation versus incorporation of the intact

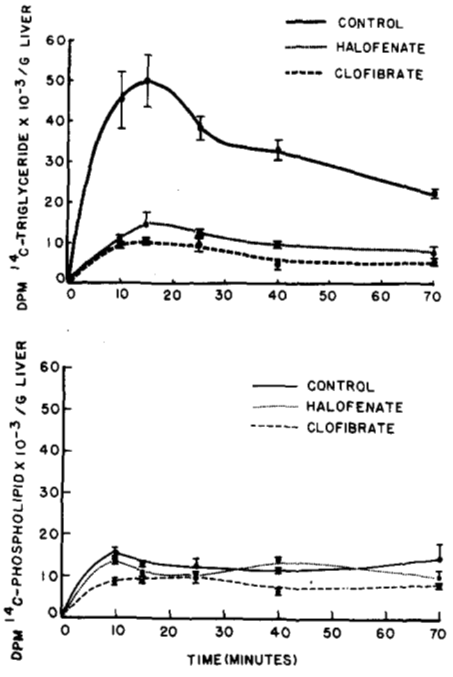

Fig. 5. Appearance in liver (per g) of ^{14}C-labeled phospholipids (bottom graph) and ^{14}C-labeled triglycerides (upper graph) after intravenous injection of 20 μCi of [U-^{14}C]glycerol per kg (7.4 mCi/mmole) into control and drug-fed rats. Rats were fed for 14 days a fat-free diet containing no drug (controls), 0.10% halofenate (HFA), or 0.25% clofibrate (CPIB). Within each treatment group four different rats were killed at each time. The values are means. The bars are standard errors.

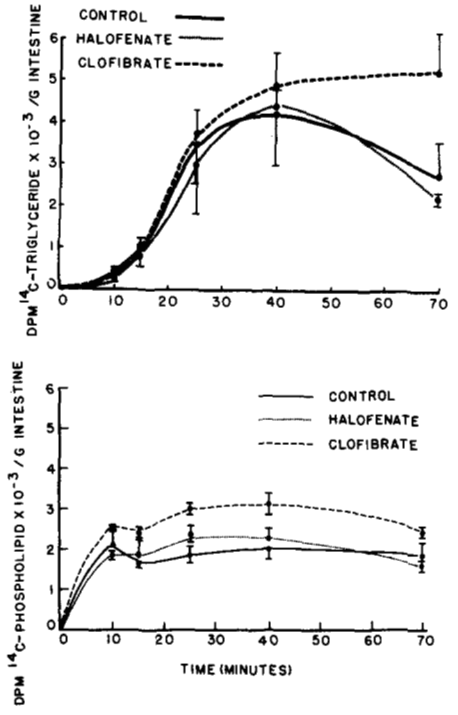

Fig. 6. Appearance in small intestines (per g) of ^{14}C-labeled phospholipids (bottom graph) and ^{14}C-labeled triglycerides (upper graph) after intravenous injection of 20 μCi of [U-^{14}C]glycerol per kg (7.4 mCi/mmole) into control, 0.10% halofenate (HFA), or 0.25% clofibrate (CPIB) fed rats. Within each treatment group four different rats were killed at each time. The values are means. The bars are standard errors. Intestinal and liver ^{14}C-labeled lipid measurements were done in the same rats.

glycerol molecule. Within 15 min after intravenous injection of [U-^{14}C]labeled glycerol into both control and treated rats, peak levels of ^{14}C radioactivity were observed in hepatic triglycerides and phospholipids (**Fig. 5**). Less than 2% of this ^{14}C was present in the fatty acid component of these molecules and no radioactivity was recovered in tissue sterols. In control rats, the hepatic triglycerides were about 3 times more highly labeled than phospholipids. Treatment with either 0.10% HFA or 0.25% CPIB in the diet reduced hepatic labeling of triglyceride by 70–80%, expressed on a per g tissue (wet wt) basis (Fig. 5). The incorporation of [^{14}C]-glycerol into hepatic triglyceride was still decreased by 60–70% by both agents, even after taking into account the hepatomegaly produced by both drugs. Labeling of hepatic phospholipids, in contrast to triglyceride, was little affected by drug treatment. HFA had no significant effect upon incorporation of ^{14}C into phospholipids per g of liver (Fig. 5). On a relative weight basis, the apparent decrease in labeling of hepatic phospholipids in CPIB-treated rats disappears when the CPIB-induced hepatomegaly is taken into consideration.

The peak radioactivity content of intestinal triglycerides and phospholipids after injection of the [^{14}C]glycerol reached 10–15% of that seen in the liver (**Fig. 6**). Treatment with HFA did not significantly alter the incorporation of label into either intestinal triglyceride or phospholipid. Rats treated with CPIB actually appeared to incorporate more [^{14}C]-glycerol into intestinal phospholipids and, to a lesser extent, into triglyceride (Fig. 6).

DISCUSSION

Measurement of serum triglyceride formation from [2-^{3}H]-glycerol only assesses lipid formation from acylation of glycerol-3-phosphate (the ^{3}H is lost in conversion to dihydroxyacetone phosphate); whereas incorporation of [1(3)-^{3}H]glycerol would reflect lipid synthesis from the acylation of both glycerol-3-phosphate and dihydroxyacetone phosphate. Control rats injected with equivalent amounts of either [2-^{3}H]glycerol or [1(3)-^{3}H]glycerol incorporated virtually identical amounts of radioactivity into serum triglycerides. A decrease in the 2-^{3}H/1(3)-^{3}H ratio of incorporation into serum triglycerides would indicate triglyceride synthesis through the dihydroxyacetone phosphate pathway. Since we found a ratio of one, it could appear that the synthesis of triglycerides proceeds almost exclusively by acylation of glycerol-3-phosphate, a view widely held until recently (24). However, Manning and Brindley (15) show that acylation of dihydroxyacetone phosphate can account for 50–60% of the glycerol incorporated into glycerolipid by rat liver slices. Failure of earlier studies to recognize the quantitative importance of the di-

hydroxyacetone phosphate pathway stems, in part, from using [2-^{3}H]glycerol to evaluate quantitatively lipid synthesis from acylation of glycerol-3-phosphate. [2-^{3}H]glycerol will overestimate the quantitative significance of the glycerophosphate pathway because of a ^{3}H isotope effect. Bowley, Manning and Brindley (16) demonstrated that the activity of hepatic mitochondrial *sn*-glycerol-3-phosphate oxidase was over 8 times more active with [1-^{14}C]glycerol-3-phosphate than [2-^{3}H]glycerol-3-phosphate. This will result in a greater proportion of the available ^{3}H- than ^{14}C-labeled molecules being incorporated into lipid via acylation of glycerophosphate. With this fact in mind, finding a ^{3}H ratio of one for [2-^{3}H]-glycerol/[1(3)-^{3}H]glycerol incorporated into serum triglyceride suggests a major contribution of the dihydroxyacetone pathway to the synthesis of serum triglycerides in vivo.

Regardless of the pathway by which serum triglycerides are synthesized, once formed they appear to be identically handled. At about 15 min after injection of either [2-^{3}H]- or [1(3)-^{3}H]glycerol, labeled triglycerides begin to enter the blood from the liver and intestines, the only tissues recognized as contributing significantly to circulating triglyceride levels. The half-life of this entry process is 5.0–6.5 min. More than 95% of the ^{3}H-labeled triglyceride that entered the serum was incorporated into a rapidly turning-over triglyceride pool, presumably VLDL triglyceride. The serum half-life of this glycerol-labeled triglyceride was found to average about 8 min. Rat plasma triglycerides, endogenously labeled with ^{14}C-labeled fatty acid, are reported to be cleared from the circulation with a similar half-life, 4–8.5 min (25, 26). In addition to the rapidly turning-over serum ^{3}H-labeled triglyceride, radiolabeled glycerol was incorporated to a minor extent, 2–5% of total ^{3}H-labeled triglyceride, into a slowly turning-over serum triglyceride fraction ($t_{1/2}$ about 1 hr). It is recognized that the triglycerides of this latter fraction, particularly, are probably distributed in a heterogeneous population of lipoproteins.

There has been considerable discussion in the literature concerning the best modeling approach for describing serum triglyceride kinetics. After release into serum from the synthetic sites (liver and intestines), triglyceride has been found to distribute almost exclusively in serum water and to have an apparent volume of distribution approximating serum volume. Thus, if only one rate constant for triglyceride disappearance from the serum is present, a monoexponential disappearance curve would be expected. Numerous studies in human subjects have shown that if serum triglyceride is followed for a long enough time period after intravenous injection of a precursor, i.e. 24 hr, the disappearance curves become biexponential at later times. In the data by Quarfordt et al (27), for example, after injection of [^{14}C]palmitate the serum triglyceride curves in normal subjects are log-linear up to about 10 hr, after which a second much slower component becomes evident. This second component begins after the serum triglyceride curve has dropped to about 5–10% of the peak concentration. The data of Kissebah, Adams, and Wynn (28), also using [^{14}C]palmitate, are very much like those of Quarfordt et al (27). In variance with this, other investigators have reported triglyceride disappearance to be monoexponential. However, many of these studies were conducted over too short a time interval to pick up the second component or had too few points on the latter parts of the curve to differentiate the later slow component. Thus most of the serum triglyceride data in the literature confirm the presence of a multiexponential decline in serum triglyceride. Ordinarily, a biexponential serum curve such as is present for serum triglyceride would be interpreted as an exchange compartment for serum triglyceride and would fit a two compartment model. However, since triglyceride has an apparent volume of distribution approximating serum water and has been reported to distribute almost exclusively in serum water, this would not appear to be a promising model to explain a biexponential curve of this magnitude. Most of the modeling approaches reported in the literature have assumed kinetic homogeneity of serum triglyceride, i.e., only one disappearance rate constant, and have used models with exchange compartments for serum triglyceride or precursor, slow hepatic input components, or precursor recycling to produce the second (slow) component of the biexponential serum triglyceride curve. An excellent review of models using these approaches has been published by Quarfordt and associates (27).

An alternate explanation to describe the biexponential serum triglyceride data would be to assume kinetic heterogeneity of serum triglyceride, i.e., two individual rate constants for serum triglyceride disappearance. This is the approach which has been taken in the analysis of the data in this report. Because the actual physiological models are complex and contain numerous compartments (6–8 in the models just described (27)), but contain data only in the serum compartment, the resulting models have many unknowns and usually result in nonunique fits of the data. Thus, as pointed out by Quarfordt et al (27), each of the models that they proposed fit the data equally well, and no conclusions as to the most appropriate model could be determined from this approach alone. Thus, conclusions as to the most appropriate model must be based on physiological and biochemical information as well as compartmental modeling. Quarfordt et al (27) did suggest as an alternate hypothesis a model with two rate constants for elimination as proposed in this paper (their "model F") but relegated it to alternative status because of their basic assumption of serum triglyceride homogeneity. However, the fit of their data using two elimination rate constants for serum triglyceride with no triglyceride exchange or slow components was equally as good as with their other models and hence, on this basis, the approach used in our study is compatible with the data of Quarfordt et al (27).

HFA at 0.10% and CPIB at 0.25% of the fat-free diet produced almost identical effects upon serum triglyceride formation and turnover. Both drugs reduced by 75–80% the incorporation of [2-^{3}H]glycerol and [1(3)-^{3}H]glycerol into serum triglycerides without accelerating the fractional turnover rate of serum triglyceride. The decreased incorporation of the radiolabeled glycerols into hepatic and circulating triglycerides cannot be explained by the observed drug effects on plasma or hepatic free glycerol levels. Neither HFA nor CPIB appeared to significantly alter plasma or liver free glycerol levels. In contrast, Adams et al (29) reported a 25–30% de-

crease in serum free glycerol levels of chow-fed rats given CPIB for 14 days.

The decrease in hepatic glycerol-3-phosphate levels produced by CPIB, an observation reported earlier by Azarnoff (30), apparently reflects a well-recognized ability of CPIB to stimulate mitochondrial glycerol-3-phosphate dehydrogenase (31, 32). If an increased turnover rate of hepatic glycerophosphate in the CPIB-treated rats resulted in lower specific activity levels of this intermediate, less radioactivity from the injected glycerols could be incorporated into glycerolipids and thus this effect might account for some of the observed net inhibition of hepatic triglyceride formation with CPIB. Measurements of whole liver glycerol-3-phosphate specific activity were not attempted, in part, because rat liver likely possesses at least two different pools of glycerophosphate (cytoplasmic and mitochondrial) that could variably contribute to glycerolipid synthesis.

The results of the present study indicate that, in the intact normal rat, HFA and CPIB produce sustained hypotriglyceridemia by decreasing hepatic and (or) intestinal output of triglyceride. Furthermore, the target site of action for these drugs appears to be the liver since treatment with HFA or CPIB greatly decreased [U-^{14}C]glycerol incorporation in vivo into hepatic triglycerides without decreasing incorporation into intestinal triglycerides. These findings agree with the observations of Adams, Webb, and Fallon (29). They reported that treatment of the rat with CPIB decreased the incorporation in vivo of [^{14}C]glycerol into hepatic and serum glycerides and that CPIB inhibited glyceride synthesis by rat liver in vitro from ^{14}C-labeled glycerol-3-phosphate. As with the present study, they conclude that inhibition of hepatic glyceride synthesis is important in explaining the hypotriglyceridemic action of this drug. In apparent contradiction to this view, numerous reports provide evidence indicating that CPIB can lower plasma triglyceride by enhancing removal of triglyceride from the circulation. Our own earlier work with the dog (33) suggested that treatment with CPIB enhanced plasma clearance of chylomicron triglyceride and increased adipose tissue lipoprotein lipase activity. Also, Tolman, Tepperman, and Tepperman (34) reported increased adipose lipoprotein lipase activity in rats fed CPIB. In hypertriglyceridemic humans given CPIB, total splanchnic production of triglyceride was reported unchanged while plasma triglyceride levels fell, thus indicating that the reduced plasma triglyceride levels resulted from enhanced tissue clearance of circulating triglycerides (35). Similarly, using the sucrose-induced hyperlipidemic rat, Segal, Roheim, and Eder (36) observed that treatment with CPIB over an 8 day period initially increased and later slightly decreased incorporation in vivo of [^{14}C]leucine into the protein component of serum VLDL and consistently increased the turnover of the labeled VLDL protein and, presumably, triglyceride during this period. The inability of CPIB to lower plasma triglyceride in normotriglyceridemic humans (35) contrasts with its effective hypotriglyceridemic action in the normal rat and thus suggests differences in the action of this drug in the two species. Further, the nutritional status of the animal can have a great bearing on the effects of CPIB upon triglyceride metabolism. For example, in the hypertriglyceridemic rat Segal et al (36) observed that 2–4 days of treatment with CPIB were required for significant reduction of serum triglyceride, whereas only 6 hr of treatment are required to significantly lower serum triglycerides in the normal rat (29). Also, the source of dietary carbohydrate can markedly influence the effects of CPIB upon hepatic lipid metabolism (37).

It is difficult to reconcile the observations indicating that CPIB lowers plasma triglyceride by decreasing hepatic synthesis and secretion with those showing that this drug acts by promoting removal of triglyceride from the circulation. In the present study, after 14 days of treating the rat with HFA or CPIB, there was no indication of enhanced fractional turnover rates of endogenously formed ^{3}H-labeled triglyceride. However, our observations do not rule out drug effects on triglyceride turnover at earlier times, i.e., that before a sustained, steady state, reduction of plasma triglyceride has been achieved. Conceivably, at some earlier treatment time, when near normal amounts of hepatic VLDL triglyceride were still being released into the circulation, drug effects on serum clearance of labeled triglyceride could have been demonstrated.

Treatment with either CPIB or HFA clearly resulted in reduced labeling of hepatic triglyceride but not of phospholipid from [U-^{14}C]glycerol. This could suggest that the decreased labeling of hepatic triglycerides resulted from drug effects at a site subsequent to formation of diacylglycerol, a terminal common intermediate in the synthesis of triglycerides and major phospholipids (24). Drug actions in this area might involve inhibition of diacylglycerol acyltransferase or stimulation of hepatic catabolism of newly formed triglycerides. However, Lamb and Fallon (38) reported that CPIB did not inhibit diglyceride acyltransferase in rat liver microsomes and, in preliminary studies, we observed no effect of treatment with CPIB or HFA on rat hepatic lipase activities (unpublished observations). Rather, Lamb and Fallon (38) reported that CPIB reduced hepatic glycerolipid synthesis from [^{14}C]glycerol by inhibiting *sn*-glycerol-3-phosphate acyltransferase. In apparent disagreement with this observation, Daae and Aas (39) recently reported finding no inhibition by CPIB of diacylglycerophosphate formation from palmitylcarnitine in rat liver homogenates at physiologically encountered CPIB liver concentrations. Similarly, Brindley and Bowley (40) saw little effect of CPIB or HFA on the glycerol phosphate acyltransferase activity of rat liver homogenates. In contrast to direct effects upon enzymes of triglyceride synthesis, drugs might reduce hepatic triglyceride formation by restricting the availability of fatty acids for esterification. Both CPIB and HFA decrease [^{14}C]acetate incorporation into fatty acids by isolated rat hepatocytes (41). HFA is also reported to inhibit fatty acid synthesis from ^{14}C-labeled glucose (42, 43). Reduction of fatty acid synthesis, at least for CPIB, could be secondary to drug inhibition of hepatic acetyl coenzyme A carboxylase (44, 45). Also CPIB reduces circulating FFA levels in the rat (46), and hepatic triglyceride content and secretion are directly proportional to the concentration of FFA perfusing the liver (47). Interestingly, perfusion of the rat liver with high concentrations of FFA stimulated glyceride synthesis much more than phospholipid syn-

thesis (47). It is therefore tempting to speculate that the greater inhibition of hepatic triglyceride rather than the phospholipid formation by HFA and CPIB seen in the present study (Fig. 5) might be related to drug induced decreases in the availability of fatty acids for glycerolipid synthesis.

Technical assistance was provided at various times by A. Beaumont, R. Benson and G. Stonestreet. The hypolipidemic drugs were supplied by Dr. J. Noble of Ayerst Laboratories (clofibrate), New York, N.Y., and by Dr. L. Mandel of the Merck Institute for Therapeutic Research (halofenate), Rahway, N.J. This investigation was supported by grants from the Merck Institute for Therapeutic Research and the West Virginia University Computer Center.

Manuscript received 15 April 1975 and in revised form 16 September 1975; accepted 28 November 1975.

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