

# Effect of Clofibrate on Lipoprotein Metabolism in Hyperlipidemic Rats

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**ABSTRACT** The effects of clofibrate administration were studied in rats made hyperlipidemic by the feeding of diets high in sucrose. Within 12 hr of administration of clofibrate, there was a marked decrease in the concentration of serum high density lipoproteins but no change in the concentrations of the low and very low density lipoproteins. Between 2–4 days of treatment, the concentration of the very low density lipoproteins decreased whereas that of the low density lipoproteins was unchanged. In addition, the composition of the very low density lipoproteins was altered by clofibrate administration with a decrease in triglyceride and an increase in phospholipid content.

The synthesis of high density lipoproteins, as measured by the incorporation of labeled amino acids, decreased within 12 hr of treatment. The synthesis of the very low density lipoproteins was increased during the first 2 days and then decreased slightly. The synthesis of low density lipoproteins did not change. The rate of removal of very low density lipoproteins was measured at various times from 0.5 to 8 days of treatment and was found to be elevated. It was concluded that in the hyperlipidemic sucrose-fed rat, administration of clofibrate results in a reduced level of very low density lipoproteins which may be due in part to enhancement of the rate of removal of this fraction.

## INTRODUCTION

Clofibrate is widely used in the treatment of patients with hyperlipoproteinemia, but its mechanism of action is as yet not known (1). Although studies have suggested that its primary role is that of an inhibitor of cholesterol biosynthesis (2), other studies (3, 4) have shown that in rats, clofibrate also inhibits the transport

of triglyceride from the liver into the plasma. Gould, Swyryd, Avoy, and Coon (5) measured the synthesis of the protein portion of the lipoproteins both in liver slices and in intact normal rats and found decreased lipoprotein synthesis. However, the lipoproteins were separated only into two fractions—a high density fraction ( $d$  1.21–1.063) and a low density fraction ( $d$  < 1.063)—very low density lipoproteins (VLDL)<sup>1</sup> ( $d$  < 1.006) were not separated. Since in clinical experience the effect of clofibrate is greatest on VLDL (6), we felt it important to pay special attention to this lipoprotein class. We have, therefore, studied the metabolism of the protein moiety of VLDL as well as that of the other lipoprotein classes in clofibrate-treated rats. Our experiments differ from previous studies because we used rats made hyperlipidemic by sucrose feeding.

## METHODS

**Animals and diet.** Male Sprague-Dawley rats (Holtzman Co., Madison, Wis.), weighing 300–400 g were used. The semipurified high sucrose diet was obtained from General Biochemicals Div., Chagrin Falls, Ohio, and contained 20% vitamin-free casein, 60% sucrose, 4% corn oil, 1% vitamin mixture (Wooley and Sebrell [7]), 4% salt mixture (USP XIV), and 10% cellulose. The treated rats received the same diet to which 0.25% clofibrate (w/w) was added. Rats received the diet without clofibrate for 7–14 days, and thereafter received the diet containing clofibrate for periods of time varying from 12 hr to 8 days. The experiments were carried out on a schedule in which all animals in an experimental group were killed at the same time (between 9 and 11 a.m.). The rats were given food and water ad lib. throughout the experiment. They were weighed three times a week and animals losing weight were excluded from the study. Weight gain was similar in the clofibrate-treated and control animals.

**Chemical methods.** Serum lipids were extracted by the method of Folch, Lees, and Sloane Stanley (8); cholesterol was determined by the method of Abell, Levy, Brodie,

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<sup>1</sup> Abbreviations used in this paper: HDL, high density lipoprotein; LDL, low density lipoprotein; VLDL, very low density lipoprotein.

and Kendall (9); triglycerides were measured according to Van Handel's modification (10) of the method of Van Handel and Zilversmit (11); phospholipids were measured according to the method of Beveridge and Johnson (12); and protein was determined by the method of Lowry, Rosenbrough, Farr, and Randall (13). Albumin was prepared by the method of Schwert (14).

**Lipoprotein separation and analysis.** Lipoproteins were separated by the method of Havel, Eder, and Bragdon (15), into VLDL ( $d < 1.006$ ), low density lipoproteins (LDL) ( $d 1.006-1.063$ ), and high density lipoproteins (HDL) ( $d 1.063-1.21$ ). For separation of VLDL and LDL, ultracentrifugation was carried out in a Beckman model L-2 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) for 20 hr at 114,000  $g$  at 16°C in a 40.3 rotor. For HDL, centrifugation was carried out for 40 hr at 114,000  $g$ . The isolated fractions were separated by cutting the tubes with the Spinco tube cutter (Spinco Div., Beckman Instruments, Inc., Fullerton, Calif.) The lipoprotein fractions were washed by resuspending them in salt solution of appropriate density and repeating the centrifugation. In the experiment where chylomicrons were separated, 0.15 M NaCl was layered above the serum and centrifugation was carried out at 10,000 rpm for 30 min. The top layer was removed, layered under 0.15 M NaCl, and centrifuged for 30 min at 10,000 rpm. The chylomicron fraction was then subjected to ultracentrifugation for 20 hr at 114,000  $g$ , and repeated. In this study, (Table I), the VLDL fraction consisted of the  $d < 1.006$  supernate minus the chylomicrons, whereas in all other studies, it was the total  $d < 1.006$  fraction. Proteins were prepared for counting and chemical analysis as described by Roheim, Miller, and Eder (16). Portions were suspended in Cab-O-Sil obtained from Cabot Corp., Boston, Mass., and counted in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard Instrument Co., Inc., Downers Grove, Ill.) (17) for the determination of radioactivity.

**Incorporation of labeled amino acids.** Uniformly labeled L-leucine- $^{14}C$  and L-leucine-4,5- $^3H$  were obtained from Amersham/Searle Corp., Arlington Heights, Ill. and from New England Nuclear Corp., Boston, Mass. A mixture of uniformly labeled L-amino acids- $^{14}C$  was obtained from New England Nuclear Corp. The amino acids, dissolved in 0.85% NaCl solution at pH 7.4, were injected intravenously into the tail vein. After periods of clofibrate administration, ranging from 0.5 to 8 days, groups of control and treated rats were injected with 3  $\mu Ci$  of leucine- $^{14}C$ /

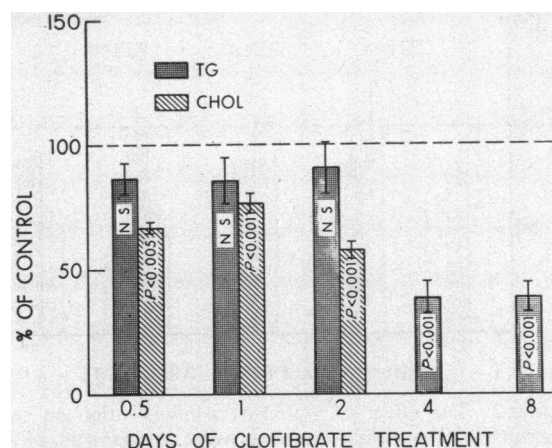


FIGURE 1 The effect of clofibrate administration on serum triglyceride and cholesterol. At each time, groups of six treated and six control rats were sacrificed. Determinations were performed on sera from each animal and the values averaged. The results are expressed as per cent of control values at each time period. The vertical lines (brackets) represent  $\pm 1$  se for the treated group. The  $P$  value is for the difference between the treated and control animals (18).

100 g of body weight; 90 min later 60  $\mu Ci$  of leucine- $^3H$ /100 g of body weight was injected into the same animals; and 60 min after this the animals were killed. This was done in order to measure incorporation of labeled amino acids in the same rat at two different times, 60 and 150 min. These time intervals were chosen because the maximal specific activity of VLDL is reached between 30 and 50 min after administration of the precursor, whereas the maximal specific activity for LDL and HDL is reached between 60 and 150 min.<sup>2</sup> Each group consisted of six to nine rats, and the serum of each rat was measured.

In another series of experiments, turnover was measured by administering 15  $\mu Ci$  of uniformly labeled amino acids- $^{14}C$ /100 g of body weight to control rats and to rats treated with clofibrate for 40 hr. Groups of six control and six treated rats were killed at 1, 2.5, 5, and 10 hr after the injection of the labeled amino acids.

## RESULTS

**Serum lipid concentration.** The effect of clofibrate administration for various periods of time on serum triglyceride and cholesterol concentration is shown in Fig. 1. The data are shown as percentages of the corresponding control values at each treatment time. The control values ranged from  $128 \pm 6$  to  $129 \pm 11$  mg/100 ml for triglyceride and from  $83 \pm 3$  to  $73 \pm 4$  mg/100 ml for cholesterol. Serum triglyceride did not differ significantly from the control until the 4th day of treatment when it decreased by 60%. This difference was also apparent on the 8th day of treatment. Serum cholesterol was signifi-

<sup>2</sup> Lasser, N. L., P. S. Roheim, D. Edelstein, and H. Eder. The serum lipoproteins of normal and cholesterol-fed rats. Submitted for publication.

TABLE I  
Effect of Clofibrate Administration for 12 Hr on  
Lipoprotein-Protein Concentrations

	mg/100 ml Serum			
	Chylomicron	VLDL*	LDL	HDL
Control (6)†	0.8 $\pm 0.1$	6.2 $\pm 0.6$	3.9 $\pm 0.3$	40.6 $\pm 1.3$
Clofibrate (6)	0.5 $\pm 0.5\%$	6.0 $\pm 0.3\%$	4.4 $\pm 0.3\%$	26.3 $\pm 2.1\%$

\* Total  $d < 1.006$  protein minus chylomicron proteins.

† Number of animals.

§ Not significantly different from control.

||  $P < 0.001$  (as cf. Control).

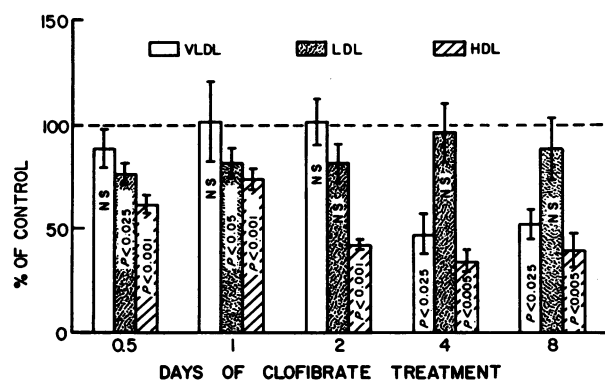


FIGURE 2 The effect of clofibrate administration on serum lipoproteins. The results are expressed as per cent of control protein concentration at each time period. Remainder similar to legend for Fig. 1.

cantly lower than the control after 12 hr of treatment, and remained low thereafter.

**Lipoprotein concentration.** We have defined VLDL as the lipoproteins separated by ultracentrifugation at  $d < 1.006$  at 114,000  $g$  for 20 hr. This fraction therefore contained chylomicrons in addition to VLDL. Since these rats were not fasted, it was necessary to determine the contribution of chylomicron protein to this fraction in sera from both clofibrate-treated and control rats. Table I shows that the chylomicron protein constitutes less than 12% of the total  $d < 1.006$  protein. Most of the protein in the  $d < 1.006$  fraction is the VLDL protein. Therefore, in the subsequent experiments we did not separate the chylomicrons, and the VLDL actually contains a small amount of chylomicron protein. In addition, Table I shows that at 12 hr there was no change in VLDL protein concentration but a marked decrease in HDL protein concentration. Changes in lipoprotein-protein concentration that occurred during 8 days of clofibrate administration are shown in Fig. 2. VLDL protein concentration did not change until after the 2nd day of treatment; by the 4th day it had decreased by 50% and remained low thereafter. The LDL protein showed no consistent change. HDL protein concentration decreased by 40% within 12 hr after clofibrate administration and progressively decreased thereafter.

**Composition of serum lipoproteins.** In addition to changes in the concentrations of the lipoproteins, certain changes in composition were also observed. 8 days of clofibrate administration resulted in a decrease of 50% in the serum concentration of triglyceride present in the VLDL fraction. VLDL protein decreased but to a lesser extent. Phospholipid was the least affected. As a result, the VLDL of the clofibrate-treated rats was relatively poor in triglyceride and rich in phospholipids (Table II). In the HDL fraction, protein and cholesterol concentrations decreased proportionately. However, the

decrease in HDL phospholipid concentration was greater than in the other components. As a result, HDL of the clofibrate-treated rat (Table II) contained less phospholipids and slightly more protein.

**Incorporation of labeled leucine into lipoproteins.** The effect of the administration of clofibrate on incorporation of either L-leucine- $^3H$  or L-leucine- $^{14}C$  into the protein portions of the various lipoproteins was measured. Radioactivity in VLDL was determined 60 min after administration of the labeled precursor; radioactivity in LDL and HDL was determined 150 min after administration of the labeled leucine. Fig. 3 shows the total incorporation of labeled amino acid into the three lipoprotein fractions after clofibrate administration. Incorporation into VLDL was increased during the first 2 days of treatment but appeared to decrease by the 4th and 8th days of treatment. Incorporation of labeled leucine into HDL decreased appreciably within 12 hr and decreased further thereafter. The specific activity (Fig. 4) of VLDL increased after administration of clofibrate while the specific activity of HDL protein was unchanged. However, the time course of labeling was unchanged by the administration of clofibrate.

In order to obtain information as to the rate of turnover of VLDL at different times after the administration of clofibrate, we employed the method described by Schimke, Ganschow, Doyle, and Arias (19) whereby two time points on a decay curve can be determined in the same protein in the same animal. In this technique one isotopic form of an amino acid ( $^{14}C$ ) is administered initially. After a specified time, the second isotopic form ( $^3H$ ) of the same amino acid is administered, and the animal killed shortly thereafter. If certain assumptions are met, the  $^3H$  counts represent an initial time point, and the  $^{14}C$  counts represent a subsequent point on

TABLE II  
Effect of Clofibrate on the Composition of Serum Lipoproteins\*

	Treatment days	Protein	Tri-glyceride	Total cholesterol	Phospholipid
% of total†					
VLDL	0	4.1	61.5	5.7	28.7
	8	4.7	49.3	6.2	39.7
LDL	0	18.5	6.7	24.0	50.7
	8	16.9	18.8	15.7	48.6
HDL	0	39.1	—§	31.0	30.0
	8	43.8	—	32.8	23.8

\* Pooled sera from 6 to 10 rats were used for the separation of lipoprotein fractions. Chemical determinations were done in triplicate.

† Sum of components.

§ Not determined due to loss of sample.

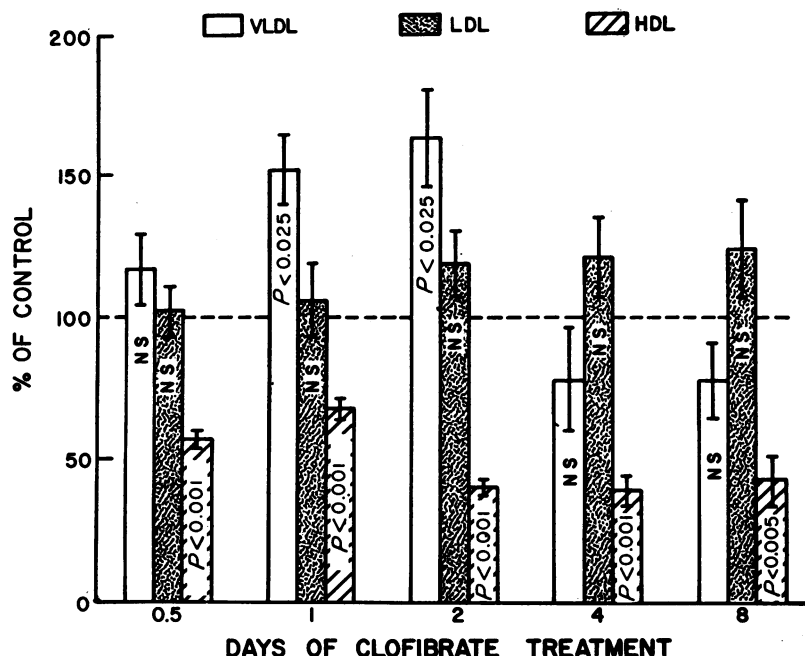


FIGURE 3 The effect of clofibrate administration on incorporation of labeled leucine into serum lipoproteins. The results are expressed as per cent of control dpm at each time period. For VLDL radioactivity due to tritium was measured; for LDL and HDL radioactivity in  $^{14}\text{C}$  was determined. Remainder similar to legend for Fig. 1.

the turnover-curve of the labeled protein. The ratio  $^3\text{H}/^{14}\text{C}$  is therefore a function of the rate of turnover. The assumptions required are: (a) at the time the animal is killed the protein is in a process of isotopic decay, (b) the isotope is not metabolized and reutilized during the course of the experiment, (c) the protein follows exponential decay kinetics, and (d) the rate of synthesis of the protein does not change during the course of the experiment. Since the specific activity of VLDL is maximal at 50 min after the injection of labeled amino acid, we measured incorporation at 60 min after leucine- $^3\text{H}$  and 150 min after leucine- $^{14}\text{C}$  were administered.

Fig. 5 shows the effect of clofibrate on  $^3\text{H}/^{14}\text{C}$  ratios of the VLDL protein. The  $^3\text{H}/^{14}\text{C}$  ratio is significantly higher in the clofibrate-treated animals during the first 4 days of clofibrate administration and elevated to a lesser extent on the 8th day. These data suggest that the administration of clofibrate increases the rate of turnover of VLDL.

Turnover was also determined by measurement of the specific activity of VLDL after the pulsed administration of labeled amino acids. Animals that had been treated with clofibrate for 40 hr and untreated animals were studied. The rats treated with clofibrate were killed at 1, 2.5, 5, and 10 hr after the injection of the labeled amino acids. The specific activity of the VLDL protein

in the clofibrate-treated rats was almost twice as high in the treated as the control rats at the peak specific activity, 1 hr after injection of the labeled amino acids (Fig. 6). The specific activity of the VLDL of the

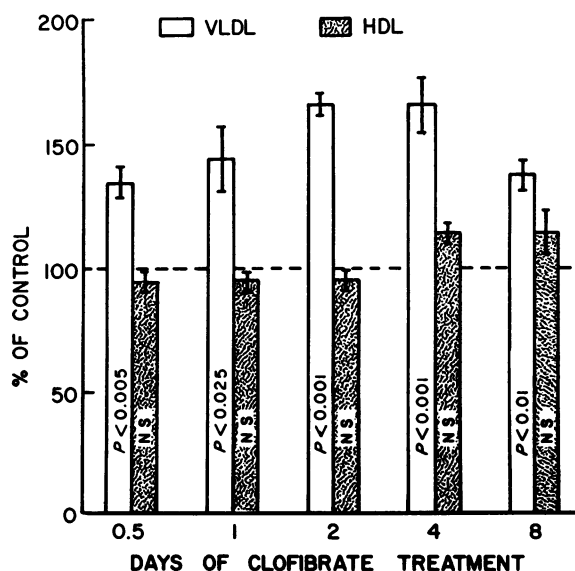


FIGURE 4 The effect of clofibrate administration on specific activity of VLDL and HDL. For details see Fig. 3.

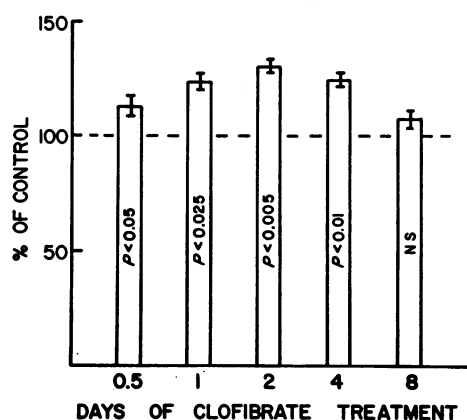


FIGURE 5 The effect of clofibrate administration on  $^3\text{H}/^{14}\text{C}$  ratios in VLDL protein in rats receiving  $^3\text{H}$  and leucine- $^{14}\text{C}$ . The results are expressed as per cent of control ratios at each time period.

treated rats decreased at a more rapid rate than did the specific activity of the VLDL in the control rats. 10 hr after the injection of labeled amino acid, the specific activity of VLDL protein was significantly lower in clofibrate-treated animals than in the control group. These results indicate increased turnover rate of the VLDL protein and are in agreement with results obtained by the double label technique of Schimke et al. (19). As a control, the specific activity curve of albumin was also determined. The albumin reached maximal specific activity in  $2\frac{1}{2}$  hr and the turnover-curve was identical in the treated and control groups. This finding suggests that no changes in amino acid pool size have occurred in rats given clofibrate.

## DISCUSSION

Clofibrate is widely used in the treatment of patients with hyperlipidemia. Accordingly, it would appear that hyperlipidemic rats would constitute a more appropriate model for the study of the mode of action of this drug. Hyperlipidemia was produced by feeding a diet high in sucrose. This results in a marked increase in the concentration of VLDL in the serum, no change in LDL concentration, and a lesser but significant increase in HDL. The synthesis of both VLDL and HDL protein is increased (20). The administration of clofibrate to sucrose-fed rats resulted in a fall in serum cholesterol concentration within 12 hr and a decrease in triglyceride concentration that did not occur until after 2 days of clofibrate administration. HDL protein concentration decreased concomitantly with the decrease in serum cholesterol. VLDL protein concentration decreased after 2–4 days of treatment. The decrease in the concentration of VLDL protein was not as great as that of triglyceride; therefore, in the treated rats, the VLDL has

a reduced triglyceride content. Recently, it has been shown that during the removal of VLDL from plasma, intermediates with reduced triglyceride content appear (21, 22). In the presence of an accelerated rate of removal of VLDL, the finding of increased amounts of such "remnants" (23) is not unexpected.

The decrease in the concentration of serum HDL is in part due to decreased synthesis of HDL. However, it would appear that changes in removal rate—which we did not measure—must also occur, since the concentration of HDL protein decreased by 40% within 12 hr after the initiation of clofibrate administration. The  $t_{\frac{1}{2}}$  of rat HDL is about 11 hr (24), so that a reduction in concentration of the magnitude that we observed would require almost complete cessation of synthesis. It is noteworthy that the rate of cholesterol synthesis is also reduced within 12 hr of clofibrate treatment. Since HDL is the primary vehicle for transport of cholesterol out of the liver in the rat, it is possible that the inhibition of cholesterol synthesis is a consequence of cholesterol accumulation in intracellular organelles. It is also possible that HDL synthesis is decreased secondarily to diminution of cholesterol available for lipoprotein formation in the liver.

VLDL synthesis, as measured by incorporation of labeled amino acids, was increased during the first 2 days of treatment. However, during this period, no change in VLDL concentration was observed, and this suggests that the rate of removal of VLDL increased. This was

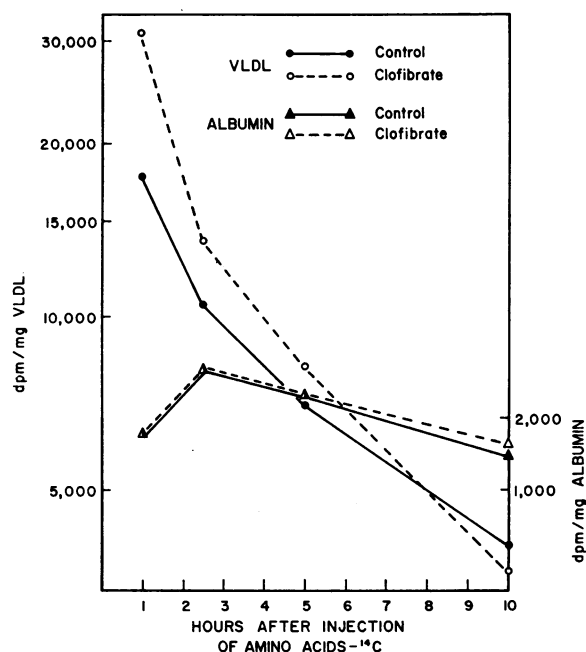


FIGURE 6 Specific activities of serum VLDL and serum albumin after administration of amino acids- $^{14}\text{C}$ .

confirmed in the study where turnover-rate of VLDL as measured by the double isotope procedure of Schimke et al. (19) was found to be increased. Turnover of VLDL was also measured by the determination of the specific activity at various time intervals after administration of labeled amino acid and was also found to be increased. Thus it appears that soon after the administration of clofibrate, both the rate of synthesis and the rate of removal of VLDL are increased. However, the increased rate of synthesis is not sustained, and when it decreases in the presence of the increased rate of removal, the concentration of VLDL in the plasma decreases.

Since serum triglyceride is a major constituent of VLDL, changes in triglyceride metabolism are in many instances indicative of changes in the metabolism of VLDL protein. A number of workers have suggested that the rate of removal of triglyceride from the serum plays a major role in determining its plasma level (25, 26). Spritz (27) and Sodhi, Horlick, and Kudchodkar (28) have shown in hyperlipidemic patients that the  $t_{1/2}$  of VLDL triglyceride was markedly shortened after administration of clofibrate. Bierman et al. (29) found a consistent decrease in endogenous triglyceride production rate after the administration of clofibrate but in addition suggested that triglyceride removal may be enhanced. In the rat, Tolman, Tepperman, and Tepperman (30) have shown that administration of clofibrate results in increased adipose tissue lipoprotein-lipase activity and have also concluded that enhanced peripheral removal of triglyceride is responsible for the effect of clofibrate.

There are, however, studies suggesting that administration of clofibrate inhibits the release of triglyceride into the plasma (3, 4, 31, 32). Gould et al. (5) have found decreased incorporation of amino acids- $^{14}\text{C}$  into mixed lipoproteins by liver slices obtained from rats treated with clofibrate for 5 days. However, since the lipoproteins were not separated, this could be due to the decreased synthesis of HDL. Lipoprotein synthesis was also measured in intact rats by injection of labeled amino acids. Incorporation into HDL and into a mixed LDL plus VLDL fraction was found to be decreased.

It should be emphasized that our studies were carried out with sucrose-fed hyperlipidemic rats (20). The use of such animals could account for the differences between our findings and those of others (3, 31-33). It is of interest that Kokatanur and Wu (34) have shown that the effect of clofibrate on acetate incorporation into lipids differs between rats fed diets containing dextrose and those fed diets in which starch was the source of carbohydrate.

Lipoproteins were separated at densities of 1.006, 1.063, and 1.21, respectively. It is recognized that under these conditions, the LDL fraction (d 1.006-1.063) con-

tains traces of HDL. However, in earlier studies we have found that in the rat fed a normal diet most of LDL appears between densities 1.040 and 1.050 (35). The density distribution of LDL of the sucrose-fed rat appeared to be similar to that of the rats fed normal diets. Since the purpose of these experiments was to measure the total amounts of these various lipoprotein fractions, it was essential that the entire fraction be isolated. Had we been primarily interested in obtaining lipoprotein fractions uncontaminated with other lipoproteins, a narrower density range would have been separated, but at the expense of total recovery of any fraction. Furthermore, these conditions favor the isolation of all the VLDL and HDL at which this study has been primarily directed.

The mechanism whereby clofibrate exerts its effect on lipoprotein metabolism is, as yet, unknown. It is of particular interest that its initial effect is on HDL synthesis and only subsequently on VLDL. Since rat HDL and VLDL contain subunit polypeptides which are immunologically related (36-38), it is tempting to speculate that the primary effect of clofibrate is on these particular subunit proteins. Because of this heterogeneity of the subunit proteins of the HDL and VLDL, it is possible that changes in the rate of turnover of the total proteins in a given fraction would reflect marked changes in the turnover of only some of the subunit polypeptides. Studies of the rate of turnover of the various subunit polypeptides are presently in progress in this laboratory.

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