Inhibition of Hormone-Stimulated Lipolysis by Clofibrate

A POSSIBLE MECHANISM FOR ITS HYPOLIPIDEMIC ACTION

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Abstract The present study was undertaken to investigate the mechanism of the antilipolytic action of clofibrate (p-chlorophenoxyisobutyrate). Clofibrate, in the dose range of 10–80 mg/100 ml, inhibited the initial rate of norepinephrine-stimulated lipolysis 17–44% in isolated rat fat cells. At a dose corresponding to therapeutic levels in vivo (10 mg/100 ml) clofibrate also inhibited hormone-stimulated lipolysis by 20–30% in fragments of human subcutaneous fat. Inhibition of lipolysis by clofibrate occurred at all concentrations of norepinephrine and ACTH (0.02–0.1 μg/ml) but did not occur with equilipolytic concentrations of dibutyryl cyclic AMP, suggesting a proximal site of action on the lipolytic sequence. Clofibrate reduced by 60% (315 ±40 vs. 120±25 pmol/g lipid; mean±SEM) the nor-epinephrine-stimulated initial rise in cyclic AMP, measured 10 min after addition of hormone. Because the antilipolytic effect occurred in the presence of glucose and without altering cellular ATP levels, the reduction in intracellular cyclic AMP levels could not be attributed to uncoupling of oxidative metabolism or to secondary effects of free fatty acid accumulation. In the presence of procaine-HCl, which blocks hormone-stimulated lipolysis without inhibiting cyclic AMP accumulation, addition of clofibrate prevented the hormone-stimulated rise in cyclic AMP. Clofibrate did not affect the activity of the low-K_m 3',5'-cyclic AMP phosphodiesterase in norepinephrine-stimulated adipocytes. These data suggest that the antilipolytic effect of clofibrate is due to its suppression of cyclic AMP production by inhibition of adenylate cyclase. The drug's hypolipi-demic action may in part be explained by its antilipolytic effect, which deprives the liver of free fatty acid substrate for lipoprotein synthesis.

INTRODUCTION

Clofibrate (ethyl p-chlorophenoxyisobutyrate) is commonly used in the treatment of different types of hyper-lipidemia (1, 2). Although its ability to lower serum lipids was recognized about ten years ago (3–7), an understanding of the mode of action of the drug remains somewhat speculative. While recent literature (8, 9) favors the view that clofibrate reduces serum triglyceride levels primarily by enhancing VLDL[1] catabolism, many authors have either demonstrated a reduction in VLDL synthesis (10, 11) or cannot exclude the possibility that reduced VLDL formation and secretion by the liver contributes to the drug's hypo-lipidemic action (8, 11–14). In addition to effects of clofibrate on VLDL synthesis and removal, a number of other less well substantiated mechanisms have been postulated to explain the drug's lipid-lowering action. These include inhibition of cholesterol and triglyceride synthesis in the liver (15–18), enhancement of the up-take and storage of cholesterol in various tissues (19, 20), and increased lipoprotein lipase activity in adipose tissue (21). Recently, studies in this laboratory (22) have reexamined these latter mechanisms in order to reappraise their significance. The results of this work (22) suggest that these latter mechanisms (15–21) may play a minor role in the hypolipidemic action of clofibrate.

[1] Abbreviations used in this paper: ACTH, Cortrosyn, a synthetic subunit of adrenocorticotropic hormone; cAMP (cyclic AMP), cyclic adenosine 3',5'-monophosphate; DBC, N_6,-O_2-dibutyryl cyclic adenosine 3',5'-monophosphate; FFA, free fatty acids; NE, norepinephrine; VLDL, very low density lipoprotein.

Clofibrate lowers serum free fatty acid (FFA) levels and inhibits the release of FFA from adipose tissue in rats (23, 24), dogs (25), and man (26). It has a similar action in norepinephrine (NE)-stimulated fat cells isolated from rat epididymal adipose tissue (22). If this antilipolytic effect of clofibrate is responsible for the reduction in plasma FFA levels, it could, by limiting substrate for hepatic VLDL synthesis, account for the observed reduction in plasma triglyceride levels. The plausibility of this mechanism is reinforced by the fact that in perfused livers, FFA uptake and VLDL production are proportional to physiological concentrations (K_m 0.3–0.4 mM) of free fatty acid in the perfusate (27, 28). To substantiate the notion that substrate (FFA) deprivation may explain the drug's hypolipidemic effect, a detailed study of its antilipolytic action was undertaken. It was observed that at concentrations corresponding to therapeutic plasma levels, clofibrate inhibited basal and hormone-stimulated lipolysis in human adipose tissue fragments, presumably by inhibiting adenylate cyclase. This finding is compatible with the view that diminished VLDL synthesis may, in part, explain the hypolipidemic action of clofibrate.

METHODS

Materials. NE bitartarate and N_6-O_2-dibutyryl 3',5'-cyclic AMP (DBC), 3',5'-cyclic AMP (cAMP), adenosine, *Crotalus atrox* snake venom, and procaine-HCl were purchased from Sigma Chemical Co., St. Louis, Mo.; cyclic [³H]AMP (20.8 Ci/mmol) was purchased from Schwarz/Mann Div., Becton, Dickinson & Co., (Orangeburg, N. Y.); collagenase, prepared from *Clostridium histolyticum,* was obtained from Worthington Biochemical Corp., Freehold, N. J.; bovine serum albumin (fraction V) was an Armour Pharmaceutical Company (Chicago, Ill.) product; sodium clofibrate was supplied by Ayerst Laboratories, Montreal, Canada. Cortrosyn, a 1–24 amino acid synthetic subunit of ACTH, was provided by Organon Inc., Canada. In the text this compound is referred to as ACTH. The enzymes used for glycerol and ATP assay were Boehringer products (Boehringer Mannheim Corp., New York). All other reagents were of analytical grade.

Incubation procedures. Adipocytes were isolated by the collagenase digestion method of Rodbell (29) with minor modifications (30) from epididymal fat pads of 12 Wistar rats (180–200 g) raised on Purina Chow (Ralston Purina Co., St. Louis, Mo.). Incubations with isolated cells or fragments of human adipose tissue were carried out in 1-oz plastic (Nalgene, Nalge Co., Nalgene Labware Div., Rochester, N. Y.). bottles in a Dubnoff metabolic shaker oscillating at 90 cycles/min at 37°C with 95% O_2-5% CO_2 gas phase. Cells (100±20 mg of adipocyte lipid) were suspended in 2 ml of Krebs-Ringer bicarbonate buffer containing 1.27 mM $CaCl_2$ and 5% dialyzed bovine serum albumin. The cells were preincubated for 10 min, after which zero time samples were taken for glycerol determinations before any additions were made. Lipolysis was induced by the addition of aqueous solutions of NE, ACTH, or DBC. At the end of the incubation, the contents of each vial were transferred to 15-ml centrifuge tubes and spun at 300 g for 15 s. The medium was separated from the cell

float by aspiration through polyethylene tubing (Intramedic PE100, Clay-Adams, Inc., Parsippany, N. J.) attached to a 20-gauge 1" needle and syringe. Glycerol was measured in deproteinized medium by the well-established enzymatic method of Garland and Randle (31). The mean basal (unstimulated) rate of glycerol release after subtracting zero time values was 1.06±0.09 μmol/g lipid/10 min (mean ±SEM, $n = 6$). Zero time glycerol levels and basal (unstimulated) rates of glycerol release were measured in each experiment. Glycerol output was used as a measure of lipolytic rate since reutilization of glycerol by adipose cells is negligible (32) in relation to the amounts produced.

Preparation of human samples. Subcutaneous adipose tissue samples were obtained from the lower end of a midthoracic incision (upper abdominal wall fat) in four patients undergoing corrective cardiovascular surgery. Patient M. B., a 23-yr-old woman, had an ostium primum defect. Patient P. M., a 20-yr-old woman, underwent ventricular septal defect repair. Patient M. C., a 61-yr-old woman, was undergoing aortic valve replacement for aortic stenosis and aortic insufficiency. Patient C. C., a 47-yr-old woman, underwent aortocoronary bypass surgery. None of the patients were obese, diabetic, or hypertensive, and all were euthyroid. Patients M. C. and C. C. were on maintenance digoxin therapy before surgery and received Lasix (furosemide, Hoechst Pharmaceuticals, Inc., Somerville, N. J.) intermittently for control of congestive heart failure. After the removal of adipose tissue (3–5 g) from the incision site, the fragments were rinsed in fresh Krebs-Ringer bicarbonate buffer to remove adherent blood. Fibrous connective tissue was cut away and 150–200-mg slices of adipose tissue were removed. Care was taken to avoid strands of dense fibrous connective tissue. Each slice was cut into 25–50 mg fragments and placed in an incubation vial containing 2 ml Krebs-Ringer bicarbonate-albumin buffer with 6 mM glucose. After 30 min preincubation, NE (1 μg/ml) and sodium clofibrate (10 and 80 mg/100 ml) were added, and samples were removed after 2 and 4 h of incubation. The data are expressed as net glycerol release by subtracting zero time values and the basal rate from stimulated values. Incubations were carried out in triplicate with single determinations of glycerol in each sample ($n = 3$). Isolated cells or fragments of human adipose tissue were homogenized with 3 ml diethyl ether and centrifuged. The supernatant organic phase was removed and the pellet rehomogenized twice. The ethyl ether extracts were pooled and a sample was evaporated to dryness in a tared vessel. The final results were expressed per unit cell or tissue lipid.

Nucleotide analysis. For the measurement of intracellular cAMP, the adipose cell float was treated with 2 ml of 5% trichloracetic acid, mixed on a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), and allowed to stand for 30 min at 0°C. After centrifugation, the supernate was extracted with diethyl ether to remove lipids and trichloracetic acid, and then lyophilized. cAMP was measured by the protein-binding assay described by Gilman (33) with minor modifications (34). The binding reactions were carried out for 90 min at 0°C in 50 mM sodium acetate buffer, pH 4, in a volume of 100 μl, with 4 μg of binding protein, 15 μg of the protein inhibitor fraction, and 4 pmol of [³H]cAMP (14.2 Ci/mmol). The counting efficiency for ³H was 36%.

ATP assays were carried out on perchloric acid extracts of adipose cells. The adipose cell float was denatured by the addition of 2 ml of 5% perchloric acid, the mixture was then thoroughly mixed on a Vortex mixer and centri-

TABLE I

TABLE I

Effect of Albumin Concentration on the Antilipolytic Action of Clofibrate in Vitro

Additions	Medium Albumin	Net glycerol release	Inhibition
	g/100 ml	*μmol/g lipid/30 min*	*%*
NE	2.5	19.53±0.69*	—
NE + clofibrate (10 mg/100 ml)	2.5	16.22±0.65‡	17
NE + clofibrate (40 mg/100 ml)	2.5	12.59±0.44‡	36
NE	5.0	28.83±1.12	—
NE + clofibrate (10 mg/100 ml)	5.0	23.56±0.86‡	18
NE + clofibrate (40 mg/100 ml)	5.0	17.97±0.70‡	38

* Mean values ±SEM (n = 8).
‡ Significantly different from corresponding NE values (P < 0.02).

Adipocytes (120 mg lipid/flask) were preincubated for 10 min in 2 ml of Krebs-Ringer bicarbonate-6 mM glucose, containing either 2.5% or 5% albumin. Lipolysis was stimulated with NE (0.1 μg/ml). Glycerol was measured in the medium after 30 min incubation, and zero time and basal levels were subtracted.

fuged for 5 min at 300 g, and the clear aqueous phase was removed for ATP assays. The perchloric acid extracts were neutralized to pH 7 with a saturated solution of KHCO₃ and centrifuged, and ATP was assayed enzymatically as described by Denton, Yorke, and Randle (35).

Phosphodiesterase assay. At the end of the 10-min incubation, cells and medium were separated by centrifugation, the medium was retained for determination of glycerol, and the cells were assayed for phosphodiesterase activity. The cell float was homogenized in 2 ml of cold 0.25 M mannitol (pH 7.5) by hand (10 downward strokes of the pestle). The homogenate was centrifuged at 2,000 rpm for 4 min (Sorvall GLC-1, Ivan Sorvall, Inc., Newtown, Conn.) and the infranatant was aspirated, made up to 2 ml with mannitol, and used as the enzyme preparation. The low-K_m phosphodiesterase activity was assayed by the procedure of Loten and Sneyd (36), as modified by Zinman and Hollenberg (37), with an additional step of stopping the phosphodiesterase activity after 10 min of incubation by immersing the tubes into boiling water for 10 min. The effect of sodium clofibrate (50 and 100 mg/100 ml) was also tested on the activity of beef heart 3′,5′-cyclic nucleotide phosphodiesterase (Sigma Chemical Co.) by the same assay.

RESULTS

Effect of clofibrate on catecholamine-induced lipolysis. NE-stimulated glycerol release was significantly inhibited (17–38%) with 10–40 mg/100 ml clofibrate (Table I). The dose of 10 mg/100 ml is comparable to that achieved in patients (8) or rats ² with hypo-

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² Smigura, F. C., M. A. D'Costa, and A. Angel. Unpublished observations.

lipidemic effects of clofibrate. In some of the subsequent experiments, higher concentrations were used.

In a time course study of the antilipolytic effect of clofibrate on NE-stimulated cells, a 40% inhibition occurred throughout (Fig. 1). This pattern was consistent and typical of six different experiments. The rates were linear up to 30 min and therefore represent initial rates of lipolysis.

Effect of clofibrate on lipolysis in human adipose tissue fragments. Clofibrate inhibited NE-stimulated lipolysis in fragments of subcutaneous adipose tissue obtained from four patients (Fig. 2). In patients M. B. and P. M. clofibrate at a concentration of 10 mg/100 ml inhibited glycerol output by approximately 20–30% (P < 0.01) at 2 and 4 h of incubation. At a concentration of 80 mg/100 ml, clofibrate inhibited glycerol output by 60% after 2 h. However, this degree of inhibition did not persist for the second 2 h of incubation. Patients M. C. and C. C. displayed lower rates (50% less) of glycerol release in response to NE than patients M. B. and P. M. However, the inhibitory effects of clofibrate were similar. A lag in hormone-stimulated glycerol output was observed in M. C. and
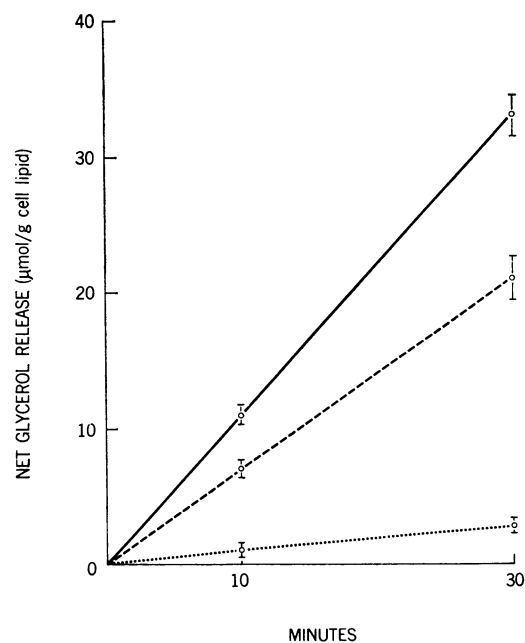

FIGURE 1 A time course study of the effect of clofibrate on the initial rate of lipolysis. Fat cells (110 mg lipid/flask) were preincubated for 10 min in 2 ml of Krebs-Ringer bicarbonate-5% albumin medium and incubated for 10 and 30 min with the following additions: none (· · ·); 0.1 μg/ml NE (———), and 0.1 μg/ml NE plus 80 mg/100 ml sodium clofibrate (– – –). The data, derived from two experiments, are the mean±SEM of six incubations for each value recorded. NE + clofibrate values differ significantly from NE alone (P < 0.01).
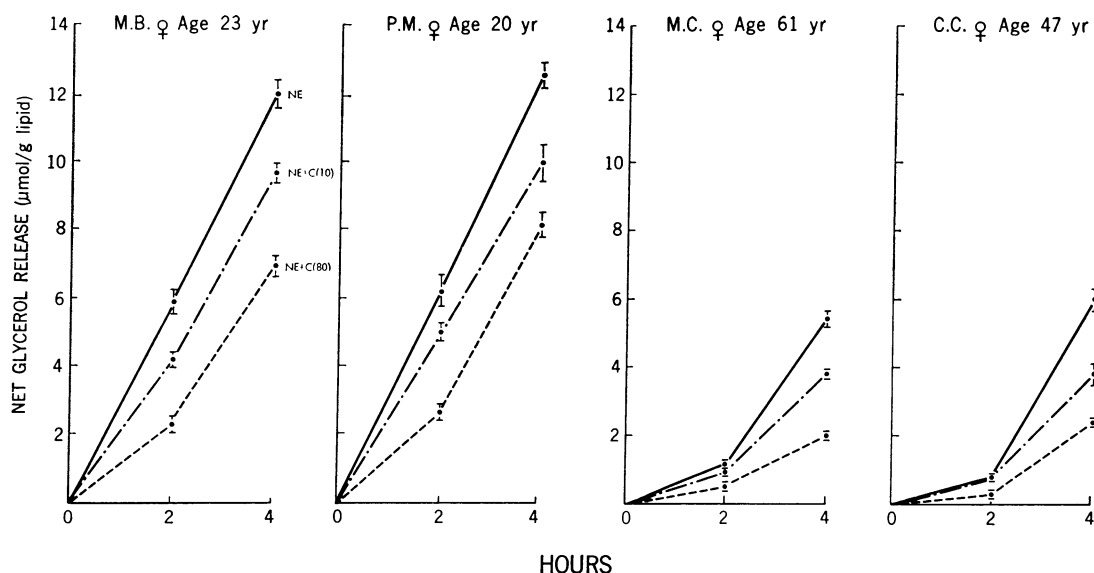
FIGURE 2 Effect of clofibrate (C) (·—·—· 10 mg/100 ml; - - - - 80 mg/100 ml) on NE (——— 1 μg/ml) stimulated lipolysis in fragments of subcutaneous adipose tissue obtained from patients undergoing elective cardiovascular surgery (see Methods for details). Each point is the mean±SEM of triplicate incubations. NE + C values differ significantly (P < 0.01) from NE alone at 2 and 4 h in patients M. B. and P. M. and at 4 h in M. C. and C. C.

C. C. The reason for this lag became apparent on examining the pattern of basal glycerol release (Table II). In both patients the basal glycerol output approached a plateau within 2 h of incubation, after which little further release of glycerol occurred. When the basal values (Table II) were summed with the hormone-stimulated increment (Fig. 2), the slopes approach linearity.

Basal glycerol release occurred in adipose tissue fragments form all four patients (Table II). In tissues from patients M. C. and C. C. clofibrate at a concentration of 80 mg/100 ml inhibited glycerol release by 30–50%. Thus both basal (Table II) and hormone-stimulated glycerol release (Fig. 2) were inhibited by clofibrate (80 mg/100 ml).

Effect of albumin on the antilipolytic action of clofibrate. It is known that clofibrate, which is anionic at physiological pH, binds to serum albumin in competition with FFA for the anionic binding sites (23, 25). Experiments were therefore designed to test the effects of clofibrate on lipolysis with two concentrations of albumin (2.5% and 5%). The lipolytic rate was 32% lower in medium containing 2.5% albumin than in 5% albumin (Table I). However, clofibrate at 10 and 40 mg/100 ml inhibited lipolysis to precisely the same degree, independent of the concentration of albumin and the lipolytic rate. These findings would indicate that the effect of clofibrate in vitro is not mediated through secondary redistribution or cellular accumulation of long-chain fatty acid anions.

Effect of glucose on the antilipolytic action of clofibrate. The presence of 6 mM glucose in the incubation medium did not affect the inhibitory action of clofibrate on lipolysis (Table III). Glucose promotes the re-esterification of FFA and prevents their uncoupling effects (38, 39). Since clofibrate reduced the initial rates of lipolysis in the presence of glucose, we concluded that the antilipolytic effect of the drug is unrelated to effects due to intracellular accumulation of fatty acids or their acyl derivatives.

TABLE II
Effect of Clofibrate (C) on Basal Lipolysis in Human Adipose Tissue Fragments

Patient	Incubation time	Glycerol release		Inhibition
		Basal	Basal + C	
	h	μmol/g lipid		%
M. B. ♀ 23 yr	2	2.21±0.46	—	—
	4	2.10±0.11	—	—
P. M. ♀ 20 yr	2	2.33±0.31	—	—
	4	2.59±0.28	—	—
M. C. ♀ 61 yr	2	3.20±0.29	1.56±0.14*	51
	4	4.37±0.52	2.10±0.19*	52
C. C. ♀ 47 yr	2	2.17±0.21	1.51±0.04	30
	4	2.44±0.18	1.57±0.19	36

* Significantly different from controls without clofibrate (P < 0.05).
Human subcutaneous adipose tissue fragments (150–200 mg) were incubated in 2 ml Krebs-Ringer bicarbonate-5% albumin buffer containing 6 mM glucose. After 30 min of preincubation, sodium clofibrate (80 mg/100 ml) was added and aliquots of medium were removed at 2 and 4 h for glycerol determination. The data are the means ±SEM of triplicate incubations from each patient.

TABLE III

Antilipolytic Effect of Clofibrate in the Presence and Absence of Glucose

Experiment	Additions	Net glycerol release	Inhibition
		μmol/g lipid/ 30 min	%
1	NE	38.2±1.1*	
	NE + clofibrate	25.3±1.0**	34
	NE + glucose	36.0±1.3	
	NE + glucose + clofibrate	22.1±0.8‡	39
2	NE	25.0±0.9	
	NE + clofibrate	15.6±0.6‡	38
	NE + glucose	29.9±1.1	
	NE + glucose + clofibrate	16.8±0.7‡	44

* Mean values ±SEM (n = 3)

‡ Significantly different from controls without clofibrate ($P < 0.02$).

Adipocytes (100 mg lipid/flask) were incubated for 30 min. Additions to the Krebs-Ringer bicarbonate-5% albumin medium were NE (0.1 μg/ml), clofibrate (80 mg/100 ml), and glucose (6 mM).

Effect of clofibrate on intracellular ATP. Basal levels of ATP remained unchanged after 10 min of incubation but declined somewhat thereafter (Fig. 3). The addition of NE caused a 15% reduction in ATP levels measured at 10 min. Such an effect by catecholamines was noted previously (39, 40). The ATP level at 30 min in NE-treated fat cells was slightly but not significantly higher than at 10 min. While the mean cellular ATP levels in clofibrate-treated cells were greater than in cells exposed to NE alone, the differences were not significant. Nevertheless, lipolysis was inhibited 40% by clofibrate addition (Fig. 3). If the changes in total cellular ATP concentrations are assumed to reflect fluctuation in the critical subcellular pool(s) involved in the lipolytic sequence, then the antilipolytic effect of clofibrate cannot be attributed to deleterious changes in the energy metabolism of the cell.

Effect of clofibrate on lipolysis induced by different lipolytic agents. Fig. 4 compares the effect of clofibrate on lipolysis induced by NE, ACTH, and DBC. Clofibrate inhibited lipolysis (50–68%) at all concentrations of norepinephrine and ACTH. However, no inhibition occurred with DBC concentrations up to 1 mM, which is the half V_{max} concentration for this cyclic AMP analogue (39). Inhibition of lipolysis with 2 mM DBC occurred (25%) but was less than that observed with hormones. It may be concluded from these data that with half-maximal concentrations of the three lipolytic agents tested, clofibrate inhibited lipolysis induced by the hormones NE and ACTH but did not inhibit that induced by DBC. These data suggest that clofibrate must affect primarily that part of the lipolytic sequence that precedes the accumulation of cyclic AMP, because the lipolytic effect of DBC, which bypasses the adeny-

late cyclase and simulates the action of cyclic AMP (41, 42), was not suppressed.

Effect of clofibrate on adipocyte cyclic AMP. The effect of clofibrate on the accumulation of intracellular cyclic AMP is shown in Fig. 5. In NE-treated cells, the cyclic AMP level rose to a peak and fell to a lower level at 30 min as was noted before (34, 43). However, in the presence of clofibrate the pattern was altered and a 60% reduction in the accumulation of cyclic AMP over control values occurred at 10 min. This reduction in the rise in cyclic AMP may explain the inhibition of lipolysis (Fig. 5), since it is the initial rise in cAMP that determines the initial rate of lipolysis (34, 43–45). At 30 min the levels in control and clofibrate-treated cells were not statistically different.

Effect of clofibrate on cAMP phosphodiesterase. The intracellular level of cAMP is determined by a net balance between its synthesis by the enzyme adenylate cyclase and its degradation by the enzyme cAMP phosphodiesterase (41, 44, 46). The influence of clofibrate upon the activity of the low K_m enzyme 3′,5′-cAMP phosphodiesterase was tested to determine whether enhanced cAMP degradation might be responsible for the reduction in cAMP accumulation. The results are shown in Fig. 6. The basal activity of the low-K_m phosphodiesterase in rat adipocytes was 1.6 nmol of adenosine formed/g lipid per min. Addition of
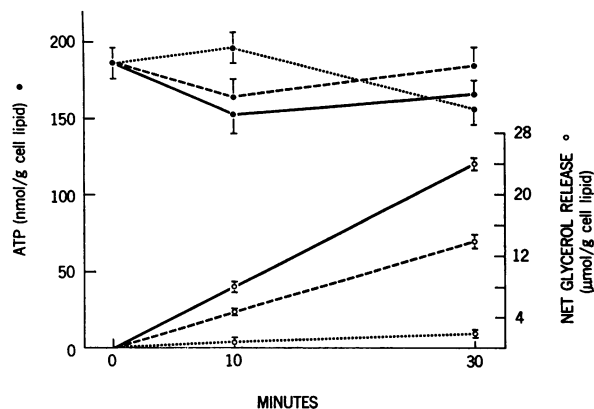

FIGURE 3 Effect of clofibrate on intracellular ATP levels and lipolysis in adipocytes. Incubation conditions were as described for Fig. 1. Glucose (6 mM) was added to the incubation medium. ATP (Closed symbols) and glycerol (open symbols) were measured in cells and medium, respectively, at zero time and after 10 and 30 min incubations. Additions were: none (· · ·); 0.1 μg/ml NE (——); and 0.1 μg/ml NE plus 80 mg/100 ml sodium clofibrate (– – –). The data (mean±SEM) were obtained from two combined experiments with triplicate incubations in each experiment (n = 6). NE + clofibrate values differ significantly from NE values ($P < 0.01$) for glycerol release.
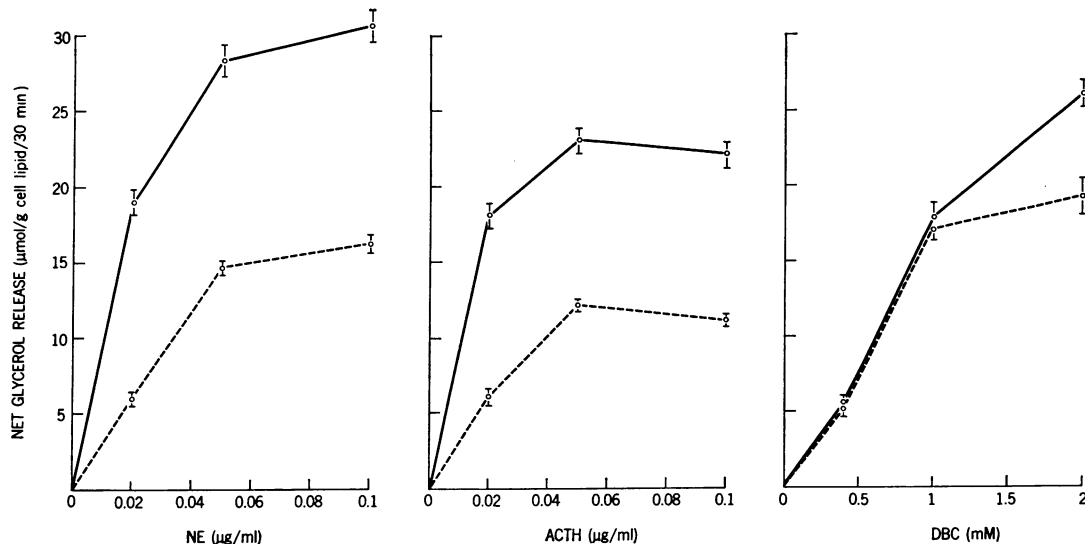
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FIGURE 4 Comparison of the clofibrate effect on lipolysis induced by different lipolytic agents. Adipocytes (90–120 mg lipid/flask) were obtained from rats weighing 190 g and preincubated for 10 min in the standard albumin medium containing 6 mM glucose. Glycerol in the medium was measured after a 30-min incubation in cells treated with hormone or DBC in the presence (- - -) or absence (——) of sodium clofibrate (80 mg/100 ml). Two experiments were carried out for each lipolytic agent tested, with triplicate incubations in each experiment. The data from the two experiments were combined (mean±SEM, $n = 6$). Values with clofibrate were significantly different from controls ($P < 0.001$) for all concentrations of NE, ACTH, and 2 mM DBC.

NE resulted in a 20% increase in phosphodiesterase activity and the expected rise in glycerol release. Addition of clofibrate (80 mg/100 ml) inhibited lipolysis (48%) but did not affect the NE-stimulated rise in phosphodiesterase activity. These findings confirm the observation that lipolytic hormones activate fat cell low-K_m phosphodiesterase (37) and show that the antilipolytic effect of clofibrate is not mediated through an enhancement of the activity of this enzyme. This conclusion is consistent with earlier experiments using purified beef heart phosphodiesterase enzyme (Sigma Chemical Co.), in which additions of clofibrate (50 and 100 mg/100 ml) had no effect on the enzyme activity in vitro (47).

Effect of clofibrate on cAMP and lipolysis in the presence of procaine. Local anesthetics are known to block hormone-stimulated lipolysis in adipose tissue (48). Preliminary studies with isolated fat cells have shown that procaine-HCl (10^{-4}-10^{-3} M) inhibits NE-stimulated lipolysis without inhibiting the accumulation of cAMP (49). Indeed, not only is there no inhibition of cAMP accumulation by procaine, but an increase in cAMP level above that with NE alone is usually seen (see below). This system was exploited to distinguish between a primary effect of clofibrate on adenylate cyclase and one that might be secondary to the lipolytic event or to FFA accumulation in the fat cell. The

results of these experiments are shown in Fig. 7. Lipolysis was inhibited 80% by procaine (NE vs. NE + P). In contrast, no inhibition of cAMP occurred. Instead the increment in cyclic AMP was more than double the value with NE alone (NE vs. NE + P). Thus, cyclic AMP accumulation was dissociated from lipolysis in the presence of procaine. The addition of clofibrate (80 mg/100 ml) to the system containing NE and procaine resulted in suppression of the rise in cAMP levels by 50% (418±40 vs. 204±35 pmol/g lipid) in the face of a 95% inhibition of lipolysis (NE + P vs. NE + P + C). Procaine had no effect on the basal rate of lipolysis or basal cAMP levels (see legend to Fig. 7). Qualitatively similar results were obtained with clofibrate at a dose of 10 mg/100 ml. However the magnitude of the reduction in cAMP levels, though significant, was less than that observed at 80 mg/100 ml. These observations suggest that the effect of clofibrate on cAMP accumulation is direct, rather than a manifestation of FFA accumulation.

DISCUSSION

The major purpose of this research was to locate the site of action of clofibrate on lipolysis. The lipolytic process in adipose tissue is briefly described. After interaction of a lipolytic hormone with its stereospecific receptor on the fat cell membrane (50), acti-
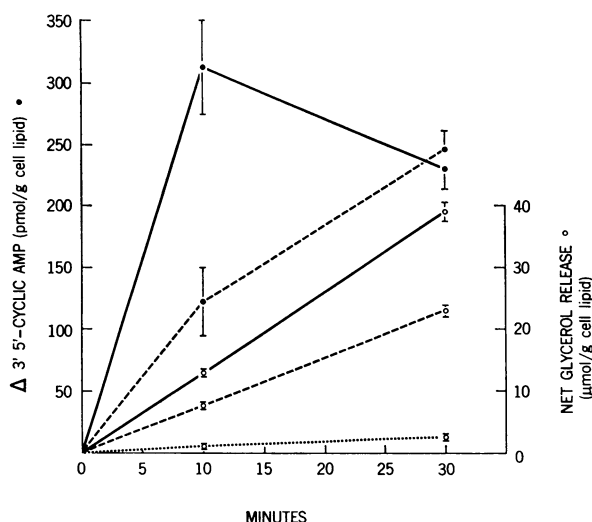
FIGURE 5 Effect of clofibrate on intracellular cyclic AMP levels in adipocytes. Cyclic AMP (closed symbols) and glycerol (open symbols) were measured in cells and medium, respectively, after 10 and 30 min incubations in Krebs-Ringer 5% albumin containing 6 mM glucose. Additions were: none (· · ·); 0.1 μg/ml NE (——); and 0.1 μg/ml NE plus 80 mg/100 ml sodium clofibrate (———). Each value (mean±SEM) was derived from three combined experiments consisting of four incubations in each experiment (n = 12). The basal (unstimulated) value for cyclic AMP was 165±27 and 153±15 at 10 and 30 min, respectively. All values with clofibrate at 10 min and those for glycerol release at 30 min are significantly different for corresponding values for NE alone (P < 0.001).

vation of membrane-bound adenylate cyclase ensues (41). Intracellular cAMP levels rapidly rise, reaching a peak concentration between 4 and 10 min, and subsequently decline (34, 43–45), although lipolysis, once initiated, continues linearly during this time. The level of cAMP achieved is the net balance between synthesis, loss from the cell, and degradation by cAMP phosphodiesterase (41, 46). cAMP, by an allosteric interaction with cAMP-dependent protein kinase, liberates a catalytic subunit that activates (phosphorylates) a triglyceride lipase (51–53). From the foregoing, it is possible that a number of different steps in the lipolytic sequence may be affected by clofibrate. The contrasting effects of the drug on lipolysis induced by DBC and hormones (Fig. 4) suggested that the primary site of action of clofibrate is at a proximal part of the lipolytic sequence, i.e., preceding the rise of intracellular cAMP. An antagonistic effect of clofibrate on the accumulation of cAMP during the initiation of lipolysis by hormones was shown (Fig. 5). This agrees with an earlier report in which very high concentrations of clofibrate (200 mg/100 ml) prevented a rise in cAMP levels in isolated fat cells in the presence of caffeine (54). However, the present investiga-

tion was carried out in the absence of phosphodiesterase inhibitors, and thus permitted interpretation of changes in cAMP levels during active lipolysis. It appears that in addition to lowering the initial rise in cAMP concentration, clofibrate altered the pattern of cAMP accumulation over the incubation period of 30 min (Fig. 5). Instead of a phasic profile, ordinarily observed with additions of hormone (34, 43–45), clofibrate changed the pattern to a depressed but progressively rising slope. This resulted in similar values for cAMP at 30 min for both NE and NE plus clofibrate-treated cells, despite continued inhibition of lipolysis by clofibrate (Fig. 5). These findings are consistent with the view that the initial rate of lipolysis is a function of the initial rate of cAMP accumulation (as measured within 10 min after exposure to hormone) rather than the levels observed later on. It is significant that the concentration of cAMP measured after a 10–15-min incubation is variable and unpredictable and often bears no relationship to lipolysis (44). This is borne out by the similarity in values for intracellular cAMP at 30 min (Fig. 5), despite substantial inhibition of lipolysis in the clofibrate-treated cells.

Since clofibrate inhibited both ACTH and NE-stimulated lipolysis, a receptor-specific interaction of clofibrate seems unlikely. Further, the reduction in the initial rate of cAMP accumulation by clofibrate did not seem to be due to an activation of the low-K_m
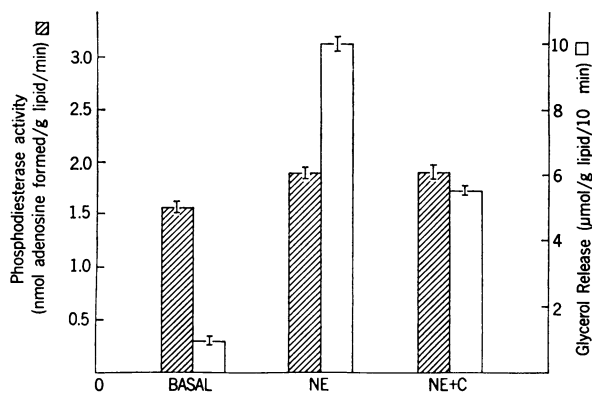

FIGURE 6 Effect of clofibrate (C) on 3',5'-cyclic AMP phosphodiesterase activity (low K_m). Rat adipocytes (120 mg lipid/flask) were preincubated for 10 min and then incubated for 10 min after additions of NE (0.1 μg/ml) and sodium clofibrate (80 mg/100 ml). Shaded bars represent enzyme activity in cells and open bars the net glycerol release into the medium. The data from two experiments were combined and represent the mean±SEM of eight incubations. Phosphodiesterase activity in NE-treated cells was significantly different (P < 0.01) from the basal but not from the NE + C value. All values for glycerol release are significantly different from each other (P < 0.001).

cAMP phosphodiesterase. This implies that the suppression of cAMP accumulation by clofibrate is brought about not by a stimulation of its degradation but by a reduction in its synthesis, i.e., inhibition of adenylate cyclase. Inhibition of this enzyme in adipose tissue of clofibrate-fed rats has been reported (55). That the effect on cAMP levels is specific and not secondary to depressed oxidative metabolism was borne out by the persistence of its antilipolytic action in the presence of glucose, which protects the cell from the uncoupling effects of FFA (38–40). Moreover, since the total ATP levels (and presumably the specific intracellular ATP pools) were unaffected by clofibrate treatment (Fig. 3), despite reported untoward effects on mitochondrial size and enzyme activities (56, 57), a defect in energy production may be ruled out.

With the aid of procaine, it was possible to demonstrate clearly that the effect of clofibrate on the adenyl cyclase system is not secondary to products of lipolysis,

FIGURE 8 Hypolipidemic effect of clofibrate: multiple sites of action. Adipohepatic cycle in the physiological regulation of plasma VLDL concentration: effects of clofibrate thereon. Dietary glucose can, under exceptional circumstances (10), serve as a precursor in hepatic VLDL-triglyceride fatty acid synthesis. In addition the gut can synthesize and secrete small amounts of VLDL (64) and therefore can also contribute to the circulating pool. However, the extent of these sources has not been quantified and they are therefore designated as minor pathways in VLDL production. LDL, low-density lipoprotein; TG, triglyceride.
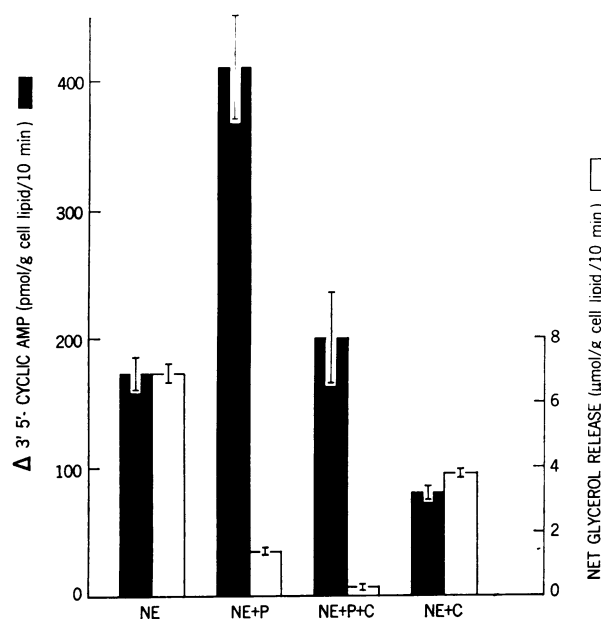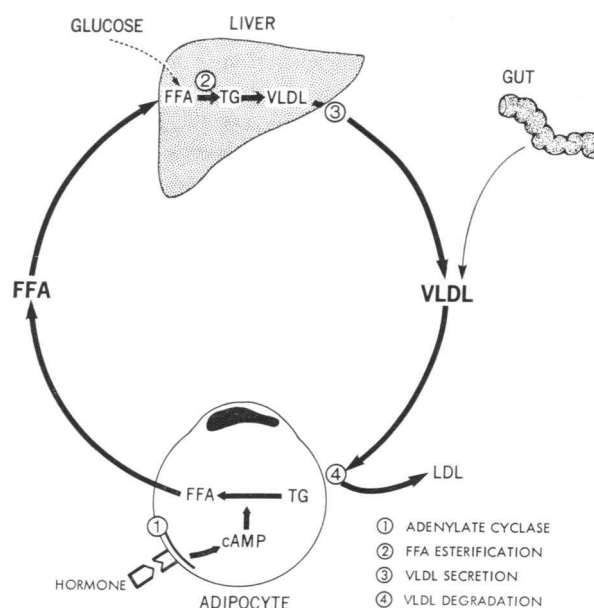
FIGURE 7 Effect of procaine-HCl (P) and clofibrate (C) on adipocyte cyclic AMP levels and lipolysis. Adipocytes (100–120 mg lipid/flask) were incubated for 10 min in standard medium containing glucose (6 mM) with additions of NE (0.1 μg/ml), procaine-HCl (10^{-3} M) and clofibrate (80 mg/100 ml). Three experiments consisting of five incubations per experiment ($n = 15$) were combined to give values (mean±SEM) for cyclic AMP (black bars) and net glycerol release (open bars). The basal (unstimulated) cyclic AMP value was 161±18 pmol/g lipid/10 min and with procaine-HCl 147±21. The corresponding values for glycerol were 1.02±0.09 and 1.07±0.04 μmol/g lipid/10 min. Values for both cyclic AMP rise and glycerol release that differed significantly ($P < 0.01$) were: NE vs. NE + P, NE + P vs. NE + P + C, and NE + C vs. NE.

because the reduction of cAMP levels occurred when FFA production was very low. The enhancement by procaine of the rise in cAMP in hormone-stimulated cells was consistently observed but cannot be explained on the basis of the present data. Procaine is known to modify intracellular calcium distribution (58). Whether the uncoupling of lipolysis from cAMP production in hormone-stimulated cells is mediated via inhibition of calcium flux has yet to be established. Nevertheless, the capacity of procaine to uncouple the proximal from the distal parts of the lipolytic sequence proved useful in locating the site of action of clofibrate and may have wider application in the study of antilipolytic agents in general.

The present work has physiological and clinical significance in that it may explain, at least in part, the mechanism of actions of clofibrate on VLDL production and turnover in certain dyslipoproteinemic states (Type IV hyperlipoproteinemia). It is significant that therapeutic amounts of clofibrate (10 mg/100 ml) inhibited NE-stimulated lipolysis by 20–30% in human fat (Fig. 2), an effect similar in magnitude to a 31% reduction in plasma FFA levels in clofibrate-treated

hypertriglyceridemic patients (8) and to a 20% reduction in plasma FFA concentration in hypertriglyceridemic patients receiving a clofibrate analogue (SU-13437) (59). Since plasma FFA are the principal lipid precursors in hepatic VLDL-triglyceride fatty acid production (10, 60) and because plasma FFA concentration determines hepatic FFA uptake and VLDL production (27, 28, 61), and generally reflects the lipolytic rate in adipose tissue, it seems reasonable that the glyceride-lowering effect of clofibrate may be related to the drug's antilipolytic action. The work of Schlierf and Dorow (62) supports this idea, as they observed that nicotinic acid prevents the overnight rise in serum triglyceride levels in hypertriglyceridemic man by inhibiting the nocturnal rise in serum FFA levels. These findings demonstrate the relevance of antilipolysis as a modality for the control of plasma triglyceride levels in hypertriglyceridemic patients and is in accord with the view that the lipid lowering effect of clofibrate is associated with a reduction in VLDL production and turnover (10, 11, 13, 59).

The above discussion does not diminish in any way the significance of increased peripheral degradation of VLDL as a mechanism responsible for the lipid-lowering action of clofibrate (2). Indeed, it can be reasoned that the antilipolytic effects of clofibrate could be responsible for increased lipoprotein lipase activity in adipose tissue (21). Patten (63) has shown a reciprocal relationship between hormone-sensitive lipase activity and lipoprotein lipase activity in rat adipose tissue. If this reciprocity obtains in human fat and lipoprotein degradation in adipose tissue is the rate-limiting step in peripheral VLDL metabolism, it is attractive to suggest that clofibrate achieves its lipid-lowering effects through these reciprocally related processes.

On the basis of the foregoing discussion, the effects of clofibrate on the physiological control of plasma VLDL levels are explained schematically (Fig. 8). The predominant and probably exclusive source of fatty acid substrate for hepatic VLDL production in both normotriglyceridemic and hypertriglyceridemic man is believed to be plasma FFA derived from adipose tissue (60). It is suggested that clofibrate reduces serum VLDL levels both by reducing its rate of production and by augmenting peripheral catabolism. Clofibrate, by inhibiting membrane-bound adenylate cyclase in the fat cell (site 1), deprives the liver of FFA substrate, thus reducing hepatic VLDL production. While clofibrate can inhibit fatty acid esterification in liver homogenates (13, 65) (site 2) and also retard triglyceride secretion (site 3) in perfused livers (12, 17) the relevance of these effects in vivo has yet to be established (8). Enhanced peripheral degradation of VLDL by clofibrate has been attributed to activation

of the rate-limiting enzyme lipoprotein lipase in adipose tissue (21) (site 4). This type of process could explain the paradoxical increase in plasma LDL levels in clofibrate-treated hyperlipidemic (Type IV) patients (9) and the enhanced disappearance of Intralipid infused into patients receiving a clofibrate analogue (66) and in rats on a diet containing clofibrate.[a] If enhanced degradation of VLDL in adipose tissue is functionally linked to inhibition of hormone-sensitive lipase in human fat, as it appears to be in rat adipose tissue (63), the importance of adipose tissue as a locus of clofibrate action will be difficult to deny.

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REFERENCES

1. Fredrickson, D. S. 1972. A physician's guide to hyperlipidemia. In Modern Concepts of Cardiovascular Disease. R. C. Schlant, editor. American Heart Association Inc., New York. 41: 31–35.
2. Havel, R. J., and J. P. Kane. 1973. Drugs and lipid metabolism. Annu. Rev. Pharmacol. 13: 287–308.
3. Thorp, J. M., and W. S. Waring. 1962. Modification of metabolism and distribution of lipids by ethyl chlorophenoxyisobutyrate. Nature (Lond.). 194: 948–949.
4. Oliver, M. F. 1962. Reduction of serum-lipid and uric-acid levels by an orally active androsterone. Lancet. 1: 1321–1323.
5. Oliver, M. F. 1963. Further observations on the effects of Atromid and of ethyl chlorophenoxyisobutyrate on serum lipid levels. J. Atheroscler. Res. 3: 427–444.
6. Hellman, L., B. Zumoff, G. Kessler, E. Kara, I. L. Rubin, and R. S. Rosenfeld. 1963. Reduction of serum cholesterol and lipids by ethyl chlorophenoxyisobutyrate. J. Atheroscler. Res. 3: 454–466.
7. Best, M. M., and C. H. Duncan. 1964. Hypolipemia and hepatomegaly from ethyl chlorophenoxyisobutyrate (CPIB) in the rat. J. Lab. Clin. Med. 64: 634–642.
8. Wolfe, B. M., J. P. Kane, R. J. Havel, and H. P. Brewster. 1973. Mechanism of the hypolipemic effect of clofibrate in postabsorptive man. J. Clin. Invest. 52: 2146–2159.
9. Wilson, D. E., and R. S. Lees. 1972. Metabolic relationships among the plasma lipoproteins. Reciprocal changes in the concentrations of very low and low density lipoproteins in man. J. Clin. Invest. 51: 1051–1057.
10. Barter, P. J., P. J. Nestel, and K. F. Carroll. 1972. Precursors of plasma triglyceride fatty acid in humans. Effects of glucose and consumption, clofibrate administration, and alcoholic fatty liver. Metab. (Clin. Exp.). 21: 117–124.

11. Bierman, E. L., J. D. Brunzell, J. D. Bagadade, R. L. Lerner, W. R. Hazzard, and D. Porte, Jr. 1970. On the mechanism of action of Atromid-S on triglyceride transport in man. *Trans. Assoc. Am. Physicians Phila.* **83**: 211–224.

12. Duncan, C. H., M. M. Best, and A. Despopoulos. 1964. Inhibition of hepatic secretion of triglyceride by chlorophenoxyisobutyrate (CPIB). *Circ. Suppl.* **30**: 7.

13. Adams, L. L., W. W. Webb, and H. J. Fallon. 1971. Inhibition of hepatic triglyceride formation by clofibrate. *J. Clin. Invest.* **50**: 2339–2346.

14. Sodhi, H. S., B. J. Kudchodkar, and L. Horlick. 1971. Effect of chlorophenoxyisobutyrate on the metabolism of endogenous glycerides in man. *Metab. (Clin. Exp.).* **20**: 309–318.

15. Avoy, D. R., E. A. Swyryd, and R. G. Gould. 1965. Effects of α-p-chlorophenoxyisobutyryl ethyl ester (CPIB) with and without androsterone on cholesterol biosynthesis in rat liver. *J. Lipid Res.* **6**: 369–376.

16. Azarnoff, D. L., D. R. Tucker, and G. A. Barr. 1965. Studies with ethyl chlorophenoxyisobutyrate (clofibrate). *Metab. (Clin. Exp.).* **14**: 959–965.

17. Gould, R. G., E. A. Swyryd, D. Avoy, and B. Coan. 1967. The effects of α-p-chlorophenoxyisobutyrate on the synthesis and release into plasma of lipoproteins in rats. *Prog. Biochem. Pharmacol.* **2**: 345–357.

18. Horlick, L., B. J. Kudchodkar, and H. S. Sodhi. 1971. Mode of action of chlorophenoxyisobutyric acid on cholesterol metabolism in man. *Circulation.* **43**: 299–309.

19. Mishkel, M. A., and W. F. Webb. 1967. The mechanisms underlying the hypolipidaemic effects of Atromid S, nicotinic acid and benzmalecene. *Biochem. Pharmacol.* **16**: 897–905.

20. Nestel, P. J., and W. Austin. 1968. The effect of ethyl chlorophenoxyisobutyrate (CPIB) on the uptake of triglyceride fatty acids, activity of lipoprotein lipase and lipogenesis from glucose in fat tissue of rats. *J. Atheroscler. Res.* **8**: 827–833.

21. Tolman, E. L., H. M. Tepperman, and J. Tepperman. 1970. Effect of ethyl p-chlorophenoxyisobutyrate on rat adipose lipoprotein lipase activity. *Am. J. Physiol.* **218**: 1313–1318.

22. Smigura, F. C., J. Farkas, and A. Angel. 1973. The effect of clofibrate on sterol synthesis and storage in various rat tissues. *Clin. Res.* **20**: 946. (Abstr.)

23. Barrett, A. M. 1966. The effect of chlorophenoxyisobutyric acid on the release of free fatty acids from isolated adipose tissue in vitro. *Br. J. Pharmacol.* **26**: 363–371.

24. Cenedella, R. J., J. J. Jarrell, and L. H. Saxe. 1968. Effects of ethyl-p-chlorophenoxyisobutyrate, clofibrate on the plasma and red blood cell free fatty acids of the rat. *J. Atheroscler. Res.* **8**: 903–911.

25. Barrett, A. M., and J. M. Thorp. 1968. Studies on the mode of action of clofibrate. Effects on hormone-induced changes in plasma free fatty acids, cholesterol, phospholipids and total esterified fatty acids in rats and dogs. *Br. J. Pharmacol. Chemother.* **32**: 381–391.

26. Rifkind, B. M. 1966. Effect of CPIB ester on plasma free fatty acid levels in man. *Metab. (Clin. Exp.).* **15**: 673–675.

27. Wilcox, H. G., W. F. Woodside, K. J. Breen, H. R. Knapp, Jr., and M. Heimberg. 1974. The effect of sex on certain properties of the very low density lipoprotein secreted by the liver. *Biochem. Biophys. Res. Commun.* **58**: 919–926.

28. Van Harken, D. R., C. W. Dixon, and M. Heimberg. 1969. Hepatic lipid metabolism in experimental diabetes. V. The effect of concentration of oleate on metabolism of triglycerides and on ketogenesis. *J. Biol. Chem.* **244**: 2278–2285.

29. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239**: 375–380.

30. Angel, A. 1970. Studies on the compartmentation of lipid in adipose cells. I. Subcellular distribution, composition, and transport of newly synthesized lipid: liposomes. *J. Lipid Res.* **11**: 420–432.

31. Garland, P. B., and P. J. Randle. 1962. A rapid enzymatic assay for glycerol. *Nature (Lond.).* **196**: 987–988.

32. Shapiro, B., I. Chowers, and G. Rose. 1957. Fatty acid uptake and esterification in adipose tissue. *Biochim. Biophys. Acta.* **23**: 115–120.

33. Gilman, A. G. 1970. A protein binding assay for adenosine 3'5'-cyclic monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305–312.

34. Desai, K., K. C. Li, and A. Angel. 1973. Bimodal effect of insulin on hormone-stimulated lipolysis: relation to intracellular 3'5'-cyclic adenylic acid and free fatty acid levels. *J. Lipid Res.* **14**: 647–655.

35. Denton, R. M., R. E. Yorke, and P. J. Randle. 1966. Measurement of concentrations of metabolites in adipose tissue and effects of insulin, alloxan diabetes and adrenaline. *Biochem. J.* **100**: 407–419.

36. Loten, E. G., and J. G. T. Sneyd. 1970. An effect of insulin on adipose-tissue adenosine 3': 5'-cyclic monophosphate phosphodiesterase. *Biochem. J.* **120**: 187–193.

37. Zinman, B., and C. H. Hollenberg. 1974. Effect of insulin and lipolytic agents on rat adipocyte low K_m cyclic adenosine 3'-5'-monophosphate phosphodiesterase. *J. Biol. Chem.* **249**: 2182–2187.

38. Hall, C. L., and E. G. Ball. 1970. Factors affecting lipolysis rates in rat adipose tissue. *Biochim. Biophys. Acta.* **210**: 209–220.

39. Angel, A., K. S. Desai, and M. L. Halperin. 1971. Reduction in adipocyte ATP by lipolytic agents: relation to intracellular free fatty acid accumulation. *J. Lipid Res.* **12**: 203–213.

40. Bihler, I., and B. Jeanrenaud. 1970. ATP content of isolated fat cells. Effects of insulin, ouabain and lipolytic agents. *Biochim. Biophys. Acta.* **202**: 496–506.

41. Butcher, R. W., C. E. Baird, and E. W. Sutherland. 1968. Effects of lipolytic and antilipolytic substances on adenosine 3',5'-monophosphate levels in isolated fat cells. *J. Biol. Chem.* **243**: 1705–1712.

42. Heersche, J. N. M., S. A. Fedak, and G. D. Aurbach. 1971. The mode of action of dibutyryl adenosine 3',5'-monophosphate on bone tissue in vitro. *J. Biol. Chem.* **246**: 6770–6775.

43. Manganiello, V. C., F. Murad, and M. Vaughan. 1971. Effects of lipolytic and antilipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells. *J. Biol. Chem.* **246**: 2195–2202.

44. Jarett, L., A. L. Steiner, R. M. Smith, and D. M. Kipnis. 1972. The involvement of cyclic AMP in the hormonal regulation of protein synthesis in rat adipocytes. *Endocrinology.* **90**: 1277–1284.

45. Schwabe, U., and R. Ebert. 1972. Different effects of lipolytic hormones and phosphodiesterase inhibitors on cyclic 3',5'-AMP levels in isolated fat cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **247**: 287–298.

46. Kuo, J. F., and E. C. de Renzo. 1969. A comparison of the effects of lipolytic and antilipolytic agents on adenosine 3',5'-monophosphate levels in adipose cells as determined by prior labeling with adenine-8-¹⁴C. *J. Biol. Chem.* **244**: 2252–2260.

47. D'Costa, M., and A. Angel. 1973. Inhibition of hormone stimulated lipolysis by clofibrate: mechanism for its hypolipidemic effect. *Clin. Res.* **21**: 884. (Abstr.)

48. Hales, C. N., and M. C. Perry. 1970. The role of ions in the hormonal control of adipose tissue. *In* Adipose Tissue. Regulation and Metabolic Functions. B. Jeanrenaud, and D. Hepp, editors. Academic Press, Inc., New York. 63–65.

49. D'Costa, M. A., and A. Angel. 1973. Antilipolytic effect of procaine: uncoupling of lipolysis from cyclic AMP accumulation in adipocytes. *Clin. Res.* **21**: 1057. (Abst.)

50. Birnbaumer, L., and M. Rodbell. 1969. Adenyl cyclase in fat cells. II. Hormone receptors. *J. Biol. Chem.* **244**: 3477–3482.

51. Corbin, J. D., and E. G. Krebs. 1969. A cyclic AMP-stimulated protein kinase in adipose tissue. *Biochem. Biophys. Res. Commun.* **36**: 328–336.

52. Corbin, J. D., E. M. Reimann, D. A. Walsh, and E. G. Krebs. 1970. Activation of adipose tissue lipase by skeletal muscle cyclic adenosine 3',5'-monophosphate-stimulated protein kinase. *J. Biol. Chem.* **245**: 4849–4851.

53. Huttunen, J. K., and D. Steinberg. 1971. Activation and phosphorylation of purified adipose tissue hormone-sensitive lipase by cyclic AMP-dependent protein kinase. *Biochim. Biophys. Acta.* **239**: 411–427.

54. Carlson, L. A., G. Walldius, and R. W. Butcher. 1972. Effect of chlorophenoxyisobutyric acid (CPIB) on fat-mobilizing lipolysis and cyclic AMP levels in rat epididymal fat. *Atherosclerosis.* **16**: 349–357.

55. Greene, H. L., R. H. Herman, and D. Zakim. 1970. The effect of clofibrate on rat tissue adenyl cyclase. *Proc. Soc. Exp. Biol. Med.* **134**: 1035.

56. Svoboda, D. J., and D. L. Azarnoff. 1971. Effects of selected hypolipidemic drugs on cell ultrastructure. *Fed. Proc.* **30**: 841–847.

57. Westerfield, W. W., J. C. Elwood, and D. A. Richert. 1972. Effect of clofibrate on the handling of dietary and liver fat. *Biochem. Pharmacol.* **21**: 1117–1125.

58. Seeman, P. 1972. The membrane actions of anaesthetics and tranquilizers. *Pharmacol. Rev.* **24**: 583–655.

59. Nikkila, E. A., and M. Kekki. 1972. Plasma endogenous triglyceride transport in hypertriglyceridaemia and effect of a hypolipidaemic drug (SU-13437). *Eur. J. Clin. Invest.* **2**: 231–238.

60. Havel, R. J., J. P. Kane, E. O. Balasse, N. Segel, and L. V. Basso. 1970. Splanchnic metabolism of free fatty acids and production of triglycerides of very low density lipoproteins in normotriglyceridemic and hypertriglyceridemic humans. *J. Clin. Invest.* **49**: 2017–2035.

61. Heimberg, M., I. Weinstein, and M. Kohout. 1969. The effects of glucagon, dibutyryl cyclic adenosine 3',5'-monophosphate, and concentration of free fatty acid on hepatic lipid metabolism. *J. Biol. Chem.* **244**: 5131–5139.

62. Schlierf, G., and E. Dorow. 1973. Diurnal patterns of triglycerides, free fatty acids, blood sugar, and insulin during carbohydrate-induction in man and their modification by nocturnal suppression of lipolysis. *J. Clin. Invest.* **52**: 732–740.

63. Patten, R. L. 1970. The reciprocal regulation of lipoprotein lipase activity and hormone-sensitive lipase activity in rat adipocytes. *J. Biol. Chem.* **245**: 5577–5584.

64. Windmueller, H. G., F. T. Lindgren, W. J. Lossow, and R. I. Levy. 1970. On the nature of circulating lipoproteins of intestinal origin in the rat. *Biochim. Biophys. Acta.* **202**: 507–516.

65. Lamb, R. G., and H. J. Fallon. 1972. Inhibition of mono-glycerolphosphate formation by chlorophenoxyisobutyrate and β-benzalbutyrate. *J. Biol. Chem.* **247**: 1281–1287.

66. Boberg, J., L. A. Carlson, S. O. Fröberg, and L. Orö. 1970. Effect of a hypolipidaemic drug (Ch 13,437) on plasma and tissue lipids, and on the intravenous fat tolerance in man. *Atherosclerosis.* **11**: 353–360.