Inhibition of adenosine 3′:5′-monophosphate accumulation in white fat cells by short chain fatty acids, lactate, and β-hydroxybutyrate

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Abstract The large increase in cyclic AMP accumulation by rat white fat cells seen in the presence of lipolytic agents plus methylxanthines and adenosine deaminase was markedly inhibited by lactate. However, lipolysis was unaffected by lactate. Octanoate, hexanoate, heptanoate, and β-hydroxybutyrate inhibited both cyclic AMP accumulation and lipolysis by rat fat cells. The mechanism by which these acids inhibit lipolysis differs from that for long chain fatty acids such as oleate. Oleate directly inhibited triglyceride lipase activity of homogenized rat adipose tissue. In contrast, octanoate, β-hydroxybutyrate, and lactate had no effect on triglyceride lipase activity. Hormone-stimulated adenylate cyclase activity of rat fat cell ghosts was inhibited by oleate and 4 mM octanoate but not by 1.6 mM octanoate, heptanoate, hexanoate, β-hydroxybutyrate or lactate. None of the acids affected the soluble protein kinase activity of rat adipose tissue. There was no stimulation by lactate, butyrate, β-hydroxybutyrate, or octanoate of the soluble or particulate cyclic AMP phosphodiesterase activity of rat fat cell homogenates. The antilipolytic action of a short chain acid such as octanoate or hexanoate was not accompanied by any drop in total fat cell ATP. The mechanism by which lactate lowers cyclic AMP but not lipolysis remains to be established.

Supplementary key words adenylate cyclase · phosphodiesterase · triglyceride lipase · protein kinase · cyclic AMP · lipolysis · fatty acids · ATP

If the accumulation of free fatty acids in the medium after the addition of lipolytic agents to rat fat cells elevates the FFA/albumin ratio above 3, lipolysis and the accumulation of cyclic AMP are inhibited (1–3). These effects are mimicked by raising the FFA/albumin ratio above 3 with oleic acid (1, 2). Adenylate cyclase activity of rat fat cell ghosts and triglyceride lipase of fat cell homogenates are also inhibited by FFA/albumin ratios above 3.

The present report is concerned with the effects of lactate, β-hydroxybutyrate, and short chain fatty acids on cyclic AMP accumulation and lipolysis. Lactate has been reported to inhibit lipolysis in adipose tissue of rats (4, 5) and dogs (6). Furthermore high levels of plasma lactate will reduce plasma FFA in pancreatectomized dogs (7). The inhibition of lipolysis in bovine (8, 9) and rat (10) adipose tissue by β-hydroxybutyrate and butyrate suggested that these acids might affect cyclic AMP accumulation and lipolysis in the same manner as long chain fatty acids. The results of this study indicate, however, that the short chain aliphatic acids act similarly to adenosine, nicotinic acid, and the prostaglandins in that they affect cyclic AMP accumulation in intact cells but do not affect the enzymatic scheme of lipolysis in broken cells.

METHODS

White fat cells were isolated by a modification of Rodbell's procedure (11) from the pooled parametrial adipose tissue of three or more 120–160 g female Sprague-Dawley rats (Charles River CD strain). The animals were given laboratory chow ad libitum and maintained at 23 ± 2°C. Krebs-Ringer phosphate buffer (NaCl, 128 mM; $CaCl_2$, 1.4 mM; $MgSO_4$, 1.4 mM; KCl, 5.2 mM; and Na_2HPO_4, 10 mM) was used in all experiments. It was prepared daily and adjusted to pH 7.4 with NaOH after addition of Armour bovine Fraction V albumin powder (#43405). In each experiment duplicate incubations at 37°C were performed in the absence of glucose with a final volume of 1 ml.

Chicken fat cells were obtained by collagenase digestion of the mesenteric fat of 6–8 week old non-laying white Leghorn pullets (4–4.5 lbs) purchased from the Morris Live Poultry Co., Providence, R.I. Approximately 4 g of tissue was obtained from a single

Abbreviation: FFA, free fatty acids.

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pullet and incubated for 90 min in 24 ml of phosphate buffer containing 4% albumin and 12 mg of collagenase.

Cyclic AMP accumulation was measured in cells plus medium after adding 0.1 ml of 2N HCl to the tubes (1 ml of buffer + cells). The tubes were then placed in a boiling water bath for 1 min and then allowed to cool to room temperature before adding 0.05 ml of 4N NaOH. The contents of the tubes were mixed and centrifuged prior to removal of 20-μl aliquots for determination of cyclic AMP. Cyclic AMP release to the medium was analyzed by taking 20-μl aliquots of the medium at the end of the incubation just prior to adding acid. Intracellular cyclic AMP was calculated by subtracting the cyclic AMP value for the medium from that for medium plus cells. The cyclic AMP standards were prepared in incubation medium containing albumin which was treated in the same manner as the unknown samples. The assay for cyclic AMP was done by a modification of the Gilman protein kinase binding procedure using rabbit muscle protein kinase (12). The free cyclic AMP was separated from the bound cyclic AMP by charcoal precipitation (13). The details of the cyclic AMP assay have been described previously along with data demonstrating that cyclic AMP values obtained by analysis of unpurified samples from fat cells are similar to those obtained after sample purification (14).

Fatty acid release was analyzed as described previously (15), with the exception that aliquots of the hexane extract of cells plus medium were not evaporated prior to titration to prevent loss of volatile acids at elevated temperature. Levels of ATP in the medium and cells were determined by fluorimetry on aliquots of neutralized perchloric acid extracts (16).

Membrane-bound adenylate cyclase activity was determined on ghosts prepared by hypotonic lysis of fat cells (17). The cells were lysed using calcium-free medium at room temperature. Protein content was assayed by the procedure of Lowry et al. (18). The ghosts (60 μg of protein per tube) were used immediately and incubated for 10 min at 37°C in a total volume of 100 μl containing 40 mM Tris buffer (pH 8.0), 5 mM $MgCl_2$, 30 mM KCl, 8 mM creatine phosphate, 0.5 U creatine phosphokinase, and 1 mM ATP. Cyclic AMP was determined on 20-μl aliquots taken after the reaction mixture was boiled for 3 min and then diluted to a volume of 1 ml. The cyclic AMP binding protein used in this assay was from the 10,000 g supernatant of homogenized bovine adrenal glands and the assay was conducted as described by Brown et al. (13) to eliminate possible interference by ATP.

Both soluble and particulate cyclic nucleotide phosphodiesterase were prepared from isolated white fat cells by a modification of the method used by Ward and Fain (19). Fat cells were isolated, washed three times with phosphate buffer containing albumin and three times with a 0.25 M sucrose–0.001 M $MgCl_2$ solution. The cells were then suspended in 3 ml of 40 mM Tris, 5 mM $MgCl_2$, 30 mM KCl, 0.25 M sucrose and homogenized in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle (1800 rpm). The homogenate was then centrifuged at 48,000 g for 30 min. The fat cake was discarded and the resulting infranatant was removed and used as the soluble preparation of the phosphodiesterase. The remaining precipitate was washed by resuspension in the Tris–sucrose solution and centrifuged at 48,000 g for 20 min. The precipitate was resuspended in 2 ml of the Tris–sucrose solution and used as the particulate preparation of phosphodiesterase.

The assay for cyclic AMP phosphodiesterase activity was a modification of that used by Thompson and Appleman (20). Aliquots of the preparations were added to an incubation medium (final volume 100 μl) having the following composition: 40 mM Tris, 5 mM $MgCl_2$, 30 mM KCl, 0.05 μCi ^{3}H-labeled cyclic AMP and enough unlabeled cyclic AMP to yield a final concentration of either 1 or 0.1 μM cyclic AMP. The phosphodiesterase preparations were incubated at 37°C for 10 min and the reaction was stopped by boiling for 3.0 min. A 0.1 ml portion of snake venom (*Ophiophagus hannah*) (1 mg/ml) was then added to the above medium and incubated at 37°C. The labeled adenosine thus formed was separated from labeled cyclic AMP by the addition of 1 ml of 1:3 (w/v) slurry of washed Dowex-1 chloride (8% cross linked, 200–400 mesh). The Dowex was prepared as described by Boudreau and Drummond (21). The contents were centrifuged at 3,500 g for 10 min and 500-μl aliquots of the supernatant fluid were added to vials containing 4 ml of Triton X-100 (1 part) and 0.4% Omnifluor in toluene (2 parts) and then counted in a liquid scintillation spectrometer.

Protein kinase activity (22) was determined using [γ-^{32}P]ATP prepared from $H_3{}^{32}PO_4$ in our laboratory (23). White fat cells were homogenized in 0.15 M KCl with three strokes of a glass homogenizer fitted with a Teflon pestle (1800 rpm). After centrifugation at 48,000 g for 30 min at 4°C, 10-μl aliquots from the soluble fraction, containing about 20 μg of protein, were assayed for protein kinase activity in the absence and presence of 0.04×10^{-6} M cyclic AMP. The assay mixture contained 1.25 μmole of potassium phosphate (pH 6.5), 0.42

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TABLE 1. Inhibition of cyclic AMP accumulation by short chain aliphatic acids

Additions	Final FFA	Glycerol Release	Intracellular Cyclic AMP
	μmoles/ml	*μmoles/g*	*nmoles/g*
None	1.4	16.0	12.3
Oleate	3.8	4.4	0.8
Octanoate	3.4	8.0	0.6
Heptanoate	3.3	12.8	0.2
Hexanoate	3.0	10.7	0.1
Lactate	1.7	18.3	0.1

Fat cells (23 mg/tube) were incubated for 20 min in the presence of 1.5 μM norepinephrine, 200 μM theophylline and 0.5 μg/ml of adenosine deaminase either in the absence or presence of added acids at a concentration of 3 mM in buffer containing 4.8% albumin (0.7 μmole/ml). The values are the means of two experiments. Basal values for glycerol release and intracellular cyclic AMP in the absence of any added agents were less than 0.1 μmole/g and 0.1 nmole/g respectively.

μmole of magnesium acetate, 0.14 μmole of [γ-^{32}P]ATP and 0.36 mg of histone in a final volume of 0.125 ml. After 20 min incubation, 50 μl of the reaction mixture was spotted on 2.3 cm Whatman No. 3 filter paper discs and dropped into cold 10% trichloroacetic acid. The discs were washed as described by Reimann, Walsh, and Krebs (24), rinsed in acetone, then dried under heat lamps. The discs were transferred to liquid scintillation vials for counting in toluene containing 0.4% Omnifluor. Incorporation of ^{32}P into histone was calculated from a zero time control value obtained by rapidly pipetting the protein kinase extract solution, withdrawing 50 μl and spotting onto the disc, then dropping the disc into the cold 10% trichloroacetic acid.

Hormone-sensitive lipase was isolated according to the procedure of Khoo and Steinberg (25). About 15 g of parametrial adipose tissue from nine female rats was minced and homogenized for 60 sec at 3°C in a Waring blender with 2 volumes of 0.25 M sucrose, 1 mM EDTA and 10 mM Tris-HCl, pH 7.4. The extract was filtered through glass wool and then centrifuged at 40,000 *g* for 30 min before the pH 5.2 fraction was prepared as described by Khoo and Steinberg (25). This was either used immediately or frozen until assayed (240 μg protein per tube) in the presence of [^{14}C]triolein.

All of the short and long chain fatty acids were dissolved in water with the addition of enough sodium hydroxide to adjust the pH of the solution to 7.4. The stock solution of sodium oleate (stored under nitrogen) was an opalescent suspension at 37°C, which was kept in a uniform dispersion by mixing the solution with a magnetic stirring bar when aliquots were removed. L(+)Lactic acid, DL-β-hydroxybutyrate, *n*-butyric acid, octanoic acid (caprylic acid, Grade 1), hexanoic acid (*n*-caproic acid, Sigma grade), heptanoic acid (Grade 1), oleic acid (Sigma grade), (−)norepinephrine hydrochloride, glucagon histone (Type II-A from calf thymus), dibutyryl cyclic AMP (N^6,$O^{2'}$-dibutyryl adenosine 3′,5′-cyclic monophosphoric acid), Tris, (hydroxymethyl)aminomethane (Trizma reagent grade), theophylline (1,3-dimethylxanthine), dithiothreitol (Cleland's reagent), triolein (glycerol trioleate, Sigma grade) and *Ophiophagus hannah* snake venom were obtained from the Sigma Chemical Company, St. Louis, Mo. Glycerol trioleate (carboxyl-^{14}C, 5–10 mCi/mmole) was purchased from ICN radioisotope Division, Irvine Cal. $H_3{}^{32}PO_4$ and [^{3}H]adenosine 3′:5′-cyclic monophosphoric acid were purchased from New England Nuclear Corp., Boston, Mass.

RESULTS AND DISCUSSION

The addition of enough oleate to elevate the ratio of FFA/albumin in the incubation medium to more than 3 results in an inhibition of lipolysis and cyclic AMP accumulation by rat fat cells (1, 2). The addition of 3 mM oleate (which gave a final FFA/albumin ratio of 5.5) markedly reduced lipolysis in the presence of norepinephrine, theophylline and adenosine deaminase (**Table 1**). Oleate also reduced the intracellular cyclic AMP measured after 20 min of incubation (Table 1). Octanoate, heptanoate, hexanoate, and lactate had similar inhibitory effects on cyclic AMP accumulation after 20 min. However, lactate at 3 mM did not inhibit lipolysis, hexanoate (3 mM) and heptanoate (3 mM) slightly reduced lipolysis, and octanoate (3 mM) reduced rat fat cell lipolysis by 50% over the 20 min incubation (Table 1).

In all studies intracellular cyclic AMP was measured since after prolonged incubation, when cyclic AMP is maximally stimulated by the combination of catecholamines or glucagon plus theophylline and adenosine deaminase, there is appreciable release of cyclic AMP to the medium (26). Adenosine deaminase elevates cyclic AMP accumulation due to lipolytic agents and presumably acts by removing adenosine, which is a potent inhibitor of cyclic AMP accumulation (26).

In other studies lipolysis and cyclic AMP were measured at 10 and 60 min after the addition of glucagon, theophylline, and adenosine deaminase (left part of **Fig. 1**). Again lactate inhibited cyclic AMP accumulation but not lipolysis by rat fat cells. Octanoate, hexanoate and β-hydroxybutyrate reduced cyclic AMP accumulation to a greater extent

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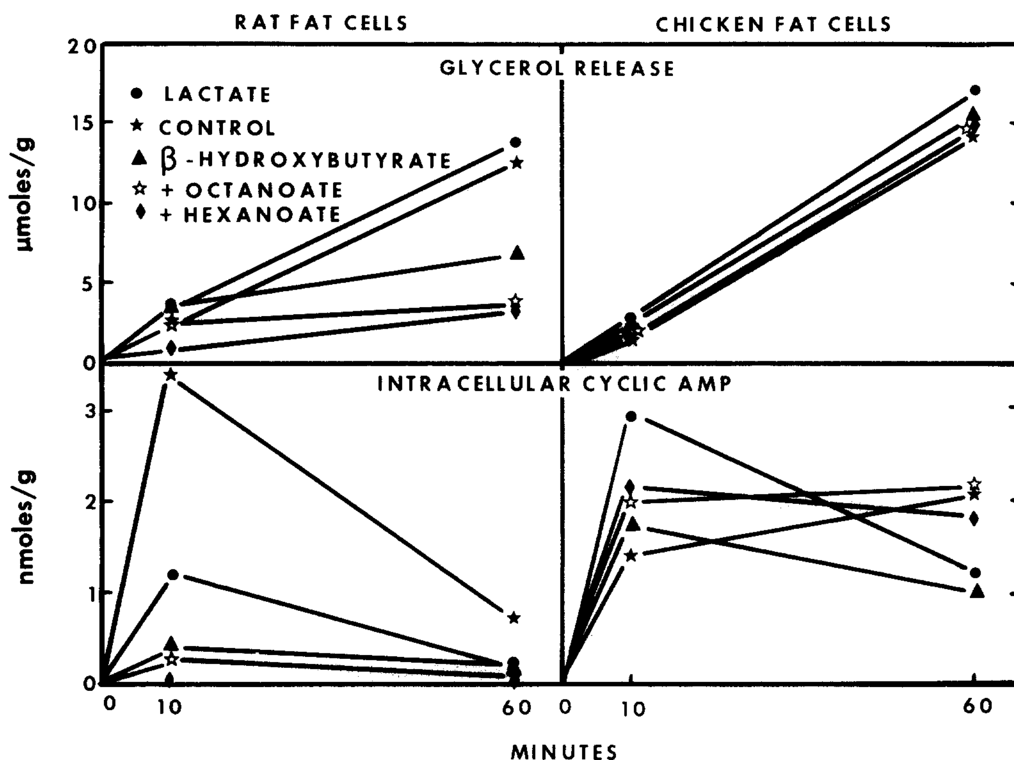

Fig. 1. Comparison of chicken and rat fat cells with respect to effects of short chain aliphatic acid. Rat fat cells (50 mg/tube) or chicken fat cells (35 mg/tube) were incubated for 10 or 60 min in 1 ml of buffer containing 4.8% albumin (0.70 μmoles/ml). Glucagon (5 μg/ml), theophylline (100 μM) and adenosine deaminase (0.5 μg/ml) were present in all tubes. The values are the means of three experiments.

than did lactate and they also inhibited lipolysis at 60 min (Fig. 1). There was a small antilipolytic effect of hexanoate after 10 min incubation.

Our results are in contrast to those in isolated canine adipose tissue (6), rat adipose tissue (4, 5), and in pancreatectomized dogs (7), where lactate inhibited FFA release from adipose tissue. Possibly the inhibitory effects of lactate reported by others resulted from using conditions where cyclic AMP accumulation was rate-limiting for lipolysis. In our studies, total intracellular cyclic AMP accumulation apparently was not rate-limiting for lipolysis since there was a large accumulation of cyclic AMP due to the addition of a lipolytic agent plus theophylline and adenosine deaminase. Furthermore, the dissociation between lipolysis and cyclic AMP accumulation is not surprising since low concentrations of norepinephrine that are able to stimulate lipolysis do not elevate cyclic AMP accumulation by rat fat cells (14, 26, 27).

Because cyclic AMP levels at 20 min may not reflect the values seen at early time periods in the presence of lactate, cyclic AMP accumulation was also measured after 2 min of stimulation by norepinephrine (**Fig. 2**). These studies were done without or with 100 μM theophylline. The data are plotted on a log scale vs. the norepinephrine concentration (log scale) to expand the lower part of the curve. At all concentrations of norepinephrine, lactate reduced the level of cyclic AMP without affecting lipolysis. Lower concentrations of norepinephrine had little effect on lipolysis or cyclic AMP in the absence of theophylline. Theophylline magnified these effects. Since even the smallest rise in cyclic AMP seen two minutes after catecholamine addition was reduced by lactate without any detectable inhibition of lipolysis, it appears that detectable increases in cyclic AMP are not required for activation of lipolysis. Another interpretation is that there are other factors besides cyclic AMP involved in the lipolytic action of catecholamines.

We have shown that in chicken fat cells neither lipolysis nor cyclic AMP accumulation was affected by concentrations of long chain free fatty acids that inhibited these processes in rat fat cells (1, 2). Similarly, short chain aliphatic acids did not inhibit cyclic AMP accumulation by chicken fat cells, and cyclic AMP accumulation by chicken fat cells at 10 min was actually enhanced by lactate (Fig. 1). These results indicate that the insensitivity of chicken fat

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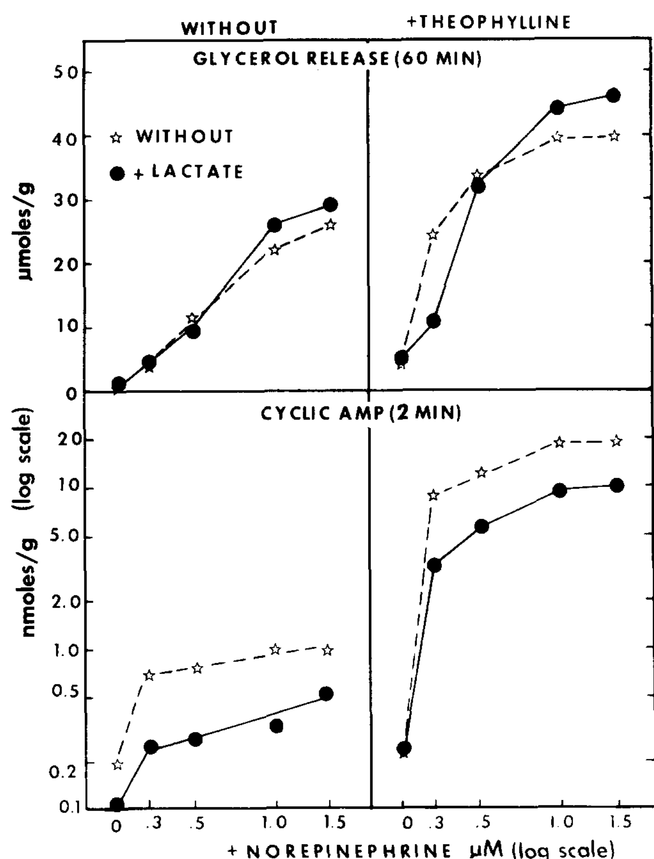

Fig. 2. Glycerol release and cyclic AMP accumulation in the presence of lactate. Rat fat cells (27 mg/tube) were incubated 2 min or 60 min in 1 ml of 3% albumin buffer. Norepinephrine was added in the absence or presence of 3 mM lactate. The values are the means of three experiments for cells incubated in the absence or presence of 100 μM theophylline. The cyclic AMP values are plotted vs. norepinephrine concentration on a log scale.

cells to inhibition of cyclic AMP accumulation by long chain fatty acids also extends to short chain aliphatic acids.

The delayed onset of the antilipolytic action of 3 mM β-hydroxybutyrate noted in Fig. 1 was also observed in the studies shown in **Table 2**. There was no inhibition by 3 mM lactate of rat fat cell lipolysis even if cells were incubated with lactate for 40 min prior to the addition of lipolytic agents. β-Hydroxybutyrate was effective in inhibiting lipolysis due to lipolytic agents only if the cells had been preincubated with it for 40 min. The antilipolytic action of β-hydroxybutyrate was not seen with an equimolar concentration of sodium butyrate. However, cyclic AMP accumulation was markedly reduced by lactate, butyrate or β-hydroxybutyrate either without or with a 40 min preincubation (Table 2).

One difference between the inhibition of cyclic AMP accumulation by short chain aliphatic acids and long chain fatty acids was the failure of short chain acids at a concentration of 1.6 mM to inhibit glucagon-activated adenylate cyclase activity of rat fat cell ghosts (**Fig. 3**). Adenylate cyclase activity of the ghosts was measured in the absence of albumin and, under these conditions, as little as 0.2 mM oleate gave over 70% inhibition.

Catecholamine-stimulated adenylate cyclase activity in the presence of short chain aliphatic acids was also investigated (**Fig. 4**). Basal activity was 0.125 nmoles/mg and was increased to 1.67 nmoles/mg in the presence of 150 μM norepinephrine. Lactate or β-hydroxybutyrate, even at a concentration of 4 mM, did not inhibit norepinephrine-activated adenylate cyclase. Octanoate at a concentration of 4 mM inhibited cyclase activity 62 ± 3%. Octanoate (4 mM) also inhibited glucagon-stimulated adenylate cyclase by 80% (unpublished experiments). These experiments were done without albumin. Octanoate also inhibited the stimulated cyclase activity by 66% in the presence of 450 μM albumin (data not shown). The inhibition of adenylate cyclase activity by 4 mM octanoate may explain the antilipolytic effect of 3 mM octanoate shown in Table 1. It is also possible that detergent effects may explain this inhibition. However, octanoate was much less effective as an inhibitor of adenylate cyclase than was oleate. Oleate at a concentration of 0.2 mM gave 70% inhibition of hormone-activated adenylate cyclase while

TABLE 2. Effect of prolonged incubation of fat cells with lactate, butyrate, or β-hydroxybutyrate

Added Acids	10 min + NE[a] and TPH[b]	40 min, then 10 min + NE[a] and TPH[b]	40 min, then 10 min + NE[a], TPH[b], and ADA[c]
Intracellular cyclic AMP, nmoles/g			
None	5.4	1.5	6.3
Lactate, 3 mM	0.8	0.6	1.0
β-Hydroxybutyrate, 3 mM	0.4	0.4	0.1
Butyrate, 3 mM	0.5	0.7	1.6
Glycerol release, μmoles/g			
None	9.6	8.3	8.3
Lactate	10.2	12.4	9.1
β-Hydroxybutyrate	8.7	5.5[d]	5.5
Butyrate	7.9	8.1	10.7

Rat fat cells (35 mg/tube) were incubated for 10 min with norepinephrine (1.5 μM) plus theophylline (100 μM) either without or with 0.5 μg/ml of adenosine deaminase. Some cells were incubated in the absence or presence of 3 mM lactate, butyrate, or β-hydroxybutyrate for 40 min prior to addition of the lipolytic agents. The values are the means of three paired experiments.

[a] NE, norepinephrine.
[b] TPH, theophylline.
[c] ADA, adenosine deaminase.
[d] The percentage inhibition seen in the three experiments ranged from 15% to 40%.

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1.6 mM octanoate was ineffective (Fig. 3). Only at an octanoate concentration of 4 mM (Fig. 4) was the same inhibition achieved as with 0.2 mM oleate.

Even at a concentration of 4 mM in the absence of albumin there was no inhibition of adenylate cyclase by either lactate or β-hydroxybutyrate. These results suggest that if lactate or β-hydroxybutyrate inhibits adenylate cyclase in the intact cells, it is by processes that are lost during the isolation and incubation of fat cell ghosts. There must be some differences in the mechanisms involved in inhibition of cyclase by lactate and by β-hydroxybutyrate as contrasted to octanoate and oleate, since inhibitory effects of the latter could be obtained on isolated fat cell ghosts. The effects of lactate and β-hydroxybutyrate are similar to those for prostaglandins of the E series, which are potent inhibitors of cyclic AMP accumulation in intact fat cells but do not affect adenylate cyclase activity of broken cell preparations (27).

Short chain aliphatic acids did not affect the cyclic AMP phosphodiesterase activities of soluble or particulate preparations from rat fat cells when examined in the presence of 0.1 or 1 μM cyclic AMP. The greatest effect of 4 mM lactate, butyrate, or β-hydroxybutyrate on either soluble or particulate cyclic AMP phosphodiesterase was a 4% increase (based on three paired experiments). It was possible to alter cyclic AMP phosphodiesterase activity by 4 but not by 2 mM octanoate. However, the effect of 4 mM octanoate was a 14% and 25% decrease in breakdown of 0.1 and 1.0 μM cyclic AMP, respectively. There is no evidence from these results that short chain aliphatic acids are able to lower cyclic AMP by increasing its degradation.

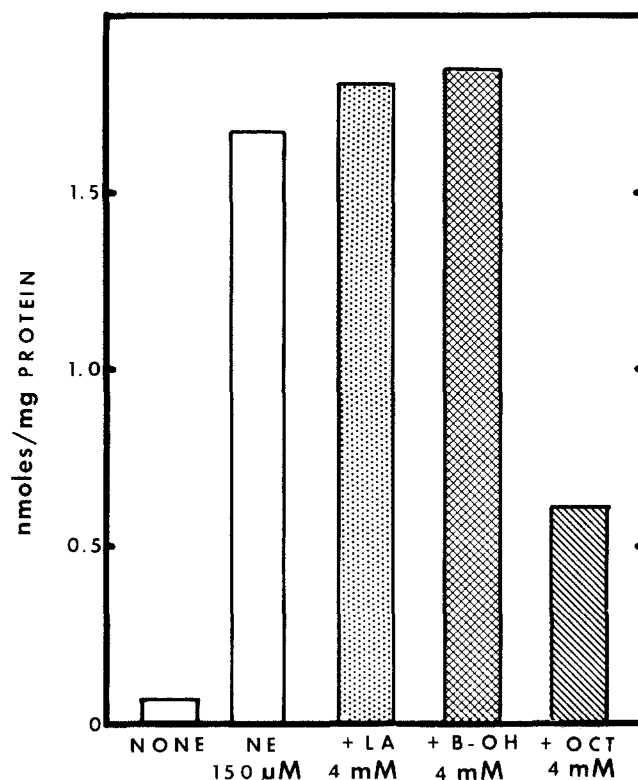

Fig. 4. Inhibition of norepinephrine-activated adenylate cyclase by octanoate. Rat fat cell ghosts (32 μg of protein per tube) were incubated without and with 150 μM norepinephrine for 5 min at 37°C. Lactate (LA), β-hydroxybutyrate (β-OH), and octanoate (OCT) were added in the presence of norepinephrine. The values are the means of four paired experiments.

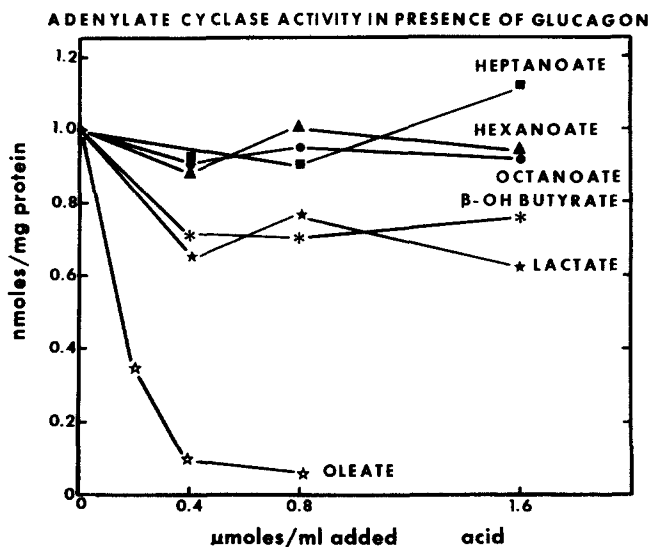

Fig. 3. Failure of short chain fatty acids to inhibit adenylate cyclase activity in the presence of glucagon. Rat fat cell ghosts (60 μg of protein per tube) were incubated in the presence of 5 μg/ml of glucagon for 10 min in the absence of added albumin. The values are the means of three paired experiments in the presence of varying amounts of oleate, different short chain fatty acids, lactate or β-hydroxybutyrate.

The soluble protein kinase activity was determined, using the supernatant fluid obtained after centrifugation of fat cell homogenates at 48,000 *g*, in the presence of various short chain fatty acids either with or without 40 nM cyclic AMP. The basal activity was 2.0 ± 0.4 nmoles of ^{32}P incorporated into histone per mg of protein over 20 min in the absence of cyclic AMP and 7.1 ± 1.1 moles of ^{32}P in the presence of cyclic AMP expressed as the means ± standard errors for five experiments. The addition of 0.8, 6.4 or 9.6 mM lactate, propionate, hexanoate, butyrate, or β-hydroxybutyrate did not inhibit protein kinase activity either in the absence or presence of added cyclic AMP. The addition of 9.6 mM octanoate resulted in a non-significant rise in basal protein kinase activity of 30 ± 40% as the mean increase in five experiments ± the standard error of the percentage increase. In the presence of

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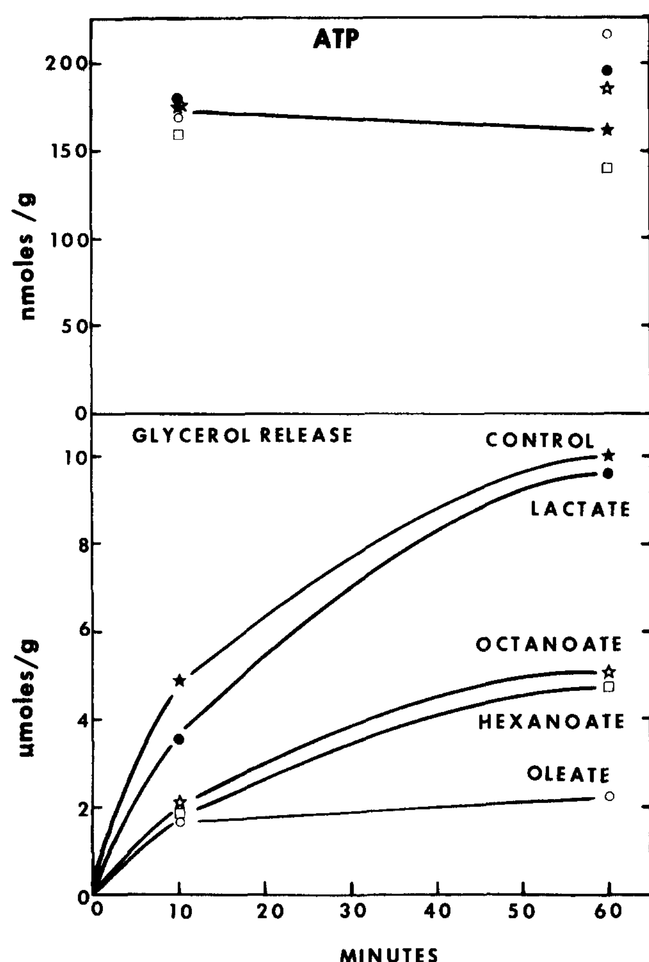

Fig. 5. Effects of short and long chain fatty acids on ATP and lipolysis. Rat fat cells (40 mg/tube) were incubated for 10 or 60 min in buffer containing 3% albumin (0.43 μmoles/ml). Glucagon (5 μg/ml), theophylline (100 μM) and adenosine deaminase (0.5 μg/ml) were present in all tubes. The values for ATP in the absence of lipolytic agents were 170 and 190 nmoles/g at 10 and 60 min respectively. Lactate, octanoate, hexanoate or oleate were added at a concentration of 3 mM at the start of the experiment. Tubes incubated in the absence of any added acids were run as controls. The values are the means of four paired experiments and none of the differences in ATP values from control were statistically significant ($P > 0.10$ by paired comparisons).

cyclic AMP there was a 13 ± 7% decrease in protein kinase activity due to 9.6 mM octanoate. These results indicate that even at a concentration of 9.6 mM there was no alteration of protein kinase activity by octanoate or other short chain aliphatic acids.

The possibility that octanoate and hexanoate might inhibit lipolysis by reducing the availability of ATP for activation of triglyceride lipase was examined by measuring total ATP. However, even after incubation of fat cells with 3 mM octanoate or hexanoate for 10 or 60 min in the presence of glucagon, theophylline, and adenosine deaminase there was no significant decrease in total ATP (**Fig. 5**). Similar negative results were seen with oleate and lactate. These results do not preclude the possibility that ATP content of a special compartment involved in lipase activation is affected by short or long chain fatty acids.

Another difference between oleate and short chain acids was the failure of β-hydroxybutyrate or octanoate to inhibit triglyceride lipase activity of the pH 5.2 precipitate from a 40,000 *g* supernatant fluid of rat fat cell homogenates (**Table 3**). Lactate was also ineffective, which was not surprising since it also failed to reduce lipolysis by intact fat cells. Octanoate at a concentration of 6.4 mM actually elevated triglyceride lipase activity. In contrast, as little as 0.8 mM oleate markedly reduced the activity of the tri-

TABLE 3. Activity of triglyceride lipase in presence of lactate, β-hydroxybutyrate, octanoate, and oleate

Additions	Without	+ Added Cyclic AMP & ATP	% Activation
	μmoles/mg protein × 1 hr		
None	3.49 ± 0.65	4.44 ± 0.94	26 ± 5
Lactate, 6.4 mM	3.88 ± 0.53	4.57 ± 0.78	15 ± 5
β-OH Butyrate, 6.4 mM	3.82 ± 0.63	4.25 ± 0.65	12 ± 3
Octanoate, 6.4 mM	5.54 ± 0.65	5.90 ± 0.93	6 ± 5
Oleate, 0.8 mM	2.08 ± 0.55	2.18 ± 0.60	8 ± 4
1.6 mM	0.59 ± 0.53	0.66 ± 0.60	12 ± 13

The pH 5.2 triglyceride lipase preparation (av 240 μg protein/tube) was incubated for 10 min at 30°C in a final volume of 0.2 ml containing 5 mM $MgCl_2$, 1 mM theophylline, 1 mM dithiothreitol, 0.5 mM EGTA (ethylene glycol bis (β-aminoethyl ether)-*N,N*-tetraacetic acid), and 50 mM Tris-HCl, pH 8.0, with or without 10 μM cyclic AMP and 0.5 mM ATP. The assay was initiated by addition of 0.6 ml of a substrate mixture containing 1 μmole of triolein emulsion (unlabeled and [^{14}C]triolein in 5% gum arabic), 20 mg of albumin either without or with added fatty acid and 40 μmoles of sodium phosphate buffer, pH 6.8, and incubated for 30 min at 30°C. The data represent the mean ± SE of three experiments. The ratio of acid to albumin was 2, 4, and 16 at 0.8, 1.6, and 6.4 mM concentrations, respectively.

TABLE 4. Inhibition of lipolytic action of dibutyryl cyclic AMP by octanoate and oleate

Additions	Basal	Δ due to: Octanoate 3 mM	Δ due to: Oleate 3 mM
	glycerol release (μmoles/g)		
None	4.9	−3.4	−3.5
Dibutyryl cyclic AMP, 0.5 mM	9.1	+1.2	−7.3
Dibutyryl cyclic AMP, 1.0 mM	44.3	−7.7	−25.4
Theophylline, 200 μM	12.7	−7.6	−11.5
Norepinephrine, 1.5 μM	28.6	−16.8	−24.2

Rat fat cells (31 mg/tube) were incubated for 1 h in buffer containing 4.8% albumin. The values are the means of three paired experiments.

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glyceride lipase both in the absence and presence of added cyclic AMP plus ATP (Table 3).

In fat cells the dibutyryl derivative of cyclic AMP activates lipolysis at millimolar concentrations. Dibutyryl cyclic AMP may work both by inhibiting cyclic AMP phosphodiesterase (28) and by deacylation to give N^6-monobutyryl cyclic AMP which is able to activate protein kinase (29). While it is not clear just how dibutyryl cyclic AMP activates lipolysis, the mechanism probably involves increases in cyclic AMP or N^6-monobutyryl cyclic AMP that activate protein kinase. Presumably agents that act at the triglyceride lipase step should be just as effective inhibitors of lipolysis due to dibutyryl cyclic AMP as that due to agents that activate adenylate cyclase. In contrast octanoate, which does not affect protein kinase or triglyceride lipase activity, should be relatively ineffective in inhibiting lipolysis due to dibutyryl cyclic AMP. The results in **Table 4** indicate that this was the case, since 3.0 mM octanoate did not inhibit the activation of lipolysis by 0.5 mM dibutyryl cyclic AMP but effectively inhibited that due to theophylline or norepinephrine. These findings are similar to those with adenosine, nicotinic acid, and prostaglandins, which are more effective inhibitors of lipolysis due to norepinephrine or theophylline than those due to dibutyryl cyclic AMP (27).

The direct inhibition of triglyceride lipase by oleate suggests that its antilipolytic action results from product inhibition when the primary binding sites on albumin for fatty acids are filled. The failure of octanoate or β-hydroxybutyrate to directly inhibit triglyceride lipase suggests that their antilipolytic action may be secondary to inhibition of cyclic AMP accumulation in free fat cells. The fact that octanoate was not inhibitory to dibutyryl cyclic AMP-stimulated lipolysis is further evidence that this aliphatic acid exerts its primary effect on adenylate cyclase (Figs. 3 & 4).

While long chain fatty acids such as oleate (1) or palmitate[1] appear to act directly to inhibit hormone-sensitive adenylate cyclase activity, this is not true of lactate or β-hydroxybutyrate. Furthermore it is not clear why lactate inhibits cyclic AMP accumulation to the same extent as octanoate and β-hydroxybutyrate but does not inhibit lipolysis. The effects of lactate and β-hydroxybutyrate are similar to those of adenosine, nicotinic acid and prostaglandins, which are potent inhibitors of cyclic AMP accumulation in intact cells but do not affect adenylate cyclase activity of broken cell preparations (27).

[1] Fain, J. N., and R. E. Shepherd. Unpublished studies.

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REFERENCES

1. Fain, J. N., and R. E. Shepherd. 1975. Free fatty acids as feedback regulators of adenylate cyclase and cyclic 3′,5′-AMP accumulation in rat fat cells. *J. Biol. Chem.* **250:** 6586–6592.
2. Malgieri, J. A., R. E. Shepherd, and J. N. Fain. 1975. Lack of feedback regulation of cyclic 3′:5′-AMP accumulation by free fatty acids in chicken fat cells. *J. Biol. Chem.* **250:** 6593–6598.
3. Rodbell, M. 1965. Modulation of lipolysis in adipose tissue by fatty acid concentration in fat cells. *Ann. N.Y. Acad. Sci.* **131:** 302–314.
4. Björntorp, P. 1965. The effect of lactic acid on adipose tissue metabolism in vitro. *Acta Med. Scand.* **178:** 253–255.
5. Dieterle, P., C. Dieterle, P. Bottermann, K. Schwartz, and J. Henner. 1969. In vitro Versuche zur antilipolytischen Wirkung von Milchsäure. *Diabetologia.* **5:** 238–242.
6. Fredholm, B. B. 1971. The effect of lactate in canine subcutaneous adipose tissue in situ. *Acta Physiol. Scand.* **81:** 110–123.
7. Miller, H. I., B. Issekutz, Jr., P. Paul, and K. Rodahl. 1964. Effect of lactic acid on plasma free fatty acids in pancreatectomized dogs. *Amer. J. Physiol.* **207:** 1226–1230.
8. Metz, S. H. M., and S. G. VandenBergh. 1972. Effects of volatile fatty acids, ketone bodies, glucose, and insulin on lipolysis in bovine adipose tissue. *FEBS Lett.* **21:** 203–206.
9. Metz, S. H. M., M. Lopes-Cardozo, and S. G. VandenBergh. 1974. Inhibition of lipolysis in bovine adipose tissue by butyrate and β-hydroxybutyrate. *FEBS Lett.* **47:** 19–22.
10. Björntorp, P. 1966. Effect of ketone bodies on lipolysis in adipose tissue in vitro. *J. Lipid Res.* **7:** 621–626.
11. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
12. Gilman, A. G. 1970. A protein binding assay for adenosine 3′,5′-cyclic monophosphate. *Proc. Nat. Acad. Sci. USA* **67:** 305–312.
13. Brown, B. L., J. D. M. Albano, R. P. Ekins, and A. M. Sgherzi. 1971. A simple and sensitive saturation assay method for the measurement of adenosine 3′:5′-cyclic monophosphate. *Biochem. J.* **121:** 561–562.
14. Fain, J. N., R. H. Pointer, and W. F. Ward. 1972. Effects of adenosine nucleosides on adenosine, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J. Biol. Chem.* **247:** 6866–6872.
15. Fain, J. N., M. P. Czech, and R. Saperstein. 1971. Growth hormone in vitro effects on lipolysis. *In*

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Methods in Investigative and Diagnostic Endocrinology, Peptides and Hormones. S. A. Berson and R. S. Yalow, editors. North Holland, Amsterdam. 267–273.

16. Fain, J. N., and J. W. Rosenthal. 1971. Calorigenic action of triiodothyronine on white fat cells: effects of ouabain, oligomycin, and catecholamines. *Endocrinology.* **89:** 1205–1211.
17. Birnbaumer, L., S. L. Pohl, and M. Rodbell. 1969. Adenyl cyclase in fat cells. I. Properties and the effects of adrenocorticotropin and fluoride. *J. Biol. Chem.* **244:** 3468–3476.
18. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
19. Ward, W. F., and J. N. Fain. 1971. The effects of dihydroergotamine upon adenyl cyclase and phosphodiesterase of rat fat cells. *Biochim. Biophys. Acta.* **237:** 387–390.
20. Thompson, W. J., and M. M. Appleman. 1971. Characterization of cyclic nucleotide phosphodiesterase of rat tissues. *J. Biol. Chem.* **246:** 3145–3150.
21. Boudreau, R. J., and G. I. Drummond. 1975. A modified assay of 3′:5′-cyclic-AMP phosphodiesterase. *Anal. Biochem.* **63:** 388–399.
22. Corbin, J. D., and E. G. Krebs. 1969. A cyclic AMP-stimulated protein kinase in adipose tissue. *Biochem. Biophys. Res. Commun.* **36:** 328–336.
23. Butcher, F. R. 1971. A rapid filter paper disk assay for picomole amounts of cyclic AMP-dependent protein kinase. *Horm. Metab. Res.* **3:** 336–340.
24. Reimann, E. M., D. A. Walsh, and E. G. Krebs. 1971. Purification and properties of rabbit skeletal muscle adenosine 3′,5′-monophosphate-dependent protein kinases. *J. Biol. Chem.* **246:** 1986–1995.
25. Khoo, J. C., and D. Steinberg. 1974. Reversible protein kinase activation of hormone-sensitive lipase from chicken adipose tissue. *J. Lipid Res.* **15:** 602–610.
26. Fain, J. N., and P. B. Wieser. 1975. Effects of adenosine deaminase on cyclic adenosine monophosphate accumulation, lipolysis, and glucose metabolism of fat cells. *J. Biol. Chem.* **250:** 1027–1034.
27. Fain, J. N. 1973. Biochemical aspects of drug and hormone action on adipose tissue. *Pharmacol. Rev.* **25:** 67–118.
28. Jarett, L., and R. M. Smith. 1974. Mode of action of N^6-$O^{2'}$-dibutyryl cyclic 3′,5′ AMP on fat cell metabolism. *Diabetes.* **23:** 29–40.
29. Neelon, F. A., and B. M. Birch. 1973. Cyclic adenosine 3′:5′-monophosphate-dependent protein kinase. *J. Biol. Chem.* **248:** 8361–8365.

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