

# Lipolytic stimulation modulates the subcellular distribution of hormone-sensitive lipase in 3T3-L1 cells

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**Abstract** 3T3-L1 cells have been a useful model system for studying adipocyte differentiation and metabolism. They acquire a hormone-sensitive lipase during differentiation (Kawamura, M., et al. 1981. *Proc. Natl. Acad. Sci. USA.* **78**: 732–735). In the present study the control of lipolysis in these cells was investigated. Basal glycerol release from cell monolayers was 437 nmol/mg protein per hr, and could be stimulated approximately 6-fold by exposure to 1  $\mu$ M isoproterenol. Subcellular fractionation of stimulated cells revealed a redistribution of triglyceride lipase activity: loss from the infranatant fraction and increase in the pellet fraction. The redistribution was dosage-dependent and reversible. Treatment of intact cells with 8-bromoadenosine 3':5' cyclic monophosphate elicited similar redistribution of the lipase activity; however, disruption and incubation of untreated cells in the presence of ATP and either cyclic AMP or the catalytic subunit from cAMP-dependent protein kinase did not. The lipase activity in the pellet fraction was increased 3- to 4-fold after maximal lipolytic stimulation of intact cells, whereas phosphorylation of the enzyme in vitro yielded 1.4- to 1.6-fold stimulation in all subcellular fractions from untreated cells. The lipase found in the particulate fraction has the same properties as the previously characterized infranatant enzyme. It is suggested that interaction of the lipase with substrate and associated intracellular membranes may be a novel feature of the regulation of lipolysis.—Hirsch, A. H. and O. M. Rosen. Lipolytic stimulation modulates the subcellular distribution of hormone-sensitive lipase in 3T3-L1 cells. *J. Lipid Res.* 1984. **25**: 665–677.

**Supplementary key words** adipocytes • lipolysis • phosphorylation

Twenty-five years ago it was shown that both ACTH (1) and epinephrine (2) could stimulate lipolysis in isolated adipose tissue. Following the observations by Rizak (3, 4) that addition of epinephrine or cyclic AMP to adipocyte homogenates stimulated the release of free fatty acids and the subsequent report of a hormone-sensitive triglyceride lipase activity (5), a role for cyclic AMP-dependent protein kinase in this phenomenon was described (6, 7). When assayed with lipid emulsions this lipase exhibited increased hydrolytic activity in the presence of cyclic AMP and cAMP-dependent protein kinase (7). The enzyme has since been purified from rat adipose tissue (8–11) and chicken adipose tissue (12). A correlation be-

tween the phosphorylation of the purified hormone-sensitive lipase by cyclic AMP-dependent protein kinase and its activity towards triacylglycerols has been shown (10, 11). These results have led to the conclusion that direct stimulation of the hormone-sensitive lipase by cAMP-dependent phosphorylation is the basis of hormone-stimulated lipolysis in adipocytes (13, 14). A recent study (15) proposed an extension of this concept to the generation of free cholesterol from its esters in adrenal cortex. However, some investigators have questioned a simple direct relationship between phosphorylation-dependent activation of hormone-sensitive lipase and lipolysis in adipocytes (16–19; review: 20). Wise and Jungas (17) reported that after rat adipose tissue had been exposed to epinephrine, lipase activity in homogenates was enhanced 3-fold with respect to endogenous substrate, but not at all toward exogenous triolein substrate. Nor could they detect any change in the lipase activity in the presence of exogenous substrate emulsions using the aqueous infranatants from treated versus nontreated tissues. In contrast, treatment of whole homogenates with cAMP-dependent protein kinase led to increased lipase activity toward added triolein emulsion but had no effect on the lipase activity on endogenous lipid substrate. Based upon these findings, Wise and Jungas (17) proposed that there is a mechanism for lipolytic stimulation in addition to that which involves phosphorylation of the hormone-sensitive lipase. Their work emphasized the importance of the substrate-lipase interaction in lipolysis, and left open the role of the infranatant-derived enzyme in the lipolytic process.

Utilizing different experimental protocols, the bulk of recovered hormone-sensitive lipase activity has been

Abbreviations: ACTH, adrenocorticotrophic hormone; FBS, fetal bovine serum; MOPS, 3-(N-morpholino)propanesulfonic acid; HEPES, N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid; PGE<sub>1</sub>, prostaglandin E<sub>1</sub>; 8-Br-cAMP, 8-bromoadenosine 3':5'-cyclic monophosphate; Iso/MIX, 1  $\mu$ M isoproterenol plus 0.5 mM 1-methyl-3-isobutylxanthine; TLC, thin-layer chromatography; LPL, lipoprotein lipase.

traced to the fat (18, 21, 22), infranatant (8–12, 23), or pellet (24, 25) fractions of adipocytes. Oschry and Shapiro (21) observed that a major portion of the hormone-sensitive lipase activity was always associated with the bulk lipid fraction, but that this enzyme was only active in whole homogenates of epinephrine-treated cells. Upon centrifugation the activity was lost and could not be regained by recombination with the infranatant fraction. However, sonication of the fat cakes from treated or untreated cells re-established the lost activity in the former and unmasked a slightly lower activity in the latter. They hypothesized that the lipid-enzyme complex is constitutive, but that water (and possibly aqueous factors) necessary for hydrolysis to occur is unavailable to the reaction site in unstimulated cells.

The 3T3-L1 cell line developed by Green and his coworkers (26, 27) differentiates in culture into adipocyte-like cells. Biochemical characterization of the cells has validated their usefulness as a model system for studying adipocyte differentiation and function (28–30). Khoo and coworkers (31) and Kawamura et al. (32) have reported the developmental acquisition of hormone-sensitive lipase during the adipocyte conversion of the 3T3-L1 cells. The present investigation of hormone-sensitive lipolysis in these cells has extended these findings by demonstrating that *a*) a significant portion of the hormone-sensitive lipase in 3T3-L1 cells is associated with the particulate fraction of the cells; *b*) the proportion of particulate-associated activity increases upon stimulation of lipolysis in the intact cells and this response is dosage dependent; *c*) increasing the cyclic AMP content of intact cells is sufficient to mimic the redistribution of the enzyme activity wrought by lipolytic agents; and *d*) the altered distribution of enzyme is reversed upon cessation of lipolytic stimulation.

## EXPERIMENTAL PROCEDURES

### Cell culture

The 3T3-L1 preadipocyte cell line, originally obtained from Dr. Howard Green, was propagated and induced to differentiate in Dulbecco's modified Eagle's medium by a slight modification of the method previously described (33). Briefly, cells at confluence (day 0) were treated with dexamethasone (0.5  $\mu$ M) and methylisobutylxanthine (0.5 mM). After 48 hr (during day 2) the cells were refed with fresh medium lacking the drugs and maintained until harvest on the day indicated.

Differentiated cells containing little or no triacylglycerol were obtained by transferring early log-phase cells to medium containing extensively dialyzed fetal bovine serum (biotin-free), and continuing the use of biotin-free medium until cell harvest (34).

In this study one 53-cm<sup>2</sup> dish contained approximately 10<sup>6</sup> cells at harvest which yielded about 1 mg of protein.

### Whole cell treatment with lipolytic drugs

Cells were harvested on days 7–10 by scraping with a rubber spatula into residual medium (0.5–1 ml/53 cm<sup>2</sup>), pooled, combined with fresh medium containing 10% FBS, and centrifuged (2 min at 500 g) in a polyethylene tube. An homogenous suspension was then made by adding fresh medium with 10% FBS to the cell pellet (1 ml per 4 × 10<sup>6</sup> cells) replacing the air in the tube with 10% CO<sub>2</sub> in air, capping, and repeatedly inverting the tube. The suspension was apportioned into new polyethylene tubes, and each was injected with 10% CO<sub>2</sub> in air and equilibrated for 10 min in a 37°C water bath, with shaking. Each aliquot then received one-ninth of its volume of either 0.15 M NaCl, or 10  $\mu$ M l-isoproterenol plus 5 mM 1-methyl-3-isobutylxanthine (Iso/MIX) in 0.15 M NaCl. At specified times, cells were centrifuged and washed with 0.15 M NaCl with or without Iso/MIX.

### Subcellular fractions

Unless otherwise indicated, washed cells (10<sup>7</sup> cells/ml) were disrupted in 50 mM MOPS buffer, pH 7.0, containing 10 mM MgCl<sub>2</sub> by ten passages through a 5/8-inch, 25-gauge needle attached to a syringe. During lysis and for a brief period following (total time, 5 min) the preparation was maintained at 37°C. This procedure produced greater than 99% cell breakage in all homogenizing media used. All subsequent procedures were carried out at 4°C or on ice. This preparation or, where indicated, the 400 g supernatant fluid was designated as the homogenate fraction.

The homogenate was centrifuged for 20 min at 40,000 g. The fat cake was carefully aspirated and the infranatant was removed. The 40,000 g pellet was resuspended in the homogenization buffer to the original volume. Needle aspiration was used to disperse the pellet. The infranatant, which often contained a small amount of redispersed fat, was again centrifuged and a clear sample was carefully withdrawn for assay.

### Solubilization of the pellet-associated lipase

For solubilization, the 40,000 g pellet was resuspended in 5 mM sodium phosphate buffer (pH 7.0) containing detergent to a protein concentration of 1 mg/ml. After 10 min on ice the suspension was centrifuged at 40,000 g for 20 min. Clear infranatant containing solubilized lipase was withdrawn from beneath a small floating lipid residue created by the procedure.

### Lipase assays

Tri[1-<sup>14</sup>C]oleoylglycerol as obtained was approximately 95 to 99% pure by thin-layer chromatography on silica

gel (35). Polyester-backed plates (Eastman no. 13179) were developed in hexane–diethyl ether–glacial acetic acid 80:20:1. Marker lipids were detected by exposure to iodine vapor. Segments of the plates were extracted and counted in Bray's liquid scintillation medium (36). To improve sensitivity and reduce levels of other radio-labeled substrates, the material, in benzene, was diluted with carrier triolein and purified by silica gel G column chromatography (37) to greater than 99.8% purity. After further dilution with carrier to obtain 1500 cpm per nmol of triolein and removal of solvents with nitrogen, the lipid was solidified at  $-20^{\circ}\text{C}$ , layered with a cold, neutralized (pH 7.0), 50 mg/ml solution of fatty acid-poor bovine serum albumin to provide 40 mg of triolein per ml, and sonicated. Maximum allowable energy from a microprobe on a Braun Sonicator was applied in 1-min bursts to produce an homogeneous emulsion. Aliquots were stored at  $-20^{\circ}\text{C}$  and thawed immediately prior to assay. Uniform results were obtained from each emulsion preparation for up to 8 weeks.

Lipase reactions were carried out in a total volume of 0.3 ml containing 2.8% neutralized fatty acid-poor bovine serum albumin, 25 mM sodium phosphate buffer, pH 7.0, and 3 mmol of emulsified tri[ $1-^{14}\text{C}$ ]oleoylglycerol per liter. After incubation for 60 min at  $37^{\circ}\text{C}$ , reactions were stopped by the addition of 1.5 ml of a mixture of chloroform–methanol–benzene 2:2.4:1 containing oleic acid (50  $\mu\text{g}/\text{ml}$ ), followed by 0.1 ml of 0.5 N NaOH (38). To extract and isolate free fatty acids, samples were vortexed for 20 sec and then centrifuged at 2000  $g$  for 5 min. A 0.5-ml portion of the alkaline aqueous upper phase containing fatty acids was transferred to scintillation vials containing 0.025 ml of 2 N  $\text{H}_2\text{SO}_4$ . The now acidified fatty acids were solubilized with vigorous mixing in 5 ml of toluene containing 20 mg of Omnifluor and sample radioactivity was determined by liquid scintillation spectrometry.

#### Activation by protein kinase

Samples containing lipase were incubated for 10 min at  $37^{\circ}\text{C}$  after the addition of sodium phosphate to 25 mM at pH 7.0, DTT to 1 mM, and  $\text{MgCl}_2$  to 10 mM, with or without ATP to 1 mM and the catalytic subunit of cAMP-dependent protein kinase (1.5  $\mu\text{g}/\text{ml}$ ). Lipase assays were then performed. Under these conditions activation was essentially complete after 5 min of incubation. As seen in Table 4, 10-min incubation in the absence of ATP and protein kinase results in a small loss of activity and does not significantly alter the effects of Iso/MIX treatment.

#### Labeling intact cells with $^{32}\text{P}$

Cells were harvested as described and washed with 0.15 M NaCl. Culture medium lacking sodium phosphate

but containing 15 mM HEPES, 5 mM  $\text{NaHCO}_3$ , and additional NaCl to maintain isotonicity was prepared and adjusted to pH 7.4. This was supplemented with 1.8% BSA that had been dialyzed and neutralized. Carrier-free  $^{32}\text{P}$ -orthophosphoric acid was added to between 0.01 mCi/ml and 0.1 mCi/ml. The cell suspension ( $2 \times 10^6$  cells/ml) was shaken at  $37^{\circ}\text{C}$  in a 50-ml polyethylene tube for 90 min. Cells were then divided into two aliquots, one of which received Iso/MIX. The incubation was then continued for 10 min, after which the cells were removed by centrifugation and washed with 0.15 M NaCl with or without Iso/MIX.

#### Labeling intact cells with $^3\text{H}$ oleic acid

[9,10- $^3\text{H}$  (N)] oleic acid in hexanes (10 mCi/ml) was diluted 1000-fold into culture medium containing 10% fetal bovine serum at  $37^{\circ}\text{C}$ . Differentiated 3T3-L1 cells were labeled with this medium for 30 hr beginning on day 6 (5 ml/53- $\text{cm}^2$  dish). This medium was then replaced with fresh medium containing no label and the cells were incubated for an additional 45 hr to allow for equilibration of the labeled oleic acid with the bulk lipid in the cells.

During the labeling period the cells incorporated 95% of the labeled oleic acid originally present in the medium. After the 45-hr equilibration period, only 6% of the incorporated label had been released to the medium. TLC analysis as described above showed greater than 98% of the label in the cellular lipid extracted by the method of Dole and Meinertz (39) migrated with triolein marker, suggesting stable incorporation of the  $^3\text{H}$ oleic acid into triacylglycerol.

#### Labeling subcellular fractions with [ $\gamma$ - $^{32}\text{P}$ ]-ATP

Small aliquots of lipase preparations were incubated for 5 min at  $37^{\circ}\text{C}$  in the presence of 7  $\mu\text{M}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP (26 Ci/mmol), 25 mM sodium phosphate, pH 7.0, 10 mM  $\text{MgCl}_2$ , and 1.5  $\mu\text{g}/\text{ml}$  of the catalytic subunit of cAMP-dependent protein kinase. Immediately following this, incubation samples were adjusted to 2% sodium dodecyl sulfate, 0.1 M Tris-HCl, pH 6.8, 28 mM  $\beta$ -mercaptoethanol, and 10% glycerol, and were incubated at  $99^{\circ}\text{C}$  for 5 min.

#### SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed in resolving gels cast with 8.5% acrylamide and 0.15% N,N'-methylene-(bis)-acrylamide. Buffers were those of Laemmli (40) with sodium dodecyl sulfate (0.1%) present in the electrode buffer, but omitted from the gel matrix.

#### Glycerol assays

Aliquots (200  $\mu\text{l}$ ) of samples withdrawn from lipolysis experiments were assayed for glycerol content by the

coupled enzyme assay of Wieland (41) as previously described (33), with the modification that NADH formed was monitored by absorption at 340 nm. Standards (5–75 nmol) were prepared in lipolysis medium (3% fatty acid-poor bovine serum albumin in Kreb's Ringer's phosphate solution).

### Protein determinations

Protein was assayed by the method of Lowry et al. (42) using bovine serum albumin as the standard.

### Materials

Cell culture medium, vitamin and amino acid components, and fetal bovine serum were purchased from Grand Island Biologicals. Dexamethasone, l-isoproterenol-d-bitartrate, ACTH, glucagon, l-methyl-3-isobutylxanthine, MOPS, HEPES, triolein, oleic acid, ATP, NAD, DTT, glycerol kinase, and  $\alpha$ -glycerol phosphate dehydrogenase were obtained from Sigma. 8-Br-cAMP was obtained from Plenum Biochemicals. PGE<sub>1</sub> and crystalline porcine insulin were provided by Dr. John Pike of Upjohn Laboratories and Dr. Mary Root of Eli Lilly and Co., respectively. Bovine serum albumin, fraction V, fatty acid-poor and Zwittergent 3-14 were from Calbiochem-Behring. Digitonin, 98% pure, was obtained from Schleicher and Schuell (Anal R brand) and Chemical Dynamics Corp. Triton X-100 and Lubrol-PX were from Rohm & Haas and ICI-America, respectively. Radiolabeled [carboxyl-1-<sup>14</sup>C]-triolein, [9,10-<sup>3</sup>H(N)]oleic acid, and carrier-free

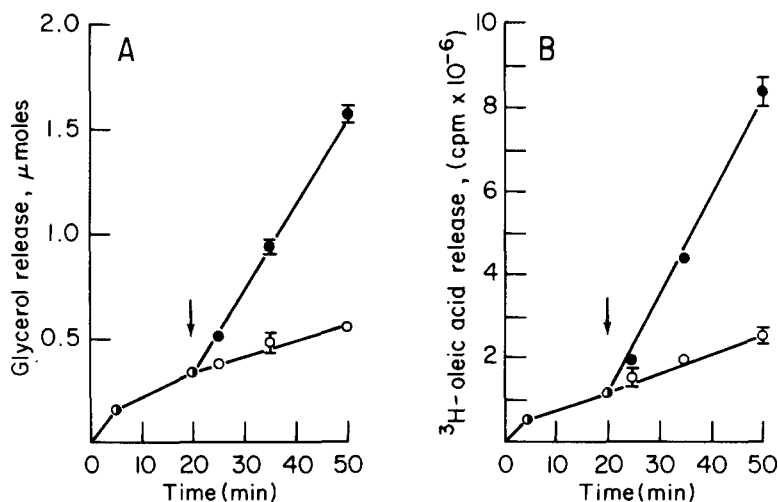
<sup>32</sup>P-orthophosphoric acid were from either New England Nuclear or Amersham. [<sup>14</sup>C]diolein was purchased from Rosechem Products. Omnifluor is a product of New England Nuclear. The purified catalytic subunit of bovine lung cAMP-dependent protein kinase was a kind gift from Dr. Dwijen Sarkar. DE-53 was purchased from Whatman, and AcA34 from LKB. Silica G was from Brinkmann.

## RESULTS

### Lipolysis

Absolute rates of glycerol release and relative rates of fatty acid release from intact differentiated 3T3-L1 cells *in situ* were determined as described in the legend to Fig. 1. An initial rapid release of both glycerol and [<sup>3</sup>H]oleic acid occurred when the cells were exposed to the Kreb's Ringer's phosphate buffer containing bovine serum albumin. After a 20-min equilibration period the basal release of glycerol stabilized at 437 nmol/mg of protein per hr. The addition of 1  $\mu$ M isoproterenol stimulated glycerol release from the cells 5.7-fold. [<sup>3</sup>H]oleic acid release increased 6.5-fold. The basal and stimulated rates of glycerol released by the monolayers of 3T3-L1 cells were significantly higher than those reported by Khoo et al. (31) and Kawamura et al. (32) for suspensions of 3T3-L1 cells.

ACTH (1  $\mu$ M) elicited the same maximal response as isoproterenol; PGE<sub>1</sub> (3  $\mu$ M) achieved about one-third to one-half of the maximal response seen with ACTH or



**Fig. 1.** Lipolysis in 3T3-L1 cells. Cells on eight 53 cm<sup>2</sup> dishes were labeled with [<sup>3</sup>H]oleic acid (Experimental Procedures) and washed with phosphate-buffered saline. Kreb's Ringer's phosphate solution (5 ml) containing 3% fatty acid-poor bovine serum albumin was then added to each dish. The cells were incubated at 37°C in air. At the times indicated, 0.5-ml samples were removed (with replacement, to maintain a constant volume) from each dish. Four dishes received isoproterenol at *t* = 20 min (arrows) and subsequent replacements to maintain a 1  $\mu$ M concentration. A, Aliquots (200  $\mu$ l) were assayed for glycerol. B, Aliquots (100  $\mu$ l) were counted directly in 5 ml of liquid scintillation solution. Each point represents the cumulative quantity released per dish averaged for the four control and four isoproterenol-treated dishes. The symbols used are: —○—, control; —●—, plus isoproterenol. Error bars show  $\pm$  the SEM.

isoproterenol. Glucagon (1  $\mu\text{M}$ ) was without effect. Insulin (100 ng/ml) abolished the response to PGE<sub>1</sub> and inhibited the response to submaximal isoproterenol stimulation.

### Effect of incubating cells with isoproterenol and MIX on the lipase activity in subcellular fractions

Treatment of cells with maximally stimulating concentrations of isoproterenol (1  $\mu\text{M}$ ) in the presence (Iso/MIX) or absence of 0.5 mM methylisobutylxanthine for 10 min significantly affected the lipase activity in all subcellular fractions (Table 1). The homogenates and pellet fractions were stimulated 1.6- and 3.7-fold, respectively, whereas the infranatant fractions lost 67% of their activity. All of the activity in the homogenate was recovered in the infranatant plus pellet fractions of both control and Iso/MIX-treated cells. However, a marked difference in the distribution of the lipase activity between the infranatant and pellet fractions was seen (Table 1).

### Effect of incubating cells with 8-Br-cyclic AMP on the lipase activity in subcellular fractions

To test whether the redistribution of lipase activity following Iso/MIX treatment of intact cells could be attributed to an increase in cyclic AMP, cells were incubated in the presence of 3 mM 8-Br-cAMP for 20 min. Under these conditions, infranatant activity was inhibited 58%; 3- and 20-min exposure to Iso/MIX gave 58% and 64% inhibitions, respectively (Fig. 2). Pellet activity was 4.3-fold stimulated after exposure of cells to 8-Br-cAMP, and 3.2- and 3.6-fold in response to 3- and 20-min treatments with Iso/MIX, respectively. Since the effects of 8-Br-cAMP are mediated directly or indirectly by cAMP-dependent protein kinase, these results argue that elevation of cAMP is sufficient to mediate the redistribution observed in response to Iso/MIX treatment of intact cells.

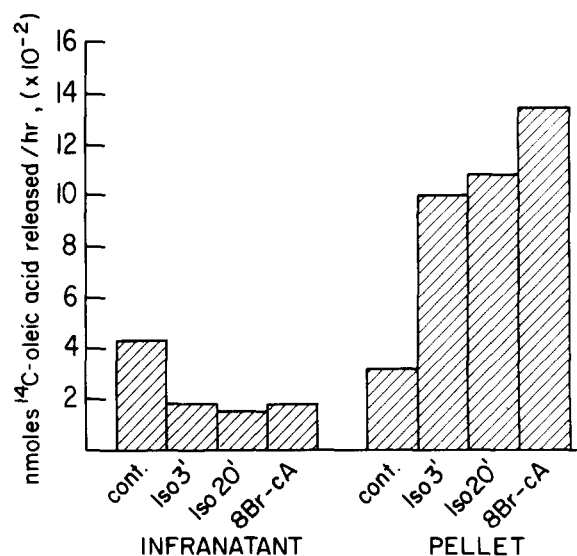


Fig. 2. Treatment of cells with either Iso/MIX or 8-Br-cAMP: effects on infranatant and pellet lipase activities. A cell suspension was divided into four portions. Twenty minutes prior to lysis one portion was treated with Iso/MIX (Iso20') and one with 3 mM 8-Br-cAMP (8Br-cA). A third portion was treated with Iso/MIX 17 min later (Iso3'); the fourth portion was untreated (Cont). Infranatant and pellet fractions from total homogenates ( $7 \times 10^6$  cells/ml) were prepared and assayed as described. Activity is per  $10^7$  cells.

### Reversibility of the effects of isoproterenol

Since the effect of isoproterenol on lipolysis in fat cells is reversible, removal of isoproterenol from the cells should lead to the reversal of changes in the activity and subcellular distribution of the lipase. The experiment presented in Table 2 shows that the isoproterenol-stimulated activities of the homogenate and pellet fractions reverted to control levels in the cells from which the isoproterenol had been removed, while the level of inhibition observed in the infranatant fraction was reduced 50%. Since cells exposed to Iso/MIX for 3- and 20-min

TABLE 1. Effect of Iso/MIX treatment of cells on triglyceride lipase activity in subcellular fractions

Subcellular Fraction	Control		Iso/MIX		Act. (Iso/MIX) <sub>b,c</sub> Act. (control)
	Activity <sup>a</sup>	Percent <sup>b</sup>	Activity <sup>a</sup>	Percent <sup>b</sup>	
Homogenate	922 ± 154	(100%)	1426 ± 137	(100%)	1.64 ± 0.12
Infranatant	592 ± 128	62 ± 7%	174 ± 32	13 ± 2%	0.33 ± 0.04
Pellet	380 ± 76	42 ± 4%	1274 ± 155	89 ± 7%	3.67 ± 0.25

In six separate experiments cells were harvested; Iso/MIX and control suspensions were prepared in four of the experiments and isoproterenol (1  $\mu\text{M}$  and 0.5  $\mu\text{M}$ ) and control suspensions were prepared in two of the experiments. The cells were lysed and total homogenates ( $10^7$  cells/ml) were used to prepare subcellular fractions as described in Experimental Procedures. Lipase assays were performed as described.

<sup>a</sup> Activity is in nmol of [<sup>14</sup>C]oleic acid released per hour per  $10^7$  cells, and is given as the mean ± SEM (N = 6).

<sup>b</sup> Percentages recovered in infranatant and pellet fractions and activity ratios are given as the mean of the values obtained in the six experiments ± SEM.

<sup>c</sup> *t*-tests applied to activity ratios show all changes to be significant at  $P < 0.001$ .

TABLE 2. Reversibility of the isoproterenol-induced changes in lipase activity

Subcellular Fraction	First Incubation	Second Incubation	Activity	Activity Ratio
Homogenate	Control	Control	1190	(1.00)
	Iso	Iso	1490	1.25
	Iso	Control	1140	0.96
Infranantant	Control	Control	602	(1.00)
	Iso	Iso	393	0.65
	Iso	Control	503	0.84
Pellet	Control	Control	420	(1.00)
	Iso	Iso	917	2.18
	Iso	Control	418	1.00

Portions of a cell suspension were incubated for 5 min (first incubation) with 0.3  $\mu\text{M}$  isoproterenol (iso), or in its absence (control). The cells were then washed in fresh medium with or without drug. They were resuspended in the same type of medium used for washing and incubated an additional 5 min (second incubation) prior to homogenization ( $6 \times 10^6$  cells/ml in 50 mM MOPS buffer, pH 7.0, 10 mM  $\text{MgCl}_2$ ), subcellular fractionation, and assay. Activity is in nmol of [ $^{14}\text{C}$ ]oleic acid released per hr per  $10^7$  cells, and is given as the mean of duplicate determinations.

periods showed essentially identical responses and, in data not presented, the effects of 0.1  $\mu\text{M}$  isoproterenol on subcellular activities are maximal within 5 min, these results reflect reversal of enzyme activation and redistribution following the removal of isoproterenol.

#### Dose-dependent response to isoproterenol

As indicated in Fig. 3, both the increase in pellet activity and the decrease in infranant activity are dose-dependent. Above 0.1  $\mu\text{M}$ , the activity in the pellet increased faster than the infranant activity decreased. The activity measured in the whole homogenate increased at concentrations of isoproterenol greater than 10 nM. The increase in homogenate activity between 10 nM and 0.1  $\mu\text{M}$  isoproterenol which was not accounted for by the changes in the pellet and infranant fractions in this experiment may have been due to an increase of enzyme in the floating bulk lipid fraction. This fraction could not be quantitatively recovered.

Maximal response as observed in subcellular fractions was often obtained with 0.5–1  $\mu\text{M}$  isoproterenol (e.g., see Table 1), however Iso/MIX treatment consistently yielded the maximal response and was therefore employed when such response was desired.

#### Comparison of Iso/MIX treatment of cells with the addition of cyclic AMP to cell homogenates

The effect of the addition of cyclic AMP and ATP during and briefly following cell lysis was assessed (Table 3) and found not to mimic the effect of exposing intact cells to Iso/MIX or 8-Br-cAMP.

When the catalytic subunit of cAMP-dependent protein kinase was included in the treatment of the homogenate,

the activity of the lipase in subsequently derived subfractions was similarly increased compared to controls (homogenate, 1.35-fold; infranant, 1.6-fold; pellet, 1.8-fold). There was, however, no alteration in the subcellular distribution of the lipase activity.

#### Addition of protein kinase to the subcellular fractions

The effect of treating infranant and pellet fractions derived from Iso/MIX-treated and control cells with ATP and cAMP-dependent protein kinase was also tested (Table 4). The isolated fractions from control cells both showed a 1.6-fold increase in activity. It is noteworthy that the stimulation of the pellet activity by protein kinase is consistently less than that which follows treatment of the cells with Iso/MIX. Fractions obtained from Iso/MIX-treated cells were unaffected by incubation with kinase; the pellet fraction was not further stimulated and there was no restoration of activity lost from the infranant.

#### Effect of pH on triglyceride lipase activity in subcellular fractions

The homogenate obtained from the Iso/MIX-treated cells showed a 2-fold stimulation of a lipase activity with a pH optimum around pH 7 (Fig. 4A). The activity found in the pellet from cells incubated in the absence of drugs was optimal at pH 6.5 (Fig. 4B), whereas the activity in comparable fractions from cells treated with Iso/MIX exhibited optimal activity at about pH 7. This pellet-

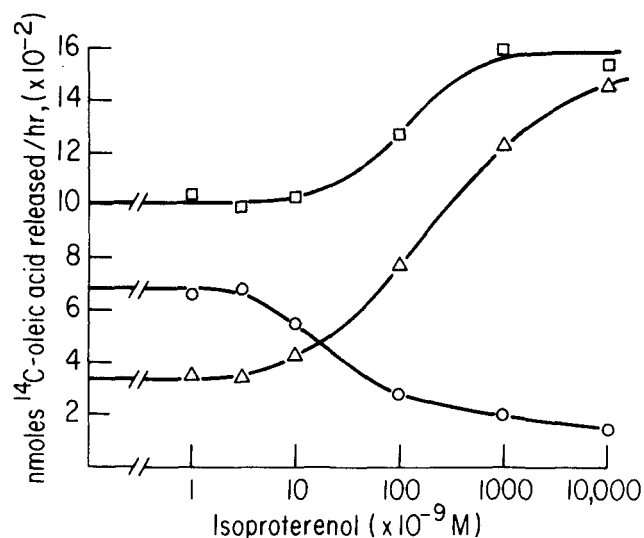


Fig. 3. Effect of the concentration of isoproterenol on lipase activity in subcellular fractions. Cells were incubated for 10 min at 37°C with the indicated concentrations of isoproterenol. They were then sedimented by centrifugation and total homogenates ( $7 \times 10^6$  cells/ml) and subcellular fractions were prepared and assayed as described. Total homogenate, ( $\square$ ); infranant, ( $\circ$ ); and pellet ( $\Delta$ ) fractions. Activity is per  $10^7$  cells.

TABLE 3. Comparison of the effects of treating cells with Iso/MIX and treating homogenates with cAMP + ATP on triglyceride lipase activity in subcellular fractions

Subcellular Fraction	Activity <sup>a</sup>				Activity Ratio: <sup>b,c</sup>	
	Control		Iso/MIX	Activity Ratio: <sup>b,c</sup>		
	No Additions	+cAMP, +ATP	No Additions	+cAMP, +ATP	Iso/MIX	
Homogenate	609 ± 114	733 ± 58	866 ± 122	1.23 ± 0.13	1.44 ± 0.07	
Infranantant	348 ± 28	449 ± 86	89 ± 8	1.28 ± 0.15	0.26 ± 0.04	
Pellet	184 ± 55	224 ± 21	652 ± 98	1.38 ± 0.53	3.72 ± 0.58	

Cells were harvested on day 7; one Iso/MIX-treated and two control suspensions were prepared as described in Experimental Procedures. The cells ( $10^7$  cells/ml) were then lysed in 5 mM sodium phosphate, pH 7.0, containing 10 mM MgCl<sub>2</sub> and, for one of the control preparations, 1.5 mM ATP and 100 μM cAMP. Each aliquot was incubated at 37°C for 10 min (during and briefly following lysis) and then received EDTA (final concentration 20 mM) and was transferred to ice. Post 400 g homogenates and 40,000 g infranantants and pellets were prepared as described. The data are average values for two separate experiments, each performed in duplicate.

<sup>a</sup> Activity is in nmol of [<sup>14</sup>C]oleic acid released per hr per  $10^7$  cells, and is given as the mean ± SEM.

<sup>b</sup> Activity (treated)/activity (control), given as the mean ± SEM.

<sup>c</sup> *t*-tests applied to the activity ratios show: cAMP + ATP: stimulation of homogenate,  $P < 0.05$ ; infranantant,  $P < 0.05$ ; pellet, not significant; Iso/MIX: stimulation of homogenate,  $P < 0.02$ ; pellet,  $P < 0.05$ ; inhibition of infranantant,  $P < 0.05$ .

associated triglyceride lipase activity in treated cells accounted for most of the activity observed in the whole homogenate. The infranantant activity, which also showed a broad pH optimum around pH 7, was inhibited ap-

proximately 75% after drug treatment in agreement with the data presented in Table 1.

The absence of a peak or shoulder in the curves above pH 7 suggests that lipoprotein lipase (LPL), which shows

TABLE 4. Effects of cyclic AMP-dependent protein kinase on subcellular fractions from control and Iso/MIX-treated cells

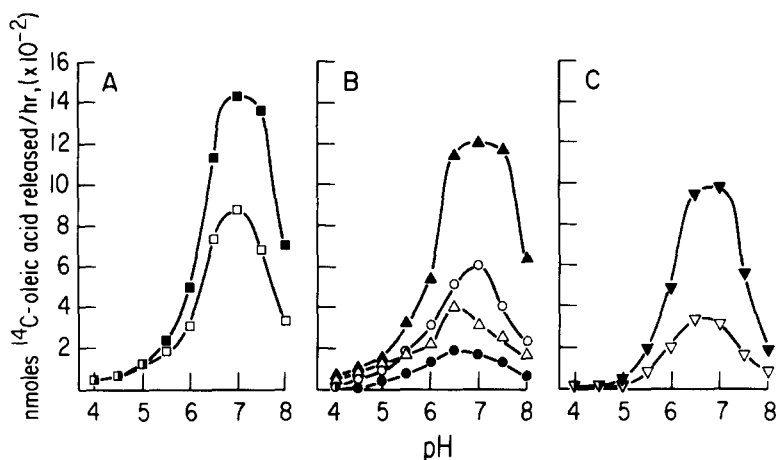
Subcellular Fraction	Cell Suspension	Fraction Incubation	Activity <sup>a</sup>	Activity Ratio:	
				Iso/MIX <sup>b</sup>	PK + ATP <sup>c</sup>
Infranantant	Control	None	409		
		Buffer	329		
		PK + ATP	515		1.6
	Iso/MIX	None	170	0.42	
		Buffer	146	0.45	
		PK + ATP	140		1.0
Pellet	Control	None	336		
		Buffer	303		
		PK + ATP	496		1.6
	Iso/MIX	None	1203	3.6	
		Buffer	1211	4.0	
		PK + ATP	1245		1.0

Control and Iso/MIX cell suspensions were prepared in two separate experiments. Subcellular fractions were prepared as in Table 1 for one experiment and as in Table 3 for the other. Aliquots of fractions were maintained on ice until assayed (fraction incubation: none), or incubated either with buffer alone (Buffer), or with added ATP plus catalytic subunit of protein kinase (PK + ATP) as described in Experimental Procedures.

<sup>a</sup> Activity is in nmol of [<sup>14</sup>C]oleic acid released per hour per  $10^7$  cells, and is given as the mean for the two experiments (each performed in duplicate).

<sup>b</sup> Activity (Iso/MIX)/activity (control).

<sup>c</sup> Activity (PK + ATP)/activity (buffer).



**Fig. 4.** The effect of pH on triglyceride lipase activity in subcellular fractions. Cells were homogenized on ice in 50 mM MOPS at pH 7.0 containing 10 mM  $MgCl_2$ . The homogenates and infranatant fluids were dialyzed at 4°C against a buffer of 5 mM MOPS and 5 mM sodium acetate at pH 6.8 and the pellets were resuspended in this buffer. Assays were performed in 50 mM sodium acetate buffer in the pH range of 4 to 6 and in 50 mM MOPS-HCl buffer in the pH range of 6 to 8. A, Homogenates ( $\square$ ,  $\blacksquare$ ); B, infranatant ( $\circ$ ,  $\bullet$ ) and pellet ( $\Delta$ ,  $\blacktriangle$ ) fractions; C, digitonin-solubilized extracts from pellets ( $\nabla$ ,  $\blacktriangledown$ ). Open symbols represent activity in fractions obtained from control cells and filled symbols represent those from Iso/MIX-treated cells. The experiment in panel C was performed on a separate cell preparation and assayed using a different batch of substrate emulsion. Activity is per  $10^7$  cells.

a pH optimum of 7.8–8.0 in these cells (43), does not significantly contribute to the activity measured under our assay conditions. Assays to which serum was added did show increments in pellet and homogenate activities which did not vary with cell treatment and were reversible with 1 M NaCl. Calculation showed LPL to be a minor component of the activity assayed in the absence of serum. Furthermore, treating homogenates with heparin did not lead to increased activity in any of the fractions.

Digitonin extracts of pellets from treated and untreated cells failed to disclose any lipase activity with an acidic pH optimum indicative of lysosomal lipase (Fig. 4C).

#### Effect of homogenization conditions on subcellular distribution of lipase

Since much of the published work on hormone-sensitive lipase has included EDTA and sucrose in homogenization buffers, the effects of these agents were investigated. Omitting  $MgCl_2$  or substituting EDTA for the  $MgCl_2$  in the basic homogenization protocol did have a quantitative effect. In control cell preparations, infranatant activity was lower and pellet activity was higher in the absence of  $Mg^{2+}$ . Thus the distribution of activity observed for the basal state was shifted strongly to the pellet fraction (infranatant:pellet; 65:35 (plus  $Mg^{2+}$ ); 30:70 (plus EDTA)). In contrast, after Iso/MIX stimulation, absence of  $Mg^{2+}$  in the homogenization buffer yielded higher infranatant activity and slightly lower pellet activity displacing the distribution towards the infranatant fraction (infranatant:pellet; 15:85 (plus  $Mg^{2+}$ ); 20:80 (plus EDTA)). Therefore the redistribution effect seen was

greatly diminished in the absence of  $Mg^{2+}$ . The MOPS/ $Mg^{2+}$  homogenization buffer was also compared to the 10 mM Tris-HCl/1 mM EDTA/0.25 M sucrose, pH 7.4, buffer system used by Kawamura et al. (32) in their studies of these cells. The results showed quantitative rather than qualitative differences, similar to those described above.

#### Properties of the pellet-associated lipase

After lipolytic stimulation of 3T3-L1 cells, the major portion of the triglyceride lipase activity was always associated with the pellet. For this reason the activity in the pellet fraction was partially purified and its properties were compared with those reported for the infranatant hormone-sensitive lipase of rat epididymal fat cells.

The pellet-associated lipase could not be effectively extracted by washing. When pellets were repeatedly washed with isotonic buffers containing either divalent metal ions or EDTA (buffer–pellet 70:1, v/v), each wash successively solubilized 20–30% and 10–20% of the activity remaining in pellets derived from control and Iso/MIX-treated cells, respectively. Increasing NaCl concentration caused increasing inhibition of the activity (above 0.5 M) without affecting solubilization. Several detergents were tested for ability to solubilize the enzyme. Triton X-100, Lubrol-PX, and Zwittergent 3-14, when used in the range of detergent–protein 1:2 to 2:1 (w/w) (see Experimental Procedures), solubilized 50–100% of the lipase activity present in the suspensions. However, the activities measured were elevated and both the difference between control and treated cell preparations and the ability to stimulate activity by phosphorylation were lost.



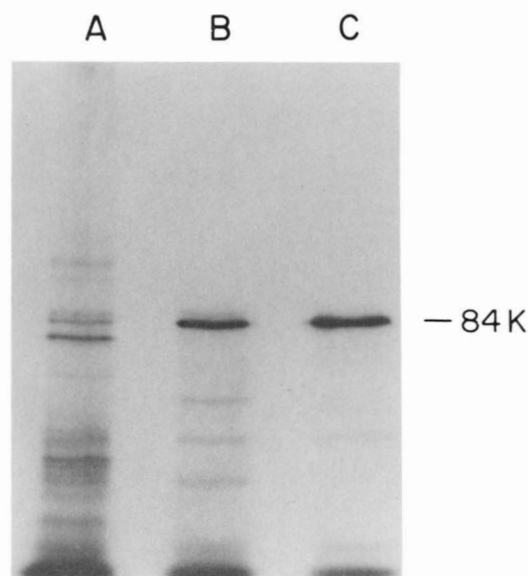
Digitonin (1 mg/ml) solubilized approximately 80% of the triglyceride lipase activity from the pellets of control or Iso/MIX-treated cells with preservation of both the difference in enzyme activity and the ability of cyclic AMP-dependent protein kinase to stimulate the triglyceride lipase activity from untreated cells. Only 40% of the protein was concomitantly solubilized yielding a 2-fold purification of the lipase.

The activity of this digitonin-solubilized lipase as a function of pH is shown in Fig. 4C. This result is similar to the data previously obtained for the infranatant enzyme from rat epididymal fat (10, 44) and from this cell line (32).

The digitonin-solubilized material obtained from control cells was fractionated by chromatography on DEAE-cellulose (DE-53). Triglyceride lipase activity was eluted from the resin between 40 and 65 mM NaCl at pH 7.9 in 5 mM sodium phosphate containing 0.1% digitonin. Fractions were adjusted to pH 7.0 immediately after collection. Two- to 4-fold increases in specific activity accompanied 30–45% recoveries. This activity could be stimulated 2-fold by treatment with catalytic subunit of cAMP-dependent protein kinase in the presence of ATP. In some experiments a second peak of lipase activity eluted between 150 and 200 mM NaCl; however, this activity could not be stimulated by protein kinase treatment and was not further purified. The enzyme activity eluted from DE-53 was concentrated 7-fold in dialysis tubing packed with dry Sephadex G-200 and applied to a column of Ultragel Aca-34 equilibrated and eluted with 25 mM sodium phosphate buffer containing 0.1 M NaCl and 0.1% digitonin. The enzyme eluted from this gel slightly behind IgG, in close agreement with the Stoke's radius obtained for the solubilized infranatant enzyme from rat epididymal fat (8). Activity was assayed using [ $^{14}\text{C}$ ]diolein emulsions prepared by ethanol dispersion (44) which yielded 30- to 50-fold greater activity than the triolein emulsion described.

#### Phosphorylation of the hormone-sensitive lipase in the pellet fraction

When either the pellet suspension, digitonin-solubilized pellet fraction, DE-53 eluate, or Aca34 fraction was incubated with the catalytic subunit of protein kinase in the presence of [ $\gamma\text{-}^{32}\text{P}$ ]-ATP, a labeled band,  $M_r = 84,000$ , was detected by SDS-PAGE. A similar result has been reported for infranatant hormone-sensitive lipase of rat epididymal fat (8–11) and 3T3-L1 cells (31, 32). In each case the  $^{32}\text{P}$  incorporated into the band, correlated with the specific activity of the lipase in the fraction. **Fig. 5** shows the result obtained for the digitonin-solubilized fraction, the DE-53 eluate, and the subsequent Aca34 fraction from a single enzyme preparation. Only a minor portion of the total protein comigrated with the radio-



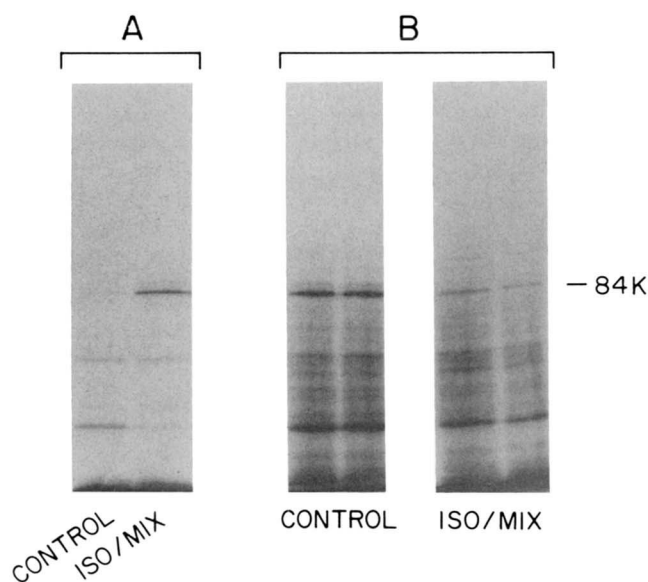
**Fig. 5.** Radioautography of samples from different steps in the purification of hormone-sensitive triglyceride lipase. Aliquots of a digitonin-solubilized pellet fraction (A), a 7-fold concentrate of the subsequent DE-53 eluate (B), and a 6-fold concentrate of the Aca34 fraction (C) were labeled using [ $\gamma\text{-}^{32}\text{P}$ ]-ATP and catalytic subunit of cAMP-dependent protein kinase as described. Approximately equal amounts of TCA-precipitable radioactivity were applied to each lane. SDS-PAGE was performed as described in Experimental Procedures. The dried gel was radioautographed at  $-20^\circ\text{C}$  using Kodak AA-5 film. Exposure time was 90 hrs.

labeled band at  $M_r = 84,000$ . A band with the same mobility was seen if cells were labeled with  $^{32}\text{P}_i$  and treated with Iso/MIX before the fractions were prepared (see Experimental Procedures). As with the subcellular fractions labeled with [ $\gamma\text{-}^{32}\text{P}$ ]-ATP, the 84,000-dalton band was more highly labeled after DE-53 fractionation.

Treatment of the cells with Iso/MIX increased phosphate incorporation into the 84-kdalton band. This was demonstrated directly in treated cells preincubated with  $^{32}\text{P}_i$  (**Fig. 6A**) and indirectly by the decreased ability to label the band with [ $\gamma\text{-}^{32}\text{P}$ ]-ATP and protein kinase in solubilized pellet fractions from treated cells (**Fig. 6B**).

#### DISCUSSION

During this study of lipolysis in the 3T3-L1 cell line, it was observed that triglyceride lipase activity translocated from the cytosolic to a particulate compartment in response to lipolytic stimulation of the intact cell (Table 1). Redistribution was invariably obtained, independent of the conditions employed for homogenization. It was dependent upon the concentration of the lipolytic agonist (**Fig. 3**) and was reversed upon withdrawal of the stimulus (Table 2). These properties correlate with the known characteristics of lipolysis. The ability of 8-Br-cAMP to



**Fig. 6.** Effect of Iso/MIX treatment of cells on the phosphorylation state of the lipase in the pellet fraction. After treating cells with Iso/MIX, and homogenization and digitonin extraction of the 40,000 g pellets as described, samples were partially purified on DE-53. Resin which had been equilibrated with 1 mg/ml digitonin in 5 mM sodium phosphate at pH 7.9 was suspended in two volumes of that buffer and gently stirred in a polypropylene tube. Digitonin-soluble extract was added dropwise (20 volumes of extract (0.4 mg protein/ml) per volume of packed resin). After 30 min the resin was recovered in a 5000 g pellet and washed twice with six volumes of 20 mM NaCl in the equilibration buffer. Enzyme activity was eluted in two volumes of the same buffer containing 75 mM NaCl. In (A) the cells were labeled with  $H_3^{32}PO_4$  prior to Iso/MIX treatment and the DE-53 eluates were immediately adjusted with sodium dodecyl sulfate buffer concentrate (see Experimental Procedures). In (B) the cells were not pre-labeled and the DE-53 eluates were exposed to  $[\gamma\text{-}^{32}P]\text{-ATP}$  and catalytic subunit of cAMP-dependent protein kinase (see Experimental Procedures); samples were run in duplicate. Electrophoresis was performed as described. The dried gels were radioautographed at  $-20^\circ\text{C}$  using Kodak SB-5 film for 66 hr (A) and 24 hr (B).

mimic the effects of Iso/MIX (Fig. 2) suggests that elevation of cyclic AMP in the cells is sufficient to bring about the redistribution of the enzyme. 8-Br-cAMP is also lipolytic when added to intact cells (data not shown). In the pellet fraction, the increase in triglyceride lipase activity observed in response to Iso/MIX treatment of intact cells was about 5 times that obtained by direct phosphorylation of this fraction from control cells (Table 4). These data suggest that elevation of cyclic AMP may affect the hormone-sensitive lipase in a more complex manner than previously considered.

Preferential release of fatty acid from a newly synthesized acylglycerol pool in adipose tissue (45) and cells (46) has been reported. Acylglycerol synthesis is catalyzed by acyltransferases which are tightly associated with the microsomal fraction in adipocytes (47). Similar microsomal localization has been shown in 3T3-L1 cells for acyltransferases, fatty acid CoA ligase, and phosphatidate phos-

phohydrolase (48, 49). Thus the redistribution of the activated (phosphorylated) hormone-sensitive lipase may play an important role in the control of lipolysis. This concept is compatible with previous reports using mature adipocytes. The neutral lipid stores in mature adipocytes are intimately associated with membranous structures (50, 51). Similar ultrastructural features have been shown in 3T3-L1 cells by Novikoff and co-workers (52). Benjamin and Clayton (53) showed that membranes could be found in the "fat cake" obtained from homogenates of rat adipocytes. The small surface to volume ratio of the lipid stores in mature adipocytes and the consequent large lipid to lipid-associated-membrane ratio, and the ability of some membrane structures to partition with the bulk lipid phase may have obscured the present findings in previous studies using mature cells and tissue. In fact, if we allow for such considerations, the observations of Khoo et al. (54) in rat adipocytes are compatible with our findings. They noted that 35–40% of the triglyceride lipase activity in control cell homogenates and 45–50% of that in homogenates of epinephrine-treated cells were lost to the fat cake upon fractionation. Furthermore, they noted that "recovery of activity in the  $S_1$  (16,000 g infranant) fraction was significantly and consistently lower in homogenates prepared from epinephrine-treated cells than in homogenates prepared from control cells." Their conclusion that the enzyme is cytosolic is warranted only if the not inconsiderable activity that partitions with the fat is disregarded.

Significant but variable amounts of triglyceride partitioned with the pellet in our preparations; in the absence of exogenous substrate, the pellet fraction was the most lipolytically active fraction (data not shown). Furthermore, the presence of this lipid in the pellet appears necessary for the lipase redistribution since it failed to occur when 3T3-L1 cells were grown in biotin-free medium. The lipase activity and distribution in untreated cells that had been grown in this medium were essentially the same as in untreated, normally grown cells, demonstrating that the enzyme was produced in the absence of substrate. Following Iso/MIX treatment of the biotin-free grown cells, a small (40%) increase in lipase activity in all subfractions was observed. This effect was similar to that observed after incubation of the homogenate with cyclic AMP and ATP. Perhaps, by analogy with the "glycogen particle," a substrate primer is required to seed a constellation of lipid-metabolizing enzymes.

After maximal lipolytic stimulation of 3T3-L1 cells, the major portion of the hormone-sensitive lipase was invariably found associated with the pellet fraction derived from the cells. The enzyme in untreated cells was more difficult to localize, its distribution in homogenates being fairly dependent upon homogenization conditions. Ka-

wamura et al. (32) proposed that the activity they observed in the pellet fraction (derived from untreated cells) may have been due to contaminating infranatant enzyme; however, significant activity in our preparations persisted through repeated washings—the enzyme from treated cells being more resistant to removal. Treating control cell homogenates with cyclic AMP and ATP activated the lipase without significantly altering either its subcellular distribution in 3T3-L1 cells (Table 3) or its interaction with endogenous substrate in rat adipocytes (17). The hormone-sensitive lipase isolated from the infranatant fraction of rat epididymal fat by Huttunen, Aquino, and Steinberg (55) is part of a large lipoprotein complex of density 1.08 to 1.09 and  $M_r = 7 \times 10^6$ , composed of 48% protein, 45% phospholipid, and 6% cholesterol. Further purification requires dissociation of this complex by detergents (8–11) or apolipoprotein A-I which possesses detergent-like properties (56). An association of this enzyme-containing lipoprotein complex with a substrate lipid-membrane complex in the intact unstimulated cell could be disrupted by homogenization. Thus the “translocation” of activated lipase from a cytosolic compartment to a particulate compartment might well be a convenient but artifactual representation of the shift from a weak, catalytically inactive interaction of the enzyme-containing lipoprotein complex described by Huttunen et al. (55) to a strong, catalytically active association with the lipid-membrane complex.

A similar translocation of CTP:phosphocholine cytidyltransferase in the regulation of phosphatidylcholine biosynthesis has recently been observed by Pelech and co-workers (57). They found that treating cultured rat hepatocytes with oleate, which stimulates the formation of phosphatidylcholine, resulted in the redistribution of the enzyme from the cytosol to a microsomal fraction.

The enzyme in subcellular fractions from both control and stimulated cells showed optimal activity around pH 7 and the magnitude of the effect on redistribution was also greatest when assayed at this pH (Fig. 5). This result, in agreement with Kawamura et al. (32), argues against lysosomal lipase playing a major role in hormone-stimulated lipolysis as proposed by Hulsmann and Stam (19).

The properties of the pellet fraction enzyme from 3T3-L1 cells are similar to those described for the hormone-sensitive lipase isolated from rodent adipocyte infranatant fractions with respect to phosphorylation in intact cells by lipolytic treatment (9, 58), Stoke's radius of the detergent solubilized form (8), apparent molecular weight of the  $^{32}\text{P}$ -labeled enzyme on SDS-PAGE (9–11), pH-activity profile (10, 44), and stimulation by cyclic AMP-dependent phosphorylation (6–8, 10, 17, 38, 44). This suggests that the enzyme previously described in the soluble fraction of rodent adipocytes is the same as that

responsible for the lipase activity observed to redistribute upon activation of lipolysis in 3T3-L1 cells. ■

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