Activation of hormone-sensitive lipase in extracts of adipose tissue

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ABSTRACT Rat adipose tissue was homogenized in 0.154 M KCl, and the supernatant fluid, obtained after centrifugation at 15,000 *g*, was extracted with benzene to remove triglycerides. Most of the lipase activity in the extracted fluid was precipitated with ammonium sulfate between 15 and 40% saturation. The specific activity of the lipase in this fraction was about three times that in the benzene-extracted supernatant fluid. The specific activity of the monoglyceride esterase was increased to a lesser extent.

Lipase activity in the benzene-extracted fluid and in the ammonium sulfate fraction was increased 15–45% by incubation with 0.3 mM ATP, 10 mM $MgCl_2$, and 0.03 mM cyclic AMP for 10 min before assay. None of these compounds alone or in combinations of two was as effective as all three together.

The specific activity of the 15–40% ammonium sulfate fraction prepared from fat cells exposed to epinephrine and glucagon was greater than that from portions of the same cell pool not exposed to hormones. In addition, the already elevated lipase activity in preparations from hormone-treated cells was not enhanced by incubation with ATP, $MgCl_2$, and cyclic AMP. Thus, it seems probable that the lipase activity in the ammonium sulfate fractions represents, at least in part, hormone-sensitive lipase.

SUPPLEMENTARY KEY WORDS monoglyceride esterase · cyclic AMP

THE so-called hormone-sensitive lipase of adipose tissue has proven difficult to characterize and to purify due at least in part to the fact that most of the enzyme activity tends to be associated with the large amount of triglyceride present in this tissue (1). Our earlier attempts to use solvents to remove lipid from homogenates or fractions thereof in order to obtain an enzyme free of triglyceride whose activity would be dependent on added substrate, led to extensive loss of activity and (or) an insoluble product (2). More recently, we found that when homogenates were prepared from fat pads depleted of stored triglyceride by prolonged fasting of donor animals, the fluid fraction obtained after centrifugation at 15,000 *g* contained very little lipid. With such extracts, hydrolysis of triolein-^{3}H was proportional to the amount of enzyme added. In addition, the lipase activity was enhanced by the addition of ATP, $MgCl_2$, and cyclic AMP, as originally reported by Rizack (3). In practice, however, it was difficult to obtain fat pads sufficiently depleted of glycerides to be usable before the rats became moribund. By starting with a similar supernatant fluid from homogenates of adipose tissue from fed rats and extracting it with benzene, we obtain a preparation which is essentially free of triglyceride and from which the bulk of the activity can be precipitated by ammonium sulfate between 15 and 40% saturation with about a three-fold increase in specific activity. Some characteristics of the lipase activity in this fraction and the effects of ATP, $MgCl_2$, and cyclic AMP on it are reported below.

MATERIALS AND METHODS

Lipase activity was assayed as previously described (4). Oleic acid-^{3}H-labeled triolein was purchased from Nuclear-Chicago Corporation, Des Plaines, Ill. It was purified by chromatography on Florisil F-100 (5), stored in anhydrous benzene, and repurified whenever necessary to maintain radiopurity (>99.8%). Unlabeled triolein (99% pure; Hormel Institute, Austin, Minn.)

Abbreviations: cyclic AMP, cyclic 3′,5′-adenosine monophosphate; ACTH, adrenocorticotrophic hormone.

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was purified on Alumina and stored in hexane. Mixed phospholipid prepared from rat liver was stored in benzene. Serum albumin (fraction V from bovine serum; Armour Pharmaceutical Co., Chicago, Ill.) treated by the method of Chen (6) to remove free fatty acids, was dissolved in 0.1 M phosphate buffer, pH 7.4, and the pH was adjusted to 7.4 with NaOH. The stock solution contained 200 mg of albumin per ml. The substrate was prepared by mixing appropriate volumes of the solutions of triolein and phospholipid such that the ratio of phospholipid to triolein was 1:5 (w/w). After the solvents were completely removed under N_2, 3.75 ml of potassium phosphate buffer, 0.1 M, pH 7.4, was added, and the mixture was sonified without cooling for 1.5 min. After the sonified mixture had cooled to room temperature, 1.25 ml of the stock albumin solution was added.

Enzyme preparations in potassium phosphate buffer, pH 7.4, with or without other additions in a total volume of 100 μl were incubated at 30°C for 10 min before addition of 100 μl of the substrate mixture (about 80,000 cpm). 10 min later the incubation period was terminated by the addition of 1.1 ml of carbonate buffer (Fisher Certified Standard Buffer, pH 10; Fisher Scientific Company, Pittsburgh, Pa.) and 3.2 ml of a mixture of chloroform–heptane–methanol 303:240:338. After mixing and centrifugation, 0.5 ml of the upper aqueous phase was added to 15 ml of scintillation mixture (toluene–ethylene glycol monomethyl ether 6:4 (v/v) containing naphthalene, 80 g/liter, diphenyloxazole, 4 g/liter, and *p*-bis [2-(5-phenyloxazolyl)] benzene, 50 mg/liter for radioassay.

The assay for monoglyceride esterase activity, referred to as monoglyceride lipase in earlier publications (1, 2), was similar to that previously described (7). Glycerol-^{3}H-labeled monoolein synthesized from glycerol-^{3}H and oleyl chloride was purified on silica gel (8). Unlabeled monoolein was purchased from the Hormel Institute. After evaporating the solvent from an appropriate quantity of monoolein (usually 10 μmoles of unlabeled carrier plus 3×10^6 cpm of monoolein-^{3}H), 4 ml of phosphate buffer, 0.1 M, pH 7.4, containing 10 mM sodium taurodeoxycholate (Sigma Chemical Co., St. Louis, Mo.) was added. The mixture was agitated using a Vortex mixer until it was clear; sometimes slight warming was required to accelerate solution of the monoolein. Assays were carried out in a total volume of 200 μl containing 100 μl of the substrate and other additions as indicated. After incubation at 30°C for 10 min, 3.2 ml of chloroform–methanol 3:2 (v/v), and 1.35 ml of a solution of KH_2PO_4, 20 mg/ml, was added. Following mixing and centrifugation, 0.5 ml of the upper phase was taken for radioassay. With the amounts of tissue fractions used in these studies, hydrolysis of monoolein was proportional to protein concentration in the assay system.

Lipase and esterase activities are expressed in milliunits equal to 1 nEq of ester hydrolyzed per min. All assays were carried out in duplicate, and every type of experiment was repeated several times with essentially the same results. The specific lipase and esterase activities, however, varied considerably from one preparation to another and, therefore, rather than attempt to combine data from several different preparations, data from individual representative experiments are reported.

All stock solutions used in preparation of substrates were stored at −20°C or −70°C. Protein was determined by the method of Lowry, Rosebrough, Farr, and Randall (9).

RESULTS

Preparation of Ammonium Sulfate Fractions

Fat pads, removed from rats (150–200 g) that had been allowed free access to food and water until they were decapitated, were homogenized in 2 volumes of 0.154 M KCl; the homogenate was then centrifuged for 30 min at 15,000 *g* (4°C). The clear pink fluid below the floating fat cake was carefully aspirated with a syringe and needle and mixed thoroughly with 2 volumes of redistilled benzene at room temperature in a glass-stoppered conical centrifuge tube. After centrifugation at 200 rpm for 5 min, the benzene phase was removed, and the same extraction procedure was repeated twice more. After the last extraction and aspiration of the bulk of the benzene, the remaining solvent was removed from the aqueous phase in a stream of nitrogen gas at 10–15°C. Particulate material was removed by centrifugation of the enzyme preparation at 15,000 *g* for 30 min (4°C). As shown in Table 1, about 15% of the protein of the supernatant fluid was removed in this step. Although it might appear from Table 1 that there was relatively little loss of lipase activity with benzene extraction, this probably is not the case since the original supernatant fluid contained triglyceride in amounts that would significantly decrease the specific activity of the labeled substrate employed in the lipase assay. Benzene extraction removed 85–90% of the lipid (hydroxamate-reactive ester) that was present in the untreated fluid (Table 2). The extracted lipid consisted chiefly of triglyceride with small amounts of lower glycerides and free fatty acids.

The clear fluid obtained after benzene extraction was fractionated by the addition of solid ammonium sulfate. The precipitates collected in the indicated concentrations of ammonium sulfate, were suspended in 0.05 M potassium phosphate, pH 7.4, with EDTA and dithiothreitol, each 0.5 mM, and dialyzed for 1–2 hr against two changes of the same solution. The clear supernatant fluid obtained after centrifugation at 15,000 *g* for 30 min (4°C) was assayed. In the experiment summarized in

TABLE 1 LIPASE ACTIVITY IN AMMONIUM SULFATE FRACTIONS

	Fraction	Total Protein		Lipase Activity		Esterase Activity	
		Before Dialysis	After Dialysis	Total	Per mg of Protein	Total	Per mg of Protein
		mg		*mUnits*			
1	Supernatant fluid, 15,000 *g*	37.1	38.6	162	4.2	178	4.6
2	Benzene-extracted fraction 1	31.7	32.3	133	4.1	136	4.2
	Ammonium sulfate fractions from fraction 2						
3	0–15%	0.8	0.4	3.6	9.0	2.2	5.6
4	15–40%	10.0	8.4	115	13.7	58	6.9
5	40–100%	18.0	16.7	8.4	0.5	26.7	1.6
	Sum of 3, 4, and 5	28.8	25.5	127.0		86.9	

The supernatant fluid was prepared from 3.5 g of tissue.

TABLE 2 REMOVAL OF LIPID FROM SUPERNATANT FLUID WITH BENZENE

Fraction	Lipid Ester	
	Expt. 1	Expt. 2
	nEq/mg protein	
Fluid before extraction	373	292
Fluid after extraction	36	29
Benzene extract	318	252

Supernatant fluid fractions (15,000 *g*) were prepared and extracted with benzene as described in the text. Samples of the fluid before and after benzene treatment were extracted as described by Dole (10), and appropriate samples of the isooctane phase were used for determination of hydroxamate-reactive ester (11). Values for the benzene extract are expressed per mg of protein in the fresh supernatant fluid.

Table 1, about 80% of the protein present in the benzene-extracted fluid was recovered in the three ammonium sulfate fractions after dialysis. Most of the lipase activity, usually 80–90%, was found in the fraction collected between 15 and 40% saturation, with about a three-fold increase in specific activity. The specific activity of monoglyceride esterase in this fraction was 50–100% greater than in the benzene-extracted fluid. Recovery of esterase activity in the experiment shown in Table 1 was unusually low. Most often it was in the range of 80–100%. The 15–40% ammonium sulfate fraction could be lyophilized or stored for several weeks at −80°C (after freezing it in a dry ice–ethanol bath) without loss of lipase activity or responsiveness to the cofactors as described below.

For most studies, the 15–40% ammonium sulfate fraction was prepared as described above. Similar fractions prepared from 100,000 *g* supernatant fluid had somewhat lower lipase activity (total and per mg of protein). Centrifugation of both types of ammonium sulfate fractions for 1 hr at 100,000 *g*, however, sedimented small amounts of high specific activity lipase, more from the 15,000 *g* fluid preparation, and yielded supernatant fractions with comparable lipase activity.

With the 15–40% ammonium sulfate fraction, hydrolysis of triolein-^{3}H was proportional to the amount of protein added over the range of 40–340 μg of protein; a similar proportionality was demonstrated for the benzene-extracted supernatant fluid. The effect of substrate concentration on lipase activity in this fraction is shown in Fig. 1. With amounts of enzyme protein below 200 μg per assay, 30 μg of substrate was used in a number of early experiments. More recently we have regularly used 60 μg of triolein-^{3}H per assay. A 10 min incubation period was used in these and all other experiments. The rate of lipolysis was constant for at least 15 min in the assay system.

Effect of ATP, Mg^{++}, and Cyclic AMP on Lipase Activity

In preliminary experiments with extracts of adipose tissue from fasted rats, it appeared that the magnitude of

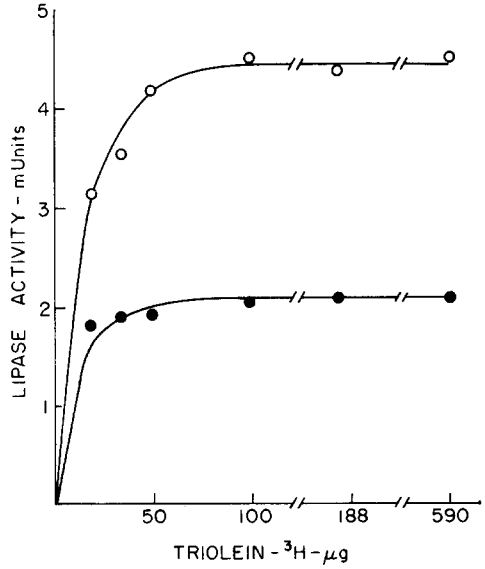

FIG. 1. Effect of substrate concentration on lipase activity. Each assay contained 168 (●) or 337 (○) μg of enzyme protein (15–40% ammonium sulfate fraction).

stimulation produced by ATP, $MgCl_2$, and cyclic AMP was greater when the initial lipase activity was permitted to decline by incubation before addition of cofactors (Table 3). Lipase activity declined about 30% in 3 hr in Experiment 2, and after incubation with ATP, $MgCl_2$, and cyclic AMP for 6 min it had returned to the zero-time level. The combination of three cofactors was usually more effective than any one or two alone in raising lipase activity to or above the original level. With the benzene-extracted supernatant fluid and the 15–40% ammonium sulfate fraction, however, as shown in Table 4, incubation at 30°C or dialysis at 4°C led to inactivation of lipase that was not apparently reversed by the subsequent incubation with cofactors. In fact, the percentage effect of the cofactors tended to decrease also, particularly with incubation at 30°C. Other experiments on the effect of cofactors on lipase activity were, therefore, carried out using only a 10 min incubation period, with or without cofactors, before assay of lipase activity.

Neither ATP, $MgCl_2$, nor cyclic AMP added individually over a wide range of concentrations influenced lipase activity in the 15–40% ammonium sulfate fraction or in the benzene-extracted supernatant fluid. In the latter, as shown in Fig. 2, maximal enhancement of lipase activity was achieved with 0.3 mM ATP, 10 mM $MgCl_2$, and 0.03 mM cyclic AMP. Despite the fact that the stimulation of lipase activity was seldom greater than 40%, these findings were consistently reproducible. We subsequently, therefore, used these concentrations of cofactors to test for stimulation of lipase activity in fractions prepared in various ways from the benzene-extracted supernatant fluid. As shown in Table 5, ATP, $MgCl_2$, and cyclic AMP together were more effective than any combination of two in stimulating lipase activity in the ammonium sulfate fraction. The percentage effect of these three cofactors on lipase activity in the 15–40% ammonium sulfate fraction was similar to or greater than that in the extracted supernatant fluid from which it was made. No stimulation of the small amounts of lipase activity in the other ammonium sulfate fractions was demonstrated.

The effect of pH on lipase activity and stimulation by cofactors is shown in Fig. 3. In this experiment, the same pH was used for the incubations before and during assay.

TABLE 3 LIPASE ACTIVITY IN SUPERNATANT FLUID FROM HOMOGENATES OF FAT PADS FROM FASTED RATS

Additions	Percentage of Zero-Time Lipase Activity	
	Expt. 1	Expt. 2
None	95 ± 5.2	71 ± 1.8
$MgCl_2$	115 ± 1.5	90 ± 3.7
ATP	101 ± 1.2	83 ± 1.3
Cyclic AMP		78 ± 2.4
$MgCl_2$, ATP	96 ± 6.6	91 ± 1.2
$MgCl_2$, cyclic AMP		98 ± 4.9
$MgCl_2$, ATP, cyclic AMP	135 ± 0.9	110 ± 5.2

Rats were fasted for 7 days before they were killed. Fat pads (ca. 45 mg each) were homogenized in 9 volumes of 0.154 M KCl. After centrifugation (15,000 *g*, 30 min, 4°C) only a very small amount of floating fat was visible. The clear fluid fraction was stored 1–2 days at −20°C before use. Samples (50 μl) of supernatant fluid were incubated in a total volume of 90 μl containing bovine serum albumin, 40 mg/ml, pH 7.4, for 45 min in Expt. 1, and 3 hr in Expt. 2, after which additions were made as indicated in a volume of 10 μl. The incubation continued for 6 min, and three samples of 20 μl each were then taken from each tube for assay of lipase activity. The concentrations of $MgCl_2$, ATP, and cyclic AMP were, respectively, 20 mM, 20 μM, and 20 μM in Expt. 1, and 20 mM, and 2 μM in Expt. 2. The data are presented as percentages of the activity at zero time without incubation, mean ± SEM (n = 3). The activity of these preparations was 0.5–0.8 mUnits/mg protein.

TABLE 4 INACTIVATION OF LIPASE AND EFFECT OF COFACTORS

Expt.	Treatment	Relative Lipase Activity			
		Time	Basal	Plus Cofactors	Cofactor Effect
		hr			%
1	Incubation, 30°C, phosphate buffer, 0.05 M, pH 7.4	0	100*	145	+45
		1	76	104	+37
		2	58	74	+28
		3	47	61	+30
		3.5	46	55	+19
2	Dialysis, 4°C, phosphate buffer, 0.05 M, pH 7.4; EDTA, 0.5 mM; dithiothreitol, 0.5 mM	0	100†	136	+36
		3	86	109	+27
		6	72	100	+39
		22	68	82	+21

Two different enzyme preparations were used in these experiments. After treatment as indicated, samples were incubated for 10 min with or without cofactors before assay of lipase activity. The concentrations of cofactors during this 10 min period were: $MgCl_2$, 10 mM; ATP, 0.3 mM; cyclic AMP, 0.03 mM.

* 3.2 mUnits/mg of protein (benzene-extracted supernatant fluid).

† 15.6 mUnits/mg of protein (15–40% ammonium sulfate fraction).

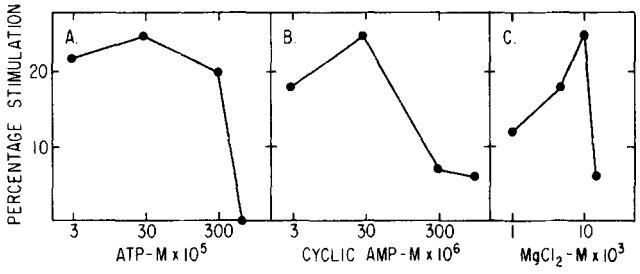

FIG. 2. Effect of concentration of ATP, $MgCl_2$, and cyclic AMP on lipase activity. Samples of benzene-extracted 15,000 *g* supernatant fluid (0.2 mUnits/mg of protein in the absence of cofactors) were incubated with cofactors for 10 min before addition of substrate and assay of lipase activity. Percentage increase in basal (no cofactors) lipase activity is reported. In *A* and *B*, the concentration of $MgCl_2$ was 10mM. In *A* and *C*, the concentration of cyclic AMP was 0.03 mM. In *B* and *C*, the concentration of ATP was 0.3 mM.

TABLE 5 EFFECT OF ATP, $MgCl_2$, AND CYCLIC AMP ON LIPASE ACTIVITY

Additions	Relative Lipase Activity	
	Frozen Enzyme	Lyophilized Enzyme
None	100	90
ATP, $MgCl_2$	111	97
ATP, cyclic AMP	87	66
$MgCl_2$, cyclic AMP	86	87
ATP, $MgCl_2$, cyclic AMP	119	124

The enzyme preparation was a 15–40% ammonium sulfate fraction, one portion of which had been lyophilized immediately after preparation and another stored at −70°C. The activity of the latter was 5.1 mUnits/mg of protein = 100% in the Table. The concentrations of ATP, $MgCl_2$, and cyclic AMP were, respectively, 0.3 mM, 10 mM, and 0.03 mM, during 10 min of incubation before addition of substrate and assay of lipase activity.

Lipase activity was maximal at pH 7.2–7.5, whereas the optimum for cofactor activation was apparently nearer pH 8.

We have only briefly investigated other media and conditions for preparation of homogenates. Results of two of these experiments are summarized in Table 6. Omission of benzene extraction resulted in preparations which were only slightly or not at all stimulated by cofactors. Homogenates prepared in water yielded fractions comparable in activity with those prepared in potassium chloride. From homogenates prepared in other media, we obtained ammonium sulfate fractions with lower specific activity and (or) responsiveness to cofactors. The use of ice-cold water or potassium chloride solution for homogenization appeared to offer no advantage over the room temperature media routinely used (data not shown).

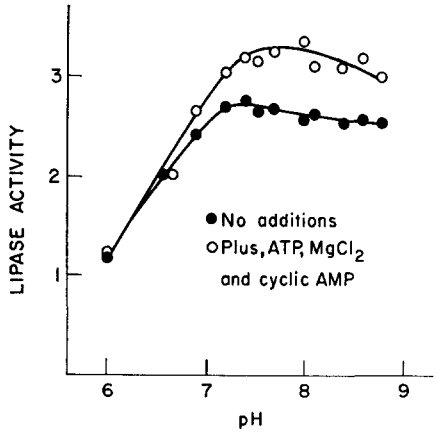

FIG. 3. Effect of pH on lipase activity and effect of cofactors. Samples of 15–40% ammonium sulfate fraction (11 mUnits/mg of protein) were incubated with or without 0.3 mM ATP, 10mM $MgCl_2$, and 0.03 mM cyclic AMP for 10 min at the indicated pH before assay of lipase at the same pH. Lipase activity in arbitrary units.

TABLE 6 EFFECT OF HOMOGENIZING MEDIUM ON LIPASE ACTIVITY IN 15–40% AMMONIUM SULFATE FRACTIONS

Expt. No.	Homogenization Medium	Protein	Lipase Activity		Cofactor Effect
		mg	*mUnits/mg protein*	*mUnits*	%
1	0.15 M KCl*	6.2	17.6	119	+ 7
	0.15 M KCl	5.6	14.6	80	+12
	Water*	6.1	25.8	158	+ 8
	Water	6.2	22.7	141	+15
2	Water	6.9	19.1	132	+14
	0.25 M Sucrose	5.5	24.6	135	0
	0.1 M NaF	4.4	10.0	44	+ 3
	0.01 M $MgCl_2$	3.9	10.2	40	+29

Homogenates were prepared at room temperature, each with 2.5 g tissue and 5 ml of medium. All media except the $MgCl_2$ contained 1 mM EDTA and 1 mM DTT. Ammonium sulfate was added as a saturated solution (pH 7.5) immediately after extraction without the usual attempt to remove residual benzene.

* No benzene extraction.

Attempts to separate with ammonium sulfate fractionation, the lipase activity from a hypothetical activating system that might be necessary for demonstration of cofactor stimulation, have been unsuccessful. As shown in Table 7, a large fraction of the lipase activity was precipitated by 30% ammonium sulfate, but the percentage stimulation by cofactors was the same in this fraction as it was in the 30–45% fraction. Corbin and Krebs (12) recently reported the presence of a cyclic AMP-stimulated protein kinase in a 30–45% ammonium sulfate fraction from fat cells. Addition of a fraction prepared as described by these authors did not enhance the percentage effect of the cofactors on lipase activity in a fresh 15–40% ammonium sulfate fraction prepared in the usual fashion. Neither did it restore activity or increase the effect of cofactors in the similar fractions which had been inactivated by dialysis for 20 hr as described in Table 4. The protein kinase preparation had very low lipase activity, and this was unaffected by ATP, $MgCl_2$, and cyclic AMP. When added to the 15–40% ammonium sulfate fraction, it slightly decreased the apparent rate of lipolysis with and without cofactors, perhaps due to dilution of the substrate with unlabeled triglyceride contained in the kinase preparation, but did not alter the percentage stimulation by the cofactors.

In early studies it was found that dithiothreitol, 0.5 mM, and EDTA, 0.5 mM, retarded, although they did not prevent, the apparently irreversible loss of lipase activity that occurred at 4°C or 30°C. These components were, therefore, usually included in dialysis media and in some experiments (Table 6) in homogenizing media. When enzyme preparations were incubated for 3 hr without these agents, subsequent incubation with 1 mM dithiothreitol for 10 min increased lipase activity, with and without cofactors, and esterase activity by 75–85%. Simi-

TABLE 7 EFFECT OF COFACTORS ON LIPASE ACTIVITY IN AMMONIUM SULFATE FRACTIONS

Expt. No.	Ammonium Sulfate Fraction	Protein	Lipase Activity	
			Basal	Plus Cofactors
		mg	*mUnits/mg protein*	
1	15–30%	2.7	24.9	34.2
	30–45%	7.1	4.0	5.8
2	15–30%	7.9	21.0	25.0
	30–45%	20.6	3.5	4.3

Ammonium sulfate fractions were prepared from benzene-extracted supernatant fluid (50,000 *g* in Expt. 1, 100,000 *g* in Expt. 2), and dialyzed as described in the text. In both experiments, the 15–30% and the 30–45% fractions contained about 6% and 16%, respectively, of the protein in the fluid.

TABLE 8 EFFECT OF LIPASE ACTIVITY IN AMMONIUM SULFATE FRACTIONS FROM FAT CELLS TREATED WITH ACTH AND GLUCAGON

Expt. No.	Cells Incubated for 5 min	Lipase Activity		Esterase Activity
		Basal	Plus Cofactors	
		mUnits/mg protein		
1	Without hormones	1.7	2.2	8.1
	With epinephrine and glucagon	3.2	3.3	9.7
2	Without hormones	0.9	1.1	7.2
	With epinephrine and glucagon	1.9	2.0	9.4

The concentration of epinephrine was 10 μg/ml and of glucagon 10 μg/ml. See text for description.

lar incubation with *N*-ethylmaleimide decreased lipase activity with and without cofactors, about 60%, and decreased esterase activity by 40%.

Caffeine (0.2 mM and 1 mM), theophylline (1 mM and 1.5 mM), sodium pyrophosphate (2 mM and 10 mM), AMP (0.5 mM and 1.25 mM), ADP (0.3 mM and 1 mM), and sodium fluoride (0.5 mM) had no significant effect on lipase activity, with or without cofactors, or on esterase activity.

Relationship of Lipase Activity Assayed to Hormone-Sensitive Lipase

Since most experiments were done with homogenates of whole fat pads, it was important to demonstrate that the lipase system studied was, in fact, present in the adipose cells. Fat cells were prepared as described by Rodbell (13), washed three times in Krebs-Ringer phosphate medium, suspended in an equal volume of the same medium, and frozen rapidly in a dry ice–ethanol bath. Benzene was then added, and after the cells had thawed, extraction and fractionation with ammonium sulfate were carried out. The specific activity of lipase and esterase, in these preparations, benzene-extracted fluid and ammonium sulfate fractions, was similar to that in the analogous fractions from whole adipose tissue as was the effect of the cofactors on lipase activity.

Several experiments were carried out in order to determine whether the amount of lipase activity in the fractions studied was influenced by treatment of the cells with epinephrine or other lipolytic hormones before freezing them. Data from two such experiments are presented in Table 8. It was striking that although the lipase activity in preparations from control cells was stimulated by the cofactors as usual, there was no effect of cofactors on the already elevated activity of the fractions from hormone-treated cells. In addition, the specific activity of the lipase in the ammonium sulfate fraction from hormone-treated cells was always greater than that from control cells. While lipase activity was approximately doubled by hormone treatment, monoglyceride esterase activity was increased only 20–30%. In other studies, we repeatedly found that the esterase activity was not stimulated by ATP, $MgCl_2$, and cyclic AMP under conditions in which lipase activity in the same fractions was enhanced 30–40%.

DISCUSSION

Although the 15,000–100,000 *g* supernatant fluid from homogenates of adipose tissue contains only a small portion of the total lipase activity (1), we have used this fraction as a source of hormone-sensitive lipase activity because most of the lipid could be removed from it with benzene without extensive loss of enzyme activity or formation of an insoluble product, and because the lipase activity in this fraction was consistently enhanced 15–45% by a combination of ATP, $MgCl_2$, and cyclic AMP. Lipase activity in the floating particulate fractions of homogenates prepared in 0.15 M KCl, which represents as much as 90% of the total homogenate activity, remained associated with large particles after benzene extraction, and no effects of cofactors on these preparations were demonstrable. We have repeatedly found that the lipase activity (total and per mg of protein) was greater in benzene-extracted supernatant fluid and in the 15–40% ammonium sulfate fractions from fat cells treated with so-called lipolytic hormones than it was in similar fractions prepared from samples of the same fat cells not exposed to hormones. Since the already elevated lipase activity in fractions from hormone-treated cells was not enhanced by addition of the cofactors which invariably stimulated lipolysis in preparations from control cells, we believe that the enzyme we are studying is the hormone-sensitive lipase.

It seems probable that the lipase activity in the 15–40% ammonium sulfate fraction is the same as that studied by Rizack (3). The magnitude of the stimulation by cofactors was, however, greater in the latter system. This

may be due in part to the fact that the concentrations of cofactors that we have employed are not truly optimal. The concentration of cyclic AMP, 0.03 M, is similar to that found to be optimal by Rizack, but that of ATP is higher, and of $MgCl_2$ lower. It will be noted that the concentrations of cyclic AMP and of ATP do not appear to be as critical in our system as in that of Rizack. We found, however, as he did, that sufficiently high concentrations of either ATP, $MgCl_2$, or cyclic AMP abolished activation. In an attempt to increase the magnitude of the cofactor effect, we have tried to lower the basal (without cofactor) activity but, as reported above, the loss of activity produced by prolonged dialysis or by incubation at 30°C of the ammonium sulfate fraction was for the most part irreversible. We observed no stimulation of lipase activity by caffeine or theophylline whether or not cofactors were present, whereas Rizack reported that these methylxanthines were stimulatory under all conditions. This difference may well be due to the low level of cyclic AMP phosphodiesterase activity in our preparations. Less than 10% of the added cyclic AMP was degraded in 10 min.

In preliminary experiments with the fluid fraction from homogenates of tissues from fasted rats, we had found that maximal activation occurred after about 10 min of incubation with cofactors and declined thereafter. This time course of activation was similar to that reported by Rizack (3). The lipase activation system which was studied in this laboratory several years ago (14) also required 6–10 min for maximal activation, after which lipase activity declined.[1] In contrast, with the benzene-extracted supernatant fluid or with the 15–40% ammonium sulfate fraction, in current studies, activation was maximal in less than 3 min, and the level of lipase activity then remained unchanged during further incubation for 15 min in the presence of cofactors. The failure to observe reversible inactivation on incubation of these fractions and the apparent stability of lipase activity in the presence of cofactors lead us to believe that in the course of preparation, perhaps in the extraction with benzene, the lipase inactivating system has been lost, whereas evidence from Rizack's studies and from those in our laboratory supports the view that such a system exists in fresh homogenates of adipose tissue.

Thus far, attempts to separate the lipase itself from a hypothetical activating system have been unsuccessful. We have found, however, that after incubation with ATP, $MgCl_2$, and cyclic AMP, activation of the lipase is maintained through dialysis (2 hr) or passage through columns of Sephadex G-75 or DEAE-Sephadex. These observations, together with the finding that the lipase from hormone-treated cells remains activated through ammonium sulfate fractionation and dialysis, lend support to the view that activation involves a chemical change in the lipase molecule, such as phosphorylation or possibly adenylation. Whether or not the protein kinase found by Corbin and Krebs (12) in fat cell extracts plays a part in lipase activation remains to be established. Our failure to observe enhanced activation by addition of a fraction prepared as described by these authors certainly does not weigh against this possibility.

The monoglyceride esterase activity, as previously reported (1, 2), was not greatly altered by exposure of cells to so-called lipolytic hormones, nor was it influenced by ATP, $MgCl_2$, and cyclic AMP. In addition, whereas lipase activity was concentrated in the 15–40% ammonium sulfate fraction, the specific activity of monoglyceride esterase in this fraction was only slightly greater than that of the whole benzene-extracted fluid. It should be pointed out that monoglyceride esterase activity may well be associated with more than one enzyme protein. This activity was distributed through several ammonium sulfate fractions, and in pilot experiments we have found that it is also distributed in many fractions when the 15–40% ammonium sulfate fraction is chromatographed on DEAE-Sephadex.

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