Rat adipose tissue lipases: hormone-sensitive lipase activity against triglycerides compared with activity against lower glycerides

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SUMMARY The hormone-sensitive lipase of rat epididymal adipose tissue is tightly bound to the endogenous tissue lipids which float during centrifugation of homogenates. This endogenous lipid appears to provide the enzyme with saturating levels of triglyceride substrate. A method is described for extracting virtually all of this endogenous substrate with ether, after which about 25% of the original hormone-sensitive enzyme activity can be recovered in an insoluble fraction that now sediments. When assayed with triolein-C^{14}, the activity of this fraction prepared from tissues incubated with epinephrine is twice that of preparations from tissues incubated without hormone.

The activity toward monoglycerides and diglycerides, both in the whole homogenate and in the ether-extracted preparation, is more than an order of magnitude greater than that toward triglycerides at equal substrate concentrations. Recovery of activity toward lower glycerides was excellent in the ether-extracted preparations, occasionally almost quantitative. Activity against monoolein or diolein was not affected by preincubation of the tissue with epinephrine.

The properties of the enzymes in the ether-extracted preparation suggest that the hormone-sensitive triglyceride lipase activity is distinct from the monoglyceride and diglyceride lipase activities. Whether the latter two are referable to the same or different enzymes cannot yet be decided.

STUDIES RECENTLY REPORTED from this laboratory have indicated the presence in rat adipose tissue of at least two lipases distinct from lipoprotein lipase (1). One of these, a so-called *hormone-sensitive* lipase, increases strikingly in activity when the intact, preincubated tissue is briefly exposed to catecholamines, ACTH, thyroid-stimulating hormone, or glucagon and is presumably related closely to the activity studied previously in a number of laboratories (2–5). The other enzyme, recoverable in acetone powders, is at least an order of magnitude more active in the hydrolysis of monoglycerides than it is in the hydrolysis of triglycerides. Its activity does not change appreciably when the tissue is preincubated and exposed to hormones. The two activities are further distinguished by differences in pH optima, temperature–activity curves and responses to inhibitors (1). In rat and in rabbit adipose tissue homogenates, both enzyme activities are found predominantly in the fatty layer that floats to the top of the tube during centrifugation. The triglyceride-splitting enzyme system appears to be saturated with respect to substrate, no increase in rate of FFA release being obtained on addition of any of several triglyceride preparations.

The present paper describes a procedure for separating the hormone-sensitive, triglyceride-splitting enzyme from the bulk of its endogenous substrate, thus obtaining a preparation whose activity is a function of the concentration of added substrate. It is shown that this activity against added substrate is, like that of unfractionated adipose tissue preparations, stimulated by prior exposure of the intact tissue to several "lipolytic" hormones. This activity is further characterized and contrasted with activities against mono- and diglycerides, which are also present in the defatted homogenate fractions.

MATERIALS AND METHODS

Enzyme Preparations

Epididymal fat pads from Sprague-Dawley rats (150–250 g) were used as the source of adipose tissue. The

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animals were fed ad libitum on Ralston Purina rat chow. Adipose tissue was homogenized at room temperature by hand in a glass tissue grinder in 0.1 M phosphate buffer, pH 7.0 (2 ml/g of fat). In some studies this homogenate, without further treatment, was centrifuged at 15,000 × *g* for 30 min at 2°. This separated the homogenate into a top layer containing the bulk of the fat, a clear middle layer, essentially free of fat, and a pellet of sediment at the bottom. Most of the lipolytic activity was associated with the top layer of fat, in agreement with previous results (1).

Several procedures were explored in an effort to separate the lipolytic activity from the large amount of endogenous fat with which it associates so closely. The following procedure was highly effective in that it removed over 95% of the fat, and allowed recoveries of 40% or more of the lipolytic activity of the whole homogenate against mono- and diglycerides and about 25% of the activity against triolein-C^{14}. The whole adipose tissue homogenate was extracted at room temperature with 3–5 volumes of peroxide-free ether in a glass-stoppered 15 ml centrifuge tube. After separation of the phases, the ether was aspirated and the procedure was repeated twice. After the third extraction, the tube was centrifuged at 1500 rpm for a few minutes, after which the ether was carefully aspirated. Then nitrogen was bubbled through the homogenate until no odor of ether could be detected. This ether-extracted homogenate was then exposed to differential centrifugation at +2°. Three fractions were recovered. Sediment 1 was spun down at 1200 × *g* for 15 min; sediment 2 was recovered by centrifuging the supernatant fraction decanted from sediment 1 at 15,000 × *g* for 30 min; and finally sediment 3 was collected from the second supernatant fraction by centrifuging at 105,000 × *g* for 1 hr. For most enzyme assays, however, and unless otherwise noted, the ether-extracted homogenate was centrifuged immediately and only once at 105,000 × *g* for 1 hr, and the sediment was resuspended for assay (hereafter referred to simply as *ether-extracted sediment*). The sedimented pellets were resuspended in 0.1 M phosphate buffer, pH 7.0, so that 1 ml of suspension represented approximately 500 mg of original adipose tissue. Protein was measured by the method of Lowry et al. (6).

Assay of Lipolytic Activity

Assays were carried out in 12-ml centrifuge tubes containing 0.5 ml of 20% bovine serum albumin (pH adjusted to 6.8), 0.4 ml of 0.5 M phosphate buffer (pH 6.8), distilled water, substrate, and enzyme in a final volume of 2 ml. Immediately after addition of enzyme and mixing, 1 ml of the incubation mixture was pipetted into 5 ml of Dole's extraction mixture (7), modified by substituting isooctane for heptane. After incubation for varying times at 37°, the reaction was terminated by addition of 5 ml of Dole's mixture to the assay tube. Free fatty acids (FFA) were determined by titration (7). The difference in fatty acid content between the incubated sample and the zero-time sample was taken as a measure of the lipolytic activity of the tissue preparation. Glycerol was determined enzymatically (8) or by Korn's modification (9) of the method of Lambert and Neish (10).

The lipolytic activity of the ether-extracted sediment (and of the whole ether-extracted homogenate in the experiments summarized in Table 5) against highly purified triglycerides was too low to be measured accurately by titration. Instead, a radioactive assay utilizing triolein-C^{14} or tripalmitin-C^{14} was employed (label in the fatty acid moieties). These were diluted with nonradioactive triglycerides and used at final concentrations comparable to those used for mono- and diglyceride emulsions. Incubations were performed as described above, and the same extraction procedures used. FFA were extracted from an appropriate sample of the isooctane phase using alkaline ethanol as described by Borgström (11). The alkaline ethanol was then acidified and the FFA were re-extracted into isooctane. The separated triglycerides and fatty acids were quantitatively transferred to scintillation counting vials. The isooctane was evaporated and 15 ml of 0.5% diphenyloxazole in toluene was added. Samples were counted in a Packard liquid scintillation spectrometer. The FFA radioactivity recovered from the incubated sample was corrected for that found in the zero-time mixture. The fraction of triglyceride radioactivity released was multiplied by the total number of microequivalents of fatty acid in labeled triglyceride substrate at zero-time to derive "μeq of FFA formed."

The results obtained by this procedure were checked on several occasions by using thin-layer chromatography (TLC) to separate FFA and triglycerides and to rule out any significant accumulation of radioactivity in lower glycerides. After ascending chromatography (hexane–diethyl ether–glacial acetic acid, 70:30:1), the triglyceride and fatty acid spots were located by exposing the plate to iodine vapor. After the iodine had been removed by sublimation, the circumscribed silicic acid areas were transferred quantitatively by careful scraping into counting vials. Fifteen milliliters of a thixotropic scintillation counting suspension (34.7 g of Cab-O-Sil, 5 g of 2,5-diphenyloxazole, and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene per liter of toluene) was added and the samples were counted as before. Assays performed according to this chromatographic procedure gave results that agreed with those

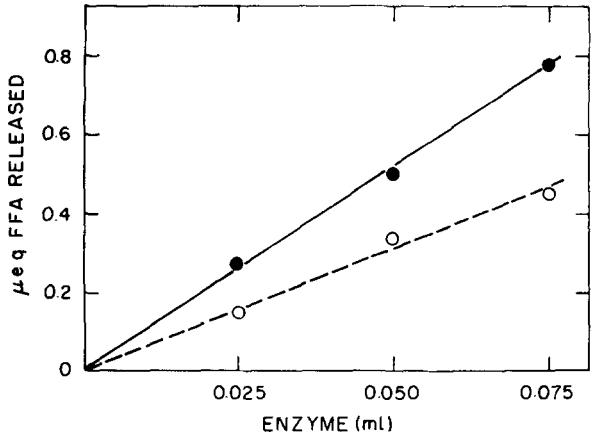

FIG. 1 FFA production as a function of enzyme concentration with mono- and diolein as substrates. Incubated for 10 min with indicated volume of resuspended sediment from ether-extracted homogenate. Each milliliter of enzyme preparation corresponds to approximately 500 mg of adipose tissue. ○ = diolein, 1 mg/ml. ● = monoolein, 1 mg/ml.

obtained by extraction. Tissue suspensions boiled for 10 min were used in control experiments.

Substrate Preparations

Monoolein, monopalmitin, diolein, dipalmitin, triolein, tripalmitin, and trilinolein (purity >99%) and triolein (purity about 90%) were purchased from the Hormel Institute, Austin, Minn. Monolaurin and dilaurin were purified on silicic acid from commercial preparations. All of the commercial diglyceride preparations were found by TLC to contain predominantly the 1,3-isomer.

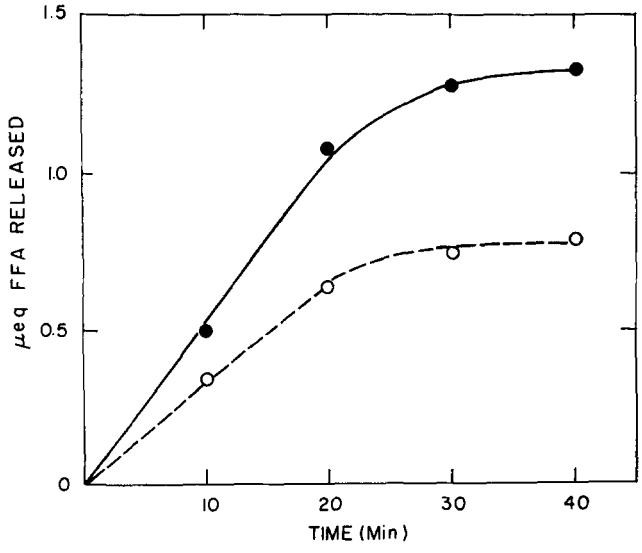

FIG. 2 FFA production as a function of incubation time with mono- and diolein as substrates. Each assay tube contained 0.05 ml of resuspended sediment from ether-extracted homogenate (equivalent to approximately 25 mg adipose tissue) in a total volume of 1 ml. ○ = diolein, 1 mg/ml. ● = monoolein, 1 mg/ml.

A sample of 1,2-diolein was generously provided by Dr. F. H. Mattson, Research Division of Procter and Gamble Co., Cincinnati, Ohio. TLC showed this to contain only a very small amount of the 1,3-isomer. Triolein-C^{14} and tripalmitin-C^{14} were obtained from New England Nuclear Corporation, Boston, Mass. The radioactive triglycerides were purified on silicic acid before use. It was shown by TLC that at least 99.5% of the radioactivity was located in the triglyceride region.

For use as substrates, the glycerides were emulsified in water, usually in a concentration of 4 mg/ml. Emulsification was accomplished by ultrasonication for a few minutes (Model S-FJ Sonifier, Branson's Instruments, Inc., Stamford, Conn.). The emulsions could be stored at −15° and after thawing they were again subjected to ultrasonication before use. Bovine serum albumin (Fraction V) was purchased from the Armour Pharmaceutical Co., Kankakee, Ill.

RESULTS

Assay Conditions for Lipolytic Activity in 105,000 × g Sediment of Ether-Extracted Homogenates

As shown in Fig. 1, there was a direct proportionality between enzyme concentration and enzyme activity, using 99% pure mono- and diolein as substrates. In Fig. 2 is shown the time course of lipolysis using these substrates. The rates of hydrolysis were constant for about 20 min and then declined. At 30 min almost 50% of the monoolein had been hydrolyzed and about 25% of the ester bonds in the diolein had been hydrolyzed. In most subsequent assays of activity against mono- and diglycerides, the incubation time used was 10 min.

If bovine serum albumin was excluded from the assay mixture, the activity decreased by about 75%. In some assays, the ratio of free fatty acid to glycerol released was measured by using the enzymatic method for glycerol determination (8) and was found to be about 1 and 2 for hydrolysis of mono- and diolein, respectively.

In preliminary studies, it was found that impure commercial triglyceride preparations, for example 90% pure triolein (Hormel Institute), gave a small but significant release of fatty acids. However, a maximum of only 5–6% of the fatty acid equivalents of the substrate could be released even using high concentrations of enzyme and prolonged times of incubation. Furthermore, the fatty acid:glycerol ratio was always significantly less than 3. When this triolein was subjected to TLC, it was found to contain diolein as a significant impurity, and this was probably being hydrolyzed rapidly and almost completely (see below).

There was virtually no hydrolysis of triolein purified by silicic acid chromatography or of the 99% pure triolein purchased from the Hormel Institute. The amount of fatty acid released was too low to be accurately measured by titration. This was also true for 99% pure tripalmitin and trilinolein. With the more sensitive isotopic method already described, however, activity against triolein-C^{14} was readily measured. As shown in Fig. 3, the release of oleic acid-C^{14} was proportional to the amount of enzyme added (30-min incubations). Figure 4 shows the time course of this hydrolysis. In most assays, only 2–6% of the label was released as free fatty acid. In control incubations with boiled enzyme preparations, there was virtually no release of fatty acid (<0.05%).

Distribution and Recovery of Lipolytic Activity

Freshly prepared adipose tissue homogenates were centrifuged at 15,000 × *g* for 30 min at 2°. The fat-poor "middle layer" (below the floating fat cake) and the sediment were separately assayed for lipolytic activity against mono- and diolein. Only about 10–15% of the activity of the whole homogenate against these substrates was recoverable in each of these fractions. Vaughan et al. (1) have previously shown that most of the "hormone-sensitive lipase" activity against triglycerides and also most of the activity against monostearin is similarly associated with the floating fat layer of adipose tissue homogenates after centrifugation.

The unfractionated ether-extracted homogenate retained 40% or more of the activity of the untreated homogenate against mono- and diolein and 22% of the original activity against triolein-C^{14}. When this ether-extracted homogenate was centrifuged at 105,000 × *g* for 1 hr, most of the activity was found in the sediment, less than 10% remaining in the supernatant fraction (Table 1). Most of the sedimentable activity came down with centrifugation for only 15 min at 1200 × *g*. The lighter sediments (15,000 × *g* and 105,000 × *g*) had specific enzyme activities not grossly different from that of the 1200 × *g* sediment, but they contained much less protein. The 105,000 × *g* supernatant fraction, on the other hand, contained over 40% of the total protein of the homogenate and the specific enzymatic activity was very low.

Even though the extraction procedures removed over 98% of the endogenous fat, the unfractionated ether-extracted adipose tissue homogenates often showed considerable "blank" lipolysis, with a release of up to 0.6 μeq of FFA per hr/mg protein in the absence of added substrate. The FFA:glycerol ratio was 2.4. On the other hand, in the 105,000 × *g* sediment of ether-extracted homogenates, which was used for most assays, this blank lipolysis was very small (about

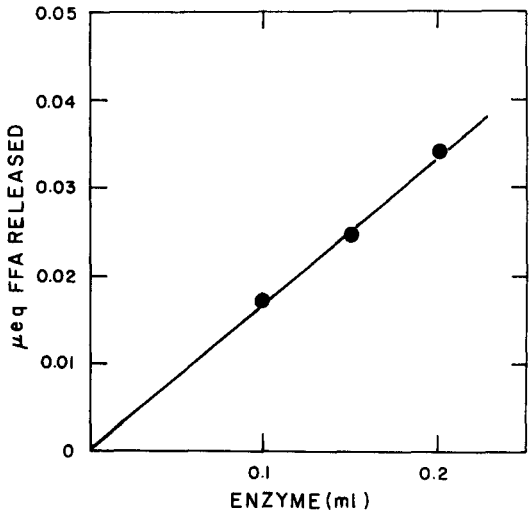

FIG. 3 Assay of activity against triolein-C^{14}. Incubated for 30 min with indicated amount of resuspended sediment from ether-extracted homogenate (approximately 500 mg adipose tissue represented in each milliliter of suspension).

0.060–0.12 μeq/hr per mg protein). The total hydroxamate-positive ester remaining in the unfractionated ether-extracted homogenate was about 40 μmoles/g of tissue treated. The 105,000 × *g* sediment retained only 9 μmoles/g of tissue treated.

Comparison of Activities Against Mono-, Di- and Triglycerides

In Table 1, it may be seen that the activity against mono- and diolein in the ether-extracted adipose tissue homogenate was 30–50 times greater than that against triolein. As is evident also in Table 1, the activities against mono-, di-, and triolein partitioned very similarly upon differential centrifugation.

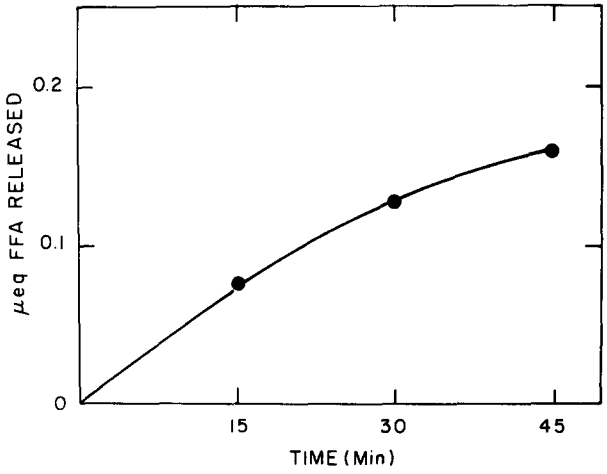

FIG. 4 Time course of hydrolysis of triolein-C^{14}. Resuspended sediment (0.25 ml.) from an ether-extracted adipose tissue homogenate incubated with 1 mg of triolein-C^{14} in a total volume of 1 ml for indicated times.

TABLE 1 DISTRIBUTION OF LIPOLYTIC ACTIVITY IN FRACTIONS DERIVED FROM ETHER-EXTRACTED HOMOGENATES OF ADIPOSE TISSUE

Ether-Extracted Homogenate	Specific Activity			Total Activity*		
	Monoolein	Diolein	Triolein-C^{14}	Monoolein	Diolein	Triolein-C^{14}
	μeq/hr/mg protein					
Whole homogenate	12.5	7.7	0.20	100	100	100
Sediment 1 (1,200 × *g*)	14.0	9.3	0.37	44	47	72
Sediment 2 (15,000 × *g*)	21.6	5.4	0.23	12	4.6	7.2
Sediment 3 (105,000 × *g*)	11.3	6.7	—†	6.7	6.7	—†
Supernatant fraction‡	1.6	1.1	0.03	6.8	6.8	7.0
105,000 × *g* sediment (1–3)‡	20.5	14.4	0.20	87	99	53.5

All substrates were used at a final concentration of 1 mg/ml. Activity against the lower glycerides was determined by titration of FFA released; activity against triolein-C^{14} was determined from measurement of percentage of triglyceride radioactivity released as described under Methods.

* Relative to that of the unfractionated ether-extracted homogenate, arbitrarily set equal to 100.

† Activity below level of accurate measurement.

‡ Whole ether-extracted homogenate (prepared from a different pool of fat pads) immediately centrifuged at 105,000 × *g* for 1 hr instead of being successively centrifuged in steps as above.

Stability of Enzyme Preparations

The ether-extracted sediment lost its lipolytic activity over a few days when stored in the frozen state. The activity against triolein decreased by about 90% after only 2 days at −15°, while the activity against mono- and diolein decreased about 50% over the same time. After a week, virtually no activity against any of the glyceride substrates remained.

The temperature stability of the system was similar for activity against mono-, di-, and triolein. Incubating the tissue preparation at 60° for 10 min resulted in loss of all activity. Heating at 50° for 10 min destroyed about 50% of its activity against all three glycerides.

The effect of pH on hydrolysis of mono-, di-, and triolein was investigated in the pH range from 5.5 to 9.5. This range was covered using phosphate and tris–HCl [tris = tris(hydroxymethyl)aminomethane] buffers. In Fig. 5 are shown the results for mono- and diolein, and in Fig. 6 the results for triolein. For mono- and diolein, the optimal pH was around 6.5–7.0. The enzyme was more active in tris–HCl buffer than in phosphate buffer at the same pH (7.5). In contrast, the hydrolysis of triolein-C^{14} in tris–HCl buffer was only one-fourth that in phosphate buffer of the same pH (7.5). The pH optimum for hydrolysis of triolein-C^{14} was at 6.5. Although the pH optima for triglyceridase activity and for lower glyceridase activities are not very different in these ether-extracted preparations, the shapes of the curves are clearly different, that for triglyceridase activity being much sharper.

TABLE 2 EFFECT OF NaF AND ISOPROPANOL ON LIPOLYTIC ACTIVITY OF ETHER-EXTRACTED SEDIMENT

Addition	Percentage Inhibition of Activity Against		
	Monoolein	Diolein	Triolein-C^{14}
NaF, 0.0125 M	31	84	64
0.05 M	62	91	76
0.2 M	84	92	
Isopropanol, 25 μl/ml	0	0	62

Each tube contained 125 μmoles of phosphate buffer, pH 6.8; emulsified substrate, 1 mg/ml; 50 mg of bovine serum albumin per mg of emulsified substrate; inhibitors as indicated; and enzyme (sediment 1–3, see Table 1) in a final volume of 1.0 ml. The incubation time was 10 min for mono- and diolein and 30 min for triolein-C^{14}.

Inhibitors

The lipolytic activity against all substrates was significantly inhibited by NaF at a concentration of 1.25×10^{-2} M, as seen in Table 2, although the inhibition was

TABLE 3 HYDROLYSIS OF VARIOUS SUBSTRATES BY ETHER-EXTRACTED SEDIMENT

Substrate	Relative Activity*
Monoolein	100
Monopalmitin	37
Monolaurin†	87
Diolein (mainly 1,3-isomer)‡	73
Diolein (mainly 1,2-isomer)§	43
Dipalmitin	<3
Dilaurin	37
Triolein-C^{14}	1
Tripalmitin-C^{14}	0

Each tube contained 125 μmoles of phosphate buffer, pH 6.8; 50 mg of bovine serum albumin; 1 mg of substrate; enzyme preparation (105,000 x *g* sediment of ether-extracted homogenate); and water to a final volume of 1.0 ml. Incubated 10 min, except in the case of triolein and tripalmitin, where incubation time was 30 min.

* Activity against monoolein arbitrarily set equal to 100.

† Corrected for incomplete recovery of lauric acid in the organic phase (12).

‡ Predominantly the 1,3-isomer according to TLC.

§ Contained small amount of the 1,3-isomer according to TLC.

somewhat less marked against monoolein. Isopropanol at a concentration of 25 μl/ml has been reported to inhibit the activity of "hormone-sensitive lipase" against endogenous substrate by about 75% (1). This concentration of isopropanol inhibited the activity of the ether-extracted sediment against triolein by 62%, whereas no inhibition was found when monoolein or diolein were used as substrates (Table 2).

Substrate Specificity

Table 3 shows the activity against glycerides of different fatty acid composition. It is seen that monolaurin was hydrolyzed almost as rapidly as monoolein, and monopalmitin at only about one-third that rate. 1,2-Diolein was a poorer substrate than commercial 99% pure diolein, which contained predominantly the 1,3-isomer according to TLC. The 1,2-diolein contained some 1,3-isomer and the recorded rate of hydrolysis may in part be due to splitting of this 1,3-isomer. Dipalmitin was a very poor substrate under the conditions of assay, whereas dilaurin was hydrolyzed one-half as rapidly as commercial diolein. There was no detectable release of fatty acid-C^{14} from tripalmitin-C^{14}. The rate of release of fatty acid from pure trilinolein and tricaprylin was too slow to be accurately measured.

Effect of Hormones on Lipolytic Activity

Previous studies have shown that lipolytic activity is decreased in whole homogenates made from epididymal fat pads preincubated for 1–3 hr (1, 3). If one of the so-called "lipolytic hormones," for example ACTH or epinephrine, was added to the incubation medium a

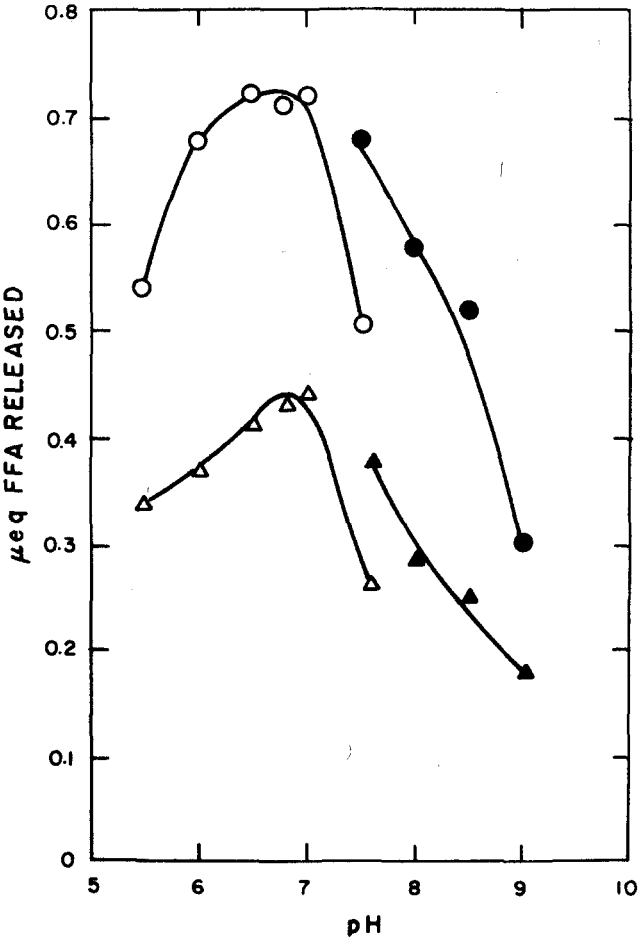

FIG. 5 Effect of pH on FFA production using mono- and diolein as substrates. Each assay tube contained 125 μmoles of buffer of indicated pH, 50 mg of bovine serum albumin, 1 mg of substrate, 0.05 ml of resuspended sediment from ether-extracted homogenate (representing about 25 mg of adipose tissue), and water to a total volume of 1 ml. Incubated for 10 min. ○● = monoolein, 1 mg/ml: △▲ = diolein, 1 mg/ml. Open symbols indicate sodium phosphate buffer, and solid symbols tris–HCl buffer.

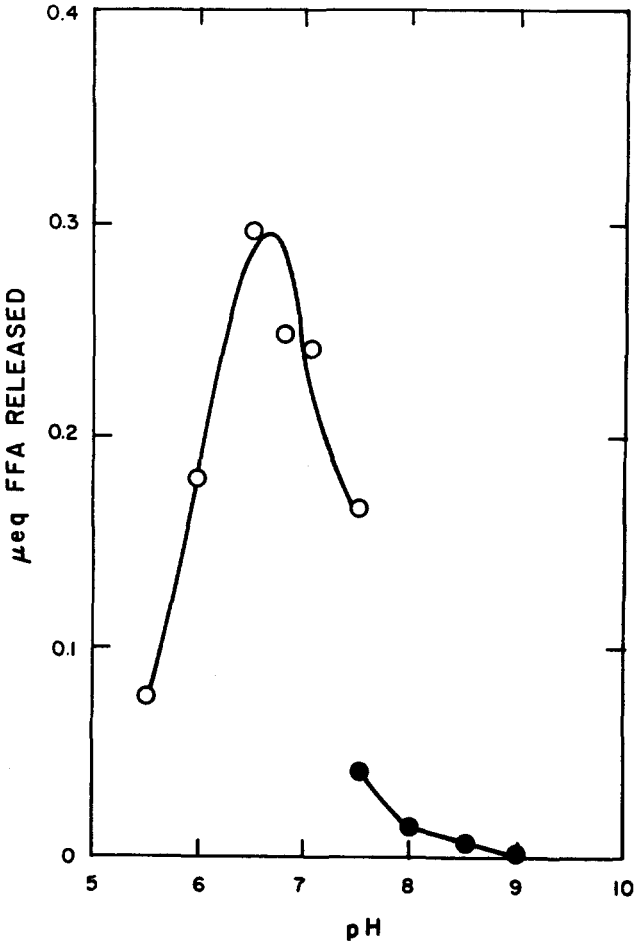

FIG. 6 Effect of pH on FFA production with triolein-C^{14} as substrate. Adipose tissue homogenate prepared in 0.154 M KCl, 2 ml/g, and ether-extracted. Each assay tube contained 125 μmoles of buffer of indicated pH, 50 mg of bovine serum albumin, 1 mg of substrate, 0.25 ml of unfractionated ether-extracted homogenate (representing about 125 mg of adipose tissue), and water to a final volume of 1 ml. Incubated 30 min. ○ = phosphate buffer; ● = tris–HCl buffer.

TABLE 4 EFFECT OF EPINEPHRINE ON LIPOLYTIC ACTIVITY BEFORE AND AFTER ETHER EXTRACTION

Expt. No.	Before Ether Extraction				After Ether Extraction			
	Monoolein Added, 1 mg/ml		Diolein Added, 1 mg/ml		Monoolein Added, 1 mg/ml		Diolein Added, 1 mg/ml	
	Control	Δ Due to Hormone	Control	Δ Due to Hormone	Control	Δ Due to Hormone	Control	Δ Due to Hormone
	μeq of FFA per g / hr							
1	207	−52	127	−9	200	−12	114	+4
2	225	−15	159	−18	234	+4	198	−70
3	180	+5	135	−2	185	+46	95	+72
4	161	+30	129	−2	206	−22	136	−2
5	153	−26	97	+37	211	+7	132	+19
Mean	186	−12	129	+1	207	+4.6	135	+4.6
SE		±13.9		±9.4		±11.6		±22.8

For each experiment four pairs of fat pads were incubated for 3 hr in 3 ml of Krebs bicarbonate medium, containing bovine serum albumin, 30 mg/ml. To one flask of each of the four pairs, 5 μg of epinephrine was added 10 min before the end of incubation. Two homogenates were then prepared, one with control and one with epinephrine-treated pads in 0.1 M phosphate buffer, pH 7.0, 2 ml/g. Samples of the homogenates were then assayed with addition of mono- or diolein respectively. Other samples of the two homogenates were ether-extracted (see text for details) and assayed for activity against mono- and diolein. Activity is expressed as μeq of FFA released per g fat tissue (wet wt) per hr.

TABLE 5 EFFECT OF EPINEPHRINE ON LIPOLYSIS IN UNTREATED AND ETHER-EXTRACTED HOMOGENATES

Expt. No.	Whole Homogenate, Endogenous Substrate		Ether-Extracted Homogenate Triolein-C^{14} Substrate (4mg/ml)	
	Control	Δ Due to Hormone	Control	Δ Due to Hormone
	μeq of FFA per g/hr			
1			2.04	+3.57
2			2.16	+1.65
3			1.23	+3.75
4	12.6	+24.3	3.27	+3.45
5	12.0	+27.9	3.21	+2.85
6	9.6	+24.3	3.36	+1.98
Mean	11.4	+25.5	2.54	+2.88
SE				±0.36

For each experiment, two pairs of fat pads were incubated for 3 hr in 3 ml of Krebs bicarbonate medium containing bovine serum albumin, 30 mg/ml. To one of each pair of fat pads epinephrine, 5 μg, was added 10 min before the end of the incubation. Fat pads were homogenized in 0.1 M sodium phosphate buffer, pH 7.0. Samples of the whole homogenate were assayed for hormone-sensitive lipase activity, as previously described (1). Another portion of the homogenate was extracted with ether. Samples of the ether-extracted homogenate were incubated for 20 min with triolein-C^{14} (4 mg/ml), albumin, and buffer. The lipolytic activity, expressed as μeq of FFA per g of tissue in the original homogenate per hour, was calculated from the percentage conversion of triolein-C^{14} to FFA-C^{14}.

few minutes before the end of incubation, the lipase activity was usually increased 2- to 3-fold as compared with the activity of a control pad incubated in the absence of hormones (1). On the other hand, the monoglyceride lipase activity did not decrease appreciably with preincubation of the fat pads nor was it significantly increased by exposure of the fat pads to lipolytic hormones.

In Table 4 is shown the effect of epinephrine on lipolytic activity toward the lower glycerides before and after ether extraction. In confirmation of the previous results, preincubation of the fat pad with epinephrine was without effect on the monoglyceridase activity of the whole untreated homogenate. Activity toward diglyceride was also unaffected. Portions of the same homogenates were ether-extracted with excellent recovery of total activities toward the lower glycerides. In the activities of the ether-extracted homogenates, there were likewise no differences attributable to the action of epinephrine. It should be noted that fatty acid production in the presence of added mono- or diglyceride has not been corrected for fatty acid production by the homogenates in the absence of added substrate. The rate of FFA production from endogenous substrate in the whole homogenate is less than one-tenth, and in the ether-extracted preparations a much smaller fraction, of that observed in the presence of added mono- or diolein.

As shown in Table 5, preincubation of the tissue with epinephrine caused a 2-fold increase in hormone-sensitive lipase activity assayed in the whole untreated homogenate. Portions of the same homogenates were extracted with ether, and lipolytic activity was determined using triolein-C^{14}.

It is difficult to know how to compare the lipolytic activities in the two preparations because of differences in the substrates used. In a few experiments, triolein-C^{14} was added to the assays of whole homogenates. The lipolytic activity based on direct measurement of FFA

production was compared with that calculated from the percentage hydrolysis of the added triolein-C^{14} assuming that it had been diluted in all of the glyceride contained in the homogenate. The activities calculated from production of FFA-C^{14} were somewhat higher, but were in the same range as those determined directly, and it seems reasonable to conclude that about 25% of the activity of the fresh homogenates toward triglyceride was recovered after ether extraction. The activities of the ether-extracted preparations from hormone-treated tissues were about 100% greater than those from control tissues. Thus, the difference due to hormone treatment was somewhat less than that observed in untreated homogenates but was nevertheless highly significant.

DISCUSSION

The present studies show that under comparable conditions of assay, the rate of hydrolysis of mono- and diglycerides by whole homogenates of adipose tissue is more than an order of magnitude greater than the rate of triglyceride hydrolysis (Tables 4 and 5). The activity toward lower glycerides behaved differently from that toward triglycerides in at least four respects. The activity of homogenates toward lower glycerides was not altered by pretreatment of the tissue with hormones, whereas the activity toward triglycerides (hormone-sensitive lipase) was increased by 100–200%. The latter activity was inhibited by concentrations of isopropanol that had no effect on the hydrolysis of lower glycerides. The pH activity curve for triglyceride lipase activity had a much sharper optimum than that for lower glyceridase activity. Finally, triglyceride lipase activity was markedly inhibited by tris buffer, whereas lower glyceridase activity was somewhat enhanced, relative to that in phosphate buffer. These findings confirm earlier studies, which showed that monoglyceride lipase activity and hormone-sensitive lipase activity could be dissociated (1), and further indicate that diglyceride lipase activity can be differentiated from hormone-sensitive lipase activity. The possibility that a single enzyme protein accounts for all of the observed lipase activities cannot be completely ruled out, but seems unlikely in view of the several differences. In none of the parameters studied were there any clear differences, however, between monoglyceride lipase and diglyceride lipase activity.

The procedure used here for separating the lipases from the large amount of fat with which they are associated is analogous to that used by Ory et al. (13) in their studies of castor bean lipase. It is of interest that the castor bean lipase, like the adipose tissue lipases, is recovered to a large extent with the fat layer that floats to the top during centrifugation. In both cases, the enzyme activity after ether extraction resides in an insoluble, sedimentable fraction, and attempts to solubilize the lipases from this particulate sediment have thus far been unsuccessful.

The recoveries of lower glyceridase activity were excellent (40–100%), but only about 25% of the hormone-sensitive lipase was recoverable. This may reflect differences in stability of the enzymes to the extraction procedure, although there were no gross differences in their stability in storage. Another possibility is that the substrate emulsions of lower glycerides are more readily accessible to the enzymes in the ether-extracted preparations than are the triglyceride emulsions.

The significance of the relatively high levels of lower glyceridase activity observed in homogenates is difficult to assess. The concentrations of lower glycerides in the epididymal fat pad immediately after excision are very low and do not change significantly during incubation with hormones that elevate the rate of lipolysis (14). These findings suggest that the initial hydrolysis of triglycerides may be the rate-limiting step and that the lower glyceridase activity is always sufficient to hydrolyze lower glycerides as quickly as they are formed. On the other hand, it must be recognized that activities were compared here with substrates added, so that the concentrations of lower glycerides were elevated significantly over the relatively low levels found in intact tissues. Under certain conditions [in vivo (15) and in perfused tissue (16)], accumulation of lower glycerides has been observed in hormone-stimulated adipose tissue. In the light of our observation that the activity of the lower glyceridase enzyme(s) is not increased along with that of the triglyceride lipase system, this may indicate that under certain conditions the rate of hydrolysis of lower glycerides becomes limiting.

It is important in studies of lipase activity in adipose tissue to be aware of the relatively high levels of lower glyceridase activity present in homogenates. Substrates used to study the hormone-sensitive triglyceridase should be completely free of lower glycerides. Since only a relatively small fraction of the total substrate added is hydrolyzed under the conditions used in most assays, the presence of even a small percentage of lower glycerides permits the possibility that a significant part of the observed activity may be due to hydrolysis of the lower glycerides.

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