Rapid assay for hormone-sensitive lipase activity of adipose tissue

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Summary A highly specific and rapid assay for hormone-sensitive lipase activity of rat adipose tissue is described. The method employs emulsified 2,3-di-*O*-oleyl-[9,10-$^3H_2$]oleoyl glycerol as a substrate; it is very sensitive and is suitable for serial sampling.

Supplementary key words triglyceride analog · glycerol diether ester · fatty acid label

A RAPID AND SENSITIVE ASSAY for the hormone-sensitive lipase of rat adipose tissue has previously been described (1). Fatty acid-labeled, emulsified triolein was used as a substrate, and the production of labeled fatty acids was measured after a simple isolation procedure (2). There are certain disadvantages with the use of a triglyceride as substrate. Rat adipose tissue contains a mono- and diglyceride-splitting enzyme(s) (e.g., a monoglyceride hydrolase) (1, 3), and the enzymatic activity against these substrates is much higher than that towards emulsified triglycerides. Since in lipase assays of the type in question the extent of hydrolysis is generally 5% or less, even a small percentage of labeled partial glycerides in the substrate can greatly diminish the specificity of the assay. Even when radioactive triglycerides are stored at −20°C in pure solvents, labeled partial glycerides of high specific activity are formed; this necessitates frequent purifications of the triglyceride. In order to overcome these difficulties we have worked out an assay with a glycerol diether ester instead of a triglyceride as a substrate. The compound contains esterified labeled oleic acid in the 1(3)-position, and the production of labeled oleic acid is measured as in the assay using triglyceride as the substrate. Some characteristics of the assay and the absence of enzymatic activity with a lipoprotein lipase or a monoglyceride hydrolase preparation from rat adipose tissue are demonstrated.

Materials. [9,10-$^3H_2$]Oleic acid and [1(3)-^{3}H]glycerol were purchased from the Radiochemical Centre, Amersham, England. Radio- and chemical purity of the acid was > 99%. The [^{3}H]glycerol was 98% pure according to the manufacturer. Oleoyl chloride (> 99% pure) was purchased from The Hormel Institute, Austin, Minn. 2,3-Di-*O*-oleyl-[9,10-$^3H_2$]oleoyl glycerol (Fig. 1) was synthesized in the following way. 2,3-Di-*O*-oleyl-1-tetrahydropyranyl glycerol was prepared from a mixture of α-tetrahydropyranyl glycerol (4), 1-oleyl-bromide, and

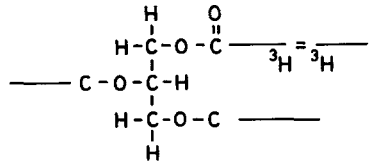

FIG. 1. Glycerol diether ester used as a substrate in the assay. [^{3}H]Oleic acid in 1(3)-position. Solid line indicates the hydrocarbon chain 18:1 in all three positions.

powdered potassium hydroxide in dry benzene by refluxing with stirring for 20 hr. The compound was then isolated as reported for the preparation of di-*O*-octadecyl glycerol (5).

The tetrahydropyranyl group was split off in methanol–ether 1:1 (v/v) by the addition of 1% (v/v) HCl with stirring for 1 hr. The glycerol diether was isolated by extraction with hexane and was purified by chromatography on alumina III (Woelm, Eschwege, W. Germany).

Usually, several grams of the glycerol diether were prepared and the material could then be used for the synthesis of a number of batches of the acylated compound (the glycerol diether can now be obtained commercially from Serdary Research Laboratories, London, Ontario, Canada). The glycerol diether was acylated with [^{3}H]oleoyl chloride (6); the glycerol diether ester was isolated and purified on a column of Florisil, using ether–hexane mixtures as eluents (7). Thin-layer chromatography revealed that the compound was more than 99.5% radiochemically pure. It was stored in benzene at 4°C.

Phospholipids were prepared from a chloroform–methanol extract of a rat liver; they were isolated as the methanol fraction from silicic acid column chromatography. Thin-layer chromatography verified that this fraction contained only mixed phospholipids. Bovine serum albumin (> 95% protein; Serva Entwicklungslabor, Heidelberg, W. Germany) was defatted by treatment with acid methanol (8).

The hormone-sensitive lipase preparation was obtained as described by others (9). A fat-free infranatant fraction from a rat adipose tissue homogenate was obtained by centrifugation (105,000 *g* for 1 hr at 2°C). The precipitate at pH 5.2 was then suspended in Tris–HCl, 0.02 M, pH 7.4, containing 10^{-3} M EDTA and 10^{-3} M dithiothreitol at a concentration of precipitate corresponding to 1.2 g of initial adipose tissue (wet wt) per ml of solution. The preparation used in the experiment described in Table 1 (termed monoglyceride hydrolase preparation) was that fraction of a benzene-extracted infranatant fraction of a rat adipose tissue homogenate which precipitated between 40 and 100% saturated ammonium sulfate (1). The lipoprotein lipase preparation was a 0.025 M NH_4OH homogenate of acetone–ether-dried adipose tissue from rats fed ad lib. (10).

Method. 13.5 mg of the labeled glycerol diether ester (approx 0.2 mCi of ^3H) and 1.35 mg of rat tissue phospholipids were mixed. The solvents were carefully evaporated under a stream of nitrogen, and 3.0 ml of 0.02 M Tris–HCl buffer, pH 7.4, containing 0.154 M NaCl was added. The mixture was subsequently sonicated with a Branson Sonifier microtip (model LS-75) for 2 min at setting 2. After the mixture reached room temperature, 1.0 ml of 20% (w/v) defatted bovine serum albumin in the same buffer solution was added, and the emulsion was sonicated in an ice bath for 2 min at setting 4. No foaming should occur during this step. The substrate mixture was sufficient for the assay of 35–38 samples. To each assay vial 100 μl of substrate (400 nmoles of ester) and enzyme preparation and buffer to a final volume of 200 μl was added. The vial was gently shaken and left at room temperature for exactly 5 min. The reaction was interrupted by the addition of an extracting solvent, and the free fatty acids were isolated as described previously (2).

Substrate containing [^3H]oleic acid-labeled triolein was prepared exactly as above with 4.7 mg of the triglyceride and 0.47 mg of phospholipids. 100 μl of this enzyme preparation contained 400 nmoles of ester.

Results. Enzyme activity was approximately linear with increasing enzyme concentration up to 80 μg of enzyme protein (Fig. 2*A*). In the interval of 80–200 μg of enzyme protein the interrelationship was no longer linear, but closely similar curves were obtained in several different experiments. Enzyme activity against [^3H]triolein was approximately linear with increasing enyzme concentrations within the same interval, but the curve deviated slightly from linearity with higher enzyme concentrations. The enzyme activity against the triglyceride substrate was always approximately twice that against the glycerol diether ester.

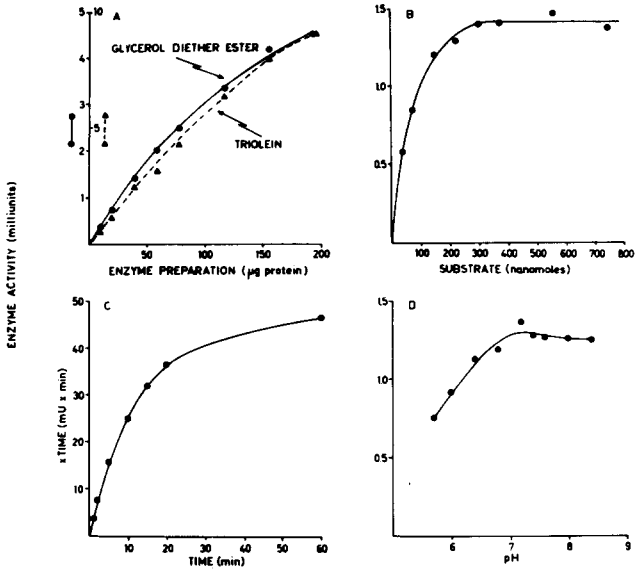

FIG. 2. Relation of lipase activity to (*A*) protein concentration of enzyme preparation (in present assay and against emulsified triolein as substrate), (*B*) substrate concentration, (*C*) incubation time, and (*D*) pH of assay. Concentrations are expressed as μg of protein or nmoles of substrate/200 μl incubation volume. 1 milliunit (mU) of enzyme activity corresponds to 1 nmole of free fatty acid produced/min/200 μl of incubation volume.

Substrate concentration of the latter was rate-limiting below 300 nmoles of substrate per incubation volume (200 μl) with the highest enzyme concentration used (Fig. 2*B*). Enzyme activity could be considered constant for the first 5 min of incubation only (Fig. 2*C*). The enzyme activity was optimal at a pH of 7.2 with little change between pH 7.0 and 8.0 (Fig. 2*D*).

Optimal concentrations of bovine serum albumin and phospholipids were determined experimentally. Exclusion of any of these two factors led to a decrease of the enzyme activity to values below 50% of those reported. The optimal conditions for sonication of the substrate mixture were obtained experimentally.

The substrate can be used for several days if stored at 4°C, but there was a gradual increase of blank values. Repeated assays of an enzyme preparation gave a standard deviation of ± 3%.

The specificity of the assay was tested in several experiments; the results are shown in Table 1. No detectable enzymatic activity was measured in the present assay with a lipoprotein lipase preparation or a preparation showing high enzymatic activity against monoglycerides in micellar solution. In other test experiments it was found that prior incubation with 1 M NaCl for 1 hr did not decrease the enzymatic activity below 90% of the control. This treatment would inhibit lipoprotein lipase activity to a much greater extent (11).

In a further test experiment the hormone-sensitive lipase preparation was activated by preincubation with optimal concentrations of 3′,5′-AMP, ATP, and Mg^{2+} (12) and subsequent assay of the lipase activity against

TABLE 1. Specificity of assay

Type of Assay	Lipoprotein Lipase Preparation	Monoglyceride Hydrolase Preparation
	nmoles of fatty acid/min/mg of protein	
Assay with glycerol diether ester	0.009 ± 0.001[a]	0 (<0.002)
Lipoprotein lipase assay	5.4[b]	
Monoglyceride hydrolase assay		4.3[b]

Enzymatic activity of lipoprotein lipase and monoglyceride hydrolase preparations measured by the present assay, a lipoprotein lipase assay (employing emulsified triolein and rat serum [10]), and a monoglyceride hydrolase assay (employing micellar monoolein [1]). Lipoprotein lipase assay, incubation time 20 min at 37°C; the two other assays, 10 min at 23°C.

[a] Mean ± SD of four samples.

[b] Mean of two samples.

TABLE 2. Activation of hormone-sensitive lipase

Substrate	Control	Lipase Preparation Preincubated with Cofactors	Increase Due to Cofactors
	nmoles of fatty acid/min (mU)		
Glycerol diether ester	0.46 ± 0.06 (5)[a]	0.59 ± 0.05 (5)	0.14 $P < 0.01$
Triolein	0.90 ± 0.09 (5)	1.12 ± 0.06 (3)	0.22 $P < 0.02$

Enzyme preparation was preincubated for 15 min at room temperature with 3′,5′-AMP, 1×10^{-6} M; ATP, 1.5×10^{-5} M; $MgCl_2$, 5×10^{-3} M; and caffeine, 1×10^{-3} M. Enzymatic activity was assayed by adding 100 μl of substrate mixture (400 nmoles of ester) to 100 μl of preincubated enzyme preparation and incubating for 5 min.

[a] Means ± SD for numbers of samples given in parentheses.

emulsified glycerol diether ester or triglyceride. The data in Table 2 show that there was a low but statistically significant increase of enzymatic activity due to the cofactors against both substrates. This is in accordance with other reports (12) and indicates that hormone-sensitive lipase activity was measured in both assays.

It can be concluded that hormone-sensitive lipase activity, but neither lipoprotein lipase nor monoglyceride hydrolase activity, is measured by the present assay. Its characteristics are very similar to an assay with emulsified triolein as a substrate, but measured enzymatic activities are lower. The reason for this is not known. The main advantage of the present assay is the higher specificity. No substrate for partial glyceride-splitting enzymes could be formed, and the necessity of frequent purifications of the substrate was avoided.

The project was supported by grants from the Swedish Medical Research Council (B-69-13X-252301), H. Jeansson's Foundation, Stockholm, A. Påhlssons Foundation, Malmö, and the Medical Faculty, University of Lund, Sweden. Miss Annica Ericson and Mrs. Birgitta Rapp provided skillful technical assistance.

Manuscript received 19 October 1970 and in revised form 18 November 1971; accepted 7 December 1971.

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