Simple assay for monoacylglycerol hydrolase activity of rat adipose tissue

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Summary A simple, sensitive, and specific assay for monoacylglycerol hydrolase activity of rat adipose tissue is described. Monoacyl[^{3}H]glycerols with different chain lengths (8–18 carbon atoms) and different degrees of unsaturation in mixed micellar solution with different detergents can be used as substrates. The [^{3}H]glycerol that is produced is isolated in a one-step liquid–liquid partition procedure. For routine purposes monooleoyl[^{3}H]glycerol was found to be the most suitable substrate. A simple method for the chemical synthesis and purification of this substrate in high yield is given. The assay allows rapid serial sampling of enzymatic activity with a high reproducibility.

Supplementary key words monooleoyl[^{3}H]glycerol synthesis · sodium mono[^{3}H]glycerate · oleoyl chloride

Enzymatic activity that catalyzes the hydrolysis of monoacylglycerols in several tissues has been reported by us (1–3) and others (4, 5). Determination of this enzymatic activity has generally been performed with long-chain monoacylglycerols in mixed micellar solution as substrate. Assay methods employing this principle have been briefly described by us in previous reports (1, 2). The present work gives a more complete description of an assay that allows simple and rapid serial sampling of monoacylglycerol-hydrolyzing activity in adipose tissue extracts and compares different monoacylglycerols as substrates in such an assay. The paper also includes a convenient procedure for the synthesis and purification of the routine substrate used, monooleoyl[^{3}H]glycerol.

Materials. [1(3)-^{3}H]Glycerol was purchased from the Radiochemical Centre, Amersham, England (98% pure according to the manufacturer), and fatty acid chlorides (>99% pure) from the Hormel Institute, Austin, Minn. Monoacylglycerols (>99% pure) were obtained from the same source or synthesized as described below. Bovine serum albumin (Serva Feinbiochemica, Heidelberg, W. Germany, >92% pure), was defatted by treatment with acid methanol (6) and dialyzed. The nonionic detergent Nonipol TD 12 (polydisperse preparation of tridecylpolyethoxyethanols with an average chain length of 12 oxyethylene units giving an average molecular weight of 728, >98% pure) was obtained from Rexoline Chemicals, Helsingborg, Sweden; Triton X-100 (polydisperse preparation of *p-t*-octylphenoxypolyethoxyethanols with an average chain length of 9–10 oxyethylene units and average molecular weight of 628, >99% pure) was from Sigma Chemical Co, St. Louis, Mo.; and sodium taurodeoxycholate (99% pure) was from Maybridge Chemical Co., Tintagel, England. All other substances used were reagent grade, and all solvents were freshly redistilled.

Monoacyl[1(3)-^{3}H]glycerols were synthesized by acylation of [1(3)-^{3}H]glycerol with the respective acyl chloride in pyridine–benzene solution (7) and purified as described below. The final yield of labeled monoacylglycerols, equilibrium mixtures of 1(3)- and 2-isomers after purification (see below), was generally 15–20% of initial glycerol radioactivity, with the remaining lipid label (40–50% after purification) in, mainly, diacylglycerol.

In order to obtain a better yield of the specific monoacylated compound, hydroxyl groups of the glycerol can be protected (8); however, with labeled glycerol used on a

milligram scale this method becomes rather inconvenient. Instead, an alternate procedure for increasing the relative yield of the monoacylated compounds has been worked out based on the higher reactivity of the alkoxide ion compared with the hydroxyls of glycerol. The following method was used. Sodium mono[^{3}H]glycerate was obtained by reacting [1(3)-^{3}H]glycerol (10 mCi, 1 mmole) with NaOH at 150°C. The glycerol was dissolved in 1 ml of methanol, 1 ml of 1 M NaOH was added, and the solvents were evaporated with dry N_2 at 150°C followed by another 30 min at 150°C. The sodium mono[^{3}H]glycerate was dried overnight in a desiccator. Dry ethyl acetate was thoroughly mixed with the compound in a closed vial, and the glycerate was subsequently acylated at room temperature for 2.5 hr with 400 μl of oleoyl chloride. The reaction was interrupted by addition of water, and the lipids were extracted with diethyl ether, which was evaporated after drying over Na_2SO_4. After the addition of unlabeled monooleoylglycerol (0.7 g), the monooleoyl[^{3}H]glycerol was purified on a silicic acid column using diethyl ether–hexane mixtures. The compound was characterized by comparison with a pure reference monooleoylglycerol on silicic acid thin-layer plates impregnated with boric acid (9) and by the equimolar production of glycerol and oleic acid upon hydrolysis. 36% of the initial 10 mCi of [^{3}H]glycerol was recovered as pure monooleoyl[^{3}H]glycerol (equilibrium mixture of 1[3]- and 2-isomers) with an additional 11% as 1,3-dioleoyl- and 5% as 1,2-dioleoylglycerol. The monooleoyl[^{3}H]glycerol obtained was >99.5% radiochemically pure by thin-layer chromatography on silicic acid and had a specific activity of 2.1 μCi/μmole. It was stored in dry, redistilled benzene at 4°C. The purity was frequently checked and it was repurified when necessary. It was sufficient for more than 5000 enzyme assays.

Enzyme source, unless otherwise indicated, was the 130-fold purified monoacylglycerol hydrolase obtained from the pH 5.2 precipitate of the fat-poor infranate (78,000 *g* for 45 min) of rat adipose tissue homogenates, which had been detergent-treated and subjected to TEAE-cellulose ion-exchange chromatography and gel chromatography on Sephadex G 150.[1] The enzyme preparation contained 34 μg of protein/ml (10) in 0.02 M Tris–HCl, pH 7.0, 0.2% (w/v) Nonipol TD 12, 10^{-3} M EDTA, 10^{-3} M dithiothreitol, and initially 26 mU of enzymatic activity (see below) per μg of protein. Enzymatic activity, however, varied between individual experiments due to inactivation when the enzyme was repeatedly frozen and thawed. In some cases the pH 5.2 precipitate referred to above was used instead of the purer preparation.

Disposable 5-ml vials with screw caps and automatic dispensing systems were used in order to increase the speed and reproducibility of the method.

Procedure. In the routine enzyme assay, labeled and unlabeled monooleoylglycerols were mixed, giving 40 μmoles of substrate with a specific activity of 0.1–0.3 × 10^6 counts/min/μmole. After removal of solvents, the substrate was solubilized by sonicating for 2 min (setting 2, Branson Sonifier, type LS 75) at 0°C in 4.0 ml of 0.8% (w/v) Nonipol TD 12 in 0.2 M Tris–HCl, pH 8.0. The resulting solution was sufficient for the assay of at least 37 samples. It could be stored at −20°C and used several times again after sonication again for 15 sec. To each assay vial was added 100 μl of substrate (1 μmole) and enzyme preparation and buffer (same solution as used for enzyme preparation, see above) to a final volume of 200 μl. The vial was gently shaken and left at 22°C for exactly 10 min. The reaction was interrupted by the addition of 3.25 ml of an extracting solvent, methanol–chloroform–heptane 1.41:1.25:1 (v/v/v) (11), followed by 1.05 ml of 2% (w/v) NaCl to obtain a two-phase system. A 1-ml aliquot of the upper methanol–water phase, which contained all the [^{3}H]glycerol produced and less than 0.3% of the monooleoyl[^{3}H]glycerol, was directly transferred to a counting vial with 10 ml of Instagel–toluene 1:1 (v/v) and counted in a Packard Tri-Carb spectrometer, model 3375, using external standardization for quenching correction.

This basic procedure was followed in all experiments. Variations of substrate and substrate concentration, type and concentration of detergent, incubation time, and temperature have been employed in different experiments and are described in the respective figure legend or table.

The liquid–liquid partition system above, used for isolation of labeled glycerol in monooleoylglycerol experiments, gave high blank values with shorter-chain monoacylglycerols. Therefore, a chloroform–methanol–water system giving a more polar chloroform lower phase (3.0

TABLE 1. Reaction rates with various monoacylglycerols as substrates

Monoacylglycerols	Relative Enzymatic Activity	
	22°C	37°C
8:0[a]	4.60 ± 0.28 (5)[b]	4.48 ± 0.32 (4)[b]
12:0		1.07 ± 0.05 (6)
18:1	1.00	1.00
18:2	0.99 ± 0.05 (5)	0.98 ± 0.06 (4)

Determination of enzymatic activity as described for the routine enzyme assay with the modifications indicated. The conditions used gave linear relationships between amount of enzyme and enzymatic activity. Substrate concentration was 5 mM for all compounds except monooctanoylglycerol, which was 10 mM. Enzyme source was the 130-fold purified enzyme preparation.

[a] Monoacylglycerols designated by chain length:number of double bonds in acyl moiety.

[b] Values are means ± SEM (number of experiments in parentheses) and are expressed relative to that of monooleoylglycerol, which was set at 1.00 at 22°C and at 37°C.

[1] Tornqvist, H., and P. Belfrage. Partial purification and characteristics of a monoacylglycerol hydrolase of rat adipose tissue. To be published.

ml of chloroform–methanol 2:1 (v/v) and 1.0 ml of 0.15 M NaCl) was employed with ^{3}H-labeled monooctanoyl- and monolauroylglycerol. In both systems, [^{3}H]glycerol was quantitatively (>99%) found in the upper phases, which contained less than 0.3% of all monoacylglycerols except monooctanoylglycerol. The partition coefficient (upper phase/lower phase at 22°C) for this compound, however, was 0.051, resulting in high blank values. The [^{3}H]glycerol produced in these experiments had to be calculated by subtraction of the monooctanoyl[^{3}H]glycerol contribution to the ^{3}H of the upper phase. With low hydrolysis rates the values obtained were less accurate than those in experiments with the other monoacylglycerols.

Enzymatic activity, specific enzymatic activity, and percentage hydrolysis were calculated with the aid of a table computer (Olivetti Programma P 602) directly from the radioactivity data. Values for enzymatic activity reported below are always above mean of controls +2 SD. 1 mU of enzyme activity represents the release of 1 nmole of glycerol/min.

Results. The acylation of the sodium monoglycerate rather than the glycerol doubled the yield of the monoacylated compound without the use of protected hydroxy groups, e.g., with isopropylidene glycerol as substrate. This is an advantage when dealing with small amounts of [^{3}H]glycerol, when the latter method becomes difficult.

We have used the nonionic detergent Nonipol TD 12 in the routine enzyme assays because this detergent was used in the partial purification of the enzyme and included in the enzyme preparations.[2] However, Triton X-100 can replace the Nonipol TD 12 in the assay with no obvious difference, while sodium taurodeoxycholate (final concentration 5 mM) gave slightly lower enzymatic activity under optimal conditions. The optimal ratio of detergent to substrate has been found to be 0.7–1.8 (mole/mole) for the nonionic detergents. Within this range, values for enzymatic activity differed by less than 10%, but at lower or higher ratios, rapid decrease of hydrolysis rates took place (50% inhibition at a ratio of 5). Sodium taurodeoxycholate was less extensively investigated, but equimolar concentrations of detergent and substrate seemed to be optimal. Bovine serum albumin, which readily binds monooleoylglycerol (13), could be used instead of detergent, and under optimal conditions (final concentration 1% [w/v]) it gave slightly lower enzymatic activity. However, from a practical point of view, both albumin and the bile acid are less convenient to use.

A number of experiments were performed to investigate differences among various monoacylglycerols as substrate.

[2] The major advantage of this detergent compared with the more commonly used Triton X-100 is its lack of UV absorbance. Such absorbance is caused by the aromatic nucleus of the latter detergent and interferes with UV absorbance by proteins, e.g., during continuous monitoring at chromatography. Both compounds have similar detergent properties, with critical micellar concentrations on the order of 10^{-4} M at 25°C (12).

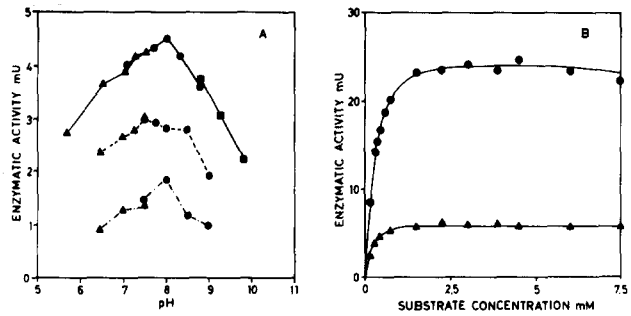

Fig. 1. Relation of enzyme activity measured under standard conditions to (*A*) pH of assay (ionic strength 0.1 M; ▲, sodium phosphate buffer; ●, Tris–HCl buffer; and ■, glycine–NaOH buffer) with a nonionic detergent (——, 0.5% Nonipol TD 12), an ionic detergent (– · –, 5 mM sodium taurodeoxycholate), or 1% (w/v) bovine serum albumin (- - -); and to (*B*) substrate concentration at two different enzyme concentrations (molar ratio of detergent to substrate was constant at 1.4). Enzyme source in *A* was the pH 5.2 precipitate fraction from the fat-poor, 78,000 *g* for 45 min supernate and in *B* was the 130-fold purified enzyme preparation (0.25 and 1.0 μg of enzyme preparation protein). Each point represents the mean of duplicates or triplicates.

These experiments included determination of optimal ratio of detergent to substrate, saturating substrate concentration, and other conditions for a linear relation between amount of enzyme and hydrolysis rate at various temperatures. It was found that monopalmitoyl- and monostearoylglycerol could not be sufficiently solubilized in mixed micelles below a temperature that completely inactivated the enzyme (45°C). The turbid suspensions of these monoacylglycerols, which were obtained by sonication, provided a good substrate for the enzyme, but enzymatic activity varied considerably among substrate preparations and was rapidly inhibited by a low concentration of detergent. Thus, for technical reasons these monoacylglycerols could not be used as substrates. The same was true for monolauroylglycerol at 22°C, although mixed micellar solubilization was obtained at 37°C. However, as shown in Table 1, enzymatic activity at this temperature was not higher than with monooleoylglycerol as substrate. Monolinoleoylglycerol was solubilized completely at 22°C and gave values for enzymatic activity similar to those of monooleoylglycerol (Table 1). Monooctanoylglycerol, on the other hand, gave a higher maximal rate of hydrolysis (Table 1). Since the apparent K_m for this compound, based on a Lineweaver-Burk plot, was 0.6 mM compared with 0.2 mM for monooleoylglycerol, the higher enzymatic activity was presumably due to the more rapid diffusion of the water-soluble octanoic acid into the aqueous environment compared with the long-chain fatty acids. A major disadvantage with the use of short-chain monoacylglycerols as substrates is the difficulty in separating the labeled reaction products from the acylglycerol.

In view of the results above, monooleoyl[^{3}H]glycerol was chosen as the best substrate to use in the routine enzyme assay. Figs. 1 and 2 present the effects of variation

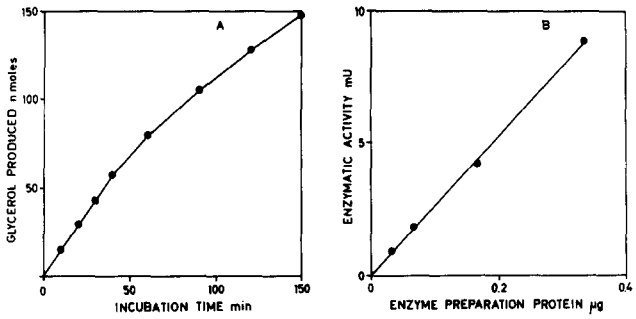

Fig. 2. Relation of enzyme activity measured under standard conditions to (*A*) incubation time and (*B*) enzyme concentration. Enzyme source was the 130-fold purified enzyme preparation. Each point represents the mean of duplicates or triplicates.

of some parameters in the routine assay. Enzyme activity plotted against pH of incubation showed a broad peak with an optimum around pH 8.0 (Fig. 1*A*). The same was found with the other detergents, but with serum albumin slightly higher values (5–10%) were obtained at pH 7.4–7.5. As shown by Fig. 1*B*, substrate concentration was not rate limiting at 5 mM, the concentration used in the routine assay. Enzyme activity was linear with time for at least 45 min of incubation (Fig. 2*A*) and with different amounts of the enzyme preparation (Fig. 2*B*). The same was true when the other detergents or serum albumin was used.

Variation of ionic strength in the incubations by addition of NaCl up to 1.0 M gave a 10% decrease of enzymatic activity. Incubation at 30°C gave a 24% and at 37°C a 40% increase of enzymatic activity.

The sensitivity was calculated as the mean of blanks (usually ca. 250 cpm) *plus* 2 SD and was found to be 0.02 mU of enzyme activity. In practice, 0.2 mU was used as the lower limit. This enzymatic activity corresponds to ca. 0.2 mg of rat adipose tissue (wet wt). The variation coefficient (relative SD) for duplicates of the enzyme preparation measured in the same assay was 2–2.5%. 100 samples could be assayed for enzymatic activity by one person in less than 2.5 hr.

The specificity of the assay was tested by incubating with a highly purified pancreatic lipase preparation (14). This preparation had an enzymatic activity of 3×10^4 mU/100 µl against a tributyrin emulsion (15) but less than 0.3 mU/100 µl in the present assay. Thus, the described method does not seem to measure lipase activity, at least not that of pancreatic lipase. The specificity in adipose tissue preparations cannot be directly determined because no pure adipose tissue lipase is available. However, hormone-sensitive lipase activity in adipose tissue preparations is low compared with monoacylglycerol hydrolase activity (2, 5). Thus, variations in the former activity, if at all measured in the present assay, would not appreciably influence the monoacylglycerol hydrolase values.

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