Assay of nanogram levels of triglyceride lipase with a radioactive substrate

WALTON H. MARSH AND PATRICK J. FITZGERALD

Department of Pathology, State University of New York, Downstate Medical Center, Brooklyn, New York 11203

Summary A simple, sensitive procedure for the determination of triglyceride lipase activity has been developed. Nanogram amounts of oleic acid hydrolyzed from commercially available [^{14}C]triolein were readily determined by the counting of the radioactivity of substrate and product after their rapid chromatographic separation on copper hydroxide-impregnated ion-exchange paper. Comparison of the relative amounts of radioactivity of the separated substrate and product gave an estimate of the percentage of hydrolysis of substrate. Comparison of results with a standard of pure lipase enables one to express the amount of hydrolysis in terms of the standard lipase. The results show that measured activity is a linear function of time up to 1 hr of incubation and of amounts of enzyme up to 125 ng. Reproducibility of the test is good.

Supplementary key words pancreatic enzymes · chromatography with copper-impregnated ion-exchange paper

MANY PROCEDURES (titrimetric [1, 2], turbidimetric [3], colorimetric [4, 5], fluorometric [6–9], and isotopic [radiometric] [10–13]) have been used to estimate the hydrolytic activity of triglyceride lipase. Recent colorimetric procedures (4, 5) have increased the sensitivity over that of titrimetric and turbidimetric methods, but they require a liquid–liquid extraction, as do the isotopic methods (11–13). The fluorometric procedures (6–9) are very sensitive, but it is difficult to compare the units of activity from the released fatty acid with those of conventional substrates, and it is not clear that fluorometrically determined esterase activity can be ascribed to that of pancreatic triglyceride lipase.

We have developed a simple, sensitive method that can detect very low levels of enzyme activity in the rat embryonic pancreas and in the in vitro organ culture of pancreatic anlagen (14–18) even when only microgram amounts of tissue are available and lipase activity is minimal. [^{14}C]Triolein was used as substrate. The radioactive oleic acid formed by triglyceride lipase action was separated from the mono-, di-, and triolein radioactive residues by a 15-min chromatographic separation on copper-impregnated ion-exchange paper (16, 17). The sensitivity of the method extends to the nanogram level and can be increased further.

Materials and methods

[^{14}C]Triolein (glyceryl tri[1-^{14}C]oleate) was purchased from Amersham/Searle, Des Plaines, Ill., 50 μCi in 0.56 ml of benzene (34.1 mCi/mmole). Purity was $> 99\%$ as judged by reversed-phase thin-layer chromatography in chloroform–methanol–formic acid–water 40:60:1:5 and by thin-layer chromatography on silica gel, using diisopropyl ether or petroleum ether–ethyl ether–acetic acid 90:10:1. The supplier stated that less than 1% radiodecomposition of this material occurs per year at room temperature.

Olive oil (highly refined, suitable for lipase determinations) was purchased from Sigma Chemical Co., St. Louis, Mo. Fatty acids were removed by adding 10 g of alumina (chromatographic grade, Merck & Co., Rahway, N.J.) to 100 ml of the olive oil (3). The suspension was mixed for 10 min and then filtered through glass wool. This procedure was repeated, and the fatty acid-free material was stored at 5°C. 1 ml (915 mg) was dissolved in benzene, and aliquots were removed for incubation as described below.

Preparation of ion-exchange chromatograph paper. Strong-

base, ion-exchange paper, SB-2 (Rohm and Haas), was purchased in 8 × 11-inch sheets from Reeve-Angel, Clifton, N.J.

Following the procedure of Anghileri (19), the papers were immersed in 5% copper sulfate for 1 hr. They were then air dried and immersed in 5% NaOH for 20 min and washed with tap water until the wash water was neutral (pH indicator paper). After immersion in distilled water the papers were again air dried and cut into strips 1.5 × 13.7 cm. The treated papers were stable for long periods when stored in the dark at room temperature.

Preparation of buffer. To a 100-ml blender container was added the following: 5.0 ml of a 3% (w/v) solution of sodium taurocholate in water (reagent grade, K & K Laboratories, Plainview, N.Y.); 1.0 ml of a 6% (w/v) water solution of purified soya phosphatides (Alcolec granules, 95% purified, American Lecithin Co., Woodside, N.Y.); 2.5 ml of 0.15 M $CaCl_2$ (reagent grade); 2 g of gum acacia (gum arabic, Will Corp., New York); 1.6 ml of 0.5 M NaOH; 20.0 ml of 0.2 M glycine; and 10 μl of Tergitol 7 (Union Carbide Corp., New York). The mixture was homogenized for 5 min at top speed (Waring Blendor Deluxe) and the pH was adjusted to 8.6. The final volume was 30 ml. This solution was faintly turbid. Its stability was not checked beyond 2 days of storage.

Incubation. All sample volumes of 100 μl or less were handled with Corning disposable micropipets. To 0.9 × 6.0-cm test tubes containing 100 μl of benzene (reagent grade) was added (by rinsing in) 1 μl of olive oil (915 μg) and 20 μl of [^{14}C]triolein (41.6 μg, 893 nCi).

Complete solution in benzene and thorough randomization of labeled and unlabeled triglyceride was achieved. This was demonstrated in early experiments in which the total olive oil in the medium was kept constant but the ratio of ^{14}C to ^{12}C was varied. The computed enzyme activity (micromoles hydrolyzed/unit time) remained constant at constant enzyme level, time, and temperature and was independent of the ^{14}C to ^{12}C ratio.

The benzene solution of olive oil was evaporated to dryness at room temperature under a gentle stream of nitrogen (about 5 min). 100 μl of buffer solution at 25°C and a small magnetic flea were added. The olive oil was emulsified by high-speed agitation with a Micro V (Cole-Parmer, Chicago, Ill.) magnetic stirrer. The test tube rack on the stirrer could hold 16 samples. This procedure and subsequent operations and incubations were carried out in a constant-temperature oven at 25°C.

100 μl of the homogenate to be tested, or a standard lipase solution, was added to the tube. Standard lipase A and B (pure porcine pancreatic lipase) was a gift from Dr. Robert Verger, Institut de Chimie Biologique, Marseilles. When volumes of standard less than 100 μl were used, the final volume was made to 100 μl by addition of 0.9% aqueous saline. Convenient standards contained 100 ng of lipase.

A zero-time sample was removed; 3-μl samples were removed at 15-min intervals while the incubation mixture was stirred. The samples were spotted and dried on the copper-impregnated ion-exchange paper. In testing the method, samples were incubated for periods of up to 2 hr and with amounts of lipase of from 25 to 200 ng (Figs. 1 and 2).

The zero-time sample gave the distribution of counts between oleic acid and unchanged triglyceride. The presence of oleic acid at zero time is ascribed to that present as contaminant in the commercial preparation and to that which is formed due to shearing during homogenization. Purification of the commercial preparation (13) and elimination of vigorous homogenization could reduce this to virtually zero. At no point in this study did radioactivity in the oleic acid fraction at zero time exceed 5% of the total count.

Chromatography and radioactivity counting. Spotting and drying of incubation medium on the strips appeared to inhibit further enzymic action, probably through the combination of known inhibition by copper salts and through drying itself. Once dried, the spotted strips were stored at room temperature until it was convenient to develop them chromatographically. Storage for several days before development did not alter apparent enzymic activities.

The sample-containing strips were placed in commercially available 2 × 20-cm screw-capped test tubes containing 3 ml of benzene–chloroform 1:2. The ascending front migrated 10 cm from the starting point in about 15 min. The papers were removed and dried in air. Under these conditions, the mono-, di-, and unreacted triglycerides migrated with an R_F of 0.95. The oleic acid freed by hydrolysis migrated with an R_F of 0.09. This degree of separation permitted ready measurement of radioactivity of the two fractions. Although end-window Geiger and liquid scintillation countings should be feasible, we chose to count the fractions using an integrating 4π gas flow strip counter (Baird-Atomic, Cambridge, Mass., model 1-372). The recorder chart speed was 8 inches/hr, and the ratemeter was operated at 1.15 kv. Under the conditions of our experiments, our average total integrated counts were 10,000 cpm.

It should be possible to determine lipase activity by measuring either substrate disappearance or appearance of product. However, to reduce variability introduced by small volume sampling and transfer, as well as variability in counting (i.e., voltage setting of ratemeter and possible fluctuation of strip speed through the counting chamber), the integrated counts of oleic acid (product)

were expressed as a percentage of the total counts (substrate + product).

Determination of lipase activity per unit of time. The percentage conversion of total radioactivity by the enzyme at each 15-min interval was expressed as the percentage converted at 1 hr, and these figures were averaged. From the known amount of triglyceride present in the incubation medium, the amount of oleic acid formed per hour could be calculated. The amount of enzyme present in the unknown specimen could be related to the amount of lipase A and B present by comparison with a previously determined standard curve using pure lipase A and B.

Results

The response to hydrolysis of labeled substrate is shown in Fig. 1. The hydrolysis rate remained linear with time for up to 1 hr.

With a fixed incubation time of 60 min, the rate of the reaction was proportional to the amount of enzyme added from 25 to 125 ng (Fig. 2).

In order to determine the precision of the test, it was run on separate days for 10 days, using a standard of lipase made from pure lipase A and B. The test was run for 1 hr, using 75 ng of enzyme. Six samples were prepared from the standard lipase; they were analyzed each day, and the results were averaged and the standard error of the mean was determined (Table 1).

We have used the method for over 2 yr without significant difficulties (16, 17).

Discussion

A number of the triglyceride lipase determinations which have been proposed in the literature have a high degree of sensitivity. Our method gains sensitivity by use of the isotopic substrate and, in addition, offers a simpler separation of the products of hydrolysis because the copper on the ion-exchange paper immobilizes the oleic acid. This permits the use of a rapidly migrating solvent front which carries with it the unreacted radioactive material, i.e., mono-, di-, and triglycerides. Anghileri's method (19) with copper-impregnated filter paper gave inadequate separation of components within 10 cm of solvent migration.

Direct counting of the material on the paper by a strip counter eliminates the need for transfer or further processing. The method could be readily adapted to liquid scintillation counting, since the radioactivity on the paper could be eluted with the scintillation solvents (e.g., toluene) and an organic base (e.g., Hyamine) employed.

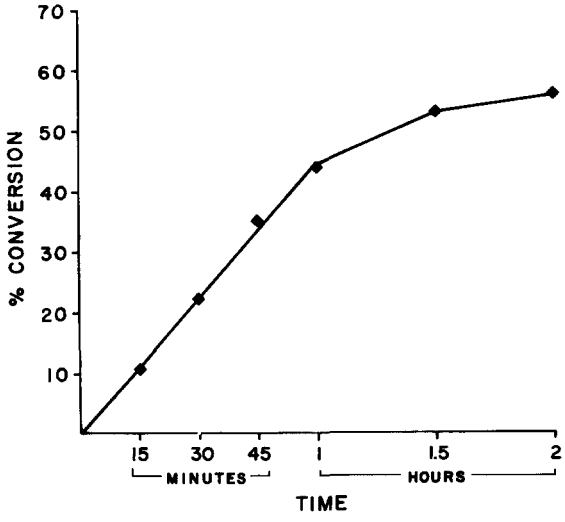

FIG. 1. With a fixed amount of lipase (125 ng), the hydrolysis rate, expressed as % [^{14}C]triolein converted to oleic acid at 25°C, is expressed as a function of time. The triglyceride used was made up of [^{14}C]triolein (41.6 μg) plus olive oil (915 μg).

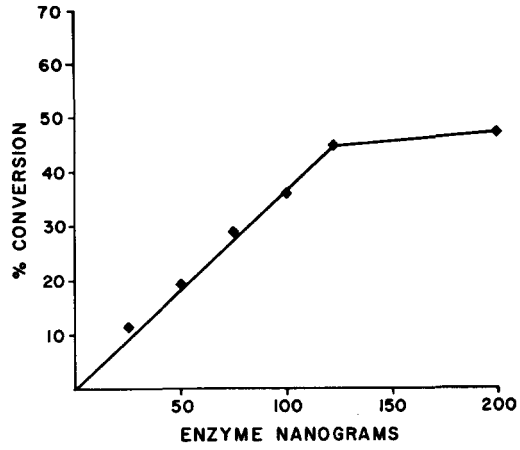

FIG. 2. For a constant incubation time of 1 hr at 25°C, the amount of hydrolysis, expressed as % triolein converted to oleic acid, is plotted against the amount of enzyme added. The triglyceride used contained [^{14}C]triolein (41.6 μg) plus olive oil (915 μg).

TABLE 1. Reproducibility of lipase determination

Day	Percentage Conversion
1	40
2	35
3	30
4	42
5	38
6	45
7	44
8	38
9	38
10	43
	Mean 39.3 ± 4.5 (SEM)

Six samples, each containing 75 μg of lipase A and B, were prepared and analyzed daily for 10 consecutive working days to determine the percentage of [^{14}C]triolein converted to radioactive reaction product. From these values a mean and SEM were computed.

We have taken zero-time samples as representative of the oleic acid contaminant present in the commercial preparation. This also has allowed us to correct for oleic acid formed by hydrolysis which occurs during homogenization. This has been shown to happen in experiments in which the sample and substrate were homogenized together.[1] Oleic acid contaminant in the commercial sample can be removed by the liquid–liquid extraction method of Kaplan (13), should it become necessary to do so.

The ratio of ^{12}C (carrier olive oil) to [^{14}C]triolein used in this study was 22:1. By maintaining a constant total triglyceride content in the incubation medium (^{12}C + ^{14}C), the enzyme saturation remains constant; however, one may increase the sensitivity of the method by decreasing the ^{12}C olive oil and to the same extent increasing the amount of [^{14}C]triolein. In this way, the total amount of triglyceride hydrolyzed remains the same under constant incubation conditions, but a larger proportion is isotopically labeled. This in turn should allow detection of smaller amounts of hydrolysis product, i.e., labeled oleic acid, and consequently lower enzyme activity levels to be assayed.

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REFERENCES

1. Cherry, I. S., and L. A. Crandall, Jr. 1932. The specificity of pancreatic lipase: its appearance in blood after pancreatic injury. *Amer. J. Physiol.* **100:** 266–273.
2. Marchis-Mouren, G., L. Sarda, and P. Desnuelle. 1959. Purification of hog pancreatic lipase. *Arch. Biochem. Biophys.* **83:** 309–319.
3. Vogel, W. C., and L. Zieve. 1963. A rapid and sensitive turbidimetric method for serum lipase based on differences between the lipases of normal and pancreatitis serum. *Clin. Chem.* **9:** 168–181.
4. Massion, C. G., and D. Seligson. 1967. Serum lipase. A rapid photometric method. *Amer. J. Clin. Pathol.* **48:** 307–313.
5. Dirstine, P. H., C. Sobel, and R. J. Henry. 1968. A new rapid method for the determination of serum lipase. *Clin. Chem.* **14:** 1097–1106.
6. Meyer-Bertenrath, J. G., and H. Kaffarnik. 1968. Simple method for determination of lipase activities. *Hoppe-Seyler's Z. Physiol. Chem.* **349:** 1071–1072.
7. Jacks, T. J., and H. W. Kircher. 1967. Fluorometric assay for the hydrolytic activity of lipase using fatty acyl esters of 4-methylumbelliferone. *Anal. Biochem.* **21:** 279–285.
8. Guilbault, G. G., M. H. Sadar, and D. Arcenaux. 1968. Evaluation of fluorometric substrates for lipase. *Anal. Lett.* **1:** 551–563.
9. Guilbault, G. G., and J. Hieserman. 1969. Fluorometric substrate for sulfatase and lipase. *Anal. Chem.* **41:** 2006–2009.
10. Chino, H., and L. I. Gilbert. 1965. Determination of lipase activity by Florisil column chromatography and liquid scintillation spectrometry. *Anal. Biochem.* **10:** 395–400.
11. Greten, H., R. I. Levy, and D. S. Fredrickson. 1968. A further characterization of lipoprotein lipase. *Biochim. Biophys. Acta.* **164:** 185–194.
12. Belfrage, P., and M. Vaughan. 1969. Simple liquid–liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. *J. Lipid Res.* **10:** 341–344.
13. Kaplan, A. 1970. Simple radioactive assay for triglyceride lipase. *Anal. Biochem.* **33:** 218–225.
14. Marsh, W. H., A. Parsa, and P. J. Fitzgerald. 1966. Comparative enzymatic activity of regenerating adult pancreas and the embryonic pancreas. *Amer. J. Pathol.* **48:** 22.
15. Parsa, I., W. H. Marsh, and P. J. Fitzgerald. 1969. Pancreas acinar cell differentiation. I. Morphologic and enzymatic comparisons of embryonic rat pancreas and pancreatic anlage grown in organ culture. *Amer. J. Pathol.* **57:** 457–487.
16. Parsa, I., W. H. Marsh, and P. J. Fitzgerald. 1970. Chemically defined medium for organ culture differentiation of rat pancreas anlage. *Exp. Cell Res.* **59:** 171–175.
17. Parsa, I., W. H. Marsh, and P. J. Fitzgerald. 1970. Pancreas acinar cell differentiation. III. Importance of methionine in differentiation of pancreas anlage in organ culture. *Amer. J. Pathol.* **59:** 1–22.
18. Marsh, W. H., and P. J. Fitzgerald. 1971. Pancreas acinar cell differentiation. IV. Assay of nanogram levels of chymotrypsin by radioactively labeled synthetic substrate. *Anal. Biochem.* **40:** 72–79.
19. Anghileri, L. J. 1965. Paper chromatography of lipids: use of cupric hydroxide impregnated paper. *J. Chromatogr.* **17:** 188.

[1] Marsh, W. H., and P. J. Fitzgerald. Unpublished results.