A method for the determination of lipoprotein lipase in postheparin plasma and body tissues utilizing a triolein-coated Celite substrate

ISRAEL POSNER and VIRGILIO BOSCH

Hospital Central de las Fuerzas Armadas, and Instituto de Medicina Experimental, Universidad Central de Venezuela, Caracas, Venezuela

SUMMARY A simple and specific method for assaying lipoprotein lipase activity is described. Postheparin plasma, heart homogenates, or extracts of acetone powder of adipose tissue were incubated with a triolein-coated Celite substrate, and enzyme activity was determined from the rate of free fatty acid (FFA) release in the incubation system. FFA release was linear for 30 min, and was proportional to protein concentration in the incubation system. FFA release was decreased by addition of deoxycholate or Triton X-100. Increasing the concentration of heparin in the incubation system caused a gradual decrease in FFA release by postheparin plasma and increases in activity of heart homogenates and adipose tissue lipoprotein lipase. The Celite substrate was found to be satisfactory for assaying pancreatic lipase activity as well.

SUPPLEMENTARY KEY WORDS adipose lipoprotein lipase · heart lipoprotein lipase

THERE ARE various methods for the assay of lipoprotein lipase (LPL) activity in postheparin plasma or in body tissues which are based on the original method of Korn (1). A common feature of many methods has been the activation of the incubation system by addition of serum or preheparin plasma, and the ability to inhibit enzyme activity with 1 M NaCl has been considered as a criterion for the specificity of the method. The outstanding difference between LPL techniques has been the substrate, e.g., chyle chylomicrons (2), commercial fat emulsions such as Ediol (1, 3) or Intralipid (4), and emulsions prepared by sonication in the presence of a detergent such as Triton X-100 (5, 6).

The administration of heparin releases into the circulation enzymes other than triglyceride lipase, and Biale and Shafrir (7), who tested the activity of postheparin plasma (PHP) with a variety of substrates, stressed the need for a careful selection of substrates; they suggested chylomicrons or pure triglycerides as being the most suitable. However, chylomicrons and triglyceride emulsions prepared by sonication are relatively unstable, and commercial fat emulsions contain mixed glycerides and considerable amounts of monoglycerides. The presence of detergents in the assay system may be undesirable, since it was shown by Doizaki and Zieve (8) that detergents can influence the stability of the substrate. The notorious instability of the substrates mentioned above, coupled with the lack of uniformity in expression of enzymatic units, has impeded the comparison of experimental results obtained in any two laboratories or even the comparison of results obtained within any one laboratory with different batches of substrate preparations (9).

In view of the above considerations, a simple assay method for LPL activity in PHP, heart, and adipose tissue utilizing a triolein-coated Celite substrate has been developed. The advantages of this method are its specificity for LPL and the ease of preparation of the substrate, which is stable and reproducible and requires neither emulsifying agents nor sonication. The substrate can efficiently be used for assaying pancreatic lipase as well.

Materials and Methods

Celite was obtained from the Arthur H. Thomas Co., Philadelphia, Pa. Sodium heparin (Eli Lilly and Co., Indianapolis, Ind.) had a concentration of 1000 units/ml or approximately 0.10 unit/μg. Triolein (Calbiochem, Los Angeles, Calif.) was >90% pure as judged by thin-layer chromatography on silica gel H with petroleum ether (bp 30–60°C)–diethyl ether–glacial acetic acid 90:10:1 (v/v/v) as the developing solvent. The major impurity in the triolein migrated with an R_F similar to that of methyl oleate; with the same solvents in a ratio of 70:20:4 (v/v/v), respectively, no traces of mono- or diglycerides could be detected even when the plate was overloaded with triolein. Bovine serum albumin, fraction V, was purchased from the Armour Pharmaceutical Co., Chicago, Ill. All reagents were analytical grade.

Preparation of Triolein-coated Celite and Incubation Buffer. 25 ml of a solution of triolein in chloroform (21.25 mg/ml) was transferred to a petri dish (10 cm in diameter), and 3 g of dry Celite was then carefully sprinkled over the entire surface of the solution. The petri dish was gently swirled in order to distribute the Celite evenly until the mixture had a gel-like appearance. The chloroform was allowed to evaporate at room temperature (1–2 hr) and the powder was broken up with a spatula and allowed to dry for another hour. The dry triolein-coated Celite was transferred to a glass-stoppered flask. Any clumps present were broken up by a vigorous shaking of the flask; this also assured the even distribution of the particles. The dry powder did not adhere to the glassware nor to the spatula and was sufficient for the assay of about 25 samples. For storage over long periods of time, the air in the flask was replaced by nitrogen and the flask was kept at 4°C.

Abbreviations: PHP, postheparin plasma; LPL, lipoprotein lipase; FFA, free fatty acids; BSA, bovine serum albumin.

The incubation buffer was prepared by dissolving 80 mg of BSA in 2.4 ml of 0.2 M Tris–HCl buffer (pH 8.0) and 0.6 ml of serum from fasting rats. The incubation buffer could be stored in the freezer for at least 3 months.

Preparation of Postheparin Plasma and Tissue Extracts. 24-hr-fasted rats were lightly anesthetized with ether and injected by heart puncture with 0.2 ml of a heparin solution containing 2 mg/ml; 10 min later blood was collected in chilled tubes by cannulating the abdominal aorta. 0.25 M sodium citrate (0.5 ml/10 ml of whole blood) was used as anticoagulant. After centrifugation at 3000 *g* for 30 min at 30°C, the plasma was withdrawn and kept in an ice bath until use. In some experiments PHP was stored at −20°C for 3 months. The 10% crude heart homogenates were prepared as described by Gartner and Vahouny (3), except that 0.025 M NH_4OH–NH_4Cl buffer (pH 8.0) was used as the homogenizing medium. Extracts of acetone powder of adipose tissue were prepared according to Greten, Levy, and Fredrickson (5), and extracts of pancreas were prepared in a similar fashion.

Enzyme Assay. 120-mg samples of the coated Celite were weighed and transferred to 25-ml stoppered Erlenmeyer flasks, and 3 ml of the incubation buffer was added to each flask. After the flasks were swirled for a few seconds in order to distribute the substrate evenly, they were placed in a Dubnoff shaker and incubated at 37°C (100 strokes per min). After temperature equilibration (ca. 10 min) 1 ml of PHP or aliquots of tissue extracts were added to each flask and incubation was continued for the desired time period. 1-ml aliquots were removed at convenient time intervals (1-ml serological pipettes were used, since narrow-tipped pipettes may on occasion become clogged with substrate) and they were immediately transferred to test tubes containing 5 ml of isopropanol–heptane–2 N H_2SO_4 40:10:1 (v/v/v) for fatty acid determinations by the method of Dole (10). The amounts of FFA released were plotted against time, and the points were joined by a straight line which passed through the origin. The slope of the curve was a measure of enzyme activity which was expressed in nmoles of FFA released per milliliter of plasma or per milligram of protein per minute, or as otherwise expressed under Results. Protein in tissue extracts was determined by the method of Lowry et al. (11).

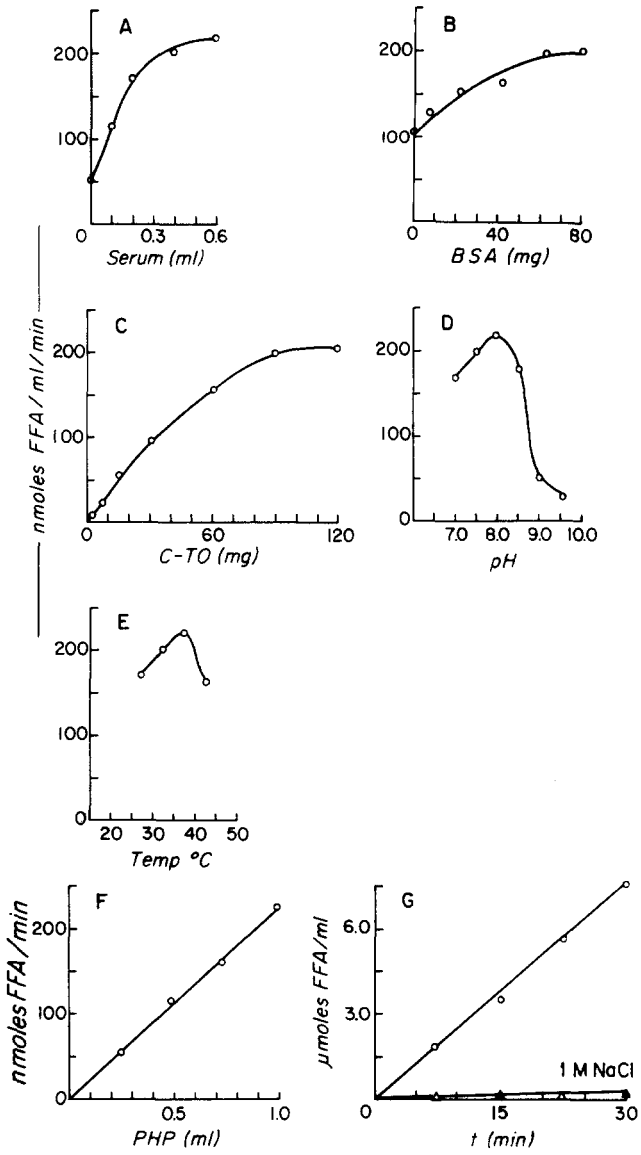

FIG. 1. Effects of: (*A*) serum concentration in the incubation buffer (ml of serum/3 ml of buffer), (*B*) bovine serum albumin (BSA) concentration in the incubation buffer (mg of BSA/3 ml of buffer), (*C*) amounts of substrate (Celite–triolein, C–TO) added to the system (1 mg C–TO ≈ 0.153 μmole of triolein), (*D*) pH of the incubation buffer, (*E*) temperature of incubation, and (*F*) postheparin plasma (PHP) concentration on the release of FFA during a 30-min incubation period of PHP. (*G*), effect of 1 M NaCl on enzyme kinetics. In experiments A–E, only the variant under study was changed; all other factors were maintained at optimal conditions as specified in the text.

Results

Lipoprotein Lipase Activity in Postheparin Plasma. The effect of varying the amounts of serum and BSA in the incubation buffer are shown in Fig. 1, *A* and *B*. As can be seen, FFA release increased with increasing amounts of serum or BSA, reaching maximal levels with amounts used in the final assay system, 0.6 ml and 80 mg, respectively. Three different lots of BSA gave identical results. By increasing the amount of substrate in the incubation medium (Fig. 1 *C*) no increment in FFA release was observed beyond 90 mg; hence, the amount employed in the final assay system, 120 mg, was not rate limiting. The optimal pH for the reaction was found to be 8.0 (Fig. 1 *D*), and the optimal temperature was 37°C (Fig. 1 *E*). As can be seen from Fig. 1 *F*, FFA re-

lease in the system was directly proportional to the amount of PHP added (in each case the volume of the incubation medium was brought up to 4 ml with preheparin plasma). Release of FFA was linear for 30 min (Fig. 1 *G*), but tended to level off somewhat after that time. As shown in the same figure, in the presence of 1 M NaCl, 98% of the enzymatic activity was inhibited.

Enzyme activity in PHP was determined under various experimental conditions. Storage of PHP from six normal rats for a period of 3 months at −20°C had only a very slight effect on LPL activity. Enzymatic activity in seven different pools (in each case PHP from six rats were pooled), each assayed with different preparations of substrate and incubation buffer, varied by less than 5%, demonstrating the satisfactory reproducibility of the method. Essentially the same results were obtained with three batches of substrate which were stored for 3 months, indicating the great stability of the substrate. Finally, three pools of PHP were shaken with untreated Celite (100 mg/ml) for 10 min at 37°C, and after centrifugation at 3000 *g* at 4°C, the supernatant PHP was assayed for LPL activity. The results showed that Celite had no effect on plasma LPL. FFA release by preheparin plasma was about 2.5% of the release by PHP. Preincubation of PHP for 60 min at 37°C caused a 60% decrease in enzyme activity. The addition of Triton X-100 (0.5 and 1.25 mg/ml of incubation system) caused decreases in FFA release (10 ± 2 and 57 ± 4%, respectively). The addition of deoxycholate (0.75 and 5.0 mg/ml) also caused decreases in FFA release (27 ± 3 and 78 ± 5%, respectively).

Lipoprotein Lipase Activity in Extracts of Adipose Tissue. An acetone powder of adipose tissue of rats fasted for 24 hr was extracted with cold NH_4OH–NH_4Cl buffer (pH 8.0); 15 mg of the powder per ml of buffer had a protein concentration of 6.5 mg/ml. 0.5–2.0 ml of extract was incubated with Celite substrate, incubation buffer, and 100 μg of heparin; the final volume of the incubation medium was adjusted to 5 ml with buffer. Since there was an unexplained lag period of 30 min during which there was little release of FFA, 30 min of incubation was taken as zero time. Samples were taken at four 30-min intervals for FFA determinations. The amount of FFA released was directly proportional to added protein, and the reaction was linear up to 120 min beyond zero time. LPL activity was completely inhibited with 1 M NaCl. When adipose tissue extracts were incubated in the absence of serum, enzymatic activity was about 30% of that observed when serum was present (Table 1).

Lipoprotein Lipase Activity of Heart Tissue Homogenates. The release of FFA by 10% heart homogenates was proportional to the amount of added protein (i.e., volume of aliquot in a total volume of 7 ml). In these studies, samples were taken after 15 min to allow for temperature equilibration, and at four 30-min intervals thereafter. As before, FFA release was linear over the entire 120 min of incubation. Enzymatic activity was completely inhibited with 1 M NaCl. As with PHP and adipose tissue, omitting serum from the incubation buffer caused a decrease of 60% in FFA release (Table 1).

TABLE 1 EFFECT OF DELETION OF SERUM FROM INCUBATION BUFFER ON LIPOPROTEIN LIPASE ACTIVITY OF HEART TISSUE HOMOGENATES AND EXTRACTS OF ACETONE POWDER OF ADIPOSE AND PANCREATIC TISSUES

Enzyme Source	Volume	Protein Concn	FFA Released With Serum	FFA Released Without Serum
	ml	*mg/ml*	*nmoles/mg of protein/min*	
Adipose tissue*	2.0	6.5	1.8	0.5
Heart homogenates*	3.0	10.0	3.9	1.6
Pancreatic tissue	0.1	0.58	3330	3450
Pancreatic tissue + 1 M NaCl	0.1	0.58	3500	3400

See text for experimental conditions.
* 100 μg of heparin was added to each incubation flask.

Triglyceride Lipase Activity in Pancreatic Tissue. 10 mg of acetone powder of pancreatic tissue was extracted in 10 ml of NH_4OH–NH_4Cl buffer (pH 8.0), resulting in a protein concentration of 57.5 μg/ml. Aliquots of up to 1 ml were incubated with substrate and incubation buffer or with incubation buffer devoid of serum. As the data in Table 1 indicate, the presence of serum in the incubation system was not required for the release of FFA, and the reaction was not affected by 1 M NaCl. FFA release was found to be proportional to protein concentration and was linear up to 1 hr of incubation.

In Vitro Effect of Heparin on Lipoprotein Lipase Activity. 1-ml aliquots of PHP, 2 ml of adipose tissue extracts, and 3 ml of 10% heart homogenates were assayed for LPL activity under the conditions specified above and in the presence of different concentrations of added heparin. The results of these experiments are presented in Fig. 2. Plasma LPL activity decreased progressively with in-

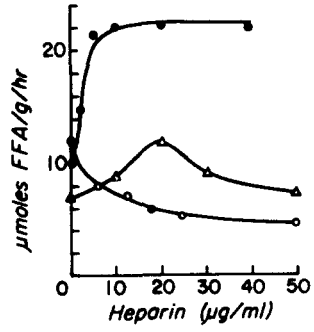

FIG. 2. Effect of heparin on the in vitro release of FFA by incubating postheparin plasma (○), heart (●), and adipose tissue (△) lipoprotein lipase. Incubation conditions are described in the text.

creasing heparin concentrations. A biphasic effect of heparin on adipose tissue LPL was observed, with a maximal increase of 60% in FFA release by 20 μg/ml. Heart LPL activity was increased more than twofold by as little as 8 μg/ml of added heparin; contrary to results with plasma and adipose tissue LPL, larger concentrations of heparin had no further effect on FFA release.

Discussion

The purpose of creating incubation conditions which would favor the encounter of a hydrophilic enzyme, LPL, and a hydrophobic substrate, triglyceride, is customarily accomplished by use of fat emulsions. In the present method this purpose was achieved by utilizing triolein-coated Celite as substrate. Triolein, dispersed over the fine particles of Celite, results in a large surface area, a condition which facilitates enzyme–substrate interactions; Celite itself has no effect on the enzymatic activity. The present method offers a number of advantages, the most salient of which are the ease of preparation of the Celite substrate and its excellent stability for long periods of time, as well as the reproducibility of experimental results. Triolein-coated Celite was kept for 3 months without any detectable changes in its substrate characteristics, and there is every reason to believe that it is stable for much longer periods when stored under proper conditions. The method is very specific for LPL: (*a*) practically no FFA was released by incubating preheparin plasma; (*b*) the only substrate available to the enzyme is a relatively pure triglyceride, free from mono- and diglycerides; (*c*) the system could be activated by the addition of serum; (*d*) 98% of FFA release could be blocked by 1 M NaCl; and (*e*) preincubation of PHP for 1 hr at 37°C caused a decrease of 60% in enzymatic activity similar to what was found by Greten et al. (12), who also showed that enzymatic activity toward mono- or diglyceride substrates was stable under these conditions. In contrast, pancreatic lipase, for which the coated Celite serves as a very satisfactory substrate, was not affected by either serum or 1 M NaCl. Optimal pH and temperature of incubation were within the ranges generally found in assaying LPL. Unlike the findings of Greten et al. (5), there was greater release of FFA at 37°C than at 27°C.

In determining LPL activity in PHP, we have found that sampling for FFA at zero time and after 15 and 30 min of incubation is quite satisfactory, since the amounts of FFA released during this time interval are sufficiently large to be determined with accuracy by the method of Dole (10). Linearity in FFA release with time has been generally observed. If preferred, 1-^{14}C-labeled triolein may be added during the coating of the Celite, and FFA release can then be determined by the methods of Greten et al. (5) or Schotz et al. (6).

In agreement with the findings of Wills (13), who had shown that large concentrations of detergents interfere with triglyceride lipase determinations, we observed that deoxycholate and Triton X-100 could cause considerable decreases in FFA release when added to our incubation system. Rather than an inhibition of enzymatic activity, the effect is probably due to interference with enzyme–substrate interactions. Fielding (14) used deoxycholate in isolating LPL from the enzyme–substrate complex during the purification of the enzyme.

The effect of heparin in decreasing FFA release by PHP was similar to that observed by Boberg and Carlson (4). The greater than twofold increase in FFA release observed during the incubation of heart tissue homogenates in the presence of heparin confirms the results of Gartner and Vahouny (3). We have no explanation for the biphasic effect of heparin on adipose tissue LPL activity. Greten et al. (5) reported that in the absence of heparin, FFA release was only 20% of the maximum, whereas Schotz et al. (6) reported that while 1 μg of heparin had no effect on FFA release, 20 μg considerably inhibited enzymatic activity.

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References

1. Korn, E. D. 1955. Clearing factor, a heparin activated lipoprotein lipase. *J. Biol. Chem.* **215:** 1–26.
2. Salaman, M. R., and D. S. Robinson. 1966. Clearing factor lipase in adipose tissue. *Biochem. J.* **99:** 640–647.
3. Gartner, S. L., and G. V. Vahouny. 1966. Heparin activation of soluble heart lipoprotein lipase. *Amer. J. Physiol.* **211:** 1063–1068.
4. Boberg, J., and L. A. Carlson. 1964. Determination of heparin-induced lipoprotein lipase activity in human plasma. *Clin. Chim. Acta.* **10:** 420–427.
5. Greten, H., R. I. Levy, and D. S. Fredrickson. 1968. A further characterization of lipoprotein lipase. *Biochim. Biophys. Acta.* **164:** 185–194.
6. Schotz, M. C., A. S. Garfinkel, R. J. Huebotter, and J. E. Stewart. 1969. A rapid assay for lipoprotein lipase. *J. Lipid Res.* **11:** 68–69.
7. Biale, Y., and E. Shafrir. 1969. Lipolytic activity toward tri- and monoglycerides in postheparin plasma. *Clin. Chim. Acta.* **23:** 413–419.
8. Doizaki, W. M., and L. Zieve. 1968. Effect of surface-active agents on post-heparin lipase activity. *Biochim. Biophys. Acta.* **152:** 713–717.
9. Boberg, J. 1970. Quantitative determination of heparin-released lipoprotein lipase activity in human plasma. *Lipids.* **5:** 452–456.
10. Dole, V. P. 1956. A relation between non-esterified fatty acids in plasma and the metabolism of glucose. *J. Clin. Invest.* **35:** 150–154.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
12. Greten, H., R. I. Levy, H. Fales, and D. S. Fredrickson.

1970. Hydrolysis of diglyceride and glyceryl monoester diethers with "lipoprotein lipase." *Biochim. Biophys. Acta.* **210:** 39–45.
13. Wills, E. D. 1955. The effect of surface-active agents on pancreatic lipase. *Biochem. J.* **60:** 529–534.
14. Fielding, C. J. 1969. Purification of lipoprotein lipase from rat postheparin plasma. *Biochim. Biophys. Acta.* **178:** 499–507.