A stable, radioactive substrate emulsion for assay of lipoprotein lipase

Peter Nilsson-Ehle and Michael C. Schotz

Research, Veterans Administration Wadsworth Hospital Center, Los Angeles, California 90073; and Department of Medicine, UCLA School of Medicine, Los Angeles, California 90024

Abstract A method is described for the assay of lipoprotein lipase, using a stable, radioactive substrate emulsion. Fatty acid-labeled trioleoylglycerol was emulsified by homogenization in glycerol with lecithin as detergent. This anhydrous emulsion was stable for at least six weeks. Substrate solutions for enzyme assay were prepared by diluting the emulsion with buffer containing serum and albumin. The fatty acid produced on hydrolysis was isolated in a one-step liquid–liquid partition system. Incubations with extracts of acetone powders from adipose tissue displayed characteristics of lipoprotein lipase activity, i.e., serum dependence and inhibition by NaCl and protamine. The method is rapid (<1 hour), sensitive and reproducible, and suitable for routine use.

Supplementary key words adipose lipoprotein lipase · tri-[^{3}H]oleoylglycerol · triglyceride · postheparin serum

A variety of substrates have been employed for the determination of lipoprotein lipase (LPL) activity, reflecting the inherent difficulties in lipase assays. Chylomicrons, a natural substrate, are relatively unstable and difficult to standardize (1). Commercially available emulsions (2–4) are stable but suffer from lack of specificity due to the presence of partial glycerides and water-soluble esters (3, 5), which may constitute substrates for esterolytic activities other than LPL (5). This problem is avoided in assays using triacylglycerol emulsions prepared by sonication in the presence of a detergent (5–7). Incorporation of radioactive tracers in such substrate preparations greatly increases the sensitivity and speed of operation (8–11); however, the instability of these emulsions precludes repeated use and necessitates daily sonication under strictly controlled conditions to minimize interassay variation (11–13). The present report describes the preparation of a stable, radioactive trioleoylglycerol (TO) emulsion, which allows repeated assay of LPL with high sensitivity, specificity, and reproducibility. A one-step liquid–liquid partition system for isolation of reaction products (14) contributes to the simplicity and rapidity of the method.

MATERIALS

Tri-[9,10-^{3}H]oleoylglycerol ([^{3}H]TO) (414 mCi/mmole), obtained from Amersham-Searle, Arlington Heights, Ill., was purified by column chromatography using 2 g of silicic acid covered by a layer of Florisil (200 mg) to improve separation of fatty acid (15). Ten mCi of [^{3}H]TO were applied to the columns in petroleum ether (bp 30–60°C) with 50 mg of unlabeled TO as carrier. After passage of 50 ml of petroleum ether, TO was eluted with 5% diethyl ether in petroleum ether (80–90% recovery). Radiopurity of the product, as determined by thin-layer chromatography, was >99.9%. Unlabeled TO (NuChek Prep, Chicago, Ill.) was purified in the same way.

The purification of TO substrates resulted in substantially lower blank values (about 300 cpm compared to about 1200 cpm when nonpurified TO was used as substrate). Also, the removal of monoacylglycerol (frequently constituting 0.5–1.2% of total acylglycerols in the commercial TO preparations) minimized the risk of interference in the assay of other lipolytic activities, e.g., monoacylglycerol-hydrolyzing enzymes (5, 11).

[9,10-^{3}H]Oleic acid, obtained from Amersham-Searle, had a stated purity of >99%. Unlabeled oleic acid was purchased from NuChek Prep. Lecithin and lysolecithin, both from egg yolk, were from Sigma Chemical Co., St. Louis, Mo. Crystalline bovine serum albumin, protamine sulfate (salmine) and heparin (160 U/mg) were obtained from Calbiochem, La Jolla, Calif. Glycerol (spectrophotometric grade, <0.05% H_2O) was obtained from Mallinckrodt, St. Louis, Mo. All other substances and solvents used were reagent grade.

Serum was obtained from fasting rats and heated to 62°C for 10 min to eliminate endogenous lipolytic

Abbreviations: LPL, lipoprotein lipase; TO, trioleoylglycerol; [^{3}H]TO, tri-[^{3}H]oleoylglycerol.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

activity (16). Acetone powders of rat epididymal adipose tissue were prepared as previously described (17). The powders were extracted (2–5 mg of powder per ml of buffer) with 0.05 M NH_4OH–NH_4Cl buffer (pH 8.1) or 0.05 M Tris-HCl (pH 8.0) containing 1M ethylene glycol (17). The supernates obtained after centrifugation for 40,000 *g-min* were employed as enzyme source in most of the experiments.

Postheparin serum, used to check stability of the concentrated substrate emulsion, was obtained from blood drawn 10 min after the injection of 10 U heparin per kg body wt into fasting rats. Lipolytic activity of postheparin serum was stable for at least 2 months at −20°C.

As reference method for the measurement of LPL activity, our earlier described assay system (12) was employed. This substrate was prepared by sonication of TO in Tris-HCl buffer, using lysolecithin as emulsifier. The final concentrations in the assay were TO, 1.47 μmoles/ml; lysolecithin, 0.085 μmoles/ml; albumin, 0.1% (w/v); and serum, 3% (v/v); and 0.1 M Tris-HCl (pH 8.0). Incubations, isolation of released fatty acid, and radioactivity measurements were performed as described below under Procedure.

PROCEDURE

Anhydrous emulsions of [^{3}H]TO, stabilized by lecithin, were prepared in glycerol (18) as follows. Labeled and unlabeled TO (stored in benzene) were mixed to give 600 mg of TO (with ca 2500 × 10^6 cpm) and 36 mg of lecithin (in chloroform) was added. The solvents were evaporated under a stream of nitrogen. The dried lipids were emulsified in 10 ml (12.5 g) of glycerol by homogenization for 5 min continuously using a Polytron PT 10–35 (Brinkmann Instruments, Westbury, N. Y.) equipped with a PT 10 ST generator at setting 7. The homogenization was carried out in a glass test tube, 1.8 × 13 cm, immersed in an ice bath. The emulsion, which is optically clear due to the similar refractive indices of the lipid and glycerol, could be stored at room temperature for at least six weeks (see Results). It will be referred to as "concentrated substrate".

Substrate solutions for assay were prepared daily by dilution of 1 volume of concentrated substrate with 4 volumes of Tris-HCl buffer 0.2 M (pH 8.0) containing 3% (w/v) bovine serum albumin and 1 volume of serum, and shaken vigorously on a Vortex mixer for 5 sec. The viscous concentrated substrate was dispensed with a Biopette (Schwartz/Mann, Orangeburg, N. Y.); the accuracy was checked by weighing (specific gravity of glycerol, 1.25). The opaque emulsion obtained was ready for use immediately and retained its substrate properties for at least 6 hr (see Results). It will be referred to as "assay substrate".

Incubations were carried out at 37°C in a total volume of 0.2 ml with 0.1 ml of assay substrate and 0.1 ml of enzyme and/or buffer. The final concentrations in the assay vials were TO, 5.66 μmoles/ml; lecithin, 0.35 μmoles/ml; albumin, 1% (w/v); and serum, 8.5% (v/v) in Tris-HCl 0.07 M (pH 8.0) containing 8.5% glycerol. Assay time was generally 30 min; however, all incubations with postheparin serum, because of its high enzymatic activity, were carried out for 15 min. The amount of enzyme assayed was selected so no more than 8% of the TO substrate was hydrolyzed during the incubations.

The fatty acid produced during the incubations was isolated using a modification of the liquid–liquid partition system described by Belfrage and Vaughan (14). The reactions were stopped by addition of 3.25 ml of methanol–chloroform–heptane 1.41:1.25:1 (v/v/v) (14), followed by 1.05 ml of 0.1 M potassium carbonate–borate buffer (pH 10.5) (11, 14). The extraction and all subsequent procedures were performed at room temperature. After vigorous mixing on a Vortex mixer for 15 sec, the tubes were centrifuged for 15 min at 3000 *g*. A 1-ml aliquot of the methanol–water upper phase, which had a volume of 2.45 ml and contained about 76% of the [^{3}H]oleic acid released (see Results), was counted in a Beckman LS 250 spectrometer using 10 ml of Instagel–toluene 1:1 as scintillator. An aliquot (0.025 ml) of the assay substrate was assayed for radioactivity after the addition of 1 ml of upper phase from control incubations without enzyme. Quenching, monitored by automatic external standardization, was constant within each experiment as well as between experiments.

Disposable glass tubes (1.3 × 10 cm) and automatic dispensing systems were used to facilitate the procedure and increase speed and reproducibility.

Enzyme activity was calculated according to the formula, enzyme activity = net cpm × (1/incubation time) × (1/sp act) × 3 × 2.45 × (1/0.76), where enzymatic activity is expressed as mU in the sample assayed. One mU of enzymatic activity is defined as the release of 1 nanomole of oleic acid per minute at 37°C. Net cpm refers to the radioactivity in 1 ml of upper phase; incubation time is given in min; sp act represents the specific activity of the substrate expressed as cpm per nmole of triglyceride. The last three factors represent the molar ratio of fatty acid released to triglyceride hydrolyzed, and the correction factors for the volume of the upper phase and partition of oleic acid in the extraction system, respectively.

This basic procedure was followed in most experi-

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH

ments. Any variation of assay conditions is described in the respective legends to the figures and tables.

RESULTS AND DISCUSSION

Characteristics of the assay are shown in **Fig. 1.** Substrate concentration was not rate-limiting at 5.7 μmoles/ml, the concentration used in the assay (Fig. 1A). The reaction had a pH-optimum of about 8 (Fig. 1B). Enzyme activity was linear with time for at least 2 hr (Fig. 1C) and with increasing amounts of enzyme preparation (Fig. 1D). A reduction in reaction rate was generally noted when about 8% of the TO substrate had been hydrolyzed. Maximal serum stimulation was obtained with 7.5% (v/v) serum (**Fig. 2A**). Optimal albumin concentration was about 1% (w/v) (Fig. 2B); total albumin concentration, including the contribution from the serum component, was thus about 1.4%.

Extraction of oleic acid from mixtures containing all substrate components and labeled oleic acid instead of labeled triacylglycerol was highly reproducible (**Table 1**), averaging 76% recovery in the upper phase under a variety of conditions. Identical results were obtained when [^{3}H]oleic acid in albumin-bound form

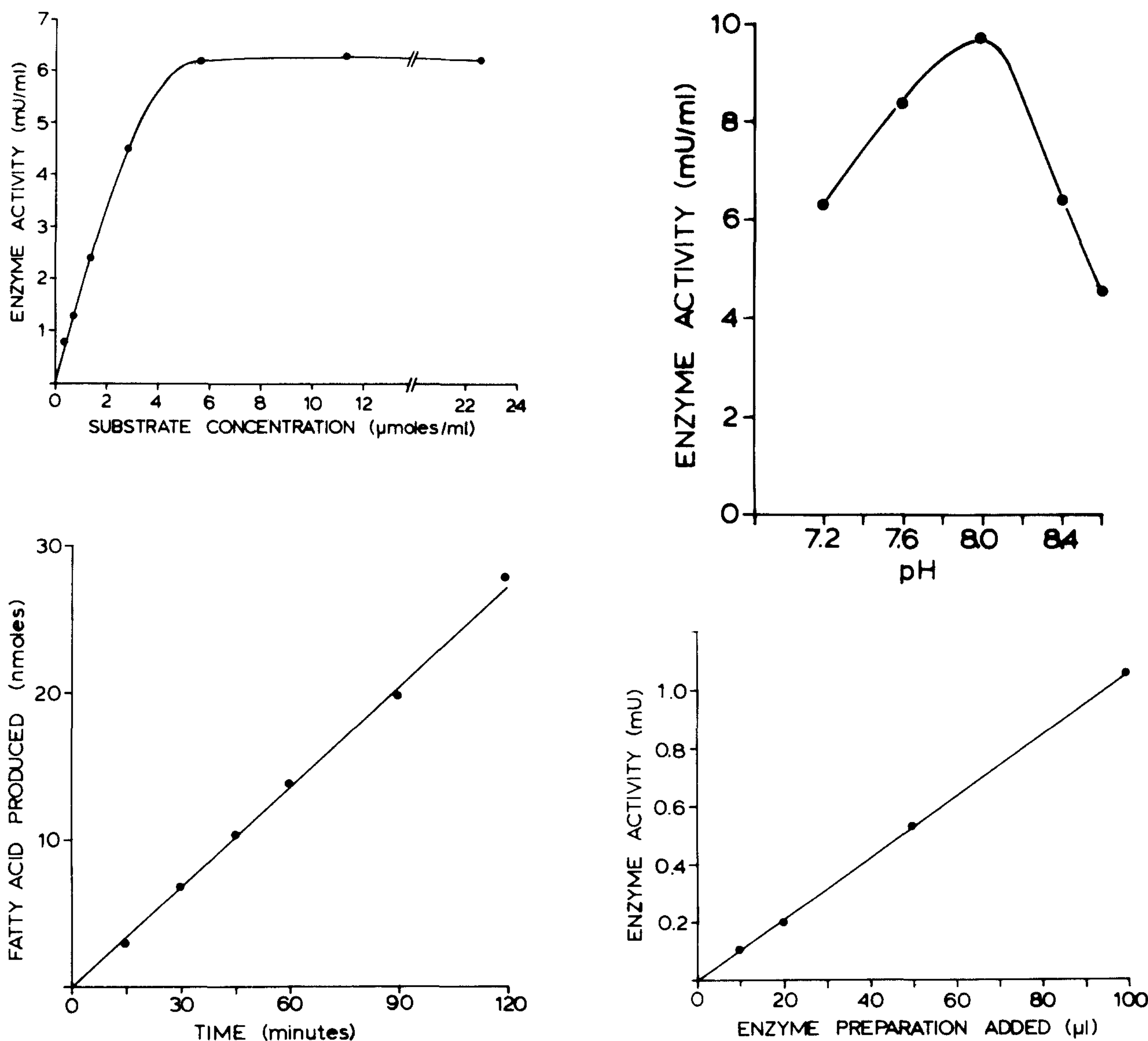


Fig. 1. Relation of enzyme activity measured under standard conditions to (A) substrate concentration (ratio substrate: detergent was constant throughout the experiment); (B) pH of assay (ionic strength constant, Tris-HCl buffer); (C) incubation time, and (D) enzyme concentration. Extracts of acetone powder from rat adipose tissue were used as enzyme source.

Downloaded from www.jlr.org by guest, on June 19, 2012

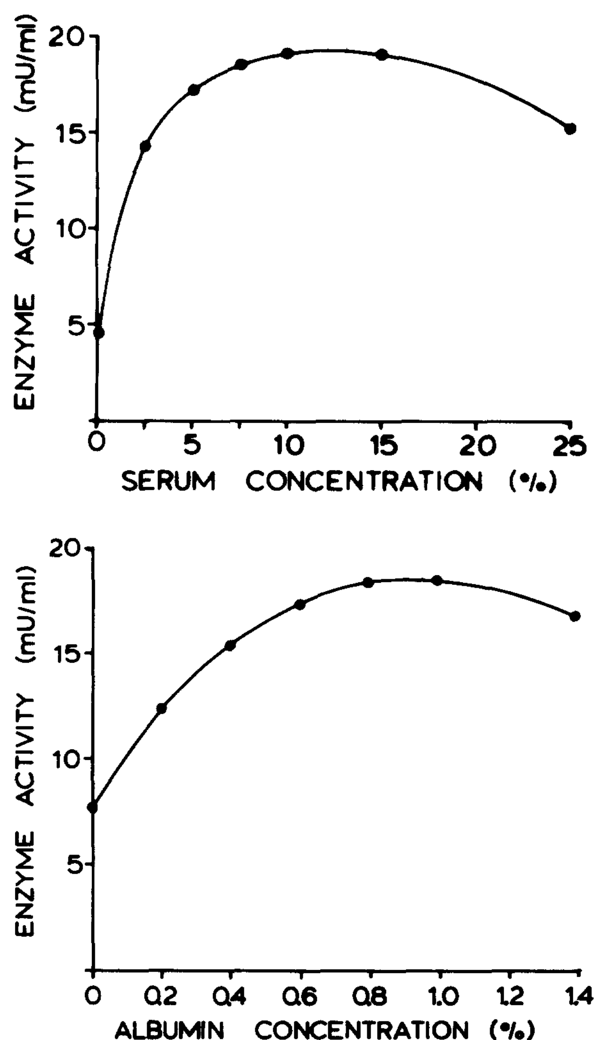


Fig. 2. Relation of enzyme activity measured under standard conditions to (A) serum concentration (albumin concentration was kept constant by successive substitution by serum for 4% (w/v) serum albumin) and (B) albumin concentration.

was added to the mixtures. It should be noted that the efficiency of extraction was significantly lower at 4°C. ($P < 0.001$).

Table 2 shows effects of some parameters generally used as criteria for LPL. Omission of serum, as well as presence of 1 M NaCl in the assay system or preincubation of enzyme with 1 M NaCl or protamine greatly reduced enzymatic activity, substantiating the specificity for LPL.

Reproducibility and precision of the assay system were satisfactory. Analysis of precision within one assay showed a coefficient of variation (relative SD) of 1.6% for determinations in the usual range of enzymatic activity (mean 1.61, range 1.58–1.65 mU, n = 8), corresponding to about 25,000 net cpm and representing hydrolysis of about 5% of the triacylglycerol substrate. With lower enzymatic activities, the coefficient of variation was 4.8% (mean 0.48, range 0.44–0.52 mU, n = 8). Interassay precision, as tested by duplicate assay of the same enzyme extract with six different substrates prepared from the same stock, showed a variation coefficient of 3.1% (mean 2.12, range 2.01–2.20 mU of enzyme activity). The preparation procedure for the concentrated substrate was also highly reproducible. Four substrate concentration curves obtained with assay substrate made by dilution from four different preparations of concentrated substrate were all essentially similar to that presented in Fig 1A. The sensitivity of the assay easily allowed determinations of about 0.06 mU of enzymatic activity (net cpm corresponding to twice the blank values). Theoretically, enzymatic activity of 0.016 mU can be determined (value > blank + 2 SD), corresponding to less than 0.5 μl of postheparin serum.

TABLE 1. Extraction of [^{3}H]oleic acid from assay mixture

Addition	Percent [^{3}H]Oleic Acid in Upper Phase
None	75.7
None; extraction at 4°C[a]	61.0
NaCl 0.15 M	74.5
NaCl 1.5 M	75.1
TRIS-HCl 0.2 M (pH 8.0)	73.5
NH_4OH-NH_4Cl 0.05 M (pH 8.1)	75.8
NH_4OH-NH_4Cl 0.05 M (pH 8.6)	77.1
Enzyme[b]	76.0
Glycerol 10% (v/v)	77.5
Ethylene glycol 1 M	77.6
Heparin 10 U/ml	76.3

Unlabeled TO (11.25 μmoles/ml) and oleic acid (1.35 μmoles/ml) with trace amounts of [^{3}H]oleic acid were emulsified by sonication with lecithin (0.70 μmoles/ml) in 0.2 M Tris-HCl (pH 8.0) containing serum (16%, v/v) and albumin (2%, w/v). A 0.1 ml portion of emulsion was mixed in quadruplicate with 0.1 ml of water containing the indicated additions. Oleic acid was extracted from the mixtures at room temperature essentially as described by Belfrage and Vaughan (15) unless indicated otherwise.

[a] Temperature of solvent mixture and buffer employed in extraction system.

[b] Supernate of ammonia extract of acetone powder from rat adipose tissue.

TABLE 2. Characterization of assay system

Condition of Assay[a]	Enzyme Activity (Relative to Complete System)
Complete system	100
NaCl (1 M)	7
Protamine sulfate (1.5 mg/ml)	69
Preincubation with protamine (1.5 mg/ml)[b]	14
Minus serum	27
Albumin (4%, w/v) substituting for serum	25

[a] Extract of acetone powder from rat adipose tissue was incubated with substrate for 30 min at 37°C under various conditions.

[b] The enzyme was preincubated with protamine sulfate for 30 min at room temperature.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH JLR

TABLE 3. Stability of concentrated substrate

Time	Blank	Enzymatic Activity Measured
days	*cpm*	*mU/ml*
1	301	53.0
2	287	54.5
8	390	51.5
15	485	54.5
22	600	50.5
28	796	53.0
35	801	51.5
42	903	51.0
Average ± SD		52.45 ± 1.45

At indicated times, assay substrates were prepared from one preparation of concentrated substrate as described in the text, and incubated for 15 min with 0.02 ml of freshly thawed postheparin serum from the same pool. Each value represents the mean of triplicate determinations.

The concentrated substrate emulsion was essentially stable for at least six weeks. Enzymatic activity recorded with different assay substrates prepared from the same concentrated substrate, using a pool of postheparin serum as enzyme source, was essentially the same over this period of time (**Table 3**), showing a variation coefficient between assays of about 2.8%. There was a slow but continuous increase in blank values, indicating some decomposition of the triacylglycerol substrate; even after six weeks, however, fatty acid radioactivity measured in blank samples corresponded to less than 0.05% of that of the TO substrate. Thin-layer chromatography of a chloroform–methanol extract of the concentrated substrate showed that 6 weeks after preparation, 99.92% of the radioactivity was associated with the TO fraction, compared to 99.98% on the day of preparation. Similar data were obtained with concentrated substrates stored at 4°C and room temperature. After the addition of buffer and serum, an assay substrate could be stored at least 6 hr without change in the enzymatic activity measured (51.0, 50.8, 51.3, and 53.9 mU of enzymatic activity per ml postheparin serum recorded after 0, 2, 4, and 6 hr, respectively).

A comparison between the new method and our earlier employed assay system using a sonicated substrate (12) demonstrated a satisfactory correlation between assays. The data in **Fig. 3,** representing single determinations with each assay system of LPL activity in fractions obtained by gel chromatography of extracts of acetone powders from rat adipose tissue (17), show a correlation coefficient of 0.96. In eight similar parallel runs correlation coefficients of 0.96–0.99 were obtained. The absolute values obtained with the two assays were similar (mean value for slope of the regression line in parallel runs was 1.06).

Increasing the time for homogenization of the concentrated substrate to 10 or 15 min did not change enzymatic activity or the shape of the substrate concentration curve. Additional sonication (with a Branson W185 Sonifier Cell Disruptor with microtip) of the concentrated substrate did not enhance the enzymatic activity recorded. In contrast to our experience with triacylglycerol emulsions prepared by sonication in buffer (12), lysolecithin could not substitute for lecithin in the present procedure. The substrate concentration curve obtained with lysolecithin-stabilized emulsions was reproducibly biphasic, indicating the presence of different populations of substrate particles. Ethylene glycol, being less viscous than glycerol and easier to dispense, was tried as a base for the concentrated substrate; however, these preparations were relatively unstable.

Zilversmit et al. (18) have demonstrated that stable, anhydrous emulsions of lipid can be prepared in glycerol by homogenization. In the present study, we have used this observation to obtain a stable substrate for lipase assay.

The method described is a modification of our earlier LPL assay using a substrate emulsion prepared by sonication (11, 12). With the sonicated emulsion, substrate saturation is obtained at about one-fourth of the TO concentration used in the present assay (11),

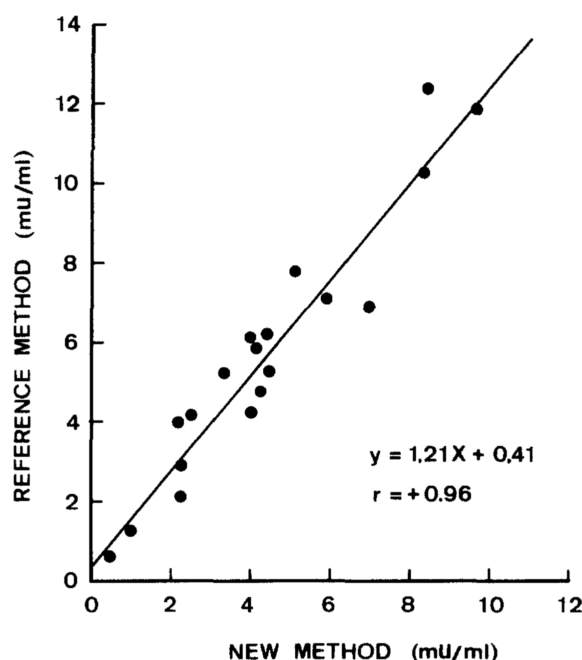
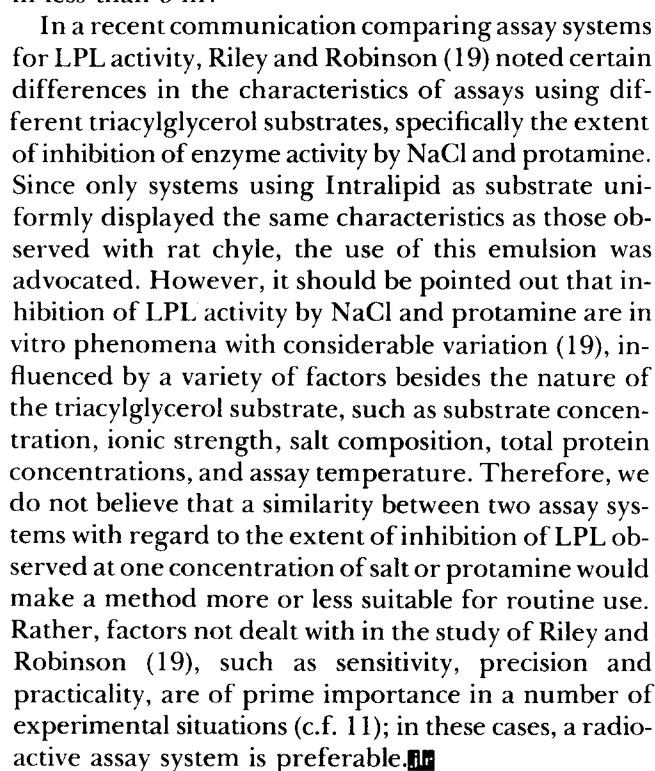


Fig. 3. Linear regression of lipoprotein lipase activities measured by assay using triglyceride substrate prepared by sonication in buffer (reference method) and assay using a triglyceride substrate prepared by homogenization in glycerol (new method). Each point represents single determinations of enzyme activity in gel chromatography fractions of an extract of acetone powder of rat adipose tissue.

Downloaded from www.jlr.org by guest, on June 19, 2012

indicating a difference in the size of the emulsion particles obtained and of the area of the total lipid–water interface. The serum and albumin concentrations required to provide optimal assay conditions were also 3–4 times higher for the method described in this communication. With these exceptions, data obtained with the two assays are similar quantitatively as well as qualitatively, as evidenced by correlation coefficients of >0.96 between parallel assay series. The intra-assay precision (± 1–4%) is comparable for the two methods (12); however, the interassay precision with the present method (± 3%) is higher than that demonstrated earlier by us (11) and others (13) for assays employing sonicated substrates (about ± 10%). From the practical point of view, the elimination of the sonication step also results in a greater speed of operation. One assay run takes 35–50 min depending on incubation time, and one person can perform 200 determinations in less than 3 hr.



In a recent communication comparing assay systems for LPL activity, Riley and Robinson (19) noted certain differences in the characteristics of assays using different triacylglycerol substrates, specifically the extent of inhibition of enzyme activity by NaCl and protamine. Since only systems using Intralipid as substrate uniformly displayed the same characteristics as those observed with rat chyle, the use of this emulsion was advocated. However, it should be pointed out that inhibition of LPL activity by NaCl and protamine are in vitro phenomena with considerable variation (19), influenced by a variety of factors besides the nature of the triacylglycerol substrate, such as substrate concentration, ionic strength, salt composition, total protein concentrations, and assay temperature. Therefore, we do not believe that a similarity between two assay systems with regard to the extent of inhibition of LPL observed at one concentration of salt or protamine would make a method more or less suitable for routine use. Rather, factors not dealt with in the study of Riley and Robinson (19), such as sensitivity, precision and practicality, are of prime importance in a number of experimental situations (c.f. 11); in these cases, a radioactive assay system is preferable.

The authors thank Dr. Donald Zilversmit for his suggested use of the glycerol-stabilized emulsion for this study. Mrs. Yona Goldberg and Mrs. Judith Nikazy provided excellent technical assistance. This study was supported in part by grants from United States Public Health Service HL16577, American Heart Association, Greater Los Angeles, Affiliate 492-IG2, and the Veterans Administration, Project 7770-01.

Manuscript received 26 December 1975; accepted 4 June 1976.

REFERENCES

1. Robinson, D. S. 1963. The clearing factor lipase and its action in the transport of fatty acids between the blood and the tissues. *Adv. Lipid Res.* **1:** 133–182.
2. Korn, E. D. 1955. Clearing factor, a heparin-activated lipoprotein lipase. *J. Biol. Chem.* **215:** 1–26.
3. Fredrickson, D. S., K. Ono, and D. L. Davis. 1963. Lipolytic activity of post-heparin plasma in hypertriglyceridemia. *J. Lipid Res.* **4:** 24–33.
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. Biale, Y., and E. Shafrir. 1969. Lipolytic activity toward tri- and monoglycerides in postheparin plasma. *Clin. Chim. Acta.* **23:** 413–419.
6. Chung, J., and A. M. Scanu. 1974. Continuous pH-stat titration method for the assay of lipoprotein lipase in vitro. *Anal. Biochem.* **62:** 134–148.
7. Fielding, C. J. 1972. Further characterization of lipoprotein lipase and hepatic postheparin lipase from rat plasma. *Biochim Biophys. Acta.* **280:** 569–578.
8. Greten, H., R. I. Levy, and D. S. Fredrickson. 1968. A further characterization of lipoprotein lipase. *Biochim Biophys. Acta.* **164:** 185–194.
9. Schotz, M. C., A. S. Garfinkel, R. J. Huebotter, and J. E. Stewart. 1970. A rapid assay for lipoprotein lipase. *J. Lipid Res.* **11:** 68–69.
10. Schotz, M. C., and A. S. Garfinkel. 1972. A simple lipase assay using trichloroacetic acid. *J. Lipid Res.* **13:** 824–826.
11. Nilsson-Ehle, P., H. Tornquist, and P. Belfrage. 1972. Rapid determination of lipoprotein lipase activity in human adipose tissue. *Clin. Chim. Acta.* **42:** 383–390.
12. Nilsson-Ehle, P. 1974. Human lipoprotein lipase activity: Comparison of assay methods. *Clin. Chim. Acta.* **54:** 283–291.
13. Krauss, R. M., R. I. Levy, and D. S. Fredrickson. 1974. Selective measurement of two lipase activities in postheparin plasma from normal subjects and patients with hyperlipoproteinemia. *J. Clin. Invest.* **54:** 1107–1124.
14. 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.
15. Carroll, K. K. 1961. Separation of lipid classes by chromatography on Florisil. *J. Lipid Res.* **2,** 135–141.
16. Henson, L., and M. C. Schotz. 1975. Detection and partial characterization of lipoprotein lipase in bovine aorta. *Biochim. Biophys. Acta.* **409:** 360–366.
17. Garfinkel, A. S., P. Nilsson-Ehle, and M. C. Schotz. 1976. Regulation of lipoprotein lipase: Induction by insulin. *Biochim. Biophys. Acta.* **424:** 264–273.
18. Zilversmit, D. B., N. K. Salky, M. L. Trumbull, and E. L. McCindless. 1956. The preparation and use of anhydrous fat emulsions for intravenous feeding and metabolic experiments. *J. Lab. Clin. Med.* **48:** 386–391.
19. Riley, S., and D. S. Robinson. 1974. Studies on the assay of clearing factor lipase (lipoprotein lipase). *Biochim. Biophys. Acta.* **369:** 371–386.

Downloaded from www.jlr.org by guest, on June 19, 2012

ASBMB JOURNAL OF LIPID RESEARCH