A simple method for the separation of triacylglycerols from fatty acids released in lipase assays

Jayme Borensztajn,[1] M. Narahari Reddy, and Alyssa R. Gladstone

Department of Pathology, Northwestern University Medical School, Chicago, IL 60611

Summary A simple procedure for the partition of triacylglycerols from albumin-bound fatty acids is described. This procedure is based on the ability of fumed silicon dioxide to remove emulsified triacylglycerols from aqueous media. The method was developed for the assay of lipoprotein lipase activity but it may be used for the assay of other lipases.—**Borensztajn, J., M. N. Reddy, and A. R. Gladstone.** A simple method for the separation of triacylglycerols from fatty acids released in lipase assays. *J. Lipid Res.* 1988. **29:** 1549–1552.

Supplementary key words chylomicrons • Liposyn • emulsified triolein

One of the methods most commonly used for the measurement of lipase activity involves the quantitation of the fatty acids hydrolyzed from a triacylglycerol emulsion. In the highly sensitive lipase assays that use radiolabeled triacylglycerols as substrate, the quantitation of the released labeled fatty acids requires that they first be separated from the unhydrolyzed labeled triacylglycerol emulsion. Among the described separation methods, liquid/liquid partition of the fatty acids from the unhydrolyzed substrate is the most rapid and practical (1–5). However, the efficiency of this separation method can be significantly affected by various additions (e.g., detergents) to the assay medium (1, 4–6). Thin-layer chromatography and anion-exchange resins have been reported to be more efficient and reliable than the liquid/liquid partition method (6, 7–11). However, these methods are labor-intensive, time-consuming and, therefore, impractical for the performance of large numbers of lipase assays. This report describes a simple and rapid procedure for the complete separation of albumin-bound fatty acids from triacylglycerol emulsions. This procedure is based on the ability of fumed silicon dioxide (Cab-O-Sil®) to precipitate lipoproteins from their aqueous environment (12, 13). We report that addition of Cab-O-Sil to a lipase assay mixture quantitatively precipitates the emulsified triacylglycerols leaving the albumin-bound fatty acids in the supernatant. This separation procedure is not affected by high protein concentrations or by the addition of detergents to the enzyme reaction mixtures. The present method was developed for the assay of lipoprotein lipase, but it may be applicable for the determination of the activity of other lipases.

MATERIALS AND METHODS

Materials

[1-^{14}C]Palmitic acid (sp act 58 Ci/mol) and tri[9,10(n)-^{3}H]oleoylglycerol (sp act 0.5–1 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin (fraction V) was obtained from Sigma Chemical Co. (St. Louis, MO). Liposyn (safflower oil emulsion) was from Abbott Laboratories (North Chicago, IL). Liquid scintillation fluid was 3a70B (Research Products Intl., Mount Prospect, IL). Cab-O-Sil (5 mesh) was obtained from Cabot Corp. (Tuscola, IL). Purified milk lipoprotein lipase was prepared as previously described (14).

Preparation of labeled triacylglycerol emulsions and albumin-bound fatty acids

Three different labeled triacylglycerol emulsions were used in this study: *a*) chylomicrons, *b*) emulsified triolein, and *c*) Liposyn. For the preparation of labeled chylomicrons, the thoracic ducts of male Sprague-Dawley rats (180–220 g) were cannulated and the animals were fed, by stomach intubation, 60 μCi of [1-^{14}C]palmitic acid mixed with 3 ml of a mixture of corn oil (1 ml) and egg yolk (2 ml). The lymph was collected during the following 12 hr at room temperature in the absence of preservatives. Large chylomicrons (S_f>400) were separated from the lymph as previously described (15) and redispersed in 0.15M NaCl for use in the lipase assays. The chylomicrons were stored at 4°C and used within 5 days of their isolation. Stable emulsions of ^{3}H-labeled triolein were prepared as described by Nilsson-Ehle and Schotz (16). Labeled Liposyn was prepared by sonication with trace amounts of labeled [^{3}H]triolein, essentially as described by Peterson, Olivecrona, and Bengtsson-Olivecrona (17) for the preparation of labeled Intralipid. Albumin-bound palmitic acid solutions were prepared as described by Spector (18).

Lipase assay composition

The composition of the lipase assay media was, with some modifications, similar to those previously used for the assay of lipoprotein lipase activity. The assay medium prepared with chylomicrons (0.145 ml) was

[1]To whom correspondence should be addressed.

based on that described by Lukens and Borensztajn (19) and contained 1.15 mg chylomicron triacylglycerols, 0.023 ml rat serum, 10% (w/v) albumin, 0.25 M Tris/HCl buffer (pH 8.5), and 0.17 M NaCl. The assay medium (0.1 ml) prepared with the stable triolein emulsion was derived from that of Nilsson-Ehle and Schotz (16) containing 1 mg triacylglycerol. The assay composition differed only in the final albumin concentration (4% rather than 1%). The composition of the assay medium (0.1 ml) containing Liposyn (1 mg triacylglycerol) was as described by Peterson et al. (17). The final volume of each assay medium was made up to 0.2 ml by the addition of 0.15 M NaCl with or without sodium deoxycholate or Triton X-100, or by the addition of purified milk lipoprotein lipase.

Separation of albumin-bound fatty acids from triacylglycerol emulsions

A slurry of Cab-O-Sil was prepared at a concentration of 4.1% (w/v) in 0.55 MNaCl. This slurry could be stored at room temperature for several weeks. The slurry was continuously and vigorously mixed during use with the aid of a magnetic stirrer in order to minimize variations in the amounts of Cab-O-Sil added to the different lipoprotein lipase assay media. Two ml of the slurry was added to the assay media (0.2 ml) and the assay tubes were vigorously mixed for 10–15 sec with the aid of a vortex mixer. The tubes were then centrifuged for 10 min at 1000 *g* in a swinging bucket bench centrifuge (Sorvall RT-6000, Hoffman Estates, Il). This centrifugation was sufficient to separate the mixture into a clear supernatant and a well-formed gel-like pellet which contained the Cab-O-Sil together with the triacylglycerol substrate. Aliquots (0.1–0.2 ml) of the clear supernatant were then taken in triplicate for radioactivity measurement.

RESULTS

Chylomicrons and very low density lipoproteins are the physiological substrates for lipoprotein lipase. However, because their radiolabeling and isolation is laborious and also because they cannot be stored for long periods in a stable form, these lipoproteins are often substituted with stable artificial triacylglycerol emulsions in lipase assays. In the present study we used lipoprotein lipase assay mixtures that contained either chylomicrons or one of two stable triacylglycerol emulsions; *a*) triolein emulsified with lecithin in the presence of glycerol or *b*) Liposyn, a 10% (w/v) preparation of safflower oil emulsified with egg lecithin that is commercially available for parenteral alimentation.

The capacity of Cab-O-Sil to separate triacylglycerols from albumin-bound fatty acids was examined in experiments in which one or the other of these lipid components was radiolabeled. In the first set of experiments, the lipoprotein lipase assay media contained ^{14}C-labeled chylomicrons or a ^{3}H-labeled triacylglycerol emulsion and albumin-bound unlabeled fatty acids; in the second set of experiments, the assay media contained unlabeled chylomicrons or triacylglycerol emulsions and albumin-bound ^{14}C-labeled palmitate. The results in **Table 1** show that addition of Cab-O-Sil to the assay media containing labeled triacylglycerols, followed by a brief centrifugation, resulted in essentially the complete removal of all three substrates from suspension. Less than 0.3% of the radioactivity originally present in the assay mixtures containing labeled triolein or Liposyn remained in the supernatants. In the mixture containing labeled chylomicrons, the radioactivity that remained in the supernatant was somewhat higher (0.7%). This was a consistent finding with different batches of chylomicrons, presumably due to the fact that a portion of the small amount of unesterified fatty acids that are normally found associated with the chylomicrons became bound to the albumin in the medium and remained in the supernatant. The results in Table 1 also show that virtually all of the albumin-bound ^{14}C-labeled fatty acids remained in the supernatant after the various triacylglycerol emulsions had been removed by Cab-O-Sil. In the case of assay mixtures containing chylomicrons or Liposyn, the separation of triacylglycerols from albumin-bound fatty acids was essentially complete. The somewhat lower recovery (92.8 ± 5.8%) of labeled fatty acids in the supernatants of assay mixtures con-

TABLE 1. Separation of triacylglycerols from albumin-bound fatty acids in lipase assay mixtures treated with Cab-O-Sil

Mixture	Radioactivity Recovered in the Supernatant Assay System		
	Triolein Emulsion	Liposyn	Chylomicrons
Labeled triacylglycerols	0.28 ± 0.07	0.07 ± 0.01	0.73 ± 0.09
Albumin-bound labeled fatty acids	92.8 ± 5.8	100 ± 4.5	96.8 ± 2.3

Lipoprotein lipase assay mixtures, prepared as described in Methods, contained either a ^{3}H-labeled triacylglycerol substrate (10^{5}–10^{6} dpm) and unlabeled albumin-bound fatty acids, or an unlabeled triacylglycerol substrate and albumin-bound labeled fatty acids (10^{5}–2×10^{5} dpm). Two ml of a 4.1% (w/v) Cab-O-Sil suspension was added to 0.2 ml of each assay mixture, and after mixing and centrifugation as described in Methods, 0.15-ml aliquots of the clear supernatant were taken for radioactivity measurement. Results are expressed as percentage of the radioactivity in the assay mixture recovered in the post-Cab-O-Sil supernatant. Each value is the mean ± SEM of six separate determinations.

taining emulsified triolein was a consistent observation and did not change with the use of different batches of the substrate. It is noteworthy that, with all three assay systems, the fatty acid recoveries in the supernatants were more efficient than in those reported using liquid/liquid partition e.g., <80% in the procedure described by Belfrage and Vaughan (1).

The effects of various additions to a lipoprotein lipase assay medium on the capacity of Cab-O-Sil to separate a triacylglycerol emulsion (Liposyn) from albumin-bound fatty acids are shown on **Table 2.** The efficiency of separation of Liposyn from albumin-bound palmitate was not significantly affected by detergents or high albumin concentration. These additions also failed to alter the separation of triacylglycerols from albumin-bound fatty acids when the substrates used were chylomicrons or emulsified triolein (not shown).

The results of the separation of triacylglycerols from albumin-bound fatty acids by Cab-O-Sil under actual assay conditions are shown in **Fig. 1.** A time course of hydrolysis of [^{3}H]triolein-labeled Liposyn by purified milk lipoprotein lipase was carried out and, under the experimental conditions used, the reaction was linear for up to 45 min of incubation, accounting for about 23% hydrolysis of the labeled substrate.

DISCUSSION

Fumed silicon dioxide (Cab-O-Sil) is an amorphous particulate material that forms reticulate aggregates when dispersed in a liquid system. In an aqueous environment, the degree of aggregate network formation depends, among other factors, on the concentration of Cab-O-Sil and on the pH of the system (20). In the present study, the pH of a 4.1% Cab-O-Sil suspension in a 0.55 M NaCl solution was about 3.0. At this concentration and pH, Cab-O-Sil forms loose aggregates and the suspended particles can be easily pipetted. Upon mixing 2 ml of this suspension with 0.2 ml of the lipase assay medium (pH 8.5), the pH of the mixture increased to about 7.4 resulting in a rapid rise in the viscosity of the system due to the reduction in the dispersion of the Cab-O-Sil particles. Large particles (e.g., lipoproteins) present in the mixture can then become trapped in the aggregate networks which are readily removed from suspension by low speed centrifugation. It is this property of Cab-O-Sil, previously used to remove lipoproteins from plasma (12, 13), that was exploited in this study to separate chylomicrons or emulsified triacylglycerol particles from albumin-bound fatty acids in lipoprotein lipase assay mixtures.

The method described in this report offers several advantages over the liquid/liquid partition method commonly used to separate emulsified triacylglycerols from albumin-bound fatty acids. *a*) It does not require the use of organic solvents; *b*) it is faster; *c*) the hydrolyzed fatty acids released into the lipase assay medium are readily partitioned from the unhydrolyzed triacylglycerols by a simple centrifugation; *d*) the recovery of fatty acids in the supernatant is more efficient; *e*) the absence of a liquid infranatant minimizes the possibility of contamination of the upper phase with unhydrolyzed substrate; and *f*) the efficiency of separation of the unhydrolyzed triacylglycerols from albumin-bound fatty acids is not affected by the presence of detergents, high protein, or high salt concentrations in the assay medium. The latter property is particularly useful when the activity of lipoprotein lipase must be differentiated from that of the hepatic triacylglycerol hydrolase in postheparin plasma (10).

TABLE 2. Effect of various additions on the separation of triacylglycerols from albumin-bound free fatty acids in lipase assay mixtures treated with Cab-O-Sil

Additions to Assay[a]	Radioactivity Recovered in the Supernatant	
	[^{3}H]Triacylglycerol	Albumin-Bound ^{14}C-Labeled Fatty Acids
	%	
None	*0.1 ± 0.02*	*100 ± 5.3*
Sodium deoxycholate (0.3%)	0.15 ± 0.02	103 ± 3.2
Triton X-100 (5.3%)	0.07 ± 0.02	93.8 ± 5.6
Albumin (4.4%)	0.11 ± 0.02	99 ± 4.8

The triacylglycerol substrate used was Liposyn. The experimental conditions were as described in the legend for Table 1. Each value is the mean ± SEM of six separate determinations.
[a]Weight/volume.

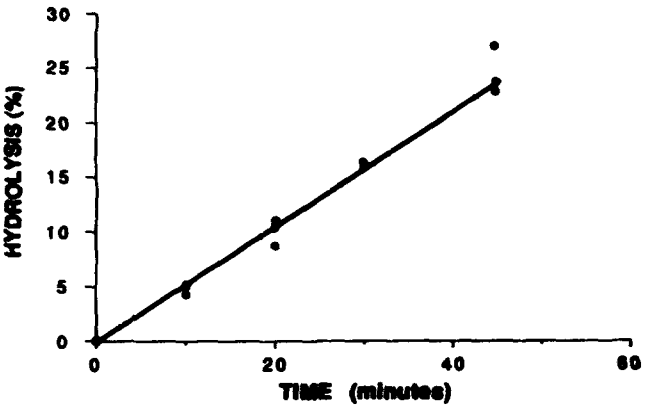

Fig. 1 Hydrolysis of ^{3}H-labeled Liposyn by purified milk lipoprotein lipase. The assay conditions were as described in Materials and Methods. Measurements were carried out in triplicate and each point represents a single determination. The linear regression equation of the measurements is $y = 0.55x - 0.352$; $r^2 = 0.987$.

The addition of 0.5 M NaCl to the assay medium, a concentration sufficient to inhibit lipoprotein lipase but not hepatic triacylglycerol hydrolase activity (5), had no effect on the efficiency of separation of the unhydrolyzed triacylglycerols from albumin-bound fatty acids (results not shown). In this study (Fig. 1), the lipoprotein lipase reaction was stopped by adding Cab-O-Sil suspended in a 0.55 M NaCl solution. This salt-mediated inhibition of the enzyme eliminated the need for the immediate removal by centrifugation of the unhydrolyzed substrate from the assay medium. Other inhibitors of the enzyme (e.g., detergents, protamine sulphate, anti-lipoprotein lipase-containing serum) could have been used in lieu of the salt.

When Cab-O-Sil is removed from an aqueous system by centrifugation, some of the liquid phase becomes trapped in the gel-like pellet. Therefore, the fraction of the total aqueous volume that is available for sampling varies with the specific conditions of separation. For example, the results of the present report were obtained by adding 2 ml of a 4.1% (w/v) Cab-O-Sil slurry to 0.2 ml of the lipase assay mixtures—conditions that we found optimal for our experimental purposes. After centrifugation at 1000 *g* for 10 min, the supernatant volume was 0.81 ± 0.04 ml (mean ± SD, n = 5) out of a total aqueous volume of 2.11 ml. In other experiments (results not shown), we used lower concentrations of Cab-O-Sil [2% (w/v)]. In these instances, the separation of the emulsified triacylglycerols from the albumin-bound fatty acids was as effective as with the higher concentration, and the supernatant volume available for sampling was increased to 1.39 ± 0.04 ml. (mean ± SD, n = 4). Thus, when larger volumes of the supernatant are desired, as for example when the specific activity of the albumin-fatty acids is low or the enzyme activity being measured is low, less concentrated suspensions of Cab-O-Sil may be used, provided that complete removal of the unhydrolized substrate is verified for different lipase assay systems.

The simplicity, efficiency, and reproducibility of the method described in this report make it suitable for routine determination of lipoprotein lipase activity in large numbers of assays.

This work was supported by the American Heart Association Grant 86 707, by the National Institutes of Health Grant HL-30192, and by the Sidney and Bess Eisenberg Memorial fund.

Manuscript received 6 April 1988 and in revised form 15 June 1988.

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