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JOURNAL OF LIPID RESEARCH

Simple liquid–liquid partition system for isolation of labeled oleic acid from mixtures with glycerides

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SUMMARY Oleic acid has a partition coefficient, upper phase/lower phase, of 1.9 (22°C) in the liquid–liquid partition system described herein. Tri-, di-, and monoolein are found almost exclusively in the lower (organic) phase. Oleic acid can be quantitatively removed from mixtures of triglyceride and partial glycerides by means of this partition system under conditions resembling those in a lipase assay.

SUPPLEMENTARY KEY WORDS triolein · diolein · monoolein

LIPASE ACTIVITY has been most commonly assayed by titration of fatty acids produced from hydrolysis of a triglyceride emulsion. The use of radioactive triglycerides greatly increases the sensitivity of the assay but isolation of the fatty acids from the glycerides is time-consuming. To overcome this disadvantage, we have developed a procedure that isolates fatty acids from the glycerides in a one-step liquid–liquid distribution. The radiolabeled fatty acids are then determined directly by liquid scintillation counting.

Materials. Oleic acid-9,10-^{3}H (The Radiochemical Centre, Amersham, Bucks., England) was purified (1) to a radiopurity of 99.5% as shown by thin-layer chromatography (2). Chemical purity was more than 99% by gas–liquid chromatography (3). Oleoyl chloride (The Hormel Institute, Austin, Minn., 99.5% pure) and tri-, di-, and monoolein (Hormel Institute, 99% pure) were used without further purification. Phosphatidyl-U-^{14}C choline (Applied Science Laboratories Inc., State College, Pa., 98% pure) was used without purification. Sodium taurodeoxycholate was obtained from Maybridge Chemical Co., Tintagel, Cornwall, England, with a stated purity of 97.5%. Bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill., Fraction V) was treated with charcoal to remove fatty acids (4). The "potassium carbonate buffer" used in the equilibrations was a potassium carbonate–potassium borate–potassium hydroxide buffer, pH 10.0, 0.05 M (Standard Buffer Solution, Fischer Scientific Co., Fair Lawn, N.J.). All other substances were reagent grade and all solvents were redistilled. All chloroform contained 0.5% (v/v) methanol.

Tri-, di-, and monooleins, ^{3}H-labeled in the oleic acid portion(s), were synthesized from ^{3}H-oleoyl chloride and glycerol according to standard procedures (5, 6). The glycerides were purified by column chromatography on Florisil (7) (Fisher Scientific Co.) to a radiopurity of more than 99.8% (triolein) and more than 99.5% (di- and monoolein). The radiopurity was frequently checked by thin-layer chromatography. Even when

Abbreviations: PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-[2-(5-phenyloxazolyl)]benzene.
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stored at −90°C under anhydrous conditions, hydrolysis occurred, which necessitated repurification every 2–3 months to maintain the purity stated above.

All pipettings were performed with Eppendorf (Eppendorf Co., Hamburg, W. Germany) automatic pipettes (up to 1 ml) and L/I Repipets (Labindustries, Berkeley, Calif.) automatic delivering systems, which greatly increased the speed and reproducibility of the method.

Procedure. The phase system for the separation of fatty acids from the glycerides was similar to that of Borgström (1) and consisted of an alkaline alcohol–water phase and an organic phase. If chloroform is included in the organic phase, however, two improvements over Borgström's system can be brought about: (*a*) free fatty acids are separated from partial glyceride products of lipolysis as well as from the triglyceride substrate, and (*b*) the free fatty acids are found in the upper phase so that sampling for radioassay is convenient and rapid. We found that a system composed of an alkaline methanol–water 1:1 (v/v) phase and a chloroform–heptane 5:4 (v/v) phase was optimal.

The lipase assay was therefore as follows. Substrate (generally, sonified triolein–phospholipid emulsions) was incubated with enzyme preparations in a phosphate buffer with bovine serum albumin in a total volume of 200 μl. Small glass test tubes (12 × 75 mm) were used. The reaction was interrupted by the vigorous addition of 3.25 ml of a methanol–chloroform–heptane mixture (1.41:1.25:1, v/v/v) and, immediately thereafter, 1.05 ml of the potassium carbonate buffer, pH 10. The solvent volumes were such as to give approximately the desired phase compositions stated above. The test tubes were then stoppered with corks lined with Teflon tape and agitated vigorously for a couple of seconds in a Vortex mixer.

After separation of the phases by centrifugation (1000 rpm, 15 min at room temperature), 0.5 ml aliquots of the upper phases were transferred to counting vials. (With head No. 381, International centrifuge, model V, 64 such tubes can be centrifuged at the same time.) Final volumes of the phases are given in Table 1. 18 ml of a toluene–PPO–POPOP scintillation mixture containing 30% (v/v) methanol (8) was added and the samples were counted in a Packard model 3375. The counting efficiency was 14% for ^{3}H with completely open window and maximal amplification, and was highly reproducible despite wide variations in concentration of salts, lipids, and protein in the incubation media. Only with some highly colored enzyme preparations was it necessary to correct for quenching.

The amount of radioactive fatty acid in the upper phase was determined from the amount in the 0.5 ml aliquot and the known final volume of the upper phase. When we desired to express lipase activity in absolute terms, the *total* amount of radioactive fatty acid could be calculated from the corresponding upper phase value and the partition coefficient of the fatty acid. The latter had to be determined in a separate experiment with labeled fatty acid and varies to some extent as discussed below.

Test experiments established the distribution of relevant lipids and the effects of various factors on these distributions. These experiments were carried out exactly as described for the lipase assay. The lipid to be tested was added in an organic solvent and the solvent was evaporated, whereupon the buffer and any other compounds were added in a total volume of 200 μl. The solvent mixtures were then added immediately, except when otherwise indicated, and the procedure described above was followed.

Results and Discussion. The distribution of labeled oleic acid, of tri-, di-, and monoolein, and of phosphatidyl choline in the phase system at 24°C is shown in Table 1. The glycerides were repeatedly extracted with fresh equilibrated upper phase in order to extract traces of labeled oleic acid before determination of the partition coefficient. The amounts of the glycerides found in the upper phase are of no practical importance in the usual lipase assay with less than 10–20% hydrolysis. The distribution of the glycerides is not affected to any important degree by additions of at least up to 10 mg of bovine serum albumin, 2 mg of rat liver phospholipids, 1 μmole of sodium taurodeoxycholate, or 20 μl of the detergent Triton X-100 [a nonionic detergent, mixture of *p,t*-octyl poly(phenoxyethoxy) ethanols] (^{3}H-triolein always < 0.001% and ^{3}H-monoolein always <0.3% in upper phase). On the other hand, the method obviously cannot be used for phospholipase assays.

From a practical point of view, it is important to know whether the distribution of the oleic acid remains constant at different temperatures and in the presence of

TABLE 1 Distribution of Oleic Acid, of Tri-, Di-, and Monoolein, and of Phosphatidyl Choline in the Solvent Mixture at 24°C

Radioactive Lipid Added	% in Upper Phase		
	Mean	SEM	n
Oleic acid, 5 μg	71.5	0.4	6
Triolein, 100 μg	0.00053	0.00015	5
Diolein, 100 μg	0.013	0.0008	6
Monoolein, 100 μg	0.24	0.008	6
Phosphatidyl choline, 20 μg	25.9	0.19	6

After equilibration the total volume of upper phase (alkaline methanol–water 1:1) was 2.45 ml, that of lower phase (chloroform–heptane 5:4) 2.05 ml. The percentage values were determined from an aliquot of the upper phase as described in the text.

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TABLE 2 Effect of Addition of Various Substances on the Distribution of Oleic Acid-^{3}H Between Phases

Additions	Amount	Fraction in Upper Phase*
None		100
Oleic acid	1 mg	102 ± 0.4 (6)
Mixture of tri-, di-, mono-olein, and oleic acid, approx. 70:15:5:10	1 mg	101 ± 0.7 (6)
	4 mg	103 ± 0.3 (6)
Rat liver phospholipids†	0.2 mg	101 ± 0.8 (6)
	0.5 mg	99 ± 0.6 (6)
	1.0 mg	99 ± 0.5 (6)
	2.0 mg	88 ± 0.7 (6)
	5.0 mg	67 ± 1.2 (6)
Bovine serum albumin, FFA-free	2 mg	100 ± 0.5 (8)
	10 mg	99 ± 0.3 (8)
	10 mg‡	103 ± 1.1 (6)
Rat serum	50 µl	98 ± 0.4 (8)
	100 µl	99 ± 0.7 (4)
	150 µl	96 ± 0.2 (4)
	200 µl	87 ± 0.9 (8)
$CaCl_2$	4 µmoles	100 ± 1.1 (6)
	20 µmoles	99 ± 0.6 (6)
Sodium taurodeoxycholate	0.1 mg	99 ± 0.5 (6)
	0.5 mg	100 ± 0.9 (6)
	1.0 mg	104 ± 0.4 (6)
	2.0 mg	101 ± 0.4 (6)
Triton X-100	0.1 mg	100 ± 0.1 (6)
	1.0 mg	91 ± 1.2 (6)
	2.0 mg	84 ± 0.3 (6)
	4.0 mg	72 ± 0.7 (6)

5 µg of oleic acid-^{3}H were used in control experiments which were performed with each set of test experiments. Final volumes of phases as in Table 1. Distribution is expressed relative to percentage of 5 µg of oleic acid-^{3}H in the upper phase in the control experiment at the same temperature. FFA, free fatty acid.

* Mean ± SEM, number of determinations within parentheses.

† Folch extract purified on silicic acid.

‡ 30 min incubation at room temperature.

various substances used in lipase assays. A series of test equilibrations at various temperatures gave the following percentages of oleic acid in the upper phase. At 4°C, 58.6 ± 0.4 (8) [mean ± SEM (number of experiments)]; 22°C, 70.0 ± 0.3 (20); 23°C, 70.5 ± 0.5 (6); 24°C, 71.5 ± 0.4 (6); 26°C, 73.1 ± 0.2 (6); and 27°C, 74.2 ± 0.2 (7). Large variations in room temperature can thus produce some variation in the distribution of oleic acid, but this can easily be controlled.

Table 2 shows the effect of the addition of various substances on the distribution of oleic acid. Although many of the small differences shown in the table are statistically significant, they are not important for practical purposes. The addition, however, of more than 1 mg of phospholipids, 150 µl of rat serum, or 0.1 mg of Triton X-100 affects the distribution markedly. The effect of rat serum is probably due to its content of

TABLE 3 Distribution of Oleic Acid-^{3}H Between Phases Under Conditions of Lipase Assay

Amount of Enzyme	Oleic Acid-^{3}H in Upper Phase*
µl	%
0	100 ± 0.6
10	99 ± 0.3
20	99 ± 0.8
50	99 ± 0.4
100	99 ± 0.4

Labeled oleic acid in trace amount was mixed with triolein and rat liver phospholipids (5:1, w/w), and the lipids were sonified in 0.1 M phosphate buffer (approximately 1.0 ml/mg of lipids), pH 7.4. The final substrate emulsion contained triolein, 0.3 µeq/ml, and bovine serum albumin, 50 mg/ml. The enzyme preparation was the supernatant fraction (15,000 *g* for 30 min) from a homogenate of epididymal fat pads from rats fasted for 5 days (320 mg of tissue in 2.0 ml of 0.154 M KCl). Each tube contained 100 µl of substrate plus enough of the enzyme preparation or 0.154 M KCl to make a total volume of 200 µl. Tubes were incubated for 5 min at room temperature (26°C). Reactions were stopped and the partition carried out as described under Procedure; final volumes of phases as in Table 1. Values expressed as fraction of radioactivity in upper phases relative to the fraction in upper phase resulting from addition of a trace of oleic acid-^{3}H to 200 µl of KCl and partitioning in the same way at the same temperature.

* Mean ± SEM for four experiments in each group.

phospholipids; the mechanism of the interference is not known. Possibly some complex formation between the potassium oleate and the detergents takes place at higher concentrations. Increasing the amount of oleic acid relative to the detergents does not change the effect.

The distribution of oleic acid in the system used for lipase assay is shown in Table 3. In an assay under these conditions, 6% hydrolysis was obtained with 100 µl of enzyme preparation. We conclude that the distribution of oleic acid is quite reproducible under conditions typical for the practical application of the method. Thus, the method works satisfactorily for the determination of labeled fatty acids in lipase assays with labeled substrate where the high concentrations of phospholipids and other additions that changed the distribution of the oleic acid would not usually be encountered. The distribution can, of course, always easily be checked with a labeled fatty acid standard. In our hands the method has proved a valuable tool in the investigation of the lipolytic enzymes of rat adipose tissue. By repeating the equilibration several times, one could use this partition system for quantitative separation of free fatty acids from mono-, di-, and triglycerides.

One of us (P.B.) gratefully acknowledges grants from Petrus and Augusta Hedlunds Foundation and the Swedish Medical Research Council which provided for the 1 yr stay at the National Institutes of Health.

Manuscript received 14 October 1968; accepted 27 January 1969.

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