

A rapid and sensitive enzymatic-radiochemical assay for the determination of triglycerides

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Summary An enzymatic-radiochemical method suitable for the determination of triglyceride levels of cells in culture is described. The method is based on the enzymatic hydrolysis of triglycerides to free fatty acids which then complex with ⁶³Ni. The method is rapid, accurate, and inexpensive. The procedure extends the sensitivity of triglyceride measurement to as low as 0.25 nanomoles. **Khoo, J.C., E. Miller, and D.I. Goldberg.** A rapid and sensitive enzymatic-radiochemical assay for the determination of triglycerides. *J. Lipid Res.* 1987. **28:** 873–877.

Supplementary key words microassay • free fatty acid • ⁶³Ni complex • cultured cells

The procedures presently available for the determination of triglycerides fall into two general categories: *a*) the measurement of glycerol liberated from triglycerides by lipolysis (1) or chemical hydrolysis (2,3); *b*) the separation of the lipid classes by thin-layer chromatography (TLC), which are then assayed either by charring of the triglycerides using concentrated sulfuric acid (4), or by gas-liquid chromatography. Although most of the methods in category “*a*” are easy to use, and are specific, they were developed for measurement of plasma triglycerides and are not sufficiently sensitive for quantification of triglycerides in small samples of cultured cells and tissues. Recently, a highly sensitive method for the determination of glycerol derived from triglycerides was reported (5). The sensitivity of this method was greatly amplified by utilizing a series of reactions (requiring up to seven enzymes) coupled with the cycling of NAD⁺ to produce a fluorescent product (6). The resulting assay, while significantly enhancing the sensitivity of glycerol determination, sacrifices the ease and simplicity of the earlier methods and requires the availability of a fluorometer. The TLC-charring method is nonspecific, requires the use of concentrated sulfuric acid at 200°C, and is no more sensitive than the former methods. Gas-liquid chromatography, while sensitive and specific, has the disadvantage of requiring expensive equipment and of being time-consuming.

We now describe a simple, reproducible, and highly sensitive method for the measurement of triglycerides. Total lipids are extracted into chloroform, and phospholipids, which may interfere with the FFA assay (7), are removed by adsorption to heat-activated silicic acid (8,9). The triglycerides are then hydrolyzed to glycerol and free

fatty acids (FFA) using commercially available triglyceride lipases which lack cholesteryl esterase activity. The FFA produced by the lipases are then quantified by a modification of the method of Ho (7), in which FFA react with ⁶³Ni to form a FFA-⁶³Ni complex that can be detected by liquid scintillation counting. The procedure extends the sensitivity of triglyceride measurement to as low as 0.25 nanomoles, and allows the ready assay of triglycerides in cultured cells harvested from one 35-mm or 60-mm petri dish.

MATERIALS AND METHODS

⁶³Nickel chloride was purchased from New England Nuclear Corp., Boston, MA. Nickel nitrate was purchased from Fisher Scientific, Springfield, NJ. Lyophilized microbial lipase (*Rhizopus arrhizus*, catalogue no. 437706) was obtained from Calbiochem, San Diego, CA. Lipase from porcine pancreas (Type VI-S, catalogue no. 0382), triolein, oleic acid, silicic acid (60–200 mesh), 1,4-bis-(4-methyl-5-phenyl-2-oxazolyl)benzene (dimethyl POPOP), and 2,5-diphenyloxazole (PPO) were obtained from Sigma Chemical Co., St. Louis, MO. [¹⁴C]Triolein (tri[¹⁻¹⁴C]oleoylglycerol) and cholesteryl [¹⁻¹⁴C]oleate were purchased from Amersham Corp., Arlington Heights, IL. Silica gel G thin-layer chromatographic plates were obtained from Analtech Inc., Newark, DE. All solvents and chemicals were of analytical grade.

Reagents

A. Standard solutions of triolein (0.01 mM and 0.1 mM) are prepared in heptane.

B. The incubation buffer contains 20 mM potassium phosphate, pH 7.0, and 0.35 mM sodium dodecyl sulfate.

C. The lipase buffer consists of 100 units of microbial lipase (more potent in the lyophilized form than in ammonium sulfate suspension) and 600 units of porcine pancreatic lipase per 100 μl of incubation buffer (reagent B).

D. Dole's extraction mixture contains isopropyl alcohol-heptane-1 N sulfuric acid 40:10:1 (10).

E. ⁶³Nickel substrate is prepared as follows:

1. One mCi of carrier-free ⁶³nickel chloride per ml water.

2. Two mmol of nickel nitrate and 0.8 ml of glacial acetic acid diluted to 100 ml with saturated sodium sulfate. Five μl of (1) are added to 2 ml of (2), yielding a specific activity of 12.5 nCi of ⁶³Ni per 100 nmol of Ni (275 dpm/nmol).

F. ⁶³Ni working solution is prepared at the time of assay. It contains 10 volumes of ⁶³Ni substrate (reagent E),

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8.5 volumes of saturated potassium sulfate, and 1.5 volumes of triethanolamine.

G. Scintillation cocktail contains 4.17 gm PPO and 56 mg POPOP per liter of toluene.

Preparation of lipid extracts

Sonicated cells (at least 200 μ g of protein) in 0.5 ml were extracted in 15 ml of chloroform-methanol 2:1 (v/v) in 50-ml screw-cap extraction tubes by the method of Folch, Lees, and Sloane Stanley (11). Two phases were partitioned by the addition of 5 ml of 0.03 N HCl, and allowed to separate overnight, after gentle inversion of the tube. The chloroform infranate was removed with a 6-in, 18-gauge stainless-steel needle attached to a 10-ml glass syringe, being careful to exclude all of the upper phase. Silicic acid, activated by heating at 120°C for 24 hr, was added to the chloroform extract at a concentration of 20 mg/ml. The suspension was then vortexed, and centrifuged at 1000 *g* for 10 min at 4°C. Equal aliquots of the chloroform infranate were added to four 10 x 75-mm tubes for assay. Control cells required aliquots of 2 ml of chloroform infranate, or the equivalent of 40 μ g of cellular protein. Aliquots of 0.25 to 2.0 ml were sufficient for extracts of macrophages previously loaded with triglycerides. Duplicate tubes labeled "a" and "b" were required in each assay.

Triglyceride Assay

Known quantities of triolein, ranging from 0.25 to 25 nmol, were added to 10 x 75-mm disposable glass tubes. These standards and sample tubes containing lipid extracts were evaporated under nitrogen. Isopropyl alcohol (15 μ l) was added to standards, to sample "a", and to reagent blanks. The tubes were then incubated at 37°C for 10 min to assure complete resolubilization of all the triglycerides. One hundred μ l of the lipase buffer (reagent C) was then added to these same tubes. The tubes were vortexed and incubated for 60 min at 37°C. The reaction was stopped by the addition of 100 μ l of Dole's extraction mixture (reagent D), followed by the addition of 100 μ l of water and 200 μ l of heptane.

The unincubated "b" sample tubes, used for the measurement of endogenous FFA, were extracted with 100 μ l of Dole's mixture, 200 μ l of water, and 200 μ l of heptane.

All the tubes were vortexed vigorously for 30 sec and were centrifuged at 1000 *g* for 5 min at 4°C. A 100- μ l aliquot of the upper (heptane) phase, containing the total FFA, was transferred to a clean 10 x 75-mm tube. Three hundred μ l of chloroform and 10 μ l of the ⁶³Ni working solution (reagent F) were added. The tubes were vortexed for 30 sec and centrifuged at 1000 *g* for 5 min at 4°C.

Aliquots of the lower (organic) phase (200 μ l), containing the FFA-⁶³Ni complex, were pipetted into scintillation vials, evaporated to dryness, and resolubilized in 10 ml of

scintillation cocktail (reagent G). (Gentle evacuation of the pipette tip, while passing through the surface film, allows the removal of 200 μ l of the lower phase without contaminating the sample.) The radioactivity was measured in a liquid scintillation counter and corrected for a reagent blank. The triglyceride contents of the chloroform extracts were determined from the triolein standard curve after subtracting the cpm of the "b" tubes from the cpm of the "a" tubes.

RESULTS

There are two crucial requirements for a triglyceride assay that is based on measurement of FFA released by hydrolysis: 1) all of the FFA released by hydrolysis must originate from triglycerides; and 2) the triglycerides must be totally hydrolyzed. In order to satisfy the first requirement it was necessary to rule out cholesteryl esters as possible sources of FFA. We found that several preparations of commercially available triglyceride lipases, including those in the enzymatic reagent set for the determination of triglycerides manufactured by Boehringer-

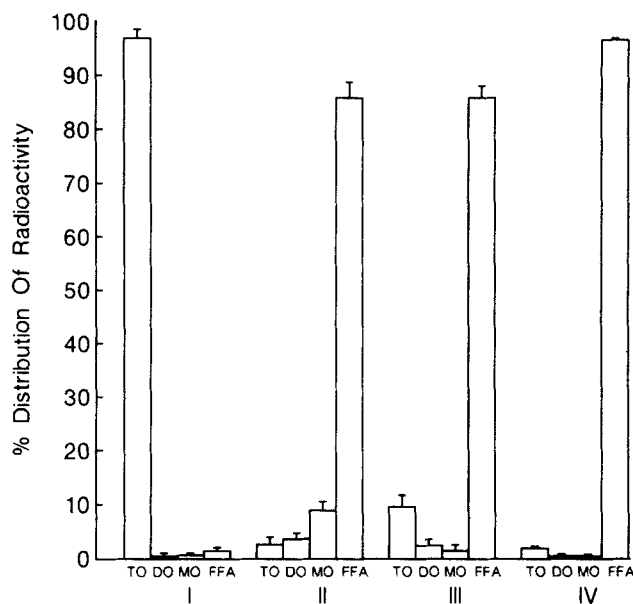


Fig. 1. The percent distribution of triolein and hydrolytic products after incubation with lipase. [¹⁴C]Triolein (10 nmol) was incubated with 0.1 ml of I) buffer alone; II) 600 units of pancreatic lipase; III) 100 units of microbial lipase; and IV) 600 units of pancreatic lipase and 100 units of microbial lipase at 37°C. After 60 min, the reaction was terminated by the addition of 0.5 ml of methanol and 1.0 ml of chloroform. The two phases were separated by the addition of 0.5 ml of 0.03 N HCl. After centrifugation, 0.5 ml of the chloroform phase was removed and evaporated under N₂. The lipid classes were separated by TLC, using a solvent system of petroleum ether-diethyl ether-glacial acetic acid 70:30:0.4, and visualized by I₂ vapor. Unlabeled carriers of triolein (TO), diolein (DO), monoolein (MO), and oleic acid (FFA) were added to each extract. Each lipid class was scraped into scintillation vials for counting. The results were obtained from three separate experiments. Each bar represents the mean \pm SD of 5 to 13 replicates.

Mannheim Diagnostics, hydrolyzed cholesteryl [$1\text{-}^{14}\text{C}$]oleate. However, neither the microbial nor pancreatic lipase used in reagent C hydrolyzed cholesteryl [$1\text{-}^{14}\text{C}$]oleate when incubated at 37°C for 1 hr. We tested four batches of microbial lipase and three of pancreatic lipase over a 5-month period and found no activity against cholesteryl [$1\text{-}^{14}\text{C}$]oleate.

To achieve total hydrolysis of the triolein, it was necessary to include *both* microbial lipase and pancreatic lipase in reagent C. To examine the extent of hydrolysis and accumulation of partial glycerides, 10 nmol of [^{14}C]triolein was incubated at various time intervals with the lipases, individually or in combination, at 37°C . The reaction was stopped every 15 min, up to 90 min. Maximal hydrolysis was obtained at 60 min for either enzyme. Addition of twice as much enzyme did not increase the yield of FFA. As shown in Fig. 1, neither lipase alone effected complete hydrolysis of the triolein to FFA. The release of FFA was $86.0 \pm 2.4\%$ and $86.2 \pm 3.1\%$ by pancreatic lipase and microbial lipase, respectively. When pancreatic lipase was analyzed alone, there was nearly complete breakdown of triolein, with a small but significant accumulation of diolein and, in particular, monoolein. The microbial lipase, in contrast, appeared to be more active towards the hydrolysis of partial glycerides. When the two lipases were used in combination, $96.8 \pm 0.3\%$ of the added radioactivity was recovered as FFA, with virtually no accumulation of monoolein or diolein. Thus, maximal amounts of both lipases were included in reagent C. Fig. 2A and B show that the calibration

curves, as measured by the FFA- ^{63}Ni complex, were linear from 0.25 to 25 nmol of triolein. Furthermore, the oleic acid recovered from the hydrolysis of triolein produced a standard curve that was superimposable on that derived from the direct measurement of pure oleic acid. Identical results were obtained using tripalmitin as a standard. However, this triglyceride is not recommended due to its low solubility.

The method was applied to the measurement of triglyceride content in resident mouse peritoneal macrophages. As shown in Fig. 3A, the method was sensitive enough to detect 0.30 nmol of triglycerides associated with $45 \mu\text{g}$ of cellular protein. Furthermore, the assay was linear with increasing concentrations of cellular extract. With lipid-laden macrophages (after a 48-hr incubation with very low density lipoprotein) as little as $5 \mu\text{g}$ of cellular protein was required (providing 0.85 nmol of triglycerides). Again, the assay was linear with increasing concentrations of cellular extract (Fig. 3B). The triglyceride contents in human skin fibroblasts and rabbit endothelial cells were 6.66 ± 0.61 and 3.98 ± 0.2 nmol/mg of protein, respectively. The triglyceride content for mouse peritoneal macrophages derived from Fig. 3A was 6.6 nmol/mg of protein.

The present method was directly compared with the widely used triglyceride reagent set of Boehringer-Mannheim Diagnostics, which is specific for the measurement of glycerol. As shown in Fig. 4, the results for the concentrations of triglycerides from 25 human serum samples were in good agreement.

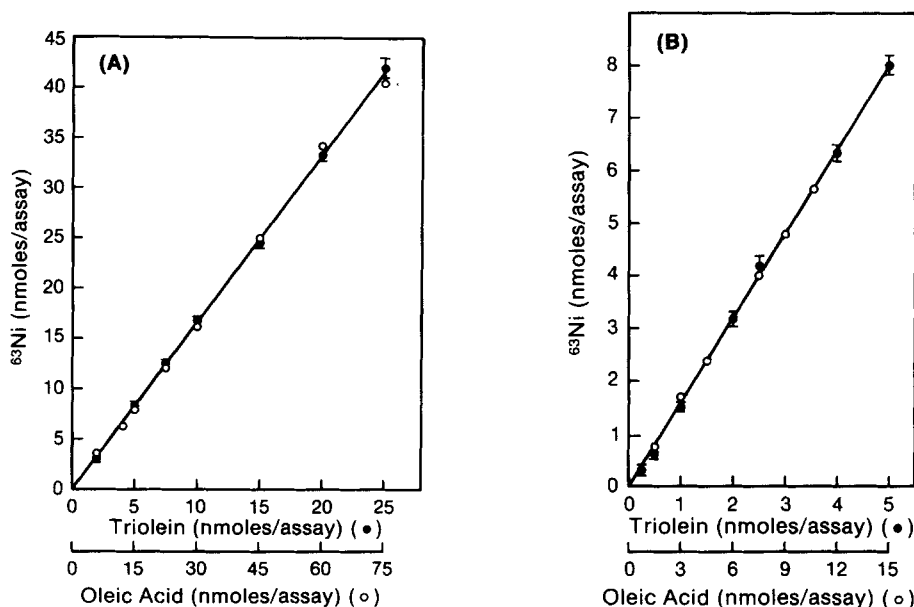


Fig. 2. Standard curves for triolein. Triolein (●) ranging from 0.5 to 25 nmol (panel A) and 0.25 to 5 nmol (panel B) was incubated with 600 units of pancreatic lipase and 100 units of microbial lipase for 1 hr at 37°C . The oleic acid released was assayed as described in Materials and Methods. Pure oleic acid (O) in heptane (volume adjusted to $100 \mu\text{l}$) was assayed directly. Each point represents the mean \pm SEM of four to nine replicates.

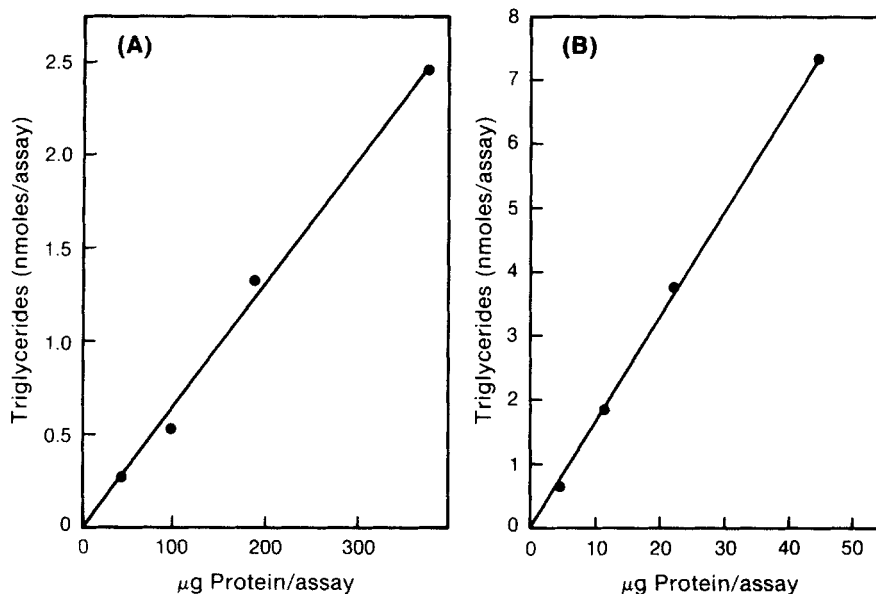


Fig. 3. The triglyceride contents in resident mouse peritoneal macrophages. (A) The triglyceride content in control cells grown in the absence of added lipoprotein. (B) The triglyceride content in lipid-laden macrophages, after a 48-hr incubation with human serum very low density lipoprotein (100 µg protein/ml media). Each point represents the mean of duplicate samples.

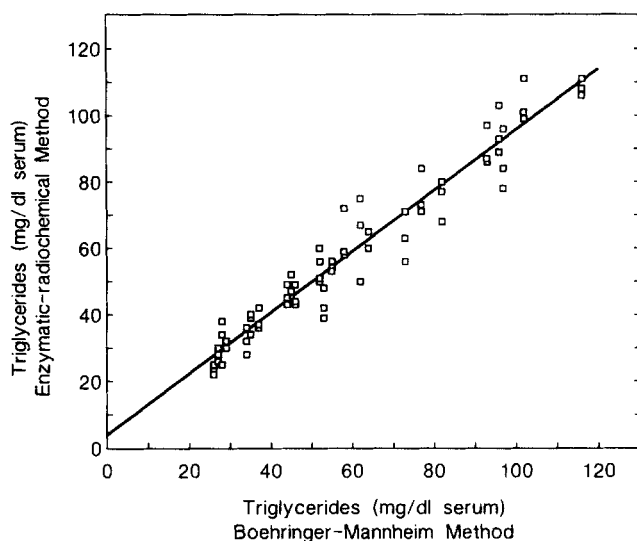


Fig. 4. Comparison of the enzymatic-radiochemical method and the Hitachi 705 automatic analyzer (using the Boehringer-Mannheim triglyceride reagent set) on the measurements of human serum triglyceride levels. Some of the serum samples were diluted by one-half with saline to obtain a lower range of triglyceride levels. The data for the enzymatic-radiochemical method were obtained from three separate experiments carried out in duplicate. The data for the Boehringer-Mannheim method were from one experiment. The equation of the regression line was $y = 3.965 + 0.9134x$, $r = 0.969$ ($P < 0.001$).

DISCUSSION

This report presents an enzymatic-radiochemical assay for the measurement of triglycerides, which is rapid, and reliable to as low as 0.25 nmol of triglycerides, as well as relatively inexpensive (currently about 8 cents per assay). If necessary, the sensitivity of the method can be increased by decreasing the concentration of unlabeled Ni used in the substrate (reagent E). The method is sufficiently sensitive for the assay of triglyceride content in cultured cells from one 35-mm or 60-mm petri dish. It is possible to assay 50–100 extracts in less than 3 hr. The method also provides simultaneous measurement of the FFA present in extracts of cells.

The increased sensitivity of this method is due to the radiochemical assay of the three FFA derived from one molecule of triglyceride rather than the measurement of one molecule of glycerol. This method is not strictly limited to triglycerides since the presence of cellular diglycerides or monoglycerides may also contribute FFA. Such lipids are normally present in small amounts, and would not produce a significant error. However, conditions may exist with some cells where the accumulation of the lipids can alter the results. We have modified the solvent system used during the formation of the FFA- ^{63}Ni complex by changing the chloroform-heptane ratio from 1.14:1, as described by Ho (7), to 3:1 in order to increase the recovery of the FFA- ^{63}Ni complex in the organic phase. Because of the strong quenching effect of chloroform on scintillation counting, it is important to evaporate the 200 µl of organic phase to dryness,

which we have accomplished by simply placing the open scintillation vials under a fume hood for 20 min. Any unbound ^{63}Ni in the aqueous phase which may have contaminated the organic phase will not be solubilized in the detergent-free, nonaqueous scintillation cocktail we have used, further increasing the accuracy of the assay.

In summary, the enzymatic-radiochemical triglyceride assay presented here is a rapid, sensitive, specific, accurate, and economical method that can readily be applied to the measurement of triglycerides in cultured cells. ■

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REFERENCES

1. Wahlefeld, A.W. 1974. Triglycerides: determination after enzymatic hydrolysis. *In Methods of Enzymatic Analysis*. Vol. 4. H. U. Bergmeyer, editor. Verlag Chemie International, Florida. 1831-1835.
2. Fletcher, M. J. 1968. A colorimetric method for estimating serum triglycerides. *Clin. Chim. Acta.* **22**: 393-397.
3. Kessler, G., and H. Lederer. 1965. Fluorometric measurement of triglycerides. *In Automation in Analytical Chemistry*. L.T. Skegg, editor, Mediad, New York. 341-344.
4. Kabara, J. J., and J. S. Chen. 1976. Microdetermination of lipid classes after thin-layer chromatography. *Anal. Chem.* **48**: 814-817.
5. Nemeth, P. M., O. E. Hitchins, L. Solanki, and T. G. Cole. 1986. Fluorometric procedures for measuring triglyceride concentrations in small amounts of tissue and plasma. *J. Lipid Res.* **27**: 447-452.
6. Kato, T. S., J. Berger, J. A. Carter, and O. H. Lowry. 1973. An enzymatic cycling method for nicotinamide-adenine dinucleotide with malic and alcohol dehydrogenases. *Anal. Biochem.* **53**: 86-97.
7. Ho, R. J. 1970. Radiochemical assay of long-chain fatty acids using ^{63}Ni as tracer. *Anal. Biochem.* **36**: 105-113.
8. Borgström, B. 1952. Investigation on lipid separation methods. Separation of phospholipids from neutral fat and fatty acids. *Acta Physiol. Scand.* **25**: 101-110.
9. Van Handel, E., and D. B. Zilversmit. 1957. Micromethod for the direct determination of serum triglycerides. *J. Lab. Clin. Med.* **50**: 152-157.
10. Dole, V. P., and H. Meinertz. 1960. Microdetermination of long-chain fatty acids in plasma and tissues. *J. Biol. Chem.* **235**: 2595-2599.
11. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**: 497-509.