Fluorometric procedures for measuring triglyceride concentrations in small amounts of tissue and plasma

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Summary It has been previously shown that triglycerides can be specifically hydrolyzed by lipase from *Rhizopus arrhizus* in the presence of hog liver esterase and sodium dodecyl sulfate. The glycerol produced can then be measured by sequential reactions with glycerokinase, pyruvate kinase, and lactate dehydrogenase: glycerol and ATP are converted to glycerol-3-phosphate and ADP by glycerokinase; the ADP reacts with phosphoenolpyruvate and pyruvate kinase to yield pyruvate; the pyruvate is converted to lactate with lactate dehydrogenase, and the cofactor NAD^+ is simultaneously reduced to NADH. This report describes procedures by which either the disappearance of NADH or the appearance of NAD^+ was determined fluorometrically, with 10- to 100-fold greater sensitivity than by spectrophotometry. In addition, enzymatic cycling of NAD^+ was used to increase the sensitivity of the assay over 1000-fold, and thereby provided accurate measurement of less than 1 ng of triglyceride. Results obtained from the three fluorometric methods were highly correlated with an automated periodate oxidation method using serum samples and lipid extracts of muscle tissue. **—Nemeth, P. M., O. E. Hitchins, L. Solanki, and T. G. Cole.** Fluorometric procedures for measuring triglyceride concentrations in small amounts of tissue and plasma. *J. Lipid Res.* 1986. **27:** 447–452.

Supplementary key words pyridine nucleotides • enzymatic cycling • skeletal muscle

There are situations in which the standard methods for measuring concentration of triglycerides (1, 2) do not provide adequate sensitivity, particularly in the case of small clinical samples or with tissues containing very low triglyceride concentrations. This report describes three variations of an enzymatic method, which permits accurate measurement of triglycerides in the range of 0.3 ng to 10 μg. Modifications of the method are described to extend the sensitivity to detect 4 pg (approximately 4 femtomoles) of triglyceride.

The method incorporates a miniaturization of the chloroform–methanol extraction method of Folch, Lees, and Sloane Stanley (3) (in the case of tissue samples), the spectrophotometric assay described by Wahlefeld (2), the fluorometric assays of Burch et al. (4), Lowry and Passonneau (5), and the enzymatic cycling methods of Kato et al. (6) and Lowry (7). In a single reaction step, triglycerides in plasma or in tissue extracts were enzymatically hydrolyzed and the glycerol produced was directed to a pyridine nucleotide-mediated reaction (**Fig. 1,** Reaction a).

Triglyceride concentration was determined fluorometrically by measuring either the disappearance of NADH or the appearance of NAD^+ after its conversion to a fluorescent product (5). To increase the sensitivity of the assay, the NAD^+ produced in Reaction a (Fig. 1) was amplified into the useful range of the fluorometer by enzymatic cycling (Fig. 1, Reaction b) and by an additional indicator step (Fig. 1, Reaction c) using the NAD^+ cycling method of Kato et al. (6).

The triglyceride concentrations obtained by the three fluorometric procedures, NADH disappearance, NAD^+ appearance, and NAD^+ enzymatic cycling of NAD^+, were compared to the results obtained by an automated nephlofluorometric method of the Lipid Research Clinics Program using periodate oxidation and acetylacetone following separation of neutral lipids by zeolite (8, 9). The correlation between the results, with sample sizes varying over 4000-fold to accommodate the sensitivity of the particular method, further indicates that the enzymatic hydrolysis is quantitative and specific. Above all, the report demonstrates the use of highly sensitive fluorometric methods to determine small amounts of triglycerides, thereby providing advantages over spectrophotometric methods.

MATERIALS AND METHODS

All enzymes were obtained from Boehringer (Mannheim, Germany). Substrates and cofactors were from Sigma Chemical Company (St. Louis, MO). Chloroform and methanol were HPLC grade from Fischer Scientific Company (St. Louis, MO), and all other chemicals were of analytical grade. A human plasma triglyceride standard (Q9) was obtained from the Centers for Disease Control (Atlanta, GA). Pyridine nucleotides were measured at wavelengths of 340 nm for excitation and 460 nm for emission using a Farrand A4 filter fluorometer (Farrand Optical Company Inc., Valhalla, NY).

Lipid extraction of tissue samples

Lipids were extracted from rat (Sprague-Dawley) and human tissues using the method of Folch et al. (3) with adjustments to accommodate small tissue samples. The smallest samples were 100 μg lyophilized weight or ap-

Abbreviations: NAD^+, nicotinamide adenine dinucleotide; NADH, nicotinamide adenine dinucleotide, reduced.

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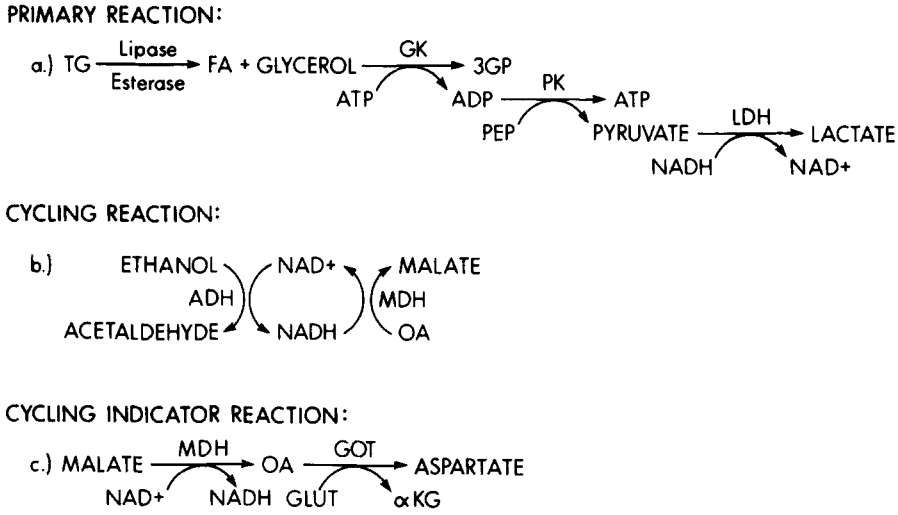

Fig. 1. Enzymatic pathways used in fluorometric assays to measure triglycerides. Abbreviations are: TG, triglycerides; FA, fatty acids; GK, glycerol kinase; 3GP, 3-glycerol phosphate; ATP, adenosine 5'-triphosphate; ADP, adenosine 5'-diphosphate; PK, pyruvate kinase; PEP, phosphoenolpyruvate; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide, reduced; NAD^+, nicotinamide adenine dinucleotide, oxidized; MDH, malate dehydrogenase; ADH, alcohol dehydrogenase; OA, oxaloacetate; GOT, glutamate oxaloacetate transaminase; Glut, glutamate; α-KG, α-ketoglutarate.

proximately 400 μg wet weight. For these samples, the following procedure was used and the amounts of reagents were increased proportionally for larger samples. The tissue was homogenized in 500 μl of methanol in 1-ml glass homogenizers (Kontes Glass Company, Vineland, NJ). The homogenate was transferred to a test tube and 1 ml of chloroform was added, rinsing the homogenizer and pestle in the process. The solution was mixed with a Vortex, and allowed to stand overnight (10 to 20 hr) at 4°C. The phases were separated by centrifugation for 5 min at 12,300 *g* after the addition of 400 μl of 0.15 M NaCl. The lipid-containing chloroform layer was removed from the bottom of the tube, passed through a 0.45-μm Nalgene filter, and stored at −20°C prior to analysis. The filtration step was found to be necessary to reduce background fluorescence, possibly by removal of residual protein.

Standard

The mixed triglyceride standard (Q9) was obtained at a concentration of 1.85 mM (164 mg/dl) and was used in the final concentrations given below (under Triglyceride Assay Procedures). Standard curves from equivalent concentrations of glycerol gave the same results as the Q9 standard.

Triglyceride assay reagents

The following concentrations were used in the assay reagent for the combined enzymatic hydrolysis of triglycerides and the determination of glycerol (Reaction a, Fig. 1): 100 mM potassium phosphate buffer, pH 7.4; 4 mM magnesium chloride; 0.1 mg/ml sodium dodecyl sulfate; 0.02% (w/v) bovine serum albumin; 0.2 mM adenosine triphosphate; 0.35 mM phosphoenolpyruvate; 6 U/ml lactate dehydrogenase; 1 U/ml pyruvate kinase; 0.6 U/ml esterase (hog liver); 0.6 U/ml glycerol kinase; 200 U/ml lipase (*Rhizopus arrhizus*); NADH in appropriate concentrations for each procedure (see below). The assay reagent was also prepared without glycerol kinase as a reagent blank.

Preparation of triglyceride assay stock reagents. Special consideration was given to the preparation of phosphoenolpyruvate, adenosine triphosphate, and NADH. For maximal stability the phosphoenolpyruvate solution, 500 mM, was prepared from a trisodium salt to maintain neutrality (or slight alkalinity) and stored at −70°C. Adenosine triphosphate, 500 mM, was neutralized with two equivalents of NaOH (2.76 g of ATP in 10 ml of 1 N NaOH) and stored at −20°C. A 20 mM stock solution of NADH was made up in 100 mM sodium carbonate buffer, pH 10.3, and was stored at −70°C in small aliquots (50 to 100 μl) that were heated to 100°C for 5 min just prior to use to destroy NAD^+.

The 1 M potassium phosphate buffer was stored at 4°C; 1 M $MgCl_2$ and 10% bovine serum albumin at −20°C; and 1 M sodium dodecyl sulfate at 25°C. Enzymes suspended in ammonium sulfate solution, as supplied by the manufacturer, are reportedly stable for about 1 year at 4°C.

NAD^+ cycling reagents. Amplification of the NAD^+ product from Reaction a (Fig. 1) was achieved in a test tube in two reaction steps (Fig. 1, Reactions b and c). The reagent for Reaction b, the cycling reaction, contained: 100 mM Tris-HCl, pH 8.1; 2 mM β-mercaptoethanol;

2 mM oxaloacetate; 300 mM ethanol; 0.02% bovine serum albumin; 15 μg/ml malate dehydrogenase; and 150 μg/ml alcohol dehydrogenase.

The reagent for Reaction c, the indicator reaction, contained: 50 mM 2-amino-2-methylpropanol buffer, pH 9.9; 10 mM glutamate, pH 9.8; 200 μM NAD^+; 2 μg/ml glutamate-oxalacetate transaminase; and 5 μg/ml malate dehydrogenase.

For the cycling reaction, it was necessary to remove the ammonium sulfate solution, in which the enzymes are supplied by the manufacturer, to reduce the sulfate concentration in the assay. This was accomplished by centrifugation of the enzyme suspensions for 3 min at 6000 *g*, followed by resuspension of the enzyme precipitate in a volume of 20 mM imidazole-HCl, pH 7.0, and 0.02% bovine serum albumin equal to the volume of ammonium sulfate solution removed.

Stock solutions, 1 M, of the buffers, β-mercaptoethanol, oxaloacetate, and glutamate and 10% bovine serum albumin were stored at 20°C.

Triglyceride assay procedures

The choice of a fluorometric procedure was dictated by the level of sensitivity desired. The optimal ranges of triglyceride measured by the three procedures are approximately 5 to 50 μg of triglyceride for NADH disappearance, 0.5 to 5 μg for NAD^+ appearance, and 0.25 to 5 ng for 1000 × NAD^+ cycling. In the case of rat muscle, which has approximately 6 μmol of triglyceride per g of tissue, the amount of the tissue used per assay was 1 to 10 mg, 0.1 to 1 mg, and 0.05 to 1 μg for the three procedures, respectively (using 885 as the average molecular weight of triglycerides). Accordingly, the standards were 5 and 10 nmoles, 0.5 and 1 nmoles, and 0.5 and 1 pmoles.

For each of the following procedures, lipid extracts from tissue and standards were pipetted into 10 × 75 mm fluorometric tubes in volumes of 1 to 10 μl and dried thoroughly at 37°C under nitrogen. Plasma samples and standards were pipetted into tubes but not dried.

NADH disappearance. Assay reagents (the complete reagent and the assay reagent without glycerol kinase for the reagent blank) were prepared containing 20 μM NADH. One ml was added directly to each of the samples and standards and incubated for 30 min at 25°C before reading the fluorescence of NADH.

NAD^+ appearance. One hundred μl of the assay reagents containing 200 μM NADH were mixed with each sample and standard, and incubated for 30 min at 25°C. The unreacted NADH was subsequently destroyed by adding 10 μl of 1 N HCl and incubating the mixture for 10 min at 25°C. The NAD^+ was converted to a fluorescent product by the addition of 1 ml of 6 N NaOH containing 10 mM imidazole base (mixed just prior to use) and incubation for 20 min at 60°C. The tubes were cooled to room temperature before the fluorescence was measured.

NAD^+ 1000× cycling. One hundred μl of the assay reagents containing 200 μM NADH was mixed with each sample and standard, and incubated for 30 min at 25°C. The reaction was stopped with 10 μl of 1 N HCl, and the tube was incubated for 10 min at 25°C. A 10-μl aliquot of the assay mixture was transferred to a fresh test tube containing 100 μl of the cycling reagent kept cold on ice. The cycle was activated by placing the test tubes into a 25°C water bath. After 1 hr, the cycling reaction was stopped by placing the tubes in boiling water for 5 min. After cooling the tubes to room temperature, 1 ml of indicator reagent was added and mixed with a Vortex mixer. The fluorescence of NADH was measured following incubation at 25°C for 15 min.

Calculations

The fluorescence attributable to triglyceride was the difference between fluorescence in the tubes containing the complete assay reagent and those containing the blank reagent. Triglyceride concentrations were then calculated from the fluorescence values of the standard curve. Concentration was expressed in μmol/g wet weight (original weight prior to lipid extraction) for tissue and μmol/ml for plasma, using 885 as the average molecular weight of triglycerides in the Q9 triglyceride standard. Linear regression analysis and Student's paired-*t* test were used to compare the plasma values obtained from the different assay procedures.

RESULTS

Plasma triglyceride concentrations

Human plasma samples (500 μl) were analyzed for triglyceride concentration by an automated chemical method using periodate oxidation. Small portions (1 to 10 μl) of the same samples were also analyzed by one or two of the fluorometric methods. **Fig. 2** gives comparative values for 27 plasma samples. There was a highly significant correlation ($P < 0.0001$) between the automated method and either the NAD^+ appearance or NADH disappearance methods over a large range of triglyceride concentrations. Eight of the samples were analyzed by both of the fluorometric procedures and were also highly correlated ($P < 0.0001$; Fig. 2 inset).

Tissue triglyceride concentrations

Lipid extracts prepared from homogenates of skeletal muscle, liver, and heart were analyzed and gave the following concentrations of triglyceride (values in μmol/g): heart, 3.96; muscle, 6.47; liver, 4.09; and adipose tissue, 555 (**Fig. 3**). The values were proportional over the twofold range of tissue weights (Fig. 3), and agreed with

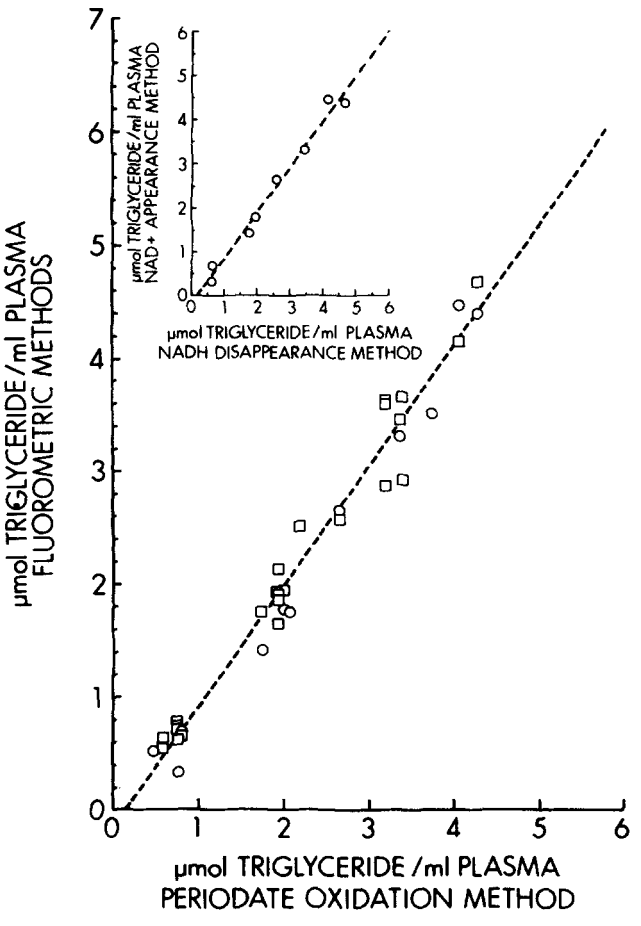

Fig. 2. Comparison of human plasma triglyceride concentrations from 27 subjects determined by fluorometric methods and an automated chemical method involving periodate oxidation and acetylacetone. Square symbols indicate the comparison of the automated method and the direct fluorometric determination of the disappearance of NADH; round symbols compare the automated method and the indirect fluorometric determination of NAD^+ production. The equation of the regression line (dotted line) was $y = 1.07 \times -0.17$; $r = 0.986$ ($P < 0.0001$). Eight of the samples were analyzed by all three methods; inset shows the relationship between values obtained by the two different fluorometric determinations. The equation of the regression line was $y = 1.04 \times -0.19$; $r = 0.9903$ ($P < 0.0001$).

values obtained in rat tissues of much larger sample sizes using other methods (values in μmol/g): heart, 5.9 (10); muscle, 2.9 (11) and 6.1 (10); liver, 4.8 (10) and 3.5 (12); and adipose tissue, 678 to 960 (1).

The muscle homogenate was used to compare the accuracy of assays over extreme differences in sample sizes. **Table 1** shows that similar results were obtained by either NADH disappearance or NAD^+ enzymatic cycling procedures, over a range of sample sizes of nearly 90-fold. For comparison, triglyceride concentration was determined in a much larger sample of the same tissue using the periodate oxidation method. Similar results were obtained with all three methods from sample sizes spanning a range of approximately 4000-fold.

The measurement of triglyceride concentration by the NAD^+ appearance procedure was compared to the NAD^+ *cycling method using human muscle* (**Table 2**). *In this* experiment, triglyceride concentrations were measured in homogenates of whole muscle and in homogenates of a pool of several hundred single muscle fibers dissected free of connective tissue. The non-dissected muscle sample had a somewhat lower triglyceride value than the dissected muscle (although the significance of this was not tested because of the limited number of samples available). There was, however, clear agreement with two methods on the same dissected muscle sample. Mean triglyceride concentration in human (non-dissected) muscle has been previously reported to be 13.7 μmol/g (13).

DISCUSSION

The fluorometric procedures presented here for measuring the glycerol product of triglyceride hydrolysis utilize well known features of pyridine nucleotides (12). Reduced pyridine nucleotides, such as NADH, have an absorption peak at 340 nm, while their oxidized forms do not absorb at this wavelength. Therefore, changes in oxidation or reduction can be measured by absorption changes in the spectrophotometer. Some of the absorbed

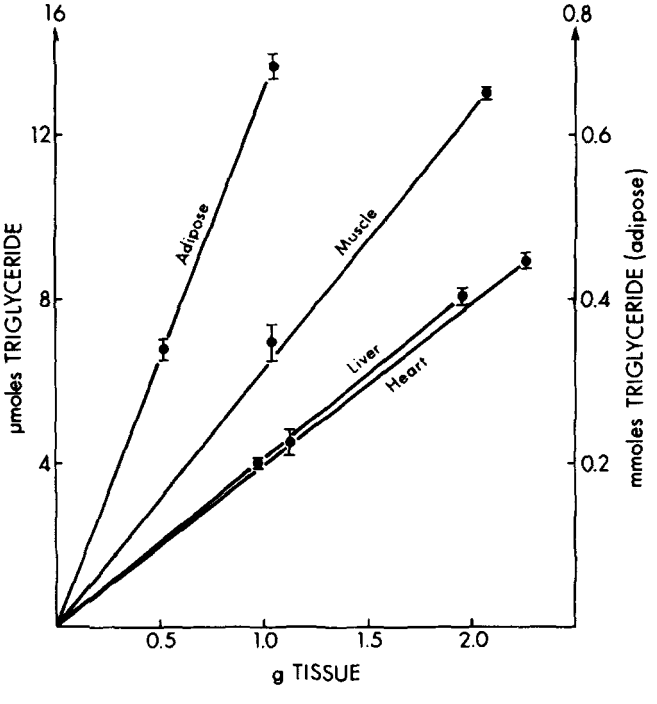

Fig. 3. Triglyceride concentrations in lipid extracts of rat tissues obtained by the fluorometric determination of NADH. Values are means ± SD of triplicate determinations from a single homogenate. The triglyceride scale on the right was adjusted to accommodate values for adipose tissue.

TABLE 1. Muscle triglyceride concentrations obtained using a wide range of sample sizes

Muscle Sample Weight	Method	Concentration
1.04 and 2.08 mg (n = 4)	NADH disappearance	6.47 ± 0.31 μmol/g
44.3 and 88.6 ng (n = 3)	NAD^+ cycling	6.82 ± 0.37 μmol/g
177.2 mg (n = 1)	Periodate oxidation	6.49 μmol/g

Measurements were made on portions of the same lipid extract of rat muscle. The first two concentrations are mean ± SD of n determinations from each sample weight and are not statistically different ($P = 0.09$). For a broader comparison, a single sample was evaluated by the automated periodate oxidation method. Values are expressed in g wet weight of muscle prior to extraction.

light is re-emitted as fluorescence and can be measured with far greater sensitivity than the absorption itself. The analytically useful range for detection of reduced pyridine nucleotides concentration in the spectrophotometer is 10 to 200 μM and in the fluorometer 0.1 to 10 μM. Fluorometric sensitivity can be further increased 6 to 10-fold (measurement of concentrations down to 10^{-8} M) by converting the oxidized pyridine nucleotide, such as NAD^+, to a highly fluorescent form with strong alkali (5).

Far greater sensitivity can be achieved by enzymatic cycling (for review, ref. 7) in which a pyridine nucleotide acts as a catalytic intermediate for two simultaneous oxidation and reduction reactions. With large amounts of enzymes and substrate, the limited amount of pyridine nucleotide cofactor is alternately oxidized and reduced (cycled) to produce a high concentration of the two main reaction products. Provided that the pyridine nucleotide level is well below the Michaelis constant of the two reactions, the procedure will linearly increase the reaction products. Cycling rates are adjusted by the enzyme concentration to yield products with a concentration in the analytical range of the fluorometer. Either one of the two products is subsequently measured. In the NAD^+ cycle illustrated in Fig. 1, the small amount of NAD^+ produced in Reaction a was "cycled" as the cofactor for the malate dehydrogenase and alcohol dehydrogenase reactions (Reaction b). While the concentration of NAD^+ did not change, both malate and acetaldehyde were increased at a rate set by the enzymes. The amplified malate concentration was measured in a separate reaction (Reaction c). The NAD^+ cycle described here permitted measurement of triglyceride concentrations 1000-fold less than direct fluorometric methods or 0.25 ng of triglyceride/sample. The NAD^+ cycle could be extended by cycling at a higher rate (with 60 μg/ml malate dehydrogenase and 600 μg/ml alcohol dehydrogenase) thereby amplifying the product 60,000-fold and providing accurate detection of 4 pg of triglyceride. In fact, with repeated cycles, up to 1,000,000-fold amplification can be achieved (6).

TABLE 2. Human muscle triglyceride concentrations obtained in dissected fibers and whole muscle

Muscle Sample Weight	Method	Concentrations
Dissected; 57.2 ng	NAD^+cycling	13.0 μmol/g
Dissected; 500 μg	NAD^+ appearance	13.3 μmol/g
Non-dissected; 500 μg	NAD^+ appearance	12.2 μmol/g

Lipid extracts of dissected and non-dissected muscle were made from different portions of the same human vastus lateralis muscle biopsy. They differ in the handling described in the text for rat muscle samples in that the tissue was freeze-dried (− 35°C at pressure less than 10^{-2} Torr for 7 days) before homogenization and extraction. Values are in g wet weight of muscle prior to extraction converted from dry weight using a factor of 4. Each value represents a single determination.

At concentrations of NADH less than 10 μM there is minimal quenching of the fluorescence and therefore the emitted light is proportional to the concentration of the nucleotide. However, a number of cations (e.g., Mg^{2+}) are known to enhance fluorescence of NADH in alkaline solution. Enhancement can be avoided by adjusting the pH below 10 or by adding EDTA. As indicated previously, alkaline pH can convert the oxidized form of the nucleotide to a fluorescent product which could interfere with the assay. In addition, high temperature decreases fluorescence. Thus, appropriate pH and temperature must be maintained. Glycerokinase and ATP in the assay can react with dihydroxyacetone and glyceraldehyde in the tissue sample to give dihydroxyacetone phosphate or glyceraldehyde-3-phosphate, respectively, and ADP, and therefore pose a limit to specificity. These compounds do not occur in the blood and are in low concentrations relative to triglycerides in most other tissues. Levels can be tested by running the reaction without lipase and esterase.

The increased sensitivity of the methods described here over conventional spectrophotometric methods may be of great value in lipid research when extremely small triglyceride concentrations must be detected or when the available tissue sample is very small. This is most common in the cases of small clinical samples of blood (e.g., cord blood, pediatrics) or of tissue obtained by biopsy. It is becoming increasingly advantageous to be able to measure even smaller samples, such as those of microdissected single cells (14). The amplification method may also be applicable to the determination of diglycerides and monoglycerides, although a preliminary separation from triglycerides would be required. In this regard, particularly in view of the current interest in the determination of diglycerides generated in response to stimulation via cell surface receptors (for review, ref. 15), this high resolution method should prove to be a powerful tool.

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REFERENCES

1. Eggstein, M., and E. Kuhlman. 1974. Triglycerides and glycerol: determination after alkaline hydrolysis. *In* Methods of Enzymatic Analysis. Vol. 4. H. U. Bergmeyer, editor. Verlag Chemie International, Florida. 1825–1831.
2. 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.
3. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
4. Burch, H. B., O. H. Lowry, L. Meinhardt, P. Max, Jr., and K-J. Chyu. 1970. Effect of fructose, dihydroxyacetone, glycerol and glucose on metabolites and related compounds in liver and kidney. *J. Biol. Chem.* **245:** 2092–2102.
5. Lowry, O. H., and J. V. Passonneau. 1972. A Flexible System of Enzyme Analysis. Academic Press, New York.
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. Lowry, O. H. 1980. Amplification by enzymatic cycling. *Molec. Cell. Biochem.* **32:** 135–146.
8. Kessler, G., and H. Lederer. 1966. Fluorometric measurement of triglycerides. Automation in analytical chemistry. Technicon Symposium, 1965. Technicon, Tarrytown, NY. 341–344.
9. Lipid Research Clinics Program. 1975. *In* Manual of Laboratory Operations, Lipid and Lipoprotein Analysis. DHEW Publication No. (NIH) 75-628. Vol. 1.
10. Connellan, J. M., and C. J. Masters. 1964. Fatty acid components of rat tissue lipids. *Biochem. J.* **94:** 81–84.
11. Barclay, J. K., and W. N. Stainsby, 1972. Intramuscular lipid store utilization by contracting dog skeletal muscle in situ. *Am. J. Physiol.* **223:** 115–119.
12. Cole, T. G., H. G. Wilcox, and M. Heimberg. 1982. Effects of adrenalectomy and dexamethasone on hepatic lipid metabolism. *J. Lipid Res.* **23:** 81–91.
13. Fröberg, S. O. 1973. Muscle triglycerides. *Acta. Med. Scand.* **193:** 463–468.
14. Hurley, B. F., P. M. Nemeth, W. H. Martin III, J. M. Hagberg, G. P. Dalsky, and J. O. Holloszy. 1986. Muscle triglyceride utilization during exercise: effect of training. *J. Appl. Physiol.* **60:** 562–567.
15. Berridge, M. J. 1984. Inositol triphosphate and diacylglycerol as second messengers. *Biochem. J.* **220:** 345–360.