notes on methodology

A sensitive radioenzymatic assay for glycerol and acylglycerols

Peter B. Schneider

Thorndike Memorial Laboratory and Nuclear Medicine Unit, Department of Medicine, Beth Israel Hospital and Harvard Medical School, Boston, MA 02215

Summary A sensitive radioenzymatic assay for glycerol and acylglycerols is described. The assay depends on the quantitative phosphorylation of glycerol to glycerophosphate by glycerol kinase using [γ-^{32}P]ATP as a substrate. The ^{32}P content of the formed glycerophosphate is determined and gives a measure of the original glycerol content. Acylglycerols can be determined by prior hydrolysis to glycerol. The assay is sensitive to about 0.1 nmol of glycerol and can be extended to 100 nmol. The assay can be applied to the determination of acylglycerols separated by thin-layer chromatography in amounts as low as 0.5 nmol. The assay is particularly useful in the determination of the specific activity of ^{14}C- or ^{3}H-labeled glycerol moeities.

Supplementary key words glycerol kinase · [γ-^{32}P]ATP · specific activity determination · thin-layer chromatography

Glycerol and the acylglycerols are compounds of widespread biological significance and their assay is of corresponding importance. Currently the most sensitive glycerol assays are enzymatic and are based on the conversion of glycerol to glycerophosphate with glycerol kinase followed by oxidation of the glycerophosphate with glycerophosphate dehydrogenase. The latter step is accompanied by the reduction of NAD to $NADH_2$, which can be measured either spectrophotometrically (1, 2) or fluorometrically (3). Acylglycerols can be determined by hydrolysis and measurement of the released glycerol (3). These assays have sensitivities of about 2 nmol of glycerol.

This report describes a radioenzymatic assay for glycerol with a sensitivity of about 0.1 nmol and its adaptation to the measurement of acylglycerols.

The assay is based on the quantitative phosphorylation of glycerol by glycerol kinase utilizing [γ-^{32}P]ATP. The ^{32}P-labeled glycerophosphate formed in the reaction is separated by cellulose thin-layer chromatography followed by determination of its ^{32}P content, which reflects the amount of glycerol originally present. If the glycerol is labeled with ^{14}C or ^{3}H, the ratio of those isotopes to ^{32}P in the final glycerophosphate gives a measure of glycerol specific activity that is essentially independent of sample recovery.

Materials

All chemicals were reagent grade and solvents were redistilled. Tripalmitin (mp 61–62°C), obtained from Fisher Chemical Co., Fairlawn, NJ, chromatographed as a single spot on silica H thin-layer plates developed with hexane–ethyl ether–acetic acid 60:40:1.5 (v/v/v). Bovine serum albumin low in fatty acids was obtained from Sigma Chemical Co., St. Louis, MO. Cellulose MN (Brinkmann, Westbury, NY) and silica gel H (Analabs, North Haven, CT) was used for thin-layer chromatography. Omnifluor scintillator is the product of New England Nuclear Corp., Boston, MA.

Glycerol kinase (*E. coli*) was obtained from Worthington Biochemical Co., Freehold, NJ, and prepared for use by dissolving 0.2 mg of the enzyme in 0.03 ml of 0.1 M ethylene glycol followed by the addition of 0.07 ml of saturated ammonium sulfate solution. The resulting suspension contained about 80 U/ml and was found to be stable for several months under refrigeration.

[γ-^{32}P]ATP was prepared enzymatically from ^{32}P-labeled orthophosphate by the method of Glynn and Chapell (4) modified in that the labeled ATP was purified by absorption on charcoal. The reaction mixture was acidified to pH 2 with 4 M HCl and added to activated charcoal (300 mg Norit for 50 μmole ATP). After washing the charcoal thrice with 5 ml of 0.01 M HCl–0.001 KH_2PO_4, the ATP was eluted with five portions of 5 ml of ethanol–1 M NH_4OH 1:1 (v/v). The solution was dried under vacuum and the ATP redissolved in 1 mM Tris-HCl buffer (pH 7.4).

Assay procedures

Glycerol assay. The assay of 0–10 nmol of glycerol is carried out in a small test tube in a total volume of 0.1 ml allowing 0.05 ml for the assay medium. Each 0.05 ml of assay medium contains 10 μmol Tris-HCl buffer (pH 9), 0.2 μmol EDTA, 0.5 μmol $MgCl_2$, 1 μmol β-mercaptoethanol, 0.5 mg fatty acid-free bovine serum albumin, 16 mU glycerol kinase, 20 nmol ATP, and sufficient [γ-^{32}P]ATP to provide a convenient specific activity of 1000–2000 cpm/nmol (0.5–1 Ci/mol). The albumin is not necessary

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

for the assay of glycerol itself but must be present to avoid interference from fatty acids released by hydrolysis of acylglycerols (see below). For the assay of larger amounts of glycerol (5–100 nmol), the glycerol kinase is increased to 80 mU per assay tube and the amount of ATP is increased to at least twice the maximum expected glycerol in the assay while the specific activity of the ATP may be reduced to a convenient value.

The components of the assay medium including the nonradioactive ATP but *not the* [γ-^{32}P] *ATP* are mixed in bulk for the appropriate number of tubes in the run and allowed to stand at room temperature for at least 15 min to permit the reaction with unlabeled ATP of any glycerol present in these reagents, thereby reducing the blank. The [γ-^{32}P]ATP is then added and 0.05 ml of the assay medium is added to 0.05 ml of sample.

After at least 15 min of reaction at room temperature, 0.01 ml from each assay tube is taken with a microsyringe for the chromatographic separation of the ^{32}P-labeled glycerophosphate formed. The thin-layer plates (20 × 20 cm) are prepared from a slurry of cellulose MN in 0.01 M EDTA applied at a thickness of 0.5 mm. Lanes of 1.5–2 cm width are scored on the dried plates to prevent cross migration. A marker mixture of 0.1 μmol each of DL-α-glycerophosphate and K_2HPO_4 is applied to each side lane and the samples are applied to the remaining lanes as a series of contiguous spots which are then dried in a stream of warm air. The plates are developed by the ascending method in methanol–1 M ammonium acetate (pH 8.5)–0.2 M EDTA 70:20:0.5 (v/v/v). The plates are dried in a stream of warm air and, with the center lanes masked, the two side marker lanes are sprayed with the Hanes–Isherwood reagent for phosphorus (5) and exposed to ultraviolet light to develop the color. The glycerophosphate band of each lane, as determined from the marker compound (R_f 0.35) is scraped into a scintillation counting vial to which is added 2 ml of methanol containing 2.5% (v/v) 4 M HCl and, a few minutes later (to allow solution of the glycerophosphate), 8 ml of toluene with 0.5% Omnifluor. Radioactivity is determined in a liquid scintillation counter set for ^{32}P. (If ^{14}C or ^{3}H specific activity of the original glycerol is to be determined, a channel is set for those isotopes). The amount of glycerol in the sample is calculated by comparing the observed ^{32}P activity to that found for a blank and a glycerol standard that have been carried through the assay. The glycerol standard solution is prepared by weight from desiccated reagent grade glycerol (Fisher Chemical Co.).

Acylglycerol assay. The acylglycerol sample is dried in a small test tube and dissolved in 0.05 ml benzene plus 0.1 ml of 0.1 M KOH in methanol. The sample is hydrolyzed by heating in a water bath at 50–55°C for at least 30 min. If, during this time, the solvent does not completely evaporate the sample is further dried under a stream of nitrogen or clean air. A 0.05 ml portion of 0.1 M HCl is then added, to partially neutralize the KOH, and is followed by 0.05 ml of the assay medium. The assay for glycerol is then completed as above. The addition of 0.05 ml of HCl for neutralization is sufficient to bring the hydrolyzed mixture into range for final adjustment by the buffer contained in the assay medium. The addition of larger amounts of HCl may result in over-neutralization, since the KOH is partially (and variably) neutralized by absorption of CO_2 from the air during hydrolysis and drying (especially if air is used). There is a tendency for the hydrolysis mixture to creep slightly up the side of the tube but this is generally visible after drying and the sample can be captured by gentle swirling of the assay medium.

Acylglycerol assay after thin-layer chromatography. The lipid sample may be chromatographed on silica gel thin layers in any appropriate solvent. The desired acylglycerol band is detected either by spraying the entire plate with rhodamine B or by comparison with a standard run and stained in a separate lane (other detection methods may work but have not been evaluated for possible interference). The band is scraped into a test tube to which is added 4 ml of 2-propanol–hexane–water 75:25:1 (v/v/v). After mixing, 4 ml of water is added and the phases are allowed to separate. These volumes are adequate for a silica gel band size of about 4 cm^2 or less but may have to be increased for larger amounts. The upper (organic) layer, measuring 1.1 ml, is transferred quantitatively with the help of several small rinses of hexane to an assay tube in which it is dried, hydrolyzed, and assayed as above. Alternatively, an aliquot of the upper phase may be taken for assay if corrected for its total volume of 1.1 ml.

Results

The linearity of the assay for glycerol in the range of 0.05–5 nmol is shown in **Fig. 1.** With adjustment of the assay conditions for greater amounts of glycerol (see Methods), a linear assay for 1–100 nmol can be obtained. Assay blanks are equivalent to 0.05–0.1 nmol of glycerol. Replicate determinations (five at each level) showed a standard deviation of 1% at 0.5 and 5.0 nmol.

Using the hydrolytic procedure a linear standard

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

ASBMB JOURNAL OF LIPID RESEARCH JLR

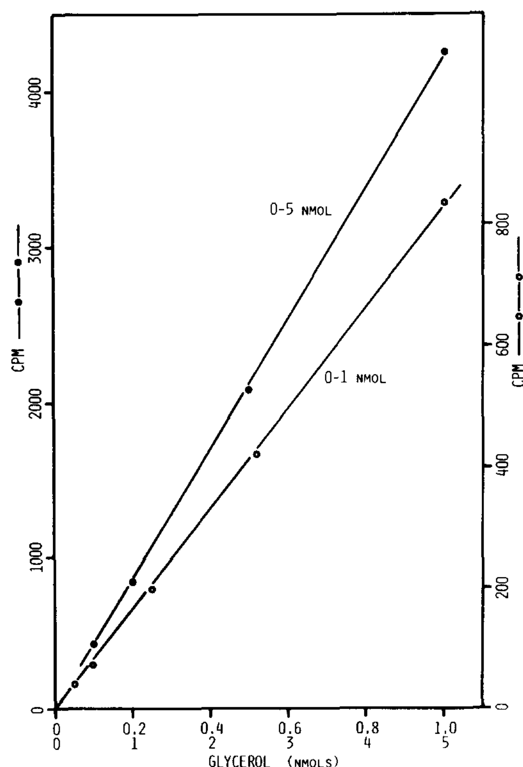

Fig. 1. Glycerol assay standard curve, 0–5 nmol. The 0–1 nmol portion of the curve is plotted with an expanded scale on the right-hand ordinate. Each point is the mean of duplicate determinations. Analysis of duplicates showed a standard deviation for these curves of 0.005 nmol for the range 0–1 nmol and 0.015 nmol over the entire range.

curve with tripalmitin can be obtained in the low range of 0.1–5 nmol and 1–100 nmol for the high range. Assay blanks are equivalent to 0.1–0.2 nmol of glycerol. A standard deviation of 3% at levels of 0.5 and 5 nmol of tripalmitin was shown by replicate determinations (10 at each level).

For the assay of acylglycerols after separation by thin-layer chromatography, a linear response up to 100 nmol can be obtained as above but the sensitivity of the assay is limited predominantly by the blank of 0.5–0.8 nmol. This blank is constructed by carrying a blank lane of the silica gel thin layer through the assay. To minimize this blank the thin-layer plate should be prewashed with two runs of acetone in a direction at right angles to the direction of the final lipid separation to sweep any glycerides to an unused side. Care must be taken to avoid contamination with adventitious glycerides at all steps. We have found, for example, that a fingerprint eluted from a glass surface may contain 5 nmol of glyceride. Another unexpected source of a high blank can be the tubing used to conduct air for the evaporation of solvents at various steps of the assay. Tygon tubing used that way may contribute 1–2 nmol to the blank and some rubber tubing is also unacceptable. We have found washed Fluran tubing (Fisher) or a washed white rubber tubing (A. H. Thomas Co., Philadelphia, PA) acceptable. On the other hand, for higher level assays where a blank of 3 nmol may be tolerated no special precautions need be taken and one may even forego the redistillation of reagent grade solvents.

Replicate determinations (10 at each level) of chromatographed tripalmitin showed a standard deviation of 6% at 5 nmol and 30% at 0.5 nmol. Recovery of tripalmitin carried through thin-layer chromatography and assay was 101% ± 10% (SD).

Fig. 2 demonstrates the application of the assay to the determination of amount and specific activity of [^{3}H]glycerol-labeled liver triglyceride. Lipids were extracted from rat liver slices that had been incubated with [2-^{3}H]glycerol. Various amounts of lipid extract corresponding to 0.05–1 mg wet weight of liver were run in duplicate on silica gel H thin

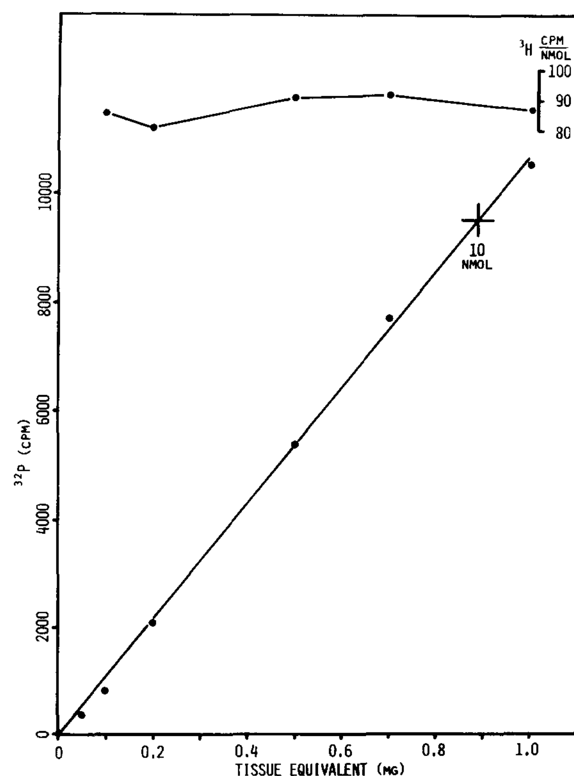

Fig. 2. Assay and specific activity determination of [^{3}H]glycerol-labeled rat liver triacylglycerol. The abscissa represents the sample size in terms of the wet weight of liver from which it was extracted. The ordinate is the ^{32}P activity observed in the assay of each sample. The ^{32}P activity corresponding to 10 nmol of glycerol intersects the line at a tissue equivalent of 0.9 mg. The upper curve is the specific activity measured for each indicated sample size. Each point is the mean of duplicate determinations. Analysis of duplicates indicates a standard deviation of 0.2 nmol over the entire assay range. For the specific activity determinations the standard deviation is 4.4 cpm/nmol.

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

layers in petroleum ether–ethyl ether–acetic acid 60:40:1.5 (v/v/v) (cholesteryl esters overlap triglycerides in this system but do not interfere with the assay). The triglyceride bands were scraped and assayed as above. Measurement of the ^3H as well as the ^{32}P in the final glycerophosphate product allowed calculation of specific activity. Comparison with a glycerol standard showed the triglyceride content of 1 mg of liver to be 11 nmol. The specific activity determinations are shown at the top of the figure and the relative constancy of the value over the range of samples illustrates the independence of this determination from absolute recovery of lipid. At the lowest level (i.e., 0.05 mg of liver) specific activity determination was limited by the low total ^3H activity in the sample which was only about 35 cpm above background.

Interference. A variety of potentially interfering compounds were tested by measuring the effect of 50 nmol of the substance on the assay of 5 nmol of glycerol. Glucose, glucose-1-phosphate, glucose-6-phosphate, glyceric acid, 3-phosphoglyceric acid, and adenosine diphosphate had no effect; 50 nmol of DL-glyceraldehyde and dihydroxyacetone, which may serve as substrates for the enzyme (6) and whose phosphorylated products overlap glycerophosphate on chromatography, contributed an apparent 0.2 and 1.8 nmol, respectively, to the assay. DL-α-Glycerophosphate contributed 1 nmol when present at a level of 50 nmol (it is not clear whether this is due to glycerol as an impurity or due to an enzyme-catalyzed exchange reaction).

Palmitic acid in amounts up to 300 nmol had no effect on the assay but the mixed fatty acids released by the hydrolysis of liver triglycerides were strongly inhibitory. However, the addition of 0.5 mg of fatty acid-free bovine serum albumin to the assay will overcome the inhibition due to the hydrolysis products of up to 40 nmole of rat liver triglycerides.

Cholesteryl esters may overlap triglycerides in some chromatography systems and hence may be brought into the assay to release cholesterol and fatty acids upon hydrolysis. Cholesterol at levels of 50 nmol does not inhibit the assay and the inhibition due to fatty acids can be counteracted as above. Similarly, glyceryl-1-octadecyl ether, a representative product of alkoxyglycerides that may cochromatograph with acylglycerols, did not inhibit the assay or raise the blank when present at levels of 50 nmol.

Supported by grant AM-10733 from the National Institute of Arthritis, Metabolism, and Digestive Diseases. We are indebted to Mr. Alan Mannarino for expert technical assistance.

Received 7 June 1976 and in revised form 22 October 1976; accepted 30 November 1976.

REFERENCES

1. Boltralik, J. J., and H. Noll. 1960. A specific sensitive assay of glycerol and L-α-glycerophosphate. *Anal. Biochem.* **1:** 269–277.
2. Moller, F., and M. W. Roomi. 1974. An enzymatic spectrophotometric glycerol assay with increased basic sensitivity. *Anal. Biochem.* **59:** 248–258.
3. Chernick, S. 1969. Determination of glycerol in acyl glycerols. *In* Methods of Enzymology, Vol. XIV. J. M. Lowenstein, editor. Academic Press, New York. 627–630.
4. Glynn, I. M., and J. B. Chappell. 1964. A simple method for the preparation of ^{32}P-labeled ATP of high specific activity. *Biochem. J.* **90:** 147–149.
5. Hanes, C. S., and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. *Nature.* **164:** 1107–1112.
6. Hayashi, S., and E. C. C. Lin. 1967. Purification and properties of glycerol kinase from *E. coli. J. Biol. Chem.* **242:** 1030–1035.

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