A 2-Phase Liquid Scintillation Assay for Glycolipid Synthetases

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ABSTRACT

Glycolipid synthetases can be assayed conveniently by incubating the lipid substrate with the radiosugar-labeled nucleotide in a small plastic scintillation vial. At the end of the incubation period, water and perchloric acid are added, then *n*-butanol, then a toluene-based scintillation cocktail. The radioactive lipid partitions into the scintillation fluid, leaving excess sugar nucleotide in the aqueous phase. Only a small fraction of the total radioactivity in the aqueous layer is detectable. This method is illustrated for ceramide:UDP-glucose glucosyltransferase. The approach should be applicable to other lipid synthetases that can be assayed with a radioactive hydrophilic substrate.

In the many widely used radiometric enzyme assay techniques, it is necessary to separate the radioactive product of enzyme action from the radioactive substrate. In the case of lipids, where the labeled substrate may be nonlipoidal, solvent partitioning is commonly done, typically with chloroform/methanol/water. The chloroform layer must be evaporated to dryness (after multiple washings) before the lipid can be counted by liquid scintillation, since chloroform is a quenching agent.

Potter (1) used a partition method to separate the labeled acetate formed by acetylcholinesterase, in which the solvent–toluene/isoamyl alcohol–was not a quencher and could then be added to a scintillation fluid directly. This approach was improved by Sankaran and Pogell (2), who simply incubated the assay mixture in a scintillation vial, partitioned the labeled product directly into a scintillation fluid, and counted the entire assembly in the normal way. Because the water in the lower phase absorbed most of the β-radiation coming from the unused tritiated substrate, the observed background activity in the scintillation fluid was not too high. In the case of ^{14}C, as opposed to ^{3}H, there was enough penetration of the water layer to raise the background to an unpleasant level and the lower layer had to be removed. A few additional examples of the scintillation partitioning method have been published since then (3-7) but none have involved lipids, which ought to be particularly suited to the approach. In our initial attempts to use the approach, however, we found excessive differences between duplicate samples and variable drift of observed activities as a function of time. These were alleviated by centrifuging, which presumably brought down small amounts of the lower phase that were adhering to the walls of the vial in contact with scintillation fluid. Additional improvement was obtained by denaturing the proteins with perchloric acid.

Another problem was the size of the boiled-enzyme blank, which resulted in part from radiation entering the scintillation fluid from the lower phase. This was reduced by lowering the specific activity (sp act) of the lower phase with added water.

MATERIALS AND METHOD

The assay medium for ceramide:UDP-GLC glucosyltransferase contained liposomes made from N-octanoyl sphingosine, lecithin, and cerebroside sulfate (8) as well as uridine diphospho[^{3}H]glucose (190,000 cpm), Tris-Cl pH 7.4, dithioerythritol, EDTA, $MgCl_2$ and ATP. The enzyme source was liver from Harlan ICR mice, homogenized in 4 vol of water and diluted further with water to contain 50 mg/ml of tissue (9). The total incubation vol was 0.2 ml and the mixture was incubated for 1 hr at 37 C in a 7-ml polyethylene scintillation vial ("Mini-Vial").

At the end of the incubation period, the vials were placed in ice and 0.2 ml of 5.6% perchloric acid was added to the first vial. This was vortexed briefly and left for a few minutes while the other vials were processed similarly. Now 0.4 ml of water and 0.4 ml of *n*-butanol were added to the first vial, which was vortexed for 30 sec. After the other vials were processed the same way, 3.6 ml of scintillation liquid (18 mg of PPO and 1.1 mg of dimethyl POPOP in toluene) was added and the vials were vortexed for 30 sec again.

The vials were then centrifuged in an angle-head rotor (GSA, DuPont/Sorvall) for 10 min at 10,000 rpm (8,500 × g–max). Rubber stoppers were placed in the rotor cups to keep the vials high enough for easy retrieval. The vials were then inserted into glass holders, using plastic collars, and loaded into a scintillation counter.

RESULTS AND DISCUSSION

In our previously described assay procedure (8), the system was incubated in a glass test tube and biosynthesized cerebroside was extracted with chloroform/methanol. Residual radioactive precursor was removed by several partitioning steps, most of the chloroform-rich layer was then evaporated to dryness in a scintillation vial and the cerebroside was dissolved in a scintillation fluid containing water and Beckman BBS-3. Comparison of [^{3}H]cerebroside (10) in this counting system and in toluene/butanol (90:10, v/v) showed that the latter yielded 12% higher readings. Toluene alone, however, dissolved only part of the labeled cerebroside and low activities were obtained.

When the toluene/butanol mixture was added to incubation vials, however, erratic activities were seen which tended to increase fairly rapidly. Various attempts at stabilization were made based on the idea that variable amounts of lower phase were clinging to the vial walls. Centrifugation did not help, possibly because sufficient speed could not be achieved with the thin-walled vials. The addition at the end of the incubation period of 100 mg of silica gel, which could have made the droplets of liquid denser, proved effective in stabilizing the activities, but this method was dropped because of the work involved in weighing out the powder. Also effective was the addition of aqueous perchloric acid to yield 2.8% acid, the concentration recommended for protein precipitation. Alumina (100 mg) and 0.2 ml of 1 N NaOH were ineffective. Centrifugation improved the degree of replication, apparently because variable amounts of precipitated protein and aqueous phase adhered to the vial walls. However, fairly high centrifugal force was required. The observed activities were now stable for 3 days (but toluene evaporates through the plastic vial upon prolonged storage).

The perchloric acid not only stabilized the counts, but it also increased them, probably because denaturing the proteins improved the extraction of the radioactive lipid from the membranes in which it had been formed. The extra water was added (0.4 ml) simply to reduce the amount of radiation entering the upper phase. Adding more would reduce the blank activity even further, but we felt that vortexing too full a vial might produce losses in the threads of the vial cap.

The butanol was added before adding the toluene because we thought this might help extract the radioactive lipid from the precipitated membranes. Conceivably, adequate extraction could be obtained even when the butanol is premixed with the toluene by lengthening the vortexing step.

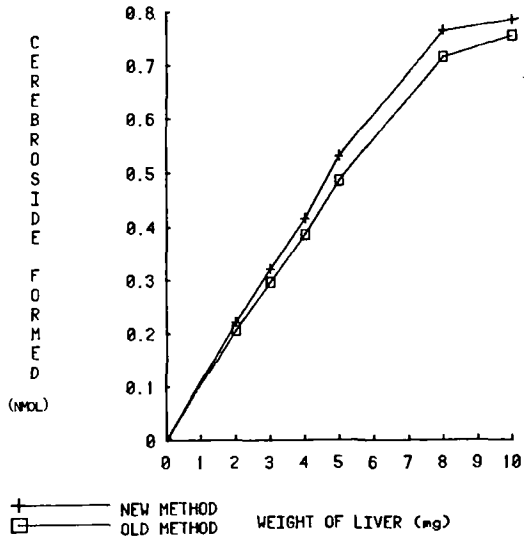

FIG. 1. Amount of glucocerebroside formed by various amounts of liver homogenate from a 26-g male mouse in 0.2 ml of incubation medium. The upper curve shows the data obtained with the new 2-phase counting system and the lower curve shows the data obtained with the earlier glass incubation system.

It is possible that the method could be made more convenient by adding the 0.4 ml of water together with the perchloric acid (i.e., 0.6 ml of 3.7% acid).

As in our previous study (8), the activity of the transferase was found to be proportional to the weight of liver up to about 7 mg (Fig. 1). An unexpected finding is that the samples incubated in the plastic vial gave higher enzyme activities (nmol product). Examination of the incubation tubes showed that most of the homogenate membranes had aggregated on the walls of the glass tube in the form of a ring. This did not happen in the plastic vials. It seems likely that the better suspension in the plastic vials yielded more efficient reactivity of the substrates with the enzyme. The data in Figure 1 also show that the relatively large amount of protein in the lower layer or in the liquid/liquid interface did not interfere with the extraction of the lipid into the upper phase.

A comparison of the 2 assay methods with 5 mg of liver (in triplicate) showed that the observed activities with the chloroform/methanol method were 65 ± 11 cpm for the zero-time blank and 2807 ± 25 cpm for the incubated sample. With the new method, the

activities were 295 ± 6 cpm for the blank and 3439 ± 12 cpm for the incubated sample (corrected for the blank activity). The improvement in variability was typically observed. The blank activity with the new method was distinctly higher (8.6% of the observed activity vs 2.3% with the older method) but this is partially offset by the higher observed activity with the new method. The higher activity results not only from the use of a better counting solvent and higher enzyme activity, but also from the fact that some of the radioactive lipid must be discarded in the chloroform/methanol partitioning method to avoid transfer of upper layer. The blank value of 295 cpm constitutes 0.15% of the total incubated tritium.

Attempts were made to lower the blank by floating a disk of polyethylene at the interface between the scintillation and aqueous layers, but these were ineffective.

The method described in this paper minimizes the exposure of personnel to radiochemicals and organic solvents, reduces the time required for processing by over 40%, and reduces variability and the danger of spillage during transfer. All of the radioactivity used in the incubation remains in the original tube, making disposal easier.

The assay method has also been used in this laboratory for ceramide:UDP-gal galactosyltransferase. It should be suitable for other lipid synthetases, such as the enzymes that make lecithin (labeled CDP-choline or S-adenosyl methionine), fatty acids (labeled acetyl-CoA or malonyl-CoA), or phosphatidylethanolamine (labeled CDP-ethanolamine). Hydrolase assays, using a lipid labeled in its polar moiety (e.g., glucose-labeled cerebroside for glucosidase), could also be handled by this method but a lower precision must be expected because the calculations would require subtracting one large number from another. Still, such reactions usually go relatively well and an appreciable degree of hydrolysis can be obtained even while maintaining substrate saturation.

ACKNOWLEDGMENT

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REFERENCES

1. Potter, L.T. (1967) J. Pharmacol. Exp. Ther. 156, 500-506.
2. Sankaran, L., and Pogell, B.M. (1973) Anal. Biochem. 54, 146-152.
3. Fonnum, F. (1975) J. Neurochem. 24, 407-409.
4. Johnson, C.D., and Russell, R.L. (1975) Anal. Biochem. 64, 229-238.
5. Wu, P.H., and Dyck, L.E. (1976) Anal. Biochem. 72, 637-642.
6. Dunant, Y., and Hirt, L. (1976) J. Neurochem. 26, 657-659.
7. Pollard, H.B., Stupak, S.S., Pazoles, C.J., and Creutz, C.E. (1979) Anal. Biochem. 99, 281-282.
8. Vunnam, R.R., and Radin, N.S. (1979) Biochim. Biophys. Acta 573, 73-82.
9. Brenkert, A., and Radin, N.S. (1972) Brain Res. 36, 183-193.
10. McMaster, M.C., Jr., and Radin, N.S. (1977) J. Labelled Comp. Radiopharm. 13, 353-357.

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