

## notes on methodology

### A simplified assay method for galactosyl ceramide $\beta$ -galactosidase

NORMAN S. RADIN and RAMESH C. ARORA

*Mental Health Research Institute, University  
of Michigan, Ann Arbor, Michigan 48104*

**SUMMARY** In a previously described method for determining the activity of cerebroside galactosidase, the enzyme preparation was incubated with an emulsion of cerebroside which had been labeled in the galactose moiety. The liberated galactose was separated from the emulsion by liquid-liquid partitioning, but the presence of detergent necessitated the use of careful agitation and a backwash in order to reduce the contamination of the aqueous layer with excess emulsified substrate. This problem is eliminated by adding a large amount of lipid to reduce the emulsifying power of the detergent. It may be that other lipid hydrolase assays, based on the same principle, would benefit by this approach. Some additional improvements in the assay system are described.

**SUPPLEMENTARY KEY WORDS** castor oil

**THE RECENT** finding (1) implicating cerebroside galactosidase as the primary defect in globoid leukodystrophy has led us to publish details of improvements in the assay method for this enzyme.

In our previously described assay procedure (2), we used galactose-labeled cerebroside as substrate and, after incubation with enzyme, partitioned the incubation mixture to remove the freed galactose from remaining substrate. The upper phase was then dried and assayed for radioactivity by liquid scintillation counting. It was found that excessive and variable amounts of labeled cerebroside entered the upper layer unless the degree of shaking during partitioning was kept very low; even with careful manipulation a backwash with lower phase was necessary. This limitation, applied to more than a few tubes, resulted in slow, tedious processing. We wish to report a technique for simplifying and speeding the partition step, based on the assumption that the detergent in the substrate emulsion was causing some cerebroside-detergent micelles to escape from the lower phase. The problem was solved by adding a very large amount of lipid after incubation, to destroy the micelle-forming ability of the detergent.

While any lipid would be suitable, a nonpolar lipid should be most effective. Moreover, use of a nonpolar

lipid simplifies the chromatographic recovery of unused substrate since only a relatively small column should suffice (3).

It was also felt that the substrate micelles had to be dissociated before the cerebroside molecules could be displaced by the added lipid. This would probably require forming a single phase containing the incubation mixture, chloroform, the added lipid, and a solvent mutually miscible with chloroform and water. Trials with corn oil and methanol showed that very large volumes of solvent would be needed, so that drying the upper phase would be a lengthy step. A slightly more polar lipid and a slightly more lipophilic solvent were used in the procedure described below.

**Procedure.** The substrate is prepared by evaporating to dryness a mixture of solutions of  $^3\text{H}$ -labeled cerebroside (1 mg, 350 cpm/nmole), Tween 20 (10 mg), Myrj 59 (5 mg, from Atlas Chemical Industries, Inc., Wilmington, Del.), and oleic acid (3.5 mg). The cerebroside is stored in chloroform-methanol and the other components in benzene, at  $-20^\circ\text{C}$ . The dry residue is warmed and sonicated in a bath with 5 ml of aqueous sodium taurocholate (20 mg) and Tris base (1.65 mg).

The incubation mixture consists of enzyme (up to 0.4 ml), substrate (0.5 ml), and citrate buffer (0.1 ml, 1 M, pH 4.5). When a particulate enzyme preparation is being assayed, it is difficult to ensure delivery of equal aliquots. We stir the suspension magnetically while withdrawing portions and, if frozen enzyme suspension is being assayed, rehomogenize the suspension after it thaws.

The incubation mixtures are shaken for 3 hr at  $37^\circ\text{C}$  in  $20 \times 150$  mm screw cap test tubes and the reaction is stopped with 4 ml of a mixture of isopropanol-castor oil-chloroform 6:1:1 (v/v/v). The liquids are mixed well on a Vortex mixer, left 15 min, and 5 ml of water containing 0.1 mg of carrier galactose is added. The liquids are again mixed, 6 ml of chloroform is added, and the mixture is agitated. After a brief centrifugation, 5 ml is carefully removed from the upper layer (7 ml total) and transferred to a scintillation vial for counting. The solvent is removed on a  $50-60^\circ\text{C}$  water bath with the aid of a strong air stream. The residue is dissolved in 0.5 ml of water, and 11 ml of a scintillation solution containing Beckman BioSolv BBS-3 (9% in toluene) is added (4).

The blank activity with this method and our present batch of labeled cerebroside (5) is about 62 cpm, which is 0.15% of the total activity in the incubation tube. In our modified procedure for preparing the substrate, in which the oleate is included in the initial emulsion instead of being added separately, an additional 15% of enzyme activity is observed. The emulsion can be stored at least one week.

The details of salvaging unused substrate from the lower layer of the partitioning step have not yet been

completely defined, but it is possible to remove nearly all the lipid with a silica gel (Unisil) column. The lower layer (200 ml) is evaporated to dryness with the aid of benzene, diluted with benzene to 50 ml, and passed through a column containing 2 g of Unisil. Additional lipid is removed with 50 ml of chloroform-methanol 97:3 and then the cerebroside is eluted with 50 ml of chloroform-methanol 94:6. The weight of this fraction was found to be 19 mg instead of the 2.3 mg calculated on the basis of  $^3\text{H}$  content, and TLC showed it to contain Tween and other lipids, well separated from the cerebroside.

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