New technique for enzymic hydrolysis of glycosphingolipids

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ABSTRACT A method is described for the study of glycosyl ceramide glycosyl hydrolases. Problems arising from the limited solubility of glycosyl ceramides in aqueous media were overcome by coating the substrate on a filter paper disc that had been treated with phosphatidyl choline. A comparison between the disc method and conventional dispersion of the substrate by detergents was made with two enzymes, galactosylgalactosylglucosyl ceramide galactosyl hydrolase (trihexosyl ceramide galactosyl hydrolase) from lysosomes of human and rat small intestine and human spleen, and D-galactose oxidase. In both cases enzymatic activity was greater with the paper disc method than it was with substrates dispersed by detergents. The galactose liberated by the glycosyl hydrolase was determined as the trimethylsilyl derivative of the free sugar by gas–liquid chromatography.

SUPPLEMENTARY KEY WORDS glycosyl ceramide galactosyl hydrolase · filter paper disc · lipid dispersion · D-galactose · gas–liquid chromatography · trimethylsilyl ether

In previous studies on the enzymatic degradation of glycosyl ceramides the substrates were labeled with tritium (1, 2) and the liberated sugar was determined by measuring the radioactivity in the aqueous layer after extraction of the lipid. Reactions with substrates that contain a terminal galactose unit have also been studied by the more specific but less sensitive method for determination of liberated galactose with D-galactose oxidase (3). The tracer technique is probably the most sensitive one available but the preparation of labeled substrate may be difficult and the system must be rigorously controlled to ensure that only one specific monosaccharide accounts for all the radioactivity measured.

Gas–liquid chromatography (GLC) has become a rapid and reliable method for the estimation of microgram quantities of monosaccharides (4, 5), the derivatives most frequently used being the trimethylsilyl (TMSi) ethers (6) and the alditol acetates (7). The TMSi derivatives give more complex chromatograms than the alditol acetates but the peaks are readily resolved and the ease of formation of the TMSi ethers gives them a considerable advantage when amounts of monosaccharides in the submicrogram range are to be determined. Further, the use of the TMSi derivatives in the GLC estimation of galactose and other monosaccharides liberated by specific glycosidases has already been developed for structural studies of glycoproteins (8), so that a similar technique might be appropriate with glycolipids.

The glycosyl ceramides have a very low solubility in water. In previous studies of their metabolism, detergents have been used extensively to enhance enzymatic activity (1, 2). Goldfine (9) has found that lipids can be quantitatively precipitated on discs of filter paper and has used this method in an assay for the products of lipid biosynthesis. The present report describes the use of a filter paper disc for glycolipid dispersion and its application, together with GLC, in the determination of trihexosyl ceramide galactosyl hydrolase and D-galactose oxidase activities.

METHODS

Purification of Trihexosyl Ceramide Galactosyl Hydrolase from Spleen or Small Intestine

Samples of human spleen or small intestine (30 g wet wt), obtained at autopsy, were washed with 0.15 M NaCl and freed from adipose and connective tissues by dissection. The tissue was then minced with scissors and homogen-

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Abbreviations: GLC, gas–liquid chromatography; TMSi, trimethylsilyl.

ized for about 10 min in 0.25 M sucrose (9 ml/g) containing 10^{-3} M EDTA, using an Eberbach homogenizer at maximum speed with intermittent periods of cooling to keep the mixture below 4° C. After filtration through cheesecloth to remove unhomogenized material the lysosomal fraction was prepared by centrifugation. The trihexosyl ceramide galactosyl hydrolase was purified from the lysosomes as described previously by Brady, Gal, Bradley, and Mårtensson (1).

This enzyme was obtained from samples of rat spleen or small intestine (10 g wet wt) by the same procedure, except that complete homogenization was obtained in about 5 min.

Assay for Trihexosyl Ceramide Galactosyl Hydrolase Activity

Purified phosphatidyl choline (containing not more than 4% phosphatidal choline), from bovine heart (20 μg in chloroform–methanol 2:1) was impregnated evenly on a filter paper disc (No. 1 Whatman, 8.2 mm diameter) and allowed to dry. The substrate, galactosylgalactosylglucosyl ceramide (30 μg in 30 μl of chloroform–methanol 2:1), was applied to the phosphatidyl choline-treated discs, which were then allowed to dry at room temperature. The disc was added to an incubation mixture (1.5 ml) that contained 0.5 ml of trihexosyl ceramide galactosyl hydrolase (protein concentration approximately 200 μg/ml in 0.01 M phosphate, pH 6.0) and 1 ml of 1% bovine serum albumin in 0.01 M potassium acetate (pH 5.0) containing 2.0 μg of mannitol as an internal standard for GLC. Incubations were also carried out with the ceramide substrate dispersed with Triton X-100 [a nonionic detergent, mixture of *p*, *t*-octyl poly(phenoxyethoxy) ethanols], as previously described (1). The effect of a smaller (6.0 mm diameter) paper disc on enzymatic activity was evaluated, and the relationship between enzyme concentration and amount of galactose liberated was studied over a suitable range.

3 ml of chloroform–methanol 2:1 was added to stop the reaction and precipitate protein and salts; after centrifugation, the upper layer was removed and evaporated to dryness. Since the residue contained significant amounts of inorganic material that tended to interfere with the silylation reaction, excess (250 μl) of a silylating reagent (either pyridine–hexamethyldisilazane–trimethylchlorosilane 5:1:1 or bis-trimethylsilylacetamide) was added. After 20 min, 1 ml of chloroform was added (10) and the salts were removed by extraction with water (3 × 0.5 ml), the aqueous phase being discarded at each step. After the mixture had been clarified by centrifugation, the chloroform layer was evaporated to dryness under a stream of nitrogen and additional silylating reagent (12 μl) was added. Samples (3 μl) were injected directly onto a column (2 m by 3.2 mm I.D.) of 3% methyl silicone polymer (OV-1) maintained isothermally at 160°C in a Hewlett–Packard Model F & M 402 gas chromatograph equipped with a flame ionization detector.

D-Galactose Oxidase Reaction with Trihexosyl Ceramide

A Galactostat kit (Worthington Biochemical Corp., Freehold, N.J.) was used as a source of enzyme; to increase the sensitivity of the assay, we added only 3 ml of 6% (v/v) methanol in water to the chromogen vial. The solution was transferred to the enzyme vial and the mixture was diluted to 5 ml with 0.01 M phosphate, pH 7.0. The galactosylgalactosylglucosyl ceramide substrate (30 μg) was either coated onto a paper disc or dispersed by detergent (Triton X-100 in phosphate, pH 7.0) and in each incubation, 0.4 ml of the enzyme–chromogen solution and 0.35 ml of buffer were used. We followed the course of oxidation of the terminal D-galactose residue by measuring the increase in absorbance at 420 nm.

Measurement of Protein Concentration

Biuret reagent (Sigma Chemical Co., St. Louis, Mo.) (0.15 ml) was added to the enzyme preparation (0.3 ml) and after vigorous shaking the optical density at 310 nm was measured. Bovine serum albumin (400 μg/ml) in the same buffer was used as the standard.

RESULTS

Human spleen and small intestine were obtained approximately 5–10 hr after death whereas the rat tissue was obtained directly after the rats had been killed. Trihexosyl ceramide galactosyl hydrolase prepared from rat tissue gave a single band on disc electrophoresis at pH 8.5 and had similar enzymatic activity, pH optimum, and substrate specificity to that previously reported by Brady *et al.* (1). Analogous preparations from human spleen or intestine had much lower specific activities (Table 1) than

TABLE 1 ACTIVITY OF TRIHEXOSYL CERAMIDE GALACTOSYL HYDROLASE PREPARATIONS WITH GALACTOSYLGALACTOSYLGLUCOSYL CERAMIDE AS SUBSTRATE

Source of Enzyme	Substrate Dispersed with a Detergent	Substrate on Phosphatidyl Choline-Treated Disc
	mμmoles galactose/mg protein per hr	
Human small intestine	5.0	8.7
Human spleen	4.7	9.6
Rat small intestine	207	315
Rat spleen	—	95
Bovine liver (crude β-galactosidase preparation)	2.1	3.9

The above results are the mean of three determinations. Liberated galactose was estimated by GLC (see Fig. 2).

those from rat tissue; this we attribute to the unavoidable time lapse between death and purification of the enzyme from tissues.

The use of the phosphatidyl choline-treated disc gave a higher activity for the trihexosyl ceramide galactosyl hydrolase (Fig. 1) than in the standard method in which the substrate was dispersed with a detergent. The addition of detergents such as sodium cholate or Triton X-100 to the disc incubations did not increase the activity of the enzyme preparations. Decreasing the surface area of the disc from 1.03 to 0.56 cm^2 approximately halved the enzyme activity and the larger size was optimal for the 1.5 ml system. Enzymatic hydrolysis of the trihexosyl ceramide using the disc technique showed the usual direct relationship between enzyme concentration and amount of galactose liberated (17 mμmoles of D-galactose liberated per 0.1 mg of protein per 4 hr, in the range 0.03–0.10 mg of protein in the 1.5 ml system).

Replacement of the phosphatidyl choline on the paper discs with sphingomyelin or with a crude polar lipid fraction did not result in any diminution of the enzymatic activity of the glycosyl hydrolase fractions, but total omission of the polar lipid resulted in a 50% loss of activity. This suggests that the polar lipid might be important in the spacial orientation of the oligosaccharide units of the substrate.

Analysis of liberated galactose by GLC of the TMSi derivative (Fig. 2) gave satisfactory quantitative results down to 0.2 μg (1 mμmole), which was approximately 10 times the sensitivity of the D-galactose oxidase method. The TMSi derivative of D-mannitol, which gives a single peak on GLC, was used as the internal standard and the detector response of TMSi galactose relative to that of

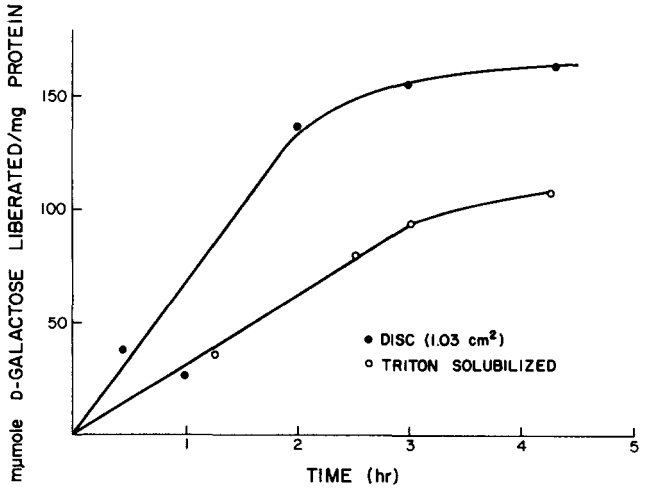

FIG. 1. Comparison of the rate of liberation of galactose from trihexosyl ceramide by rat intestinal trihexosyl ceramide galactosyl hydrolase with the filter paper disc and detergent dispersion techniques. Liberated galactose was estimated by GLC (see Fig. 2). Each point is the mean of three determinations.

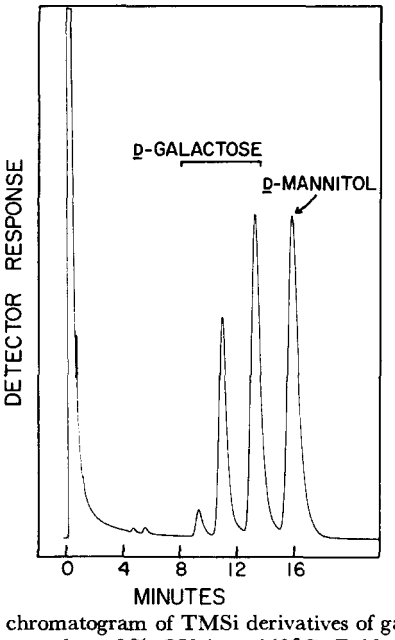

FIG. 2. Gas chromatogram of TMSi derivatives of galactose and mannitol, separated on 3% OV-1 at 160°C. Evidence was obtained by combined GLC–mass spectrometry that the two major peaks of galactose corresponded to pyranose anomers while the small peak with the earliest retention time was a furanose form.

TMSi mannitol was determined as described previously (4, 5). A factor of 1.25 was used to convert the ratio of the peak areas into the amount of galactose liberated. No unequal losses of galactose and mannitol derivatives occurred during the water–chloroform partitioning of the TMSi ethers, but further silylating reagent was added after this step as an extra precaution.

A comparison of the disc and conventional dispersion techniques was also made for the oxidation of the terminal D-galactose residue of trihexosyl ceramide with D-galactose oxidase (Fig. 3). The activity of this enzyme was also greater when the lipid was dispersed on a paper disc than it was with standard dispersion with detergents.

DISCUSSION

Two of the major problems in the study of lipid glycosyl hydrolases lie in the dispersion of the water-insoluble substrates and the detection of submicrogram amounts of liberated monosaccharides. It was found that by coating the glycolipid substrate on a phosphatidyl choline-impregnated paper disc, the use of detergents in the incubation mixture could be avoided. This can be advantageous since some detergents may contain enzyme deactivating agents and the detergents themselves may interfere with subsequent chromatographic analysis. The use of GLC to determine the amount of liberated galactose was simple, reliable, and rapid, and excellent quantitative results were obtained provided the solvents were rigorously purified and inorganic material was removed as described.

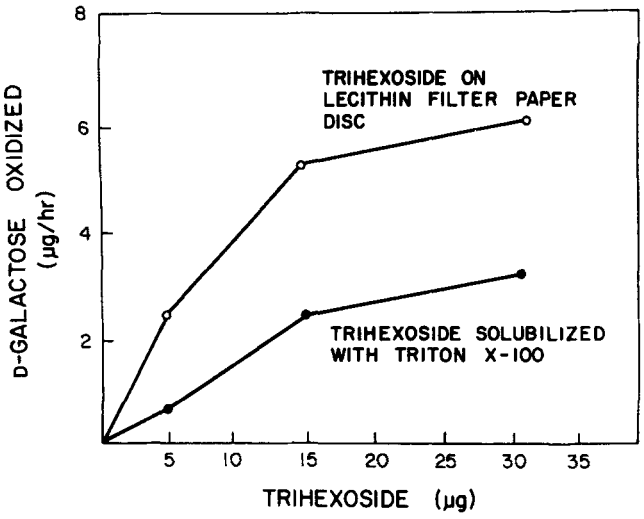

FIG. 3. Comparison of the rates of oxidation of terminal D-galactose unit of trihexosyl ceramide by D-galactose oxidase with the filter paper disc and detergent dispersion techniques.

The activity of D-galactose oxidase with a galactolipid substrate was expected since the enzyme has been shown (Dawson, G., and J. R. Clamp, unpublished findings) to oxidize the terminal D-galactose residue of lactose, stachyose, lacto-*N*-tetraose, and lacto-*N*-fucopentaose II but to be inactive toward disubstituted D-galactose residues. We have shown that D-galactose oxidase is active in the hydrolysis of galactosylgalactosylglucosyl ceramide, lactosyl ceramide, and digalactosyl ceramide and that the activity is enhanced by the use of the paper disc method for dispersion of the lipids. The value of the D-galactose oxidase method for the estimation of enzymatically liberated galactose is lessened by this lack of substrate specificity, and great care must be taken to remove the glycolipid substrates when this assay is used.

In Fabry's disease the trihexosyl ceramide galactosyl hydrolase studied here has been shown to be absent (11). There are also other inborn errors of glycolipid metabolism that involve the absence of specific glycosyl hydrolases. This GLC method combined with the filter paper disc technique may provide a more rapid and reliable method for in vitro metabolic studies of such defects.

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