PREPARATION AND CHARACTERISATION OF TRITIATED DERIVATES OF GALACTOSYLCERAMIDE AND GLUCOSYLCERAMIDE

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Revised November 1, 1976

SUMMARY:

Radiolabelled glucosylceramide and galactosylceramide have been prepared by the catalytic tritiation of a mixture of the corresponding unlabelled sphingolipids, and purified by preparative thin-layer chromatography. The localisation of the label was determined by degrading the individual monohexosyl ceramides with aqueous methanolic hydrochloric acid and measuring the radioactivity content of the liberated fragments. Under the particular experimental conditions described, more than 98% of incorporated tritium was found in the ceramide portion of each sphingolipid, while the greater part (>80%) of this radioactivity was detected in the long-chain fatty acid moieties.

The method offers a convenient means of preparing labelled substrates suitable foruse in metabolic studies. The data suggest that to reduce costs, mixtures rather than individual sphingolipids, can be tritiated.

Key Words: Tritium - galactosylceramide - glucosylceramide - hydrolysis.

0362-4803/78/0114-0017$01.00/0

The biochemical diagnosis of a number of the lipid storage disorders depends primarily on the demonstration in either white cells or fibroblasts of a deficiency in activity of any one of a number of lysosomal sphingolipid hydrolases. Non-specific fluorogenic substrates offer a convenient and sensitive means of assaying for enzyme activity but, in some instances, the natural sphingolipid substrates must be employed due to the lack of specificity of the fluorogenic substrates [1, 2].

As the radioactive sphingolipids are not, as yet, available commercially, they must be prepared in the laboratory. Although some workers have succeeded in reductively tritiating the ceramide portion of the sphingolipid molecule [3, 4, 5], more frequently two other techniques have been employed. The first of these relies on the introduction of a tritium atom into the hydrophilic (carbohydrate) moiety of oxidation of a terminal galactose by galactose oxidase and subsequent reduction with tritiated sodium borohydride [6, 7]. Alternatively a can be introduced into hydrophilic residue by organic synthesis [8, 9, 10]. The former method is only applicable to those sphingolipids which possess a terminal galactose or N-acetyl-galactosamine group. Furthermore, the specific activity of the final product is dependent on the activity of the galactose oxidase, an enzyme which can vary considerably in activity depending on its source. The latter method, on the other hand, is time consuming, too complicated for use in most clinical chemistry laboratories and has generally been applied to the synthesis of those sphingolipids posessing a single hydrophilic moiety, e.g., glucosylceramide.

The catalytic tritiation of native sphingolipids, obtained either commercially or isolated from natural sources in the laboratory, offers an excellent, simple, but relatively expensive alternative to both methods [5, 11, 12, 13]. Although some sphingolipids have been successfully tritiated by this technique, information concerning the tritiation of the monohexosyl ceramides, glucosyl and galactosyl ceramide is scanty,

and the products have not always been adequately characterised [5]. The preparation of two such derivatives, and the chemical and radiochemical characterisation of both compounds is described in detail. Because of the expense entailed in the tritiation procedure, both lipids were mixed prior to tritiation.

MATERIALS:

Galactosylceramide, from bovine brain, was purchased from Sigma, St. Louis, U.S.A. Glucosylceramide was isolated from freeze-dried Gaucher spleen as described below.

METHODS:

Isolation of glucosylceramide from spleen.

Glucosylceramide was isolated from a Gaucher spleen by a combination of column and preparative thin-layer chromatography techniques [14]. The identity of the isolated lipid was confirmed by chromatography on silica gel G-borate plates [15] and by detection, by gas-liquid chromatography, of methyl glucoside released after methoanolysis with 0.75 N hydrogen chloride in methanol for 22hr at 80° [14]. Assays of isolated glucosylceramide were based on a hexose determination [16] (assuming a molecular weight of approximately 780).

Tritiation and purification of lipids.

Tritiation:

The tritiation procedure, described in detail below was carried out at the Radiochemical Centre, Amersham, U.K.

A mixture of 60mg galactosylceramide and 6mg glucosylceramide was dissolved in 2.5ml chloroform-methanol (2:1, v/v) and 0.1ml pyridine,

then stirred in the presence of tritium gas (1 atmosphere, 10Ci) and 10% palladium on charcoal catalyst (10mg) for 4 hours. The mixture was filtered through a solvent resistant Millipore filter and the filtrate was evaporated to dryness in a rotary evaporator at 40^{o}. Labile tritium was removed by repeated evaporation of a methanolic solution of the lipid residue. The crude product was taken up in 25ml of methanol. The yield of crude products was 13 m Ci.

Purification:

Despite the fact that relatively pure lipids were subjected to the tritiation procedure, a thin-layer chromatographic examination of the final products revealed the presence of not only the parent sphingolipid, but also of other compounds which migrated more rapidly than the latter in the solvent systems used. This suggested that considerable destruction of the lipids had taken place. To remove these contaminants, preparative thin-layer chromatography of each compound was carried out. Because of the gradual breakdown of the purified high specific activity lipids on storage, presumably due to radioautolysis, aliquots of the crude sphingolipid solutions were purified as required.

The two isomeric glycolipid species were difficult to resolve by chromatographic procedures on normal silica gel plates but impregnation of the latter with borate greatly facilitates the separation of one glycolipid species from the other [15]. Aliquots of the crude sphingolipid solution (0.5 mCi) were spotted as 10cm bands, under nitrogen, onto washed plates of silica gel H borate. After applying glucosyl- and galactosylceramide markers on the edges of the plate, chromatograms were developed in chloroform-methanol-water (95:6:6 or 65:25:4, v/v). Lipid zones were located and eluted with 3 x 5ml amounts of chloroform-methanol (2:1). The eluted lipids were then rechromatographed on normal silica gel H plates in the same solvent system. After evaporation to dryness in a rotary evaporator at 40^{o} the residue was dissolved in 10ml chloroform-methanol (2:1, v/v). Specific activity deter-

minations were based on the hexose content of the eluates [14]. Approximately 25% of the total radioactivity applied to the plate was recovered in the eluted lipids.

Hydrolysis of radiolabelled sphingolipids.

Duplicate aliquots of the purified ^{3}H-sphingolipid solutions (3:11 μCi galactosylceramide and 3.6 μCi (glucosylceramide) were hydrolysed with methanol-hydrochloric acid reagent [17]. The products were partitioned in chloroform-methanol-water (8:4:3, v/v) and the resulting upper aqueous and the lower chloroform-rich layers were evaporated to dryness under nitrogen at 40°. The residues were dissolved in 2ml chloroform-methanol (2:1, v/v). Aliquots of the upper and lower phases were chromatographed on silica gel H plates in chloroform-methanol-2.5 mol/l ammonium hydroxide (40:15:1, v/v).

Characterisation of glycolipid carbohydrates after tritiation

To ascertain whether the tritiation had caused any change in lipid-bound carbohydrate the following experiment was performed: 2.5ml aliquots of the purified ^{3}H-glycolipid solutions were evaporated to dryness under nitrogen at 40°, and then hydrolysed with anhydrous methanolic hydrogen chloride [14]. The released carbohydrates were examined by gas-liquid chromatography [14].

The latter procedure was carried out using a Varian 2740 instrument. Carbohydrates were first converted to trimethylsilylether [14] derivates prior to chromatography on a 1.52m stainless steel column packed with 1.5% OV-101 on 100-120 mesh chromosorb G; oven temperature 160°; nitrogen flow rate - 20ml/min.

RESULTS AND DISCUSSIONS

The tritiated lipids migrated with the same chromatographic mobilities, in at least three different solvent systems, as the corresponding

non-tritiated compounds, but it appeared likely that there were considerable alterations in the relative proportions of the different molecular species [18] of each compound. The radiochemical and chemical purity of the purified lipids, assessed by thin-layer chromatography, was at least 96%. The specific activities of glucosylceramide and galactosylceramide were 4.3×10^6 and 0.93×10^6 dpm/nmole respectively. Based on gas chromatography data, the tritiation procedure did not appear to induce changes in glycolipid carbohydrates.

To ascertain the intramolecular localisation of the label, and to ascertain further the chemical identity of each compound, the sphingolipids were degraded by treatment with aqueous methanolic hydrochloric acid into their component long-chain fatty acids, bases and water-soluble residues. The released fragments were partitioned in a chloroform-methanol-water mixture (8:4:3, v/v). The bulk of the radioactivity (>80%) released after hydrolysis was present in the lower chloroform-rich layer (Table 1). A major component in this fraction migrated with the same chromatographic mobility as a methyloleate standard. On the other hand, the upper aqueous layers contained much lower amounts of radioactivity. A large proportion of the radioactivity in this fraction migrated in the sphingosine/dihydrosphingosine region of the chromatograms. Based on the TLC data, the ratio of tritium incorporated into long-chain bases to tritium introduced into the fatty acid moieties was 0.21 for both compounds.

It is thus apparent that a much greater proportion of tritium was incorporated into the fatty acid part of the sphingolipid molecule. The absence of significant amounts of radioactivity in areas of the chromatograms other than those occupied by methylesters of fatty acids and long-chain bases indicated that little label was incorporated into the hydrophilic portion of the sphingolipid molecules. This was confirmed for both lipids by estimating the amounts of radioactivity in

the methylglycoside and free sugar zones of thin-layer chromatograms (<2%).

Catalytic tritiation thus offers a very convenient means of preparing high specific activity monohexosylceramides. The process is relatively straightforward providing adequate facilities are available. Alternatively a tritiation service is offered by most of the radiochemical suppliers but is expensive if individual lipids are tritiated.

TABLE 1: Radioactivity content of fragments released from tritiated sphingolipids after acid hydrolysis.

Zones	Percent of Total Recovered Counts			
	Galactosylceramide		Glucosylceramide	
	Upper Layer	Lower Layer	Upper Layer	Lower Layer
Long-chain base	45.0	14.9	82.7	6.0
Fatty acid ester	47.0	82.0	8.5	90.5
Others	8.0	3.1	8.5	3.5
Total recovered radioactivity (dpm)	0.35×10^6	6.42×10^6	1.10×10^6	6.84×10^6

The value shown above represents the mean of single analysis carried out on duplicate hydrolysates. For other details see text.

The data presented here suggests that, to save expense, two, and probably more of these lipids can be tritiated together and then separated from each other by rapid thin-layer chromatographic techniques to produce pure compounds of specific activities sufficiently high for metabolic studies [19]. It is interesting to note that the lipid with the higher proportion of unsaturated linkages [20, 21] (i.e. galactosylceramide) did not attain the highest specific activity which suggests that other factors, such as the concentration of the individual lipids in a mixture, may play a role in determining the extent of tritium incorporation. Alternatively, the differences may be due to variations in the rate of tritiation and may not have been so marked if the reaction had been permitted to continue for a longer period. A major advantage of using catalytic tritiation stems from the fact that as all of the sphingolipids obtained from the biological sources contain at least some unsaturated linkages either in the long-chain base or fatty acid, they should, theoretically, be capable of incorporating tritium in the presence of a suitable catalyst. Such a general approach applies equally to the more complex lipids, e.g. gangliosides, which contain a number of carbohydrate groups.

A possible major limitation of lipids labelled in this manner is that with the removal of the unsaturated sites the molecule may alter physico-chemical characteristics in such a way that they are less satisfactory substrates for the natural catobolic enzymes. However, while there is little doubt that the addition of tritium does, for example, alter the chromatographic properties of some of the glycolipids[18] there is, as yet, little evidence that enzymes so far studied behave differently towards the saturated and unsaturated molecular species of sphingolipids [22]. The two labelled sphingolipids whose preparation has been described in this communication have been found to be highly satisfactory substrates for white cells and fibroblast sphingo-

lipid hydrolases [19].

The localisation of tritium within each sphingolipid species is worthy of comment. It is apparent, from the data shown in Table 1 that the bulk of tritium incorporated into the monohexosylceramides is located in the fatty acid moieties. Other workers have found a greater incorporation of label in the long-chain bases [13] but this may be associated with the shorter tritiation period and the slightly higher gas pressure used in this study. Alternatively the basic nature of the solvent used may exert a directing influence on tritium incorporation.

Catalytic tritiation of other glycolipid species, notably dihexosylceramide and trihexosylceramide, reportedly also leads to an incorporation of most of the label into the long-chain base rather than into the fatty acid part of the molecule [3, 4]. These differences may also be related to differences in carbohydrate chain length with its corresponding effect on lipid polarity. Alternatively slight differences either in long-chain base or fatty acid composition could have quite pronounced effects on both the extent of tritium incorporation and its intramolecular position.

ACKNOWLEDGEMENTS:

This study was carried out in the course of a project financed by the Research Trust of the Adelaide Children's Hospital. The authors would like to thank Kay Beckman for her excellent technical assistance and Professor J.N. Davidson, Institute of Neurology, London, for providing the Gaucher's spleen extract.

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