GLIAL AND NEURONAL LOCALIZATION OF CEREBROSIDE-METABOLIZING ENZYMES

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INTRODUCTION

Galactosyl ceramide is commonly described as being present in brain only in myelin, but it has been found in microsomes[5,6,11,15], isolated axonal fragments[8], and isolated neuronal preparations[22]. It is found in gray matter too and while this could be the effect of penetration by myelinated axons, the fatty acid distribution in gray matter galactocerebroside is different from that in white matter[19,20]. Since myelin appears to be made by oligodendroglial cells, it has been generally assumed that these cells are the primary or only site of galactocerebroside synthesis. The presence of this lipid in axons and neurons could be interpreted as an artifactual contamination during the preparation process or as the result of transfer from myelin while in the living brain.

A similar picture is seen with gangliosides, which are commonly described as being present only in neurons. Nevertheless, an appreciable concentration has been observed in white matter by most analysts and it is likely that some of the low values were the result of interference in the analytical techniques[12]. There is good evidence that gangliosides occur in myelin and that the distribution of ganglioside types is different from that in gray matter[14,25,26]. They occur also in isolated neurons and glia[7,18]. As with the galactocerebroside question, one could interpret these findings as indicating a transfer *in vivo* of selected gangliosides from axons to myelin and glia.

Gangliosides are formed from glucocerebroside[2], which ordinarily is found in brain in trace concentrations[27]. One might then expect to find that glucocerebroside is formed primarily in the neurons.

The recent development[4] of sensitive methods for the assay of the hexosyltransferases which make the two hexosyl ceramides, and of methods for isolating quite pure brain cell preparations has made it possible to examine the above questions in a direct manner. We have in addition assayed the cell preparations for the galactosidase which cleaves galactocerebroside.

The following abbreviations and nomenclature will be used in this paper: UDPGlc, uridine diphospho glucose; UDPGal, uridine diphospho galactose; HFA, 2-hydroxy fatty acids; NFA, nonhydroxy fatty acids. Ceramide is the fatty acyl amide of sphingosine, together with related long chain bases. Galactosyl ceramide is galactocerebroside ('cerebroside' in the older nomenclature); glucosyl ceramide is glucocerebroside.

MATERIALS AND METHODS

Materials

Glial cell preparations, supplied by Dr. Flangas, were made by a modification of a previously described method[9,10]. The cerebrums from 16 to 17-day-old rats, taken 3 at a time, were forced through two grids, 80 and 37 μm pore size. The tissue was then suspended in 4 vol. of SMCT medium containing enough Methocel (methyl cellulose, Dow Chemical Co., Midland, Mich.) to give the solution a viscosity of 30 cp. (The SMCT medium contains 0.25 *M* sucrose, 10 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, and 50 m*M* Tris, pH 7.4.) The suspension was finely dispersed by 5–10 passes through the orifice of a plastic 35 ml Luer syringe, then diluted to 4% tissue with SMCT, yielding a viscosity of 10 cp. This suspension was passed through a microsyringe equipped with a 400 mesh grid (37 μm pores) and added to a Ti 14 zonal centrifuge rotor (Beckman Instruments) while the rotor was spinning at 3500 rev./min.

The rotor was previously filled with 400 ml of a gradient containing 20–55% sucrose, 60 ml of 55% sucrose, and 180 ml of potassium citrate, density 1.49. All the sucrose solutions contained the same salts as in SMCT. The sample was added through the center of the rotor and overlaid with 60 ml SMCT. Separation of the cells was accomplished by centrifugation for 50 min at 40,000 rev./min and unloading, by pumping potassium citrate to the periphery of the rotor. The rotor effluent was collected in 20 ml fractions, each of which was sedimented at 100,000 $\times$ *g* for 30 min after dilution with 0.25 *M* sucrose. A portion of each pellet was characterized by staining[9] and microscopy, then the remainder of the pellets were pooled by resuspension in water. This procedure yielded 4 major fractions: A, small cells (oligodendroglia with some microglia), from 27 to 30% sucrose; B, larger cells (oligodendroglia), from 30 to 33% sucrose; C, astroglia and capillary elements, from 34 to 47% sucrose; D, neurons, from 55% sucrose.

For comparison, a portion of the tissue homogenate was diluted with water, like the isolated cells. The suspended cells and homogenate were stored at —70°C about 23 days, then thawed and centrifuged at 100,000 $\times$ *g* for 30 min. The pellets were lyophilized and promptly assayed for enzyme activity. The yields of dry powder were: A, 1.92; B, 10.42; C, 1.62; and D, 0.56 mg/rat. This procedure results in loss of cytosol and some material released by osmotic shock but was shown with whole brain homogenates to give good recovery of enzyme activity.

Another set of neurons, E, was prepared in Dr. Sellinger's laboratory according to his previously described procedure[23]. The pelleted cells, from the cortices of 24 rats,

15 days old, were suspended in 10 ml of water, stored in the freezer overnight, and sedimented as above. This pellet was promptly lyophilized, yielding 0.54 mg/rat. A portion of the original tissue homogenate was treated similarly for comparison.

Enzyme assay procedures

Galactocerebroside galactosidase was assayed with galactose labeled substrate, the released galactose being measured by scintillation counting[1,21]. The galactosyltransferase which makes galactocerebroside was evaluated with HFA ceramide and labeled UDPGal[4,17]. The washed lipid extract from the incubation mixture was counted directly or purified by thin-layer chromatography on borate–silica gel plates, and the radioactive galactocerebroside band counted[17]. Glucosyltransferase was evaluated similarly, but with NFA ceramide and labeled UDPGlc[4]. Details of incubations are given in the tables. Duplicate or triplicate incubations were carried out.

RESULTS

Galactocerebroside galactosidase

It appears that all brain cells contain this hydrolase, at fairly similar levels of activity (Table I).

TABLE I

GALACTOCEREBROSIDE GALACTOSIDASE OF GLIAL AND NEURONAL CELLS

The values shown are nmoles of cerebroside hydrolyzed in a 3 h incubation at 37°C. The incubation tubes contained 2 mg of dry cellular material, 0.1 mg of labeled stearoyl psychosine, 1 mg Tween 20, 0.5 mg Myrj 59, 2 mg Na taurocholate, 0.5 mg Tris oleate, and 0.1 mmole citrate pH 4.5 in 1 ml.

Cell preparation	*Cerebrosidase activity*
A. Oligodendroglia + microglia	6.70
B. Oligodendroglia	6.36
C. Astroglia	5.73
D. Neurons	—*
Total cerebrum	5.99
E. Neurons	6.06
Total cerebrum	4.13

* Available sample insufficient for assay.

Ceramide galactosyltransferase

The enzyme which synthesizes galactocerebroside is evidently present in all the cells studied (Table II, column 3). Comparisons of specific activities cannot readily be made because of the incomplete linearity with respect to weight of dried cell prep-

TABLE II

ABILITY TO SYNTHESIZE GALACTO- AND GLUCOCEREBROSIDES IN BRAIN CELLS

The galactosyltransferase incubation tubes contained dry cellular material which was homogenized in benzene, mixed with a benzene solution of 0.2 mg HFA ceramide, then dried with a stream of nitrogen. To this was added 0.2 ml of a solution containing 0.1 *M* Tris pH 7.4, 15 m*M* $MnCl_2$, 2 m*M* EDTA, 2 m*M* dithiothreitol, 5 m*M* nicotinamide, and 0.16 m*M* [^{14}C]-UDPGal. The cells and lipid were suspended with the aid of an ultrasonic cleaning bath, incubated 90 min at 37°C, and the radioactive cerebroside purified by solvent partitioning. The glucosyltransferase was assayed similarly, but with replacement of the two substrates by NFA ceramide and UDPGlc, and the addition of 2 m*M* ATP.

Cells assayed	*Tissue weight (mg)*	*Amount of cerebroside formed*	
		Galactocerebroside (nmoles)	*Glucocerebroside (nmoles)*
A. Oligodendroglia + microglia	1	1.01	n.d.*
B. Oligodendroglia	0.5	0.51	n.d.
	1	1.01	n.d.
	2	1.91	n.d.
C. Astroglia	1	0.54	n.d.
D. Neurons	0.5	0.33	n.d.
Whole homogenate	0.5	0.93	0.77
	1	2.61	2.01
	2	3.90	4.11
E. Neurons	0.5	0.97	2.98
	1	1.40	6.17
Whole homogenate	1	2.63	3.18

* Not detectable.

arations. In general it appears that the whole homogenate exhibited a higher specific activity than any cell preparation, and that the neuronal preparations had a lower specific activity than glial cells.

To eliminate the possibility that the neurons contained an active epimerase under our conditions, thus converting labeled UDPGal to UDPGlc, we examined the radioactive product by borate thin-layer chromatography and radioautography. The product was found to be galactosyl ceramide, not glucosyl ceramide.

Ceramide glucosyltransferase

The distribution of the enzyme which makes glucocerebroside is much more specific: it appears to be in the E neuronal cell preparations only (Table II, column 4). In the case of these neurons the specific activity is about double that in the whole homogenate. Here, the ratio of glucosyltransferase to galactosyltransferase is 4.4 in the neurons and 1.2 in the homogenate (compared at the 1 mg level).

The cerebroside formed by the E neurons was shown to be of the glucosyl type, rather than the galactosyl type, again confirming the absence of epimerase. Slight

TABLE III

EFFECT OF MIXING CELL PREPARATIONS ON GLUCOCEREBROSIDE SYNTHESIS

The assay conditions were similar to those in Table II, except that the UDPGlc concentration was 0.08 m*M* and the incubation was for 120 min. The lipids obtained were not chromatographed to remove a polar impurity, which explains the small amount of activity found with glial cells. The numbers in parentheses are the activities expected (the sum of the separate activities with 1 mg of each tissue).

Tissue assayed	*Tissue weight (mg)*	*Glucocerebroside formed (nmoles)*
Whole brain	1	0.71
Whole brain	2	1.70
Whole brain + B glial cells	1 + 1	0.83 (0.74)
B glial cells	1	0.03
E neurons	1	0.38
E neurons + whole brain	1 + 1	1.16 (1.09)

radioactivity seen in the lipids obtained by incubating cells B and C was found to remain at the origin of the thin-layer plates.

We checked the possibility that some toxic material introduced into the glia by the isolation procedure prevented them from making glucocerebroside. This was done by mixing the B cells with lyophilized whole brain (not water-washed) and assaying for glucosyltransferase activity (Table III). The glial cells did not inhibit the ability of whole brain to make glucocerebroside nor did the E neurons, examined by way of comparison.

The above results with the 3 enzymes were obtained in several runs, but the actual activities differed due to differences in assay technique.

DISCUSSION

A finding that an enzyme is present in one cell type but not in another must be tempered by the appreciation that atypical cells may have been isolated, or that the process of isolation may have caused deterioration of the enzyme. It is certainly evident that peripheral cell processes are lost during isolation, and the possibility must then be considered that the subcellular distribution of the enzyme is different for the different cell types. Bearing these possible complications in mind, we tentatively conclude that ceramide glucosyltransferase is a marker enzyme for neurons. The gangliosides that are found in non-neuronal cells and membranes are then presumably the result of transfer from neurons and axons of gangliosides or some precursor of gangliosides. This type of transfer has been demonstrated for several lipids between microsomes and mitochondria *in vitro*[28] and the exchange of lipids between circulating red cells and plasma has long been known.

The finding that the enzymes for making and cleaving galactocerebroside are present in neurons, as well as glia, at somewhat similar activity levels, raises the question as to why so little galactocerebroside is found in neurons and gray matter.

It is very likely that neurons make NFA ceramide of the stearate type, since this is the precursor of gangliosides and sphingomyelin. However, this type of ceramide is a very poor precursor of NFA cerebroside *in vitro*, the HFA ceramides acting as far better galactose acceptors[16,17]. It seems probable, therefore, that neurons make HFA ceramide quite slowly, if at all, and that the galactosidase acting on neuronal cerebroside destroys nearly all that is formed. It is also possible that the cerebroside formed, for lack of a binding protein in neurons (the proteolipid protein of myelin?), is readily attacked by the hydrolase. If neurons do make some HFA ceramide, one might expect to find HFA gangliosides since the glucosyltransferase acts quite readily *in vitro* to form HFA glucocerebroside[4,24]. However, HFA gangliosides do not seem to occur in brain[13].

The finding of galactosyltransferase in neurons is consistent with the observation that mice bearing the nonmyelinating mutation 'msd' contain a marked — but incomplete — deficiency of this transferase[3]. It is likely that the transferase in the glia is completely or nearly inactive or absent, and that the activity detected in *whole* brain is simply a measure of the transferase present in the neurons.

SUMMARY

Glial and neuronal cell preparations were made from young rat cerebrum and assayed for 3 enzymes involved in sphingolipid metabolism. A galactosyltransferase which makes galactocerebroside, a primary component of myelin, was found in all cell types examined, at fairly similar levels of activity. The same distribution of activities was found for the β-galactosidase which hydrolyzes galactocerebroside. It is suggested that the very low levels of galactocerebroside found in neurons are the result of an inability of neurons to form the lipoidal cerebroside precursor, hydroxy ceramide, or a cerebroside-binding protein.

The glucosyltransferase which makes glucocerebroside, an intermediate in ganglioside biosynthesis, was found only in neurons. This may be a new marker enzyme for neurons, in contrast to other brain cells. Since gangliosides are found in non-neuronal membranes, it appears likely that they (or some intermediate in biosynthesis) are transferred from neurons.

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