The Interrelations between High- and Low-Molecular-Weight Forms of Normal and Mutant (Krabbe-Disease) Galactocerebrosidase

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Galactocerebrosidase (β -D-galactosyl-*N*-acylsphingosine galactohydrolase; EC 3.2.1.46) activity of brain and liver preparations from normal individuals and patients with Krabbe disease (globoid-cell leukodystrophy) have been separated by gel filtration into four different molecular-weight forms. The apparent mol.wts. were $760\,000 \pm 34\,000$ and $121\,000 \pm 10\,000$ for the high- and low-molecular-weight forms (peaks I and IV respectively) and $499\,000 \pm 22\,000$ (mean $\pm$ s.d.) and $256\,000 \pm 12\,000$ for the intermediate forms (peaks II and III respectively). On examination by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the high- and low-molecular-weight forms revealed a single protein band with a similar mobility corresponding to a mol.wt. of about 125 000. Antigenic identity was demonstrated between the various molecular-weight forms of the normal and the mutant galactocerebrosidases by using antisera against either the high- or the low-molecular-weight enzymes. The high-molecular-weight form of galactocerebrosidase was found to possess higher specific activity toward natural substrates when compared with the low-molecular-weight form. It is suggested that the high-molecular-weight enzyme is the active form *in vivo* and an aggregation process that proceeds from a monomer (mol.wt. approx. 125 000) to a dimer (mol.wt. approx. 250 000) and from the dimer to either a tetramer (mol.wt. approx. 500 000) or a hexamer (mol.wt. approx. 750 000) takes place in normal as well as in Krabbe-disease tissues.

Galactocerebrosidase (β -D-galactosyl-*N*-acylsphingosine galactohydrolase; EC 3.2.1.46) catalyses the hydrolysis of the terminal β -galactoside linkage of galactosylceramide (galactocerebroside) (Hajra *et al.*, 1966). Deficiency of this enzymic activity has been documented to be the basic defect in Krabbe disease (globoid cell leukodystrophy) (Suzuki & Suzuki, 1970). Deficiencies in β -galactosidase activities toward galactosylsphingosine (Miyatake & Suzuki, 1972), monogalactosyldiacylglycerol (Wenger *et al.*, 1973) and lactosylceramide when assayed in the presence of pure sodium taurocholate (lactosylceramidase I) (Wenger *et al.*, 1974; Tanaka & Suzuki, 1975) have also been observed in tissues of patients with Krabbe disease. Galactocerebrosidase appears to possess hydrolase activity toward all four of these galactolipids. The synthetic 4-methylumbelliferyl β -galactoside was also found to be a substrate for this enzyme (Tanaka & Suzuki, 1977; Ben-Yoseph *et al.*, 1977a),

Abbreviation used: G_{M1}, ganglioside, galactosyl-*N*-acetylgalactosaminyl-(*N*-acetylneuraminy)galactosyl-glucosylceramide.

although most of the liver enzymic activity toward the artificial β -galactoside was attributed to G_{M1}, ganglioside β -galactosidase (O'Brien, 1975), a distinct enzyme that is deficient in another genetic disorder of galactolipid metabolism, G_{M1}, gangliosidosis. The latter enzyme also possesses activity toward lactosylceramide when assayed in the presence of taurodeoxycholate, glycodeoxycholate or taurochenodeoxycholate (lactosylceramidase II) (Tanaka & Suzuki, 1975; Wenger *et al.*, 1975). Activities of artificial aryl β -galactosidase and lactosylceramidase II were also found in a third β -galactosidase enzyme, neutral β -galactosidase (Ho & O'Brien, 1970; Ben-Yoseph *et al.*, 1977a). Although some overlap exists between the specificities of the three distinct β -galactosidases, the hydrolysis of galactosylceramide appears to be an exclusive catalytic property of the galactocerebrosidase enzyme (Tanaka & Suzuki, 1977).

G_{M1}, ganglioside β -galactosidase of human liver was shown to occur as two molecular-weight forms that share antigenic determinants and many biochemical properties (Ho *et al.*, 1973; Norden *et al.*,

1974). The major fraction had a mol.wt. of about 70000 and the minor fraction was of mol.wt. 600000–700000. A possible dimer (mol.wt. 160000) of the low-molecular-weight form of human liver G_M , ganglioside β -galactosidase was also resolved by gel filtration, and interconversion between the high- and low-molecular-weight forms was demonstrated (Cheetham & Dance, 1976). In a recent study (Lo *et al.*, 1979) a placental enzyme that was found to be devoid of galactocerebrosidase activity, but possessed lactosylceramidase II and asialo G_M , ganglioside β -galactosidase activities, has been separated by gel filtration into two forms with mol.wts. 420000–480000 and 220000. On guanidine hydrochloride/sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the low-molecular-weight form dissociated into a single protein band corresponding to a mol.wt. of 77000 and the high-molecular-weight form into three bands corresponding to mol.wts. of 77000, 31000 and 22000. The 77000-mol.wt. subunits of both forms were found to be antigenically identical with each other. Isoelectric focusing of a crude liver preparation resolved two peaks of galactocerebrosidase activity, a minor peak at pH 4.6 and a major one at pH 4.9–5.1 (Suzuki & Suzuki, 1974). Isoelectric focusing of control fibroblast extract revealed a similar pattern of galactocerebrosidase activity, but when fibroblast extract from a patient with Krabbe disease was focused, a small band was found at pH 5.0 and the minor peak of pH 4.6 was missing (Besley & Bain, 1976).

We have previously observed that galactocerebrosidase activity of human placenta (Ben-Yoseph *et al.*, 1978) and liver (Ben-Yoseph *et al.*, 1979a) exists in several molecular-weight forms. The major high-molecular-weight form of the enzyme has been purified and no attempt was made to purify the minor forms. The present study was undertaken in order to define the interrelations of the various molecular-weight forms of galactocerebrosidase and to determine if a defective aggregation of this enzyme is involved in Krabbe disease.

Materials and Methods

Sephacrose 4B was from Pharmacia (Piscataway, NJ, U.S.A.), Cutscum was from Fisher (Pittsburgh, PA, U.S.A.) and silica gel G thin-layer plates were from Brinkman Instruments (Westbury, NJ, U.S.A.). Bio-Gel A 1.5m, Bio-Gel P-150, Bio-Beads SM-2, DEAE-cellulose (Cellex D), agarose, myosin, catalase, β -galactosidase, phosphorylase *b*, bovine γ -globulin, bovine serum albumin and ovalbumin were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Human plasma α_2 -macroglobulin and human erythrocyte carbonic anhydrase B were purified as previously described (Ben-Yoseph *et al.*,

1971, 1979b). Cyanogen bromide, acrylamide, *NN'*-methylenebisacrylamide, *NNN'*-tetramethylenediamine, sodium dodecyl sulphate, $(NH_4)_2SO_4$ and Coomassie Brilliant Blue G-250 were from Eastman (Rochester, NY, U.S.A.). Concanavalin A, ϵ -aminohexanoic-*N*-benzyloxy-carbonyl acid, Blue Dextran and 4-methylumbelliferyl β -D-galactopyranoside were from Sigma (St. Louis, MO, U.S.A.). Dihydrosphingosine, galactosyl-*N*-stearyl dihydrosphingosine and γ -globulin fraction of goat antiserum to rabbit γ -globulin were from Miles Laboratories (Elkhart, IN, U.S.A.). Oleic acid and sodium taurocholate were from Calbiochem (LaJolla, CA, U.S.A.). Complete Freund adjuvant was from Difco (Detroit, MI, U.S.A.), galactose oxidase was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.) and NaB^3H_4 (sp. radioactivity 285 Ci/mol) was from New England Nuclear (Boston, MA, U.S.A.).

Enzyme assays

Galactosylceramide was labelled with 3H at C-6 of the terminal galactose residue by the galactose oxidase/ NaB^3H_4 method (Radin *et al.*, 1969). The radiolabelled product was purified by silica gel t.l.c. in a solvent system of chloroform/methanol/water (35:20:3, by vol.) and diluted with unlabelled galactosylceramide to give a specific radioactivity of 400–600 c.p.m./nmol. Galactocerebrosidase activity was assayed at pH 4.3 in the presence of oleic acid and sodium taurocholate by the method of Wenger *et al.* (1974, 1975). 3H radioactivity was measured in a Tri-Carb liquid-scintillation spectrometer. Hydrolysis of 4-methylumbelliferyl β -D-galactoside was determined at pH 4.3 as previously described (Ben-Yoseph *et al.*, 1978) with an Aminco-Bowman spectrofluorometer with excitation at 365 nm and emission at 450 nm. Protein was assayed by the dye-binding method (Bradford, 1976) with bovine γ -globulin as a standard. Absorbance measurements were made in a Beckman double-beam spectrophotometer. Cutscum was removed before the protein assay by adsorption of the samples with SM-2 beads (Holloway, 1973).

Enzyme preparation

Liver and brain tissue specimens were obtained at autopsy from patients with Krabbe disease, patients with other lysosomal diseases and normal controls. The specimens were frozen immediately and kept at $-70^\circ C$ until used. Solubilization was carried out by homogenizing the 900–100000 g pellet (Svennerholm *et al.*, 1975) in 4 vol. of 0.1% Cutscum in 10 mM-sodium phosphate buffer, pH 6.0. The high-molecular-weight form of galactocerebrosidase was purified from normal liver by the procedure previously described for purification of the placental enzyme (Ben-Yoseph *et al.*, 1978). Fractions con-

taining the low-molecular-weight form of galactocerebrosidase obtained at the Bio-Gel A 1.5m gel filtration step were concentrated by negative-pressure ultrafiltration and the enzyme was further purified by gel filtration on a Bio-Gel P-150 column and hydrophobic chromatography on the Sepharose-bound *N*- ϵ -aminohexanoyldihydrosphingosine column. Dihydrosphingosine was coupled with ϵ -aminohexanoic acid in the same manner as described for the coupling of galactosylamine (Gordon *et al.*, 1972). This compound as well as Concanavalin A were immobilized on cyanogen bromide-activated Sepharose 4B (Axen *et al.*, 1967) yielding ligand concentrations of 0.16 and 0.82 mg/ml respectively. Galactocerebrosidase of normal brain tissue and of liver and brain tissues of patients with Krabbe disease and controls was partially purified by solubilization, Concanavalin A adsorption and DEAE-cellulose ion-exchange chromatography and separated into the various molecular-weight forms by Bio-Gel A 1.5m gel filtration.

Molecular-weight determination

Concentrated preparations of partially purified enzyme, containing up to 50mg of protein in a volume of 3 ml, were chromatographed on a Bio-Gel A 1.5m column (2cm $\times$ 110cm) in 0.14M-NaCl/10mM-sodium phosphate buffer (pH 7.0) at a flow rate of 28 ml/h. The gel-filtration column was calibrated with the following known molecular-weight markers: Blue Dextran, 2000000; human plasma α_2 -macroglobulin, 720000; bovine liver catalase, 240000; bovine γ -globulin, 150000; bovine serum albumin, 68000; ovalbumin, 45000. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was carried out on (0.7cm $\times$ 9cm) 4% gel discs of composition acrylamide/bisacrylamide (75:1, w/w) at pH 7.0 with a constant current of 8mA/gel (Weber & Osborn, 1969). The following known molecular-weight markers were used for calibration: myosin, 200000; β -galactosidase, 115000; phosphorylase *b*, 94000; bovine serum albumin, 68000; ovalbumin, 45000; carbonic anhydrase B, 30000. The gels were stained by Coomassie Brilliant Blue G-250 in methanol/water/acetic acid (5:5:1, by vol.).

Immunization

Rabbit antiserum to placenta galactocerebrosidase (Ben-Yoseph *et al.*, 1978) was utilized for immunoprecipitation (Ben-Yoseph *et al.*, 1979a) of the high- and low-molecular-weight forms of the enzyme purified from normal liver. The enzyme forms were precipitated at the optimal antigen/antibody ratio (equivalence zone). The washed immunoprecipitates, containing 0.2–0.5 mg of enzyme, were mixed with equal volumes of complete Freund's adjuvant and rabbits were immunized with

these preparations by intradermal injection in multiple sites. After a positive reaction on double-gel immunodiffusion (0.8% agarose gel in 0.14M-NaCl/10mM-sodium phosphate buffer, pH 7.0), blood was collected weekly from the marginal ear vein, the serum was separated and the γ -globulin fraction prepared (Levy & Sober, 1960) from the antiserum was kept frozen at -20°C until used.

Immunoassays

Immunoprecipitation was carried out as previously described (Ben-Yoseph *et al.*, 1979a) by adding increasing amounts of the enzyme form to a constant amount of the γ -globulin fraction of the antiserum. The ratio of antigen/antibody at the equivalence zone was calculated assuming absorption coefficients, $A_{1\text{cm}, 280}^{1\%}$, of 12.9 for galactocerebrosidase and 14.0 for γ -globulin. Quantification of the enzyme protein or the antigenically cross-reacting material was performed by single radial immunodiffusion assay as previously described (Ben-Yoseph *et al.*, 1977b). Purified enzyme forms were used for calibration and γ -globulin fraction of goat antiserum to rabbit γ -globulins was used as a second antibody for detection of the precipitin rings obtained with the first antibody.

Results

Gel filtration

The Bio-Gel A 1.5m elution patterns of partially purified galactocerebrosidase from liver preparations of a normal control and a patient with Krabbe disease are shown in Fig. 1. The enzymic activity was assayed with the galactosylceramide substrate and the enzyme protein or the antigenically cross-reacting material was quantified by the single radial immunodiffusion assay by using γ -globulin fraction of antiserum to the purified high-molecular-weight form of the normal liver enzyme. A similar pattern of cross-reacting material was obtained when antiserum to the low-molecular-weight form was used. Four forms of galactocerebrosidase were resolved from the normal as well as from the Krabbe-disease liver preparation, corresponding to apparent mol.wts. of 760000, 490000, 260000 and 122000 for peaks I, II, III and IV respectively (Fig. 2). Low residual activities were detected in the mutant enzyme forms, but the amounts of antigenically cross-reacting material were comparable with those of the normal control peaks. Decreased quantities of cross-reacting material were found in the minor peaks II, III and IV when freshly prepared crude homogenates of liver and brain from Krabbe-disease patients and controls were filtered through the Bio-Gel A 1.5m column. Refiltration of the enzyme preparations after 48–72h storage at 4°C revealed partial conversion of the major high-molecular-

Fig. 1. Bio-Gel A 1.5m gel filtration of galactocerebrosidase partially purified from liver specimens of a normal control (●, ○) and a patient with Krabbe disease (▲, △)

Galactocerebrosidase activity (●, ▲) was assayed using [^3H]galactosylceramide and antigenically cross-reacting material (○, △) was quantified by the single radial immunodiffusion assay employing γ -globulin fraction of rabbit antiserum to the purified high-molecular-weight galactocerebrosidase of normal human liver as the first antibody and γ -globulin fraction of goat antiserum to rabbit γ -globulins as the second antibody preparation.

Fig. 2. Molecular-weight determination of galactocerebrosidase peaks I-IV by Bio-Gel A 1.5m gel filtration. Known molecular-weight markers (■) were used for calibration. Abbreviations used: BGG, bovine γ -globulin; BSA, bovine serum albumin.

weight form (peak I) into the minor enzyme forms (peaks II, III and IV). Storage for longer periods of time resulted in a notable loss of enzymic activity. Incubation of the low-molecular-weight forms at different pH values and at various temperatures and ionic strengths could not demonstrate any aggregation process.

Electrophoresis

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the purified high-(peak I) and low-(peak IV) molecular-weight forms of the normal liver galactocerebrosidase revealed a single protein band for each (Fig. 3). These protein bands corresponded to a mol.wt. of about 125 000 (Fig. 4). Comparison of these results with the gel-filtration pattern of the same enzyme forms (Figs. 1 and 2) show no change in the molecular weight determined for peak IV (122 000 $\rightarrow$ 125 000), but a total conversion of peak I into a sixth of its original size (760 000 $\rightarrow$ 125 000). γ -Globulin fractions of the antisera against the purified high- and low-molecular-weight forms of the normal liver galactocerebrosidase were used for immunoprecipitation of the partially purified intermediate forms of normal liver (peak II and III) and the various mole-

Fig. 3. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of purified galactocerebrosidase peaks I and IV

Peak I of the Bio-Gel A 1.5m column was further purified by sphingosine chromatography and peak IV by Bio-Gel P-150 gel filtration and sphingosine chromatography.

Fig. 4. Molecular-weight determination of the subunit of purified galactocerebroside peaks I and IV by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Known molecular-weight monomeric markers (■) were used for calibration. Abbreviation used: BSA, bovine serum albumin.

cular-weight forms (peaks I–IV) of normal brain and of Krabbe-disease liver and brain. The washed immunoprecipitates were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and when compared with electrophoresed rabbit γ -globulin, an extra protein band was demonstrated, corresponding to a mol.wt. of 113 000–128 000. In this system, however, one cannot rule out the presence of more galactocerebroside-derived polypeptides coinciding with the γ -globulin bands.

Immunoprecipitation

Fig. 5 illustrates quantitative precipitin curves obtained with partially purified high- and low-molecular-weight forms of normal and Krabbe-disease liver galactocerebroside and the γ -globulin fraction of the homologous antiserum. The high-molecular-weight galactocerebroside of a patient with Krabbe disease and the normal control were precipitated by the antiserum to the purified high-molecular-weight form of normal liver and the antiserum to the purified low-molecular-weight form was used for immunoprecipitation of the normal and mutant low-molecular-weight enzymes. For each enzyme form, similar precipitin curves were found regardless of the enzyme source (normal or Krabbe liver). The precipitin curves of the high- and low-molecular-weight enzymes were quite different from each other, but almost an identical antigen/antibody ratio (w/w) was calculated at the equivalence zone. Using heterologous antisera for immunoprecipitation resulted in similar antigen/antibody ratios for the high- as well as the

Fig. 5. Quantitative immunoprecipitation of partially purified preparation of high- (●, ○) and low- (▲, △) molecular-weight galactocerebroside from liver specimens of a normal control (●, ▲) and a patient with Krabbe disease (○, △) by γ -globulin fractions of antisera to the purified high- and low-molecular weight forms of the normal liver enzyme.

Each form was precipitated by the homologous antiserum. Abbreviations used: Ag, antigen; Ab, antibody; Ag/Ab, ratio of antigen to antibody at maximal precipitation.

low-molecular-weight forms of the normal and the mutant enzymes. On examination by double-gel immunodiffusion, with either of the antisera, antigen identity was demonstrated between the various forms (peaks I–IV) from liver and brain of patients with Krabbe disease, patients with other lysosomal disorders and normal controls.

Enzymic activities

Partially purified high- and low-molecular-weight forms (peaks I and IV) of liver and brain galactocerebroside from a patient who died of Krabbe disease were compared with the respective molecular-weight forms purified to homogeneity from control liver (Fig. 3) or partially purified from control brain, regarding their enzymic activities toward natural (galactosylceramide) and artificial (4-methylumbelliferyl β -galactoside) substrates. The results obtained for brain normal and mutant galactocerebroside are summarized in Table 1. The residual activities determined in the Krabbe-disease galactocerebroside forms were 3–5% of control values with the natural substrate and 18–34% of control values with the artificial substrate. The artificial 4-methylumbelliferyl β -galactosidase specific activities of the high-molecular-

Table 1. *Specific β -galactosidase activities of high- and low-molecular-weight forms of galactocerebrosidase toward natural and artificial substrates*

Enzyme source	Molecular-weight form	Galactosylceramide β -galactosidase (nmol/h per mg)	4-Methylumbelliferyl β -galactosidase (nmol/h per mg)
Normal brain	High	1970	1308
	Low	320	1140
Krabbe-disease brain	High	92.5	241
	Low	10.9	390

weight forms were only slightly different from those of the low-molecular-weight forms. In contrast, the natural galactosylceramide β -galactosidase specific activities of the high-molecular-weight galactocerebrosidases of normal and Krabbe-disease brain were respectively six and eight times higher than the low-molecular-weight galactocerebrosidases. Higher specific activities toward the natural substrate, as opposed to the artificial substrate, were also observed in the high-molecular-weight form of the liver enzyme partially purified from Krabbe-disease patients as well as in the high-molecular-weight form purified to homogeneity from control (Fig. 3) when compared with the partially or highly (Fig. 3) purified, low-molecular-weight forms respectively. The ratio of natural to artificial galactocerebrosidase activity in the intermediate molecular-weight forms (peaks II and III) was found to resemble that of the low-molecular-weight enzyme.

Discussion

In the present study, four molecular-weight forms were shown to be resolved by gel filtration from partially purified galactocerebrosidases of liver and brain. Although some overlapping specificities were observed among galactocerebrosidase, G_M -ganglioside β -galactosidase and neutral β -galactosidase (Norden *et al.*, 1974; Tanaka & Suzuki, 1975, 1977; Ben-Yoseph *et al.*, 1977a), the galactocerebrosidase enzyme showed exclusive specificity to the galactocerebroside substrate. Moreover, no antigenic cross-reactivity could be demonstrated between the three β -galactosidase enzymes, and each of the anti-enzyme antisera was exclusively specific to its homologous β -galactosidase (Norden *et al.*, 1974; Ben-Yoseph *et al.*, 1977a,b, 1978). Thus, by using galactosylceramide for determination of the enzymic activity and by employing antigalactocerebrosidase antisera for quantification of the antigenically cross-reacting material we were able to measure specifically the galactocerebrosidase enzyme and it would be very unlikely that the minor low-molecular-weight forms represent other β -galactosidase enzymes.

Antigenic identity was demonstrated between the various molecular-weight forms of liver and brain galactocerebrosidase by double-gel immunodiffusion and quantitative immunoprecipitation, using antisera directed against either the high- or the low-molecular-weight forms. These findings indicate that a close relationship exists between the various molecular-weight forms of galactocerebrosidase and that the high- and intermediate-molecular-weight forms (peaks I, II and III) may be the aggregation forms of the low-molecular-weight enzyme (peak IV). The similar antigen/antibody ratios at the equivalence zone of the precipitin curves of the high- and low-molecular-weight forms (Fig. 5) support the idea that all enzyme forms bear identical antigenic determinants.

The purified preparations of liver galactocerebrosidase peaks I and IV revealed an identical single protein band of mol.wt. 125 000 on sodium dodecyl sulphate/polyacrylamide gel electrophoresis (Figs. 3 and 4), indicating that this 125 000-mol.wt. polypeptide is the monomeric form of the enzyme. Protein bands of the same size were also observed when immunoprecipitates of the intermediate molecular-weight forms of normal liver (peaks II and III) and those of the various enzyme forms of Krabbe-disease and control liver and brain were subjected to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. In the latter experiments, however, it cannot be ruled out that extra galactocerebrosidase-derived polypeptides coincide with the γ -globulin bands.

The molecular weights determined for galactocerebrosidase peaks I–IV were 760 000, 490 000, 260 000 and 122 000 respectively (Figs. 1 and 2). These results, along with the conversion of the high- and intermediate-molecular-weight forms into the low-molecular-weight enzyme on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, are consistent with the hypothesis that galactocerebrosidase peak IV is the monomer and peaks I, II and III are the hexamer, tetramer and dimer respectively. This is also in agreement with our previous observations for the placenta galactocerebrosidase (Ben-Yoseph *et al.*, 1978), which was found to have

an apparent mol.wt. of 700 000–800 000 by Bio-Gel A 1.5 m gel filtration and a mol.wt. of about 120 000 by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. The partial conversion on storage of the high-molecular-weight galactocerebrosidase hexamer into tetramer, dimer and monomer on one hand, and the absence of enzyme forms corresponding to pentamer or trimer on the other hand, led us to suggest that the aggregation process of this enzyme proceeds from monomer to dimer and from the dimer to either tetramer or hexamer.

The lower specific activity toward the natural substrate, galactosylceramide, that was found in the minor low-molecular-weight forms of the normal enzyme, when compared with the high-molecular-weight galactocerebrosidase, raised the possibility that the deficient enzymic activity in tissues of patients with Krabbe disease, is due to a defective aggregation process of the enzyme molecule. As shown by the present data, this is not the case, in comparison with normal galactocerebrosidase, the enzyme partially purified from liver of a patient with Krabbe disease demonstrated similar proportions between its molecular-weight forms (peaks I–IV) as judged from both the content of antigenically cross-reacting material and the residual galactosylceramide β -galactosidase activity (Fig. 1). The molecular weights determined for the various mutant enzyme forms were identical with those of the normal enzyme. The enzymic activities toward galactosylceramide and 4-methylumbelliferyl β -galactoside were markedly decreased in the high- and low-molecular-weight forms of Krabbe-disease brain galactocerebrosidase compared with the normal enzyme forms, though the natural activity was more decreased than the artificial one (Table 1). Similarly to the normal enzyme forms, the ratio of residual activity toward the natural substrate to that toward the artificial substrate was higher in the mutant enzyme hexamer than in the monomer form. Although some of the 4-methylumbelliferyl β -galactosidase activity of the enzyme monomer might have been contributed by contamination with other β -galactosidases, the deficiency observed in the mutant enzyme form indicates that this activity mainly represents the galactocerebrosidase enzyme. It appears that the high-molecular-weight galactocerebrosidase hexamer is the active form *in vivo*. In Krabbe disease, the catalytic defect is expressed in all the molecular-weight forms of the mutant enzyme, but the aggregation process leading from monomer to hexamer appears to be normal.

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