Monahan, J. J., Harris, S. E., Woo, S. L. C., Robberson, D. L., and O'Malley, B. W. (1976a), *Biochemistry 15,* 223-233.
Monahan, J. J., Harris, S. E., and O'Malley, B. W. (1976b), *J. Biol. Chem. 251,* 3738-3748.
Pearson, W. R., Davidson, E. H., and Britten, R. J. (1977), *Nucleic Acids Res. 4,* 1727-1735.
Ryffel, G. U. (1976), *Eur. J. Biochem. 62,* 417-423.
Ryffel, G. U., and McCarthy, B. J. (1975), *Biochemistry 14,* 1379-1385.
Sala-Trepat, J. M., Savage, M. J., and Bonner, J. (1978), *Biochim. Biophys. Acta* (in press).
Sevall, J. S., Cockburn, A., Savage, M., and Bonner, J. (1975), *Biochemistry 14,* 782-789.
Smith, M. J., Britten, R. J., and Davidson, E. H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4805-4809.
Sober, H. A. (1968), in Handbook of Biochemistry, Cleveland, Ohio, Chemical Rubber Co., p H-58.
Weiss, G. B., Wilson, G. N., Steggles, A. W., and Anderson, W. F. (1976), *J. Biol. Chem. 251,* 3425-3431.
Young, B. D., Birnie, G. D., and Paul, J. (1976), *Biochemistry 15,* 2823-2829.

Purification and Characterization of GM_1 Ganglioside β-Galactosidase from Normal Feline Liver and Brain†

Jacqueline Kaye Anderson,‡ John Edwin Mole,*,§ and Henry J. Baker¶

ABSTRACT: GM_1 ganglioside β-galactosidase (GM_1-β-galactosidase) was purified from normal cat brain and liver by a combination of classical and affinity procedures. The final preparation of brain GM_1-β-galactosidase was enriched over 2000-fold with a 36% yield. However, the product was shown to contain several components by disc gel electrophoresis. GM_1-β-galactosidase was also purified from liver with greater than a 30 000-fold enrichment and 40% yield. The liver enzyme was judged homogeneous by disc gel electrophoresis at pH 4.3, 8.1, and 8.9 and by gel chromatography. Both liver and brain GM_1-β-galactosidase(s) eluted as sharp symmetrical peaks from Sephadex G-200 with molecular weights of 250 000 ± 50 000. The apparent K_m determined for 4-methylumbelliferyl β-D-galactopyranoside (4-MU-Gal) using partially purified brain GM_1-β-galactosidase was 1.73×10^{-4} M. Liver GM_1-β-galactosidase gave a K_m with 4-MU-Gal of 3.25×10^{-4} M and for [^{3}H]GM_1 ganglioside a K_m of 4.51×10^{-4} M was calculated. The pH optima of brain and liver GM_1-β-galactosidase using 4-MU-Gal was 3.8-4.5. By contrast, liver GM_1-β-galactosidase gave a sharp activity peak at pH 4.2 with [^{3}H]GM_1 ganglioside. Inhibition by mercuric chloride and sensitivity to hydrogen peroxide and persulfate suggest the involvement of a sulfhydryl in catalysis.

Although β-galactosidases are ubiquitous in plants and animals, their functions have not been clearly defined. Earlier studies indicated that these enzymes exist in many different forms (Asp and Dahlquist, 1968; Chytil, 1965; Chester et al., 1976) and more recently the mammalian β-galactosidases have been classified according to pH optima and substrate specificities (Gray and Santiago, 1969; Asp, 1970; Toofanian et al., 1973). The acid optimal (pH 3.5-4.5) β-galactosidases preferentially hydrolyze GM_1 ganglioside and the terminal nonreducing galactose of some glycoproteins and glycolipids, while the neutral enzymes cleave primarily the galactose of lactosyl residues.

The reduced catalytic activity of an acidic pH optimum β-galactosidase, GM_1-β-galactosidase,[1] has been associated with an inherited metabolic disorder in which galactose containing glycolipids and glycoproteins accumulate in secondary lysosomes (O'Brien, 1971). During the last 5 years this important hydrolase has been extensively purified from only three tissue sources: rabbit brain (Callahan and Gerrie, 1975; Jungalwala and Robins, 1968), bovine testes (Distler and Jourdian, 1973), and human liver (Norden et al., 1974). The limited information regarding GM_1-β-galactosidase, the relatively few available purification methods for glycohydrolases generally, and the availability of a well-defined feline model for GM_1 gangliosidosis, prompted the present study. This report describes methods for rapid isolation in high yield of a GM_1-β-galactosidase from normal feline brain and liver, and the basic physicochemical properties of this enzyme.

† From the Departments of Comparative Medicine and Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294. *Received August 29, 1977.* This work was supported by Grant NS 10967 from The National Institutes of Health.

‡ Division of Clinical Immunology and Rheumatology, University of Alabama in Birmingham, Birmingham, Alabama 35294.

§ Address correspondence to this author at: Department of Microbiology, University of Alabama in Birmingham, Birmingham, Alabama 35294.

¶ Department of Comparative Medicine, University of Alabama in Birmingham, Birmingham, Alabama 35294.

[1] Abbreviations used are: Na_2EDTA, ethylenediaminetetraacetic acid, disodium salt; GM_1-β-galactosidase, GM_1 ganglioside β-galactosidase; 4-MU, 4-methylumbelliferone; 4-MU-Gal, 4-methylumbelliferyl β-D-galactoside; 4-MU-GalNAc, 4-methylumbelliferyl-β-D-*N*-acetylgalactosamide; 4-MU-Glc, 4-methylumbelliferyl β-D-glucoside; 4-MU-GlcNAc, 4-methylumbelliferyl-β-D-*N*-acetylglucosamide; 4-MU-Glcur, 4-methylumbelliferyl β-D-glucuronide; NANA, *N*-acetylneuraminic acid; PATG, *p*-aminophenyl β-D-thiogalactoside; $NaDodSO_4$, sodium dodecyl sulfate.

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Experimental Procedures

Materials

Animals. Tissue for ganglioside purification and mutant enzyme studies were obtained from a colony of cats with GM_1 gangliosidosis maintained within the Department of Comparative Medicine.[2] Tissue used for enzyme purification was obtained from normal cats unrelated to cats from the GM_1 gangliosidosis colony.

Substrates and Inhibitors. The 4-MU-glycosyl derivatives of glucose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, and glucuronic acid were products of Sigma Chemical Co. PATG was from Calbiochem. [^{3}H]GM_1 ganglioside was prepared by the procedure of Suzuki and Suzuki (1972) and Radin et al. (1969). The specific activity of the final product was 8.2 Ci/mol [^{3}H]GM_1 ganglioside.

Reagents. Galactose oxidase and lactoperoxidase were products of Worthington Chemical Co. Sephadex G-200 and Con A Sepharose were purchased from Pharmacia. DE-52 was from Reeve Angel. Cellex-E was from Bio-Rad. Sodium taurocholate was the product of Nutritional Biochemicals. K^{131}I was from Amersham/Searle.

Buffers. GC was 0.17 M glycine (ammonia free)–sodium carbonate buffer, pH 10.0. Extraction buffer was 0.10 M sodium phosphate, 0.15 M NaCl, pH 7.0. AF-1 was McIlvaine's buffer, pH 4.3, 0.1 M NaCl. AF-2 was McIlvaine's buffer, pH 7.8, 0.15 M NaCl. Buffer A was 0.01 M sodium phosphate, 0.015 M NaCl, pH 7.0. CE-1 was 0.01 M sodium phosphate, 0.015 M NaCl, 0.001 M EDTA, pH 6.0.

Methods

General. Brain and liver were obtained at autopsy from normal cats and cats affected with GM_1 gangliosidosis. Tissue was either processed immediately or stored at −20 °C. Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as a standard. GM_1 ganglioside was extracted from brain of affected cats by the method of Folch et al. (1957) and the extract chromatographed on DE-52 by the method of Winterbourn (1971). Ganglioside was assayed using NANA as a standard by the resorcinol method of Svennerholm (1957) as modified by Miettinen and Takki-Lukkainen (1959). Purity of GM_1 ganglioside was confirmed by thin-layer chromatography on Silica Gel G plates using the solvent 1-propanol–water (73:27). Protein fractions were concentrated in the cold (4 °C) by positive pressure ultrafiltration (Amicon cell equipped with a PM-10 membrane). PATG-Sepharose was prepared according to the method of Steers et al. (1971).

Enzyme Assay. Solutions (5×10^{-4} M) of the 4-MU-glycopyranoside derivatives were prepared in 0.05 M sodium citrate–sodium phosphate buffer with 0.1 M sodium chloride at pH values optimal for their respective hydrolase activities: 4-MU-Gal, pH 3.8 or 7.0; 4-MU-Glc, pH 5.5; 4-MU-Glcur, pH 4.5; 4-MU-GalNAc, pH 4.5; and 4-MU-GlcNAc, pH 4.5. Twenty μL of sample was added to 100 μL of the substrate solution, and the mixture incubated at 37 °C for either 30 or 60 min. The reaction was terminated by the addition of 5.0 mL of GC buffer. Fluorescence was determined as relative intensity (Aminco-Bowman fluorocolorimeter; excitation 355–365 nm, emission 448–458 nm) with 10 nmol of 4-MU/100 μL of GC buffer as the 100% standard. Tritiated GM_1 ganglioside was assayed by the method of Norden and O'Brien (1973).

Polyacrylamide Disc Gel Electrophoresis. Samples for pH 8.1 and 4.3 gel electrophoresis systems were dialyzed into the appropriate buffer before application to gels. Electrophoresis was performed at 4 °C. Gel electrophoresis (pH 8.9) was carried out according to the method of Orenstein (1964). Gel electrophoresis (pH 8.1) was performed following the method of Orenstein for the pH 8.9 system with the following exceptions: the reservoir buffer and polymerization buffer of the running gel were adjusted to pH 8.1 with sodium barbital; all solutions contained 0.01 M NaCl and 1.0% sodium thioglycollate and gels were preelectrophoresed for 1 h. $NaDodSO_4$ gel electrophoresis was performed as reported by Weber and Osborn (1969). After electrophoresis, gels were stained for protein according to the method of Chrambach et al. (1967) or evaluated for glycosidase activities after incubation with corresponding 4-MU-glycopyranoside substrates (10–20 min at 37 °C).

pH Optima Studies. Solutions of 4-MU-Gal were prepared at various pH values from 3.0 to 8.0. Solutions of [^{3}H]GM_1 ganglioside were prepared from pH 3.5 to 6.0. Samples were assayed as previously described.

Stability Studies. Partially purified brain enzyme was adjusted to 0.1% in Triton X-100, cetyltrimethylammonium bromide or glycerol; 0.001 M in D-galactonolactone or Na_2-EDTA; or combined (50% v/v) with the supernatant from heat-treated (100 °C for 15 min) acetone powder extract and allowed to remain at 4 °C for 3 days after which time the samples were dialyzed and assessed for hydrolytic activity against 4-MU-Gal.

The GM_1-β-galactosidase isolated from normal cat liver was reacted with mercuric chloride, hydrogen peroxide, and ammonium persulfate to final concentrations of 1×10^{-6} M, 0.001%, and 1×10^{-8} M, respectively. After 5 min of incubation at 4 °C and dialysis, samples were assayed for acid 4-MU-β-galactosidase activity.

To assess temperature sensitivity, 0.5 mL of purified GM_1-β-galactosidase (0.2 mg/mL) was added to 0.5 mL of extraction buffer or 0.5 mL of 0.001 M D-galactonolactone in extraction buffer containing 0.001 M Na_2EDTA and the mixtures were incubated at temperatures between 30 and 60 °C for 15 min. The samples were quickly cooled to 4 °C and centrifuged at 10 000 rpm for 30 min, and the supernatant was dialyzed prior to assay with 4-MU-Gal (pH 3.8).

Gel Chromatography. 4-MU-β-galactosidase(s) isolated from normal cat brain and normal cat liver were dialyzed against buffer A containing 0.001 M EDTA and applied to a column of Sephadex G-200 (2.5 cm × 105 cm) at a flow rate of 15 mL/h. Fractions of 4.5 mL were collected. Molecular weight was estimated mathematically from the elution pattern by the method of Andrews (1964) as outlined in "Sephadex-Gel Filtration in Theory and Practice" (Pharmacia Fine Chemicals, 1973).

Lineweaver–Burk Plots. GM_1-β-galactosidase, isolated from brain and liver at various stages of purification, was assessed for apparent K_m values. 4-MU-Gal in final concentrations of 0.1 to 1.0 mM and [^{3}H]GM_1 ganglioside in final concentrations of 0.07 to 0.54 mM were used to generate double-reciprocal Lineweaver–Burk plots (Lineweaver and Burk, 1934). Data were evaluated by a standard linear regression program using an SR-51 Texas Instruments calculator.

Radioiodination of GM_1-β-Galactosidase Purified from Normal Cat Liver. Lactoperoxidase Technique. The method employed for protein iodination was essentially that described

[2] Experimental animals used in this research were maintained in facilities accredited by the American Association for Accreditation of Laboratory Animal Care.

TABLE I: Purification Scheme for a GM_1-β-Galactosidase from Normal Feline Brain.

Step	Total act. (nmol of 4 MU/ 60 min)	Total protein (mg)	Spec act. (nmol of 4 MU per 60 min per mg of protein)	Total purification	Yield (%)
Triton X-100 supernatant	55 000	1100	50		100
Acetone powder extraction	50 000	270	150	3	91
pH 4.3 adjustment	48 000	200	240	5	87
Thiogalactopyranoside chromatography I	24 000	1.85	13 000	260	44
Thiogalactopyranoside chromatography II	24 000	0.5	50 000	1000	44
Concanacalin A chromatography	20 000	0.2	100 000	2000	36

by Morrison and Bayse (1970) except that final concentrations for reactants were: hydrogen peroxide, 1×10^{-4} M; potassium iodide, 9×10^{-5} M; Na_2EDTA, 1×10^{-3} M; lactoperoxidase, 7.4×10^{-9} M; $K^{131}I$, 0.15 mCi; and 12 μg of purified protein.

Galactose Oxidase-Lactoperoxidase Technique. The lactoperoxidase method was used with the exception that galactose oxidase was introduced in situ to enzymatically produce hydrogen peroxide. Final concentrations were as those previously mentioned with the deletion of hydrogen peroxide and the addition of D-(+)-galactose (1×10^{-5} M) and galactose oxidase, 0.5 mg. Appropriate blanks without GM_1-β-galactosidase were simultaneously performed to assess the possibility of nonspecific labeling. Efficiency of labeling was ascertained as cpm/mg of protein after dialysis to remove free ^{131}I.

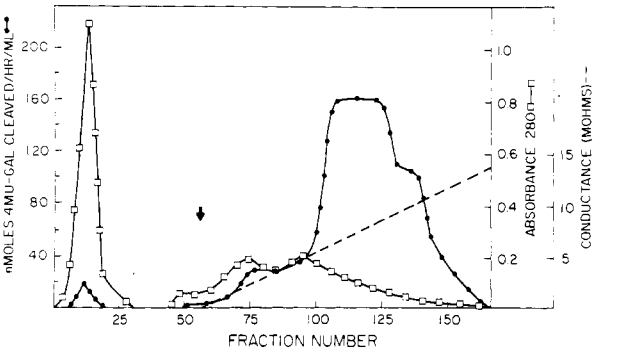

FIGURE 1: Elution profile of acid optimum β-galactosidase from normal cat liver during DE-52 cellulose chromatography. (↓) Begin gradient.

Results

Purification of a GM_1 Ganglioside β-Galactosidase from Normal Cat Brain. In order to minimize loss of activity due to enzyme instability, purifications were performed without interruption at 4 °C unless otherwise stated.

Tissue Homogenization. Normal cat brain was homogenized in aqueous 0.1% Triton X-100 (1:9 w/v). The homogenate was centrifuged at 10 000 rpm for 30 min and the supernatant retained (fraction Ia).

Acetone Precipitation. An acetone powder was prepared from fraction Ia according to the method of Morton (1955). The dried powder was extracted for 12–24 h with extraction buffer (1:9 w/v) and the mixture centrifuged. The supernatant was decanted to yield fraction IIa.

PATG-Sepharose Chromatography. Fraction iia was warmed to 25 °C and adjusted to pH 4.3 with solid citric acid. After clarification (13 000 rpm for 30 min), the supernatant was brought again to 25 °C and applied (8.0 mL/h) to a PATG-Sepharose column (0.9 cm × 30.0 cm) previously equilibrated at 25 °C with AF-1. The column was washed with 2 bed volumes of AF-1, followed by 2 bed volumes of AF-2. Two-milliliter fractions were collected. Fractions containing 4-MU-β-galactosidase activity were pooled and concentrated to 5.0 mL. The enzyme solution was again adjusted to pH 4.3 with solid citric acid and reapplied to the PATG-affinity column. Fractions with active enzyme were pooled and concentrated to 5.0 mL (fraction IIIa).

Con A Sepharose Chromatography. Fraction IIIa was adjusted to pH 7.0 with 1.0 M NaOH, chilled to 4 °C, and applied to a Con A Sepharose column according to the method of Norden and O'Brien (1974) except that a column 2.5 × 15.0 cm was used, and the sample was applied at a flow rate of 50 mL/h in extraction buffer. The column was eluted (25 °C) using 250 mL of 0.75 M methyl α-D-mannoside in extraction buffer (40 mL/h). Fractions (5.0 mL) were collected and those containing 4-MU-Gal (pH 3.8) activity were pooled and made 0.001 M in Na_2EDTA. The sample was concentrated to 0.5 mL and quick frozen for storage at −20 °C (fraction IVa). The summary of a typical purification is presented in Table I.

Purification of a GM_1-β-Galactosidase from Normal Cat Liver. Tissue homogenization, acetone precipitation, and extraction of acetone powder were as described above.

Con A Sepharose Chromatography. Sample application and elution were as described above except for: (1) column size (8.0 × 20.0 cm); (2) flow rate, 150 mL/h; (3) the column containing bound enzyme was equilibrated with buffer A (75 mL/h). Five-milliliter fractions were collected and those containing 4-MU-Gal (pH 3.8) activity were pooled and made 0.001 M with Na_2EDTA (fraction IIIb).

DE-52 Cellulose Chromatography. A column of DE-52 (2.5 cm × 15.0 cm) was equilibrated with buffer A containing 0.001 M Na_2EDTA and fraction IIIb applied (100 mL/h). The column was washed with buffer until acid 4-MU-Gal activity was no longer detected in the eluate. A linear NaCl gradient from 0.015 M (wash buffer) to 0.20 M was used for elution (Figure 1). Fractions (14.5 mL) were collected (100 mL/h). Those fractions containing acid 4-MU-Gal activity were pooled and concentrated to approximately 50 mL (fraction IVb).

Cellex-E Cellulose Chromatography. Enzyme fraction IVb was applied (25 mL/h) to a column of Cellex-E cellulose (1.5 × 10.0 cm) equilibrated with CE-1 and washed until A_{280} was less than 0.05. Elution was effected with a linear NaCl gradient from 0.015 M to 0.20 M (Figure 2). Fractions (3.3 mL) were collected and those exhibiting 4-MU-β-galactosidase activity at pH 3.8 were pooled and concentrated to 5.0 mL (fraction Vb).

PATG-Sepharose Chromatography. This step was performed as outlined previously except that fraction Vb was applied only once to the affinity column (fraction VIb). Because elution was achieved by a change in pH, and was therefore nonspecific, the protein present in fraction 17 may indicate nonspecifically adsorbed contaminants from Cellex-E chromatography (Figure 3). A summary for the purification of liver GM_1-β-galactosidase is presented in Table II.

TABLE II: Purification Scheme for a GM_1-β-Galactosidase from Normal Feline Liver.

Step	Total act. (nmol of 4 MU/ 60 min)	Total protein (mg)	Spec act. (nmol of 4 MU per 60 min per mg of protein)	Total purification	Yield (%)
Triton X-100 supernatant	980 000	12 500	78		100
Acetone powder extraction	945 000	5 400	175	2	96
Concanavalin A chromatography	832 000	119	6 991	90	85
DE-52 cellulose chromatography	546 000	9.3	58 710	753	56
Cellex-E chromatography	416 000	1.7	244 706	3 176	42
Thiogalactopyranoside chromatography	398 000	0.07	>1 000 000	30 000	40

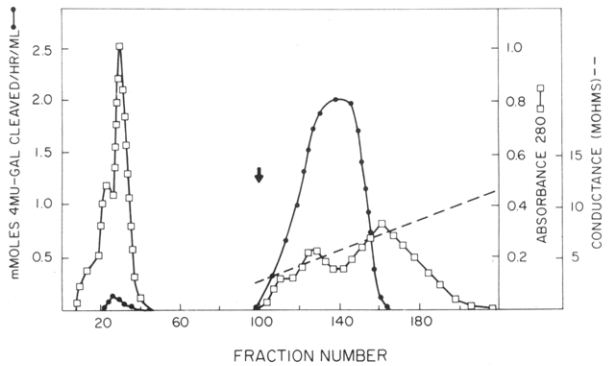

FIGURE 2: Elution profile of acid optimum β-galactosidase from normal cat liver during Cellex-E chromatography. (↓) Begin gradient.

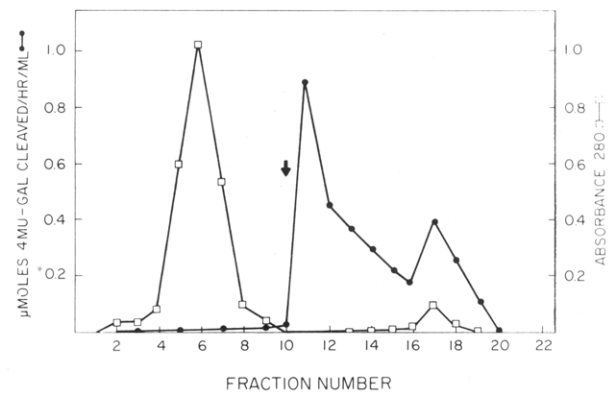

FIGURE 3: Affinity chromatography of normal feline hepatic 4-MU-β-galactosidase (pH 3.8) from Cellex-E using PATG-Sepharose. (↓) Begin elution buffer. Actual elution from the buffer change is evident in fraction 17.

Characterization of GM_1-β-Galactosidase Isolated from Brain and Liver. Purity. Normal feline brain enzyme isolated by the methods described demonstrated one band on disc gel electrophoresis (pH 8.9). However, electrophoresis at pH 4.3 revealed several bands. Treatment of the enzyme with urea–$NaDodSO_4$ and subsequent disc gel electrophoresis resolved two major and three minor components. This preparation also demonstrated a 30–40% contamination with hexosaminidases. GM_1-β-galactosidase isolated from liver (Table II) migrated as a single band on disc gel electrophoresis at pH 4.3, 8.1, and 8.9 (Figure 4). Furthermore, the 4-MU-β-galactosidase activity band was coincident with the protein band on disc gel electrophoresis at pH 8.1. The purified enzyme exhibited hydrolytic activity toward [^{3}H]GM_1 ganglioside and 4-MU-Gal (pH 3.8), but did not hydrolyze 4-MU-Glc, 4-MU-Glcur, 4-MU-Gal (pH 7.0), 4-MU-GalNAc, or 4-MU-GlcNAc.

pH Optima. 4-MU-β-galactosidase isolated from brain and liver displayed identical pH–activity profiles with optima at

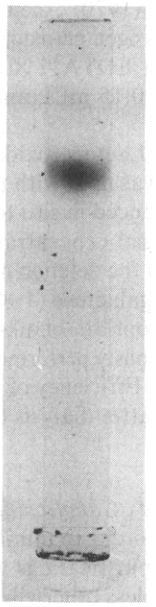

FIGURE 4: Polyacrylamide gel electrophoresis at pH 8.1 of GM_1-β-galactosidase purified from normal cat liver.

pH 4.0–4.5. In contrast, highly purified liver GM_1-β-galactosidase demonstrated a very sharp optimum at pH 4.2 with [^{3}H]GM_1 ganglioside (Figure 5).

Stability Studies. The ability of selected agents to effectively stabilize acid 4-MU-β-galactosidase activity was assessed as percentage of 4-MU-β-galactosidase activity retained: Na_2EDTA, 98%; Triton X-100, 95%; heat treated acetone powder extract, 86%; glycerol, 72%; D-galactonolactone, 68%; and cetyltrimethylammonium bromide, 57%. Due to inhibition of enzyme binding (30%) to the PATG-Sepharose column by detergent, Na_2EDTA was selected for optimal purification.

Inhibitors. The 4-MU-β-galactosidase isolated from normal feline liver was completely and irreversibly inhibited by mercuric chloride, hydrogen peroxide, and ammonium persulfate.

Temperature Sensitivity. Purified liver enzyme appeared to retain catalytic activity toward 4-MU-Gal at temperatures up to 50 °C when incubated with the reversible inhibitor, D-galactonolactone. In contrast, enzyme incubated without D-galactonolactone retained catalytic activity to 40 °C (Figure 6).

Kinetic Parameters. Apparent K_m values determined for different samples from normal cat brain and liver were between 1.62×10^{-4} and 3.25×10^{-4} M using 4-MU-Gal. Using the purified liver enzyme the K_m for 4-MU-Gal was 3.25×10^{-4} M and for [^{3}H]GM_1 ganglioside the K_m was 4.51×10^{-4} M.

Radioiodination of Purified GM_1-β-Galactosidase from Normal Cat Liver. The original method of Morrison and

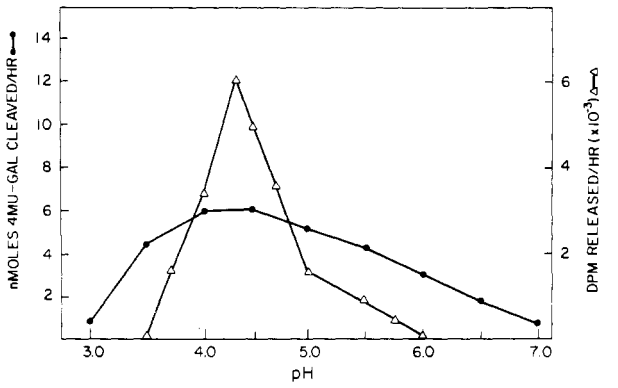

FIGURE 5: pH activity relationship of purified GM_1-β-galactosidase for 4-MU-Gal and [^{3}H]GM_1-ganglioside.

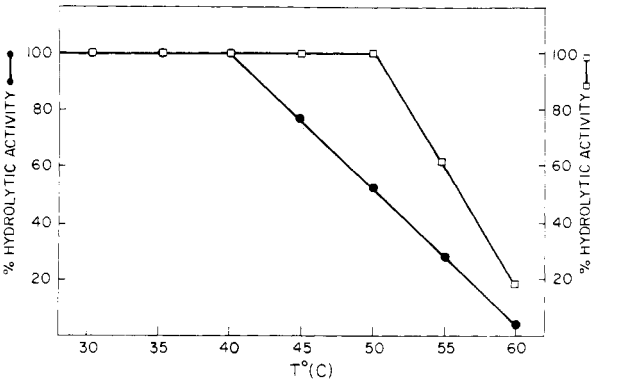

FIGURE 6: Thermal inactivation of purified feline hepatic 4-MU-β-galactosidase with (□ — □) and without (● — ●) inhibitor: 0.01 M D-galactonolactone, 15 min incubation/T °C.

Bayse, utilizing free hydrogen peroxide, was found to be unsatisfactory for radiolabeling the GM_1-β-galactosidase due to the sensitivity of the enzyme to oxidizing agents. To circumvent the denaturing effect of free hydrogen peroxide, a galactose–galactose oxidase coupled system was introduced in situ. This combination proved effective for the production of hydrogen peroxide in enzyme sparing concentrations. However, incorporation of ^{131}I irreversibly inhibited the 4-MU-Gal activity, with enzyme retaining only 55% of the original 4-MU-Gal activity after 30 min of incubation. Further reaction decreased activity to 30% and failed to increase radiolabel.

Discussion

Frequent attempts have been made to purify the acid β-galactosidases using standard biochemical techniques such as gel filtration, ion exchange chromatography, and gel electrophoresis (Alpers, 1969; Lisman and Hooghwinkel, 1973; Sato and Yamashina, 1974). The recent development of affinity chromatography has greatly simplified the purification of these enzymes (Kanfer et al., 1973, Junowicz and Paris, 1973; Bishayee and Bachhawat, 1974). However, the affinity of several proteins for the same ligand and the less than maximal coupling of the ligand during the preparation of an affinity resin has been a handicap in utilization of this method.

As procedures for separations have improved, increasing numbers of isoenzymes have been reported (Ho et al., 1973; Cheetham et al., 1975; Furth, 1965). Whether these forms are present in vivo or arise as artifacts of purification, either from cleavage by endogenous proteases or removal of particular stabilizing moieties, is unknown. The extreme lability reported for some enzymatic forms of β-galactosidases ($t_{1/2} < 1$ h) does suggest that less heterogeneity is present in the natural state. GM_1-β-galactosidase has been purified to homogeneity from rabbit brain (Callahan and Gerrie, 1975), bovine testes (Distler and Jourdian, 1973) and human liver (Norden et al., 1974). In the present paper, we have reported a novel isolation of this enzyme from feline liver and brain. The particular use of cat as a tissue source was prompted by the availability of an established feline model of human GM_1 gangliosidosis. Based on clinical, pathological, genetic, and biochemical criteria, the feline disorder appears to be nearly an exact replica of human GM_1 gangliosidosis (type 2) (Baker et al., 1971).

Comparative work is currently in progress to assess the quantities, molecular weight relationships, and substrate specificities of several glycohydrolases from the tissue of normal cats and cats affected with GM_1 gangliosidosis.

Brain was selected initially as the tissue source for the purification of GM_1-β-galactosidase. Despite the relatively small organ weight (25 g/cat), the apparent simplicity of the pH optima curve for brain encouraged its use. Through a combination of classical methods and affinity chromatography, normal feline brain GM_1-β-galactosidase was purified more than 2000-fold. The preparation eluted as a single, symmetrical peak of enzymatic activity on Sephadex G-200 chromatography and demonstrated one band on pH 8.9 disc gel electrophoresis. However, multiple bands were observed on NaDodSO$_4$ disc gel electrophoresis, suggesting that the product was heterogeneous, even after extensive purification. It is not known if the bands present on NaDodSO$_4$ disc gel electrophoresis were artifacts of purification, different molecular weight forms of acid 4-MU-β-galactosidase, or a representation of possible subunit structure. Other investigators have purified GM_1-β-galactosidases to apparent homogeneity with only a 400–600-fold enrichment of the enzyme over tissue homogenates (Callahan and Gerrie, 1975; Distler and Jourdian, 1973). The enzyme isolated by the scheme presented in Table I had a pH optimum of 4.0–4.5 (4-MU-Gal) and a molecular weight of 250 000 as estimated by Sephadex G-200 gel chromatography. This molecular weight differs from that reported for GM_1-β-galactosidase isolated from bovine testes (68 000) (Distler and Jourdian, 1973), human liver (72 000) (Norden et al., 1974), and rabbit brain (81 000 and 103 000) (Jungalwala and Robins, 1968). The existence of different molecular weight forms may suggest that the GM_1-β-galactosidase isolated from normal cat liver occurs as a tetramer; the human, bovine, and rabbit forms are single subunits, with a suggested dimer in the rabbit. Unfortunately, the quantity of purified enzyme available precluded establishing subunit structure by NaDodSO$_4$ gel electrophoresis. The partially purified enzyme was sensitive to inhibition by mercuric chloride, suggesting that a sulfhydryl group may be essential for hydrolytic activity. Sodium chloride and Na_2EDTA were shown to be effective stabilizing agents, the latter indicating that metal ions are not required for catalysis. Investigators have reported similar findings with other mammalian β-galactosidases (Ho and O'Brien, 1970; Seetharam and Radhakrishnan, 1973). The apparent K_ms for 4-MU-Gal using normal cat brain homogenate and the partially purified enzyme preparation were 1.62×10^{-4} and 1.73×10^{-4} M, respectively.

Although the final yield of 4-MU-Gal activity isolated from feline brain was 36%, the low quantity present in brain and the need for additional purification discouraged its further use as a tissue source for quantitative purification of GM_1-β-galactosidase.

Subsequently, liver was investigated as an alternative source of 4-MU-β-galactosidases. In contrast to brain, liver possesses neutral and hetero forms of the enzyme; however, it contains

acid 4-MU-β-galactosidase in comparable quantities. The larger organ size of liver encouraged its use in this study. Normal feline liver was extracted and processed as designated in Table II. The advantage of using liver over brain was to provide an easily obtainable starting material in sufficient quantity to allow a more extensive purification to be carried out. The addition of two ion-exchange columns in the purification was effective in removing approximately 80% of the contaminating hexosaminidases. The purification methodologies are notable in that an overall 30 000-fold purification with a 40% yield of total 4-MU-Gal activity was realized. The enzyme was homogeneous on pH 4.3, 8.1, and 8.9 disc gel electrophoresis and demonstrated a single, symmetrical peak of enzymatic activity on Sephadex G-200 gel chromatography.

As in brain, the isolated liver enzyme demonstrated a molecular weight of approximately 250 000 on Sephadex G-200 gel chromatography. The pH optimum for 4-MU-Gal was 4.0–4.5. Using [^{3}H]GM_1 ganglioside, a sharp optimum at pH 4.2 was observed. Liver GM_1-β-galactosidase was stabilized by sodium chloride and Na_2EDTA and irreversibly inhibited by mercuric chloride. The addition of a competitive inhibitor, D-galactonolactone, extended its temperature stability 10 °C. Apparent K_m values for samples from normal cat liver at different stages of purification varied from 1.7×10^{-4} to 3.25×10^{-4} M with 4-MU-Gal. However, these figures are similar to those reported by others (Jungalwala and Robins, 1968). The K_m for [^{3}H]GM_1 ganglioside was 4.51×10^{-4} M using the purified enzyme. Miyatake and Suzuki (1974) found a K_m value for the hydrolysis of GM_1 ganglioside by human GM_1-β-galactosidase to be 8.20×10^{-4} M. Norden et al. (1974) have also isolated a GM_1-β-galactosidase from human liver with an apparent K_m of 0.77×10^{-4} M for GM_1 ganglioside.

Purified GM_1-β-galactosidase was examined in vivo for its effectiveness in enzyme replacement therapy. However, the small amount of purified GM_1-β-galactosidase available presented difficulties in monitoring the enzyme after infusion, even though the assay procedure utilizes a sensitive fluorogenic substrate. In an attempt to introduce a sensitive tag to follow the infusion and distribution of GM_1-β-galactosidase, the enzyme was radiolabeled using a galactose–galactose oxidase system. Radiolabeling was successful; however, incorporation of iodine resulted in the simultaneous loss of hydrolytic activity. In as much as preservation of hydrolytic activity is a requisite for enzyme replacement therapy, these studies were not pursued further. Nonetheless, the feline model of GM_1 gangliosidosis (type 2) is exceptionally valuable in developing a working in vivo test system for enzyme replacement therapy in GM_1 gangliosidosis.

Unfortunately, the GM_1-β-galactosidase purified from normal feline liver was highly unstable. Because lability was evident only after the final purification step (PATG chromatography), it was most probably the result of low protein concentrations (12 μg/mL). Such nonspecific denaturation is known to occur with protein concentrations of 50 μg/mL or less.

The extreme instability of the purified enzyme ($t_{1/2} < 6$ h) and the very low amount of GM_1-β-galactosidase available from feline tissue (<70 ng/300 g of cat liver) have thus far hindered detailed kinetic or structural studies.

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References

Alpers, D. H. (1969), *J. Biol. Chem. 244,* 1238.
Andrews, P. (1964), *Biochem. J. 91,* 222.
Asp, N. G. (1970), *Biochem. J. 117,* 369.
Asp, N. G., and Dahlquist, A. (1968), *Biochem. J. 110,* 143.
Baker, H. J., Lindsey, J. R., McKhann, G. M., and Farrell, D. F. (1971), *Science 174,* 838.
Bishayee, D., and Bachhwat, B. K. (1974), *Biochim. Biophys. Acta 334,* 378.
Callahan, J. W., and Gerrie, J. (1975), *Biochim. Biophys. Acta 391,* 141.
Cheetham, P. S. J., Dance, N., and Robinson, D. (1975), *Biochem. Soc. Trans. 3,* 240.
Chester, M. A., Hultberg, B., and Ockerman, P.-A. (1976), *Biochim. Biophys. Acta 429,* 517.
Chrambach, A., Reisfeld, R. A., Wyckoff, M., and Zaccari, J. A. (1967), *Anal. Biochem. 20,* 150.
Chytil, F. (1965), *Biochem. Biophys. Res. Commun. 19,* 630.
Distler, J. J., and Jourdian, G. W. (1973), *J. Biol. Chem. 248,* 6772.
Folch, J., Lies, M., and Stanley, G. H. S. (1957), *J. Biol. Chem. 226,* 497.
Furth, A. J. D. (1965), *Biochem. J. 97,* 59.
Gray, G. M., and Santiago, N. A. (1969), *J. Clin. Invest. 48,* 716.
Ho, M. W., and O'Brien, J. S. (1970), *Clin. Chim. Acta 30,* 531.
Ho, M. W., Cheetham, P., and Robinson, D. (1973), *Biochem. J. 136,* 351.
Jungalwala, F. B., and Robins, E. (1968), *J. Biol. Chem. 243,* 4258.
Junowicz, E., and Paris, J. E. (1973), *Biochim. Biophys. Acta 321,* 234.
Kanfer, J. N., Petrovich, G., and Mumford, R. A. (1973), *Anal. Biochem. 55,* 301.
Lineweaver, H., and Burk, D. (1934), *J. Am. Chem. Soc. 56,* 658.
Lisman, J. J. W., and Hooghwinkel, G. J. M. (1973), *Neurobiology 4,* 167.
Lowry, O. H., Roebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Miettinen, T., and Takki-Lukhainen, I. T. (1959), *Acta Chem. Scand. 13,* 856.
Miyatake, T., and Suzuki, K. (1974), *Biochim. Biophys. Acta 337,* 333.
Morrison, M., and Bayse, G. S. (1970), *Biochemistry 9,* 2995.
Morton, R. K. (1955), *Methods Enzymol. 1,* 25.
Norden, A. G. W., and O'Brien, J. S. (1973), *Arch. Biochem. 159,* 389.
Norden, A. G. W., and O'Brien, J. S. (1974), *Biochem. Biophys. Res. Commun. 56,* 1973.
Norden, A. G. W., Tennant, L., and O'Brien, J. S. (1974), *J. Biol. Chem. 249,* 7969.
O'Brien, J. S. (1971), *N. Engl. J. Med. 284,* 893.
Orenstein, L. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.
Pharmacia Fine Chemicals (1973), Sephadex-Gel Filtration in Theory and Practice, Upplands Grafiska A. B., Sweden.
Radin, N. S., Hof, L., Bradley, R. M., and Brady, R. O. (1969), *Brain Res. 14,* 497.
Reisfeld, R. A., Lewis, U. J., and Williams, D. E. (1962), *Nature (London) 195,* 281.

Sato, M., and Yamashina, J. (1974), *Biochem. J. 76,* 1155.
Seetharam, B., and Radhakrishnan, A. N. (1973), *Indian J. Biochem. Biophys. 10,* 3.
Steers, E., Cautrecasas, P., and Pollard, H. B. (1971), *J. Biol. Chem. 246,* 196.
Suzuki, Y., and Suzuki, K. (1972), *J. Lipid Res. 13,* 687.
Svennerholm, L. (1957), *Biochim. Biophys. Acta 24,* 604.
Toofanian, F., Hill, F. W. G., and Kidder, D. E. (1973), *Res. Vet. Sci. 15,* 128.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Winterbourn, C. C. (1971), *J. Neurochem. 18,* 1153.

Occurrence of an Uncoupler-Resistant Intermediate Type of Phosphate–Water Oxygen Exchange Reaction Catalyzed by Heart Submitochondrial Particles†

John A. Russo, Candace M. Lamos, and Robert A. Mitchell*

ABSTRACT: The hydrolysis of ATP catalyzed by phosphorylating vesicles prepared from bovine heart mitochondria by ultrasonic disruption was studied in $H_2{}^{18}O$. Provided that an ATP-regenerating system was included to prevent accumulation of ADP due to hydrolysis, the addition of 20 mM arsenate or 0.5 mM 2,4-dinitrophenol to the incubation mixture either singly or together, had little or no effect on the number of oxygen atoms from H_2O incorporated (on the average) into each molecule of P_i formed by hydrolysis (the O:P ratio). As the ATP concentration was reduced from 2.0 to 0.05 mM, the O:P ratio increased from about 1.4 to over 2.0 and, although dinitrophenol significantly increased the ATPase activity, it did not significantly alter the O:P ratio for a given ATP level. This implies that the uncoupler does not act directly on the terminal transphosphorylation step. Companion experiments were performed in which ^{18}O label was placed either initially in H_2O or P_i. Under conditions where extensive exchange from $H_2{}^{18}O$ into P_i occurred, no ^{18}O was lost from medium P_i under identical circumstances, thus showing that the exchange was intermediate and did not involve medium P_i. Kinetic plots of v vs. v/S were nonlinear with respect to ATPase activity. The kinetic data, as well as the $P_i = H_2{}^{18}O$ exchange data, are consistent with enzyme models having multiple forms of catalytic sites. Several models are evaluated and attempts are made to distinguish between some of the simpler cases of these models.

Cohn (1953) first reported evidence for a mitochondrial catalyzed pathway whereby oxygen from inorganic phosphate (P_i)[1] equilibrated with water oxygen. Subsequent studies by other workers showed that mitochondria possessed the capacity to exchange ^{18}O from P_i into H_2O ($[^{18}O]P_i = H_2O$), from $[^{18}O]H_2O$ into P_i ($P_i = [^{18}O]H_2O$), and from $[^{18}O]H_2O$ into ATP ($ATP = [^{18}O]H_2O$). These reactions, like the $^{32}P_i$ = ATP exchange, occurred in the absence of net ATP synthesis by oxidative phosphorylation and appeared to be closely associated with a partial reaction catalyzed by the terminal phosphoryl transferase enzyme of oxidative phosphorylation, namely, the reversible formation of ATP by the reaction ADP + P_i = ATP + H_2O (for a comprehensive review of earlier work in this area, see Boyer, 1967).

As discussed in some detail (Boyer, 1967), a rapid interconversion of ADP, P_i, and ATP on the enzyme surface could produce high rates of either $P_i = H_2{}^{18}O$ or $ATP = H_2{}^{18}O$ exchange, when measured with respect to the rate of the $^{32}P_i$ = ATP exchange, but not both unless there is some additional means for incorporating ^{18}O from H_2O into ATP or P_i by a process not associated with the $^{32}P_i$ = ATP exchange. It was subsequently concluded that such a component might indeed be associated with the $P_i = H_2{}^{18}O$ exchange, particularly in view of the ability of an ATP regenerating system to suppress the $ATP = H_2{}^{18}O$ and $^{32}P_i$ = ATP exchange but still to permit $P_i = H_2{}^{18}O$ exchange to occur as ATP hydrolysis took place (Mitchell et al., 1967).

The residual $P_i = H_2{}^{18}O$ exchange activity catalyzed by submitochondrial heart vesicles, in the presence of an ATP-regenerating system, was found to be resistant to high concentrations of As_i by De Master and Mitchell (1973), despite the well-known ability of As_i to compete with P_i in P_i-requiring reactions. This observation suggested that medium P_i was not involved in the exchange, and that the incorporation of ^{18}O from H_2O had occurred with some enzyme-bound intermediate involved in ATP hydrolysis (i.e., an "intermediate" exchange). This type of intermediate has been observed with myosin (Levy and Koshland, 1959) and is distinct from the type involving medium P_i as a reactant (i.e., "medium" exchange). The data reported in the present paper confirm the existence of a $P_i = H_2{}^{18}O$ reaction of the intermediate type. This reaction is closely associated with ATP hydrolysis catalyzed by an oligomycin sensitive ATPase, but, unlike other ATP supported reactions of mitochondria, it is not inhibited by dinitrophenol. Moreover, as the concentration of ATP is decreased, the incorporation of ^{18}O into P_i due to the exchange increases from 40% to 200% (over that expected for simple hydrolysis). The implications of these findings in terms of heterogeneity of ATP enzyme complexes involved in ATP hydrolysis by submitochondrial particles are discussed.

† From The Richard M. Mc Kean Laboratory of Metabolic Studies, Department of Biochemistry, Wayne State University, Detroit, Michigan 48201. *Received March 16, 1977; revised manuscript received July 29, 1977.* This work was supported by grants from The National Institutes of Health (GM 19562) and The Michigan Heart Association.

[1] Abbreviations used are: P_i, inorganic phosphate; As_i, inorganic arsenate; dinitrophenol, 2,4-dinitrophenol; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

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