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Purification, Biochemical and Immunological Characterisation of Hexosaminidase A from Variant AB of Infantile G_{M2} Gangliosidosis

Ernst CONZELMANN, Konrad SANDHOFF, Heidi NEHRKORN, Benjamin GEIGER, and Ruth ARNON

Max-Planck-Institut für Psychiatrie, Neurochemische Abteilung, München, and
The Weizmann Institute of Science, Department of Chemical Immunology, Rehovot

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Variant AB of infantile G_{M2} gangliosidosis is a fatal disease leading invariably to death within the first few years of life, due to the excessive storage of the glycolipids G_{M2} and G_{A2} which occurs in the nervous tissue of the patient. Unlike other variants of this hereditary disease, where a deficiency of hexosaminidase A, the ganglioside-G_{M2}-degrading enzyme, could be demonstrated, the variant AB is characterized by a normal or even elevated level of this enzyme.

To examine the possibility of a mutant hexosaminidase A, well capable of hydrolyzing the fluorogenic synthetic substrates but unable to attack the ganglioside, the enzyme was isolated from a patient's tissue and characterized biochemically and immunologically in comparison with an enzyme preparation from normal control tissue. No differences between hexosaminidase A from normal and variant AB tissue could be detected indicating that the defect involved in this disease is not at the genetic level of production of either α or β chains of hexosaminidase A.

Human hexosaminidase exists in two major forms, A and B [1,2]. Inherited deficiencies of one or both of them give rise to variant forms of G_{M2} gangliosidosis [3–5]. In Tay-Sachs disease, variant B of infantile G_{M2} gangliosidosis, hexosaminidase A deficiency correlates with neuronal storage of ganglioside G_{M2}, whereas the defect of both isoenzymes A and B (variant 0) results in the simultaneous storage of glycosphingolipids G_{M2} and G_{A2} in nervous tissue and of globoside (GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1′Cer) in extracranial organs. Juvenile forms of the disease have also been described in which a partial deficiency of hexosaminidase A [6–8] (juvenile form of Tay-Sachs disease, variant B) or of both hexosaminidases A and B [9] (juvenile form of variant 0) correlates with a mild accumulation of glycolipids G_{M2} or G_{M2} and G_{A2}, respectively. In all these variants of G_{M2} gangliosidosis a deficiency of hexosaminidase A parallels an abnormal accumulation of ganglioside G_{M2}. This finding is in agreement with studies *in vitro* which demonstrate that ganglioside G_{M2} is degraded by hexosaminidase A in the presence of natural activator proteins or 2 mM taurodeoxycholate [10–12], whereas hexosaminidase B exhibits a small amount of activity and then only in the presence of a detergent such as sodium taurodeoxycholate. No activity is observed in the presence of activator proteins [12].

In variant AB an excessive storage of both glycolipids G_{M2} and G_{A2} occurs in the nervous tissue of the afflicted patients despite the fact that hexosaminidases A and B attain normal levels of activity in most organs and even have an elevated activity in brain tissue when measured with synthetic substrates [3]. Therefore the possibility was examined that hexosaminidase A from a patient afflicted with variant AB might represent a mutant enzyme which is still active on synthetic substrates but has lost its activity against the natural glycolipid substrates. The enzyme was purified by affinity chromatography from the autopsy liver of a patient. Its substrate specificity against tritium-labelled glycolipids and synthetic substrates, its kinetic and immunological properties were tested in comparison with an enzyme preparation from normal tissue.

Abbreviations. Ganglioside G_{M2}, II^{3}NeuAc-GgOse$_3$Cer or GalNAcβ1→4Gal(3←2αNeuAc)β1→4Glc1→1Cer; glycosphingolipid G_{A2}, gangliotriaosylceramide or GgOse$_3$Cer or GalNAcβ1→4Galβ1→4Glc1→1Cer.

Enzyme. β-*N*-Acetylglucosaminidase or hexosaminidase or 2-acetamido-2-deoxy-β-D-glucoside acetamido-deoxyglucohydrolase (EC 3.2.1.30). In this paper the name hexosaminidase is used since the enzyme preparation contained an *N*-acetylgalactosaminidase activity which could not be separated from the *N*-acetylglucosaminidase.

MATERIALS AND METHODS

Materials

4-Methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, -galactopyranoside, and naphthol-AS-BI-2-acetamido-2-deoxy-β-D-glucopyranoside were obtained from Koch-Light (England). Glycosphingolipids G_{M2} (16.2 Ci/mol) and G_{A2} (190 Ci/mol) were prepared as given before [3, 13]. All other reagents used were analytically pure, or of the best grade available.

Tissue Samples

Tissues were kindly supplied by Dr Ellis (London) from a patient who died at the age of 3 1/12 years with variant AB of infantile G_{M2} gangliosidosis as characterised by clinical diagnosis, lipid and enzyme analysis (see [14]). All tissues were stored at −20 °C for 3 years prior to analysis. Control tissues were obtained from patients who died from disorders not affecting the central nervous system. Fibroblasts from another patient with variant AB of infantile G_{M2} gangliosidosis [15] were kindly supplied by Dr Beratis (New York).

Enzyme Preparations

Crude liver and fibroblast hexosaminidases A and B free of each other were prepared by extraction of the sample (0.6 g of liver tissue or 250 mg of lyophilized fibroblasts) into 10 mM phosphate buffer pH 6.0 and subsequent separation of isozymes on DEAE-cellulose column [1]. The content of hexosaminidase A in the liver sample and in the fibroblast extract was 55% and 60% respectively as determined by both heat inactivation assay and by the relative activities of the peaks eluted from the ion-exchange column.

Purification of hexosaminidases A and B was performed as described earlier [12]. Protein was determined according to Lowry et al. [16] using crystalline bovine serum albumin as standard.

Enzyme Assay

Hexosaminidase activity was monitored using fluorescent substrate 4-methylumbelliferyl-*N*-acetyl-β-D-glucosaminide [17] as given before [12]. Enzyme activity was calculated according to a standard curve of 4-methylumbelliferone. One unit of enzyme activity releases 1 μmol of 4-methylumbelliferone per minute at 37 °C. For pH profile studies the enzyme and substrate samples were diluted before assay in a series of citrate buffers (0.05 M) with a pH range of 3.15 to 6.25. After incubation the actual pH value of the mixture was monitored in an aliquot sample. Assays with glycolipids G_{M2} and G_{A2} as substrates were performed as given before [12].

Heat Inactivation

Heat lability tests were carried out in 50 mM citrate buffer containing 0.01% bovine serum albumin. Enzyme samples were incubated for various periods at the temperatures indicated in Fig. 3, after which they were transferred to ice and their activity determined.

Isoelectric Focusing

Tissue homogenates (1:10) prepared in 50 mM citrate buffer, pH 4.4, were centrifuged at 100000 × *g* for 60 min. The supernatants were dialysed against 1% glycine solution and centrifuged again (100000 × *g* for 60 min). The hexosaminidases were fractionated in a minicolumn by isoelectric focusing according to Harzer [18].

Antisera

Goat antisera against pure placental hexosaminidases A and B, as well as antibody preparations specific to the A isozyme exclusively, were prepared as described previously [19]. Antisera to hexosaminidases A and B from variant AB liver were raised in rabbits by 4 injections of each isozyme (0.1 − 0.2 U per injection) with an interval of 7 − 10 days between injections. The enzymes were injected in emulsion with complete Freund's adjuvant. The titer of each antiserum was determined by its capability of binding the relevant isozyme in an antigen binding assay.

Immunodiffusion

Double Immunodiffusion in 1.5% Agarose gels was performed essentially according to Ouchterlony [20]. The gels were rinsed in phosphate-buffered saline and stained for enzymatic activity using the chromogenic substrate [21].

Radial Immunodiffusion Assay was performed in 1.5% Agarose gels containing either goat antiserum to hexosamidase B (1:500) or goat specific antiserum to hexosaminidase A (1:100), as described previously [19].

Enzyme Binding Assay

The enzyme binding assay was performed by incubating a constant amount of enzyme with increasing amounts of antiserum. The final serum concentration was kept constant by the addition of normal serum. Antiserum against the immunoglobulin G which had been employed in the first stage (rabbit antiserum to goat immunoglobulin G) or goat antiserum to rabbit immunoglobulin G was subsequently added in an amount sufficient to precipitate all the immunoglobulin G present. Following 30 min at 37 °C and about 16 h

at 4 °C the precipitate was spun down and the enzymatic activity was assayed.

Immunoadsorbents

Pure hexosaminidase A or B were coupled to cyanogen-bromide-activated Sepharose 4B according to Porath et al. [22]. 2 mg of each isozyme were coupled per 1 g of settled Sepharose gel.

Antibody Mediated Stabilization of Hexosaminidase A

Stabilization of hexosaminidase A from normal or from variant origin was performed as described previously [23].

RESULTS

Isolation and Biochemical Characterization of Hexosaminidase A from Postmortem Liver Tissue

Autopsy liver from the patient with variant AB contained hexosaminidases A (0.014 U/mg protein) and B (0.010 U/mg protein) activities which are well within the normal range. In brain tissue which exhibited a storage of glycosphingolipids G_{M2} and G_{A2} in the range reported before [3], the activities of both isoenzymes A and B were up to 0.009 and 0.006 U/mg protein, which are about twice the normal values. As shown in Fig. 1 the isoelectric points of both isoenzymes were, as expected, pH 5.0 for the A enzyme and at pH 7.1 to 7.3 for the B enzyme. Hexosaminidase B from the brain extract appeared, as is normally observed, as a broad peak after isoelectric focusing. Purification of both isoenzymes of the liver tissue (Table 1) was performed according to a procedure detailed elsewhere [12], employing chromatography on concanavalin A-Sepharose and on an affinity gel to which 6-aminohexyl-2-acetamido-2-deoxy-1-thio-β-D-galactopyranoside was bound as ligand. Specific elution of hexosaminidase activity adsorbed to the affinity gel was achieved with the competitive inhibitor of the enzymes, *N*-acetylglucosaminolactone. In the final step hexosaminidase A was separated from the B enzyme by ion-exchange chromatography and obtained as an apparently homogeneous protein with the same electrophoretic mobility as that of the normal A enzyme in 7.5% acrylamide gel at pH 8.9 employing the procedure of Ornstein [24]. The activity of hexosaminidase A isolated from pathological liver tissue exhibited a normal pH dependence (Fig. 2) and heat lability (Fig. 3) when tested with synthetic substrates. Its substrate specificity was indistinguishable from that of a normal enzyme preparation (Table 2). Michaelis-Menten values and maximal velocities obtained with 4-methylumbelliferyl derivatives of β-*N*-acetyl-glucosaminide and β-*N*-acetyl-galactosaminide

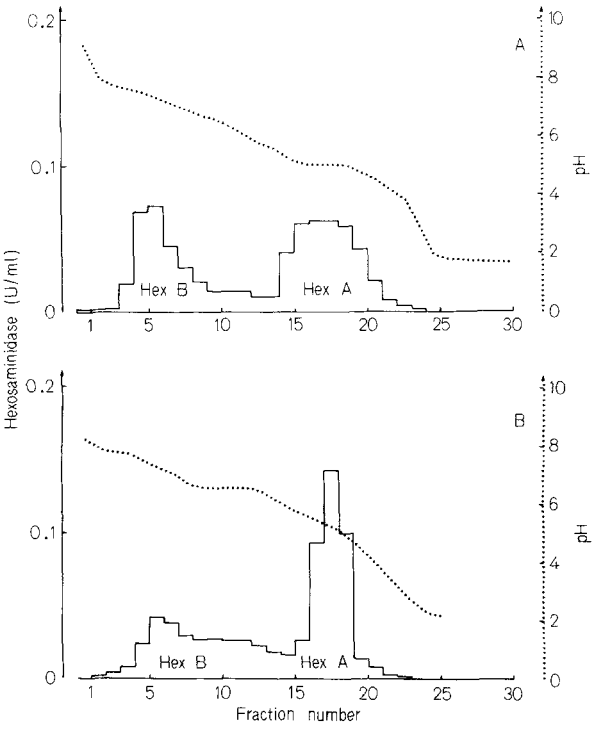

Fig. 1. *Hexosaminidase pattern in liver (A) and brain (B) tissue of a patient with variant AB of infantile G_{M2} gangliosidosis.* Curves were obtained by isoelectric focusing as described in Materials and Methods

Table 1. *Purification of hexosaminidase A and hexosaminidase B from liver of a patient with variant AB of infantile G_{M2} gangliosidosis*

Step	Enzyme activity		Purification	Yield
	U	U/mg protein	-fold	%
Homogenate	62.4	0.024	1	100
Extract	57.5	0.060	2.5	92.2
Concanavalin-A Chromatography	54.5	1.30	54.1	87.4
Affinity Chromatography	22.0	18.03	753	35.2
Hexosaminidase A DEAE-Cellulose	11.0	92.0	5885[a]	27.1[a]
Hexosaminidase B DEAE-Cellulose	5.6	–[b]	–[b]	25.6[a]
CM-Cellulose	1.2	–[b]	–[b]	5.6[a]

[a] Calculated on the basis of 62% of total hexosaminidase activity being hexosaminidase A and 38% of it being hexosaminidase B in the extract.

[b] Protein not determined due to its low concentration.

as well as with the glycosphingolipid G_{A2}, one of the storage substances of this variant disease, were as expected for the normal hexosaminidase A. Furthermore, the degradation of glycolipid G_{A2} was inhibited to the same extent by ganglioside G_{M2} for both hexosaminidase A preparations obtained from patholo-

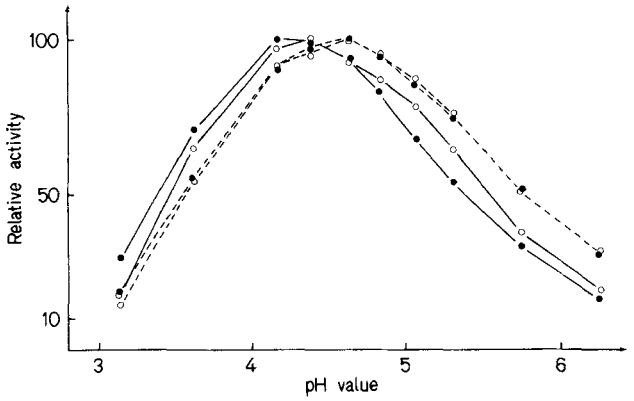

Fig. 2. *Effect of pH on the activity of hexosaminidase A purified from variant AB and normal liver tissue.* pH values were measured in the incubation mixtures. All values were performed in duplicate. Substrates and enzymes: (O——O) 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside, normal liver enzyme; (O- - -O) 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, normal liver enzyme; (●——●) 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-galactopyranoside, AB variant liver enzyme; (●- - -●) 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, AB variant liver enzyme

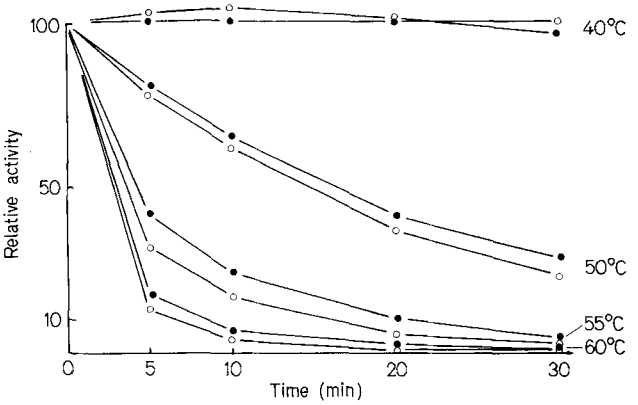

Fig. 3. *Heat lability of hexosaminidase A purified from variant AB and normal liver tissue.* Enzyme samples of 1.5 mU in 50 µl of 50 mM citrate buffer, pH 4.4, containing 0.01% bovine serum albumin were heated at different temperatures. The heat inactivation was stopped by cooling on ice after different time intervals as indicated in the abscissa. Residual hexosaminidase activity was tested under standard conditions (see Materials and Methods). (O—— O) Normal liver enzyme; (●——●) AB variant liver enzyme

Table 2. *Hydrolysis of synthetic and natural substrates by hexosaminidase A purified from variant AB and normal liver tissue (each value represents the mean of at least 3 determinations (±S.D.)*
The detergent, sodium taurodeoxycholate, when present was 2 mM

Hexosaminidase from liver of	Hydrolysis of								
	4-Methylumbelliferyl-*N*-acetyl-β-D-glucosaminide		4-Methylumbelliferyl-*N*-acetyl-β-D-galactosaminide		Glycosphingolipid G_{A2}		Ganglioside G_{m2}		
	K_m	V	K_m	V	K_m	V	K_i[a]	no detergent	with detergent
	mM	U/mg	mM	U/mg	mM	U/mg	mM	nmol × h^{-1} × U^{-1}	
Variant AB	0.80 ±0.03	160 ± 15	0.09 ±0.003	14 ± 2	0.06 ±0.01	0.20 ±0.03	0.018 ±0.006	<0.04	1.9 ±0.5
Normal	0.85 ±0.03	160 ± 15	0.09 ±0.004	14 ± 2	0.05 ±0.01	0.15 ±0.03	0.016 ±0.006	<0.04	2.2 ±0.5

[a] Inhibition constant for the degradation of glycosphingolipid G_{A2}.

gical and normal tissue. In the presence of 2 mM sodium taurodeoxycholate both preparations degraded ganglioside G_{M2}, the major storage compound, with a comparable velocity (Table 2). Freezing and thawing experiments demonstrated that hexosaminidase A purified from pathological tissue was converted to a similar extent to the B isozyme as found in the case of the normal A enzyme.

IMMUNOCHEMICAL PROPERTIES OF HEXOSAMINIDASES A AND B FROM LIVER AND FIBROBLASTS OF VARIANT AB

Immunochemical Characterization

Hexosaminidase A of normal and variant AB liver were tested in double immunodiffusion in agar gels. The antisera used were goat antisera to the A and to the B isozymes as well as specific antibodies to the hexosaminidase A unique determinants. As depicted in Fig. 4 the normal and the variant enzyme exhibit antigenic identity, namely complete cross reactivity with all three antisera.

Radial Immunodiffusion Assay

The relative antigenicity as measured with antiserum to hexosaminidase B and with specific antiserum to hexosaminidase A was established for pure hexosaminidase A of normal and of variant AB liver as well as for pure normal hexosaminidase B and crude preparations of hexosaminidase A and hexosaminidase B of the variant AB, using a serial 1:2 dilution (not

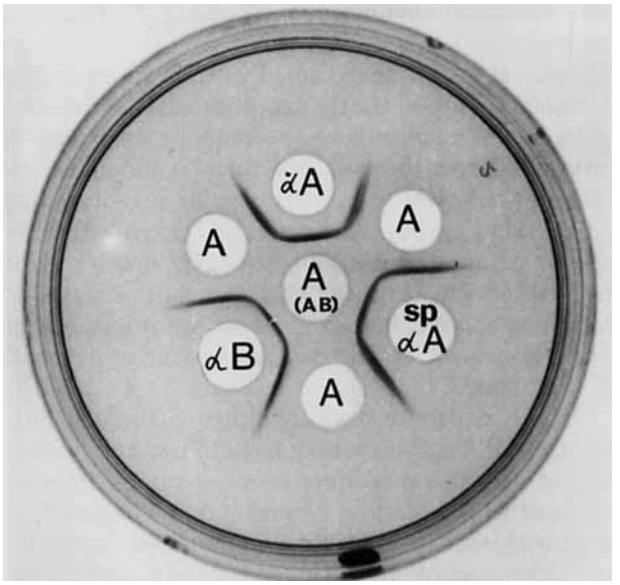

Fig. 4. *Double immunodiffusion assay of normal and variant AB hexosaminidase A (A and A(AB) respectively) with antisera against pure hexosaminidase A (αA), against pure hexosaminidase B (αB) and specific antiserum to hexosaminidase A (spαA).* The plate was stained for enzymatic activity

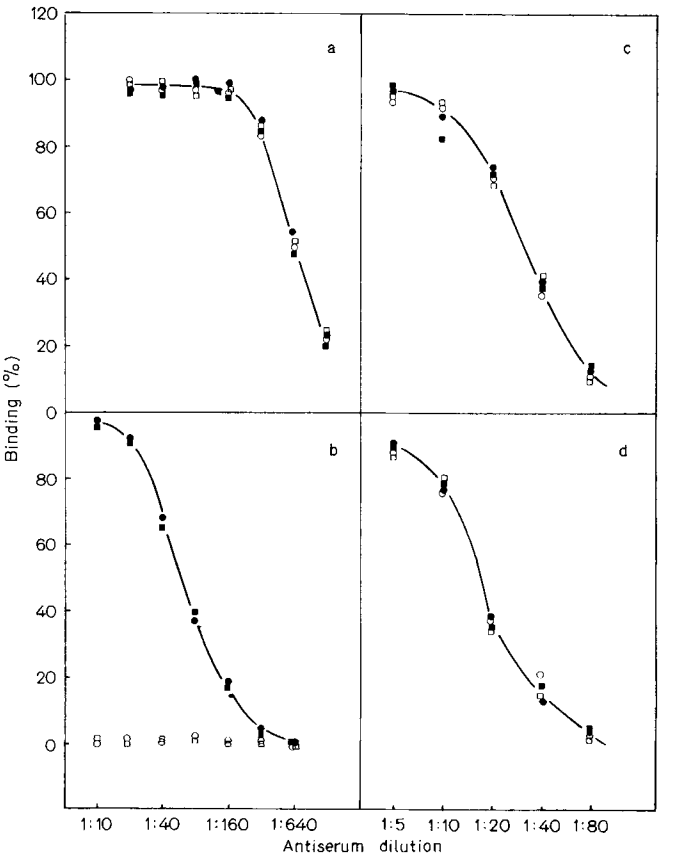

Fig. 5. *Enzyme binding assay of hexosaminidase A and hexosaminidase B of normal or variant AB origin.* Normal hexosaminidase A (●), normal hexosaminidase B (○), variant AB hexosaminidase A (■), variant AB hexosaminidase B (□). The antisera tested were: antiserum to normal hexosaminidase B (a), specific antiserum to normal hexosaminidase A (b), antiserum to hexosaminidase A of variant AB (c), and antiserum to hexosaminidase B of variant AB (d)

shown). A good correlation was obtained between the activity applied to each well (in the gel containing antiserum to hexosaminidase B) and the net area of the ring in the range of 20 – 80 mm^2. In the gel containing the specific antiserum to hexosaminidase A rings were obtained only with hexosaminidase A from the normal as well as of the variant AB liver. The relative areas of the different hexosaminidase A preparations in two gels were almost the same for the normal and for the variant enzymes. The B isozyme from either source reacted with the antiserum to hexosaminidase B but not with the specific antiserum to hexosaminidase A.

Enzyme Binding Assay

Enzyme binding assays were performed with pure liver hexosaminidase A of normal and variant AB individuals as well as with pure placental hexosaminidase B. In addition crude hexosaminidases A and B from either liver or fibroblasts were tested. Enzyme activity of all the samples was constant, about 1.0 mU per assay. The antisera employed were antiserum to hexosaminidase B and specific antiserum to hexosaminidase A (prepared in goats) and antiserum to hexosaminidase A and antiserum to hexosaminidase B of variant AB liver (prepared in rabbits). The results are shown in Fig. 5. Identical results (not shown in figure) were also obtained with crude liver hexosaminidase A and also both the B and A isozymes from fibroblasts of the AB variant. As might be concluded from the figure the response of both isozymes obtained from the variant individual with the various antisera, including antisera to the variant's enzymes is practically indistinguishable from that of the normal enzymes. It should be emphasized that in spite of the fact that the antisera to the variant enzymes were relatively weak (due to the limited amount of injected enzyme) these antisera were still capable of binding both the A and B enzymes, isolated from either normal or variant origin, almost to the same extent.

It was also found that the interaction of the various antisera with the A isozyme of the variant AB source resulted in a similar effect to that observed previously with normal hexosaminidase A, namely, in a significant stabilization of the enzymes towards heat inactivation (Fig. 6). In this experiment 50 µl of enzyme solutions were incubated for 2 h with an equal volume of normal serum and of antiserum to hexosaminidase A of either normal or variant AB origin. As depicted in the figure, both immune sera increased the stability of normal and variant hexosaminidase A preparation to an

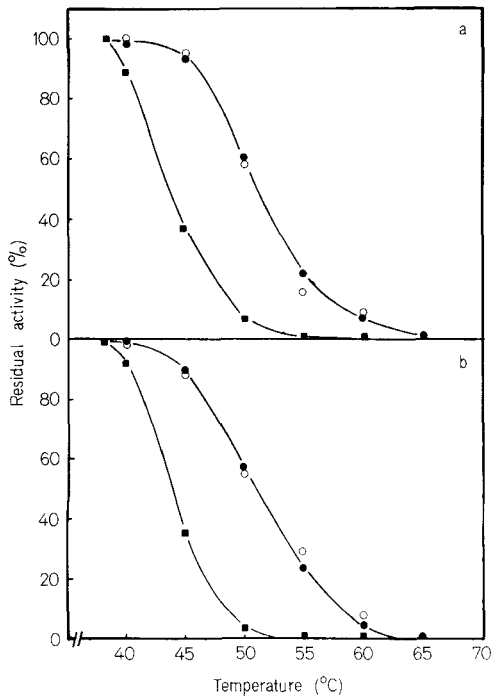

Fig. 6. *Temperature-dependent inactivation of normal (a) and of variant AB (b) hexosaminidase A in the presence of normal rabbit serum (■), antiserum to normal hexosaminidase A (●) and antiserum to variant AB hexosaminidase A (○)*

identical extent, bringing about an elevation of 9–10 degrees in the temperature giving 50% inactivation under these conditions.

Further attempts to detect possible minor antigenic sites which are unique to the variant isozymes were carried out by adsorption of antisera to the variant isozymes on immunoadsorbents containing normal hexosaminidase A or B, and a subsequent enzyme binding test with the non adsorbed fractions. It was found that the entire binding capacity was removed by the adsorption, indicating that the variant enzymes do not carry any detectable unique antigenic determinants which are not present on the normal hexosaminidases.

DISCUSSION

Neuronal storage of ganglioside G_{M2} is correlated in variant B (Tay-Sachs disease) and variant 0 of infantile G_{M2} gangliosidosis with a recessively inherited deficiency of hexosaminidase A and of the A and B isoenzymes, respectively. Studies *in vitro* showed that the A isoenzyme is capable of degrading the storage compound in the presence of detergents such as sodium taurodeoxycholate or naturally occurring activator proteins whereas the B isoenzyme exhibits a minor activity against G_{M2} ganglioside and then only in presence of sodium taurodeoxycholate [12]. Therefore the excessive neuronal storage of ganglioside G_{M2} in AB variant is still unexplained since hexosaminidase A remains active in the tissues of the afflicted patients [3,15,25]. This phenomenon could be explained in two ways. Either the patients produce a mutant hexosaminidase A which is not capable of degrading ganglioside G_{M2} but remains active on synthetic substrates, or an operational mechanism necessary for the interaction between G_{M2} ganglioside and its degrading enzyme, hexosaminidase A, for instance an activator protein, could be either missing or aberrant in this disease.

In this paper we have investigated the first possibility. The A enzyme was isolated by a simple affinity chromatographic procedure from an autopsy liver of a patient who died from variant AB of infantile G_{M2} gangliosidosis. Electrophoretic mobility, substrate specificity against synthetic and natural substrates, and kinetic data obtained with the purified enzyme all appeared to be identical to those of a normal enzyme preparation. In particular, hexosaminidase A isolated from the pathological liver was still capable of degrading normally both storage compounds G_{M2} and G_{A2} under the conditions tested. These studies gave no hint of a possible alteration of the patient's hexosaminidase A.

Recent studies have shown that the human isoenzymes A and B have a subunit in common, hexosaminidase A being a heteropolymer with the subunit structure $\alpha_2\beta_2$ and hexosaminidase B being a homopolymer with the subunit structure $\beta_2\beta_2$ [26]. This composition explains freezing and thawing experiments which result in a conversion of A to the B isoenzyme [27]. A similar conversion of hexosaminidase A from the pathological liver indicates that its composition is unchanged. To test its structure in more detail, the antigenicity of hexosaminidase A purified from the patient's liver was tested with various antisera against either the A and B isoenzymes from normal and variant AB individuals, as well as with a preparation of antibodies reactive exclusively with hexosaminidase A. Various techniques such as antigen binding, double and radial immunodiffusion, and testing of the capability of the antisera to stabilize the enzyme towards heat inactivation, demonstrated antigenic identity between the respective isozymes of the normal and variant AB sources.

It is concluded that the hexosaminidases produced by these patients are not different from those of normal individuals. Thus the defect involved in the disease is not at the genetic level of production of either α or β chains of hexosaminidases. It seems likely, therefore, that the second alternative mentioned above, namely a deficiency in an activator protein which is required for the activity of the enzyme *in vivo*, might be the cause of variant AB of G_{M2} gangliosidosis.

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E. Conzelmann, K. Sandhoff, and H. Nehrkorn, Neurochemische Abteilung, Max-Planck-Institut für Psychiatrie, Kraepelinstraße 2, D-8000 München 40, Federal Republic of Germany

B. Geiger and R. Arnon, Department of Chemical Immunology, The Weizmann Institute of Science, P.O. Box 26, Rehovot, Israel