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ACID GLYCOHYDROLASE IN CHINESE HAMSTER WITH SPONTANEOUS DIABETES

II. *N*-ACETYL-β-D-HEXOSAMINIDASE IN PLASMA AND TISSUES

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Summary

Excessively high activity of *N*-acetyl-β-D-hexosaminidase (2-acetamido-2-deoxy-β-D-glucoside acetamidodeoxy-glucohydrolase, EC 3.2.1.30) was found in the plasma of hereditary diabetic XA line animals, which however showed similar activity of this enzyme in both 12 000 × *g* supernatant and precipitate fractions of kidney homogenates as the nondiabetic M line animals. 0.1% Triton X-100 extracts of kidney, spleen, hind leg muscle, cheek pouch and spinal cord of XA and M line animals also showed similar activities of this enzyme whereas the XA animal liver extracts showed significantly higher activity than the M extracts. On a Sepharose CL-6B column, plasma *N*-acetyl-β-D-hexosaminidase was eluted as two major peaks at 0 and 0.05 M NaCl (isozyme B1 and B2). Both isozymes showed pH optima between 3.5 and 4.0 and the same Michaelis constants for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide at pH 4.5, i.e. 0.18 mM. Isozymes from XA and M animals showed identical properties. *N*-acetyl-β-D-hexosaminidase in the liver extracts was separated into 3 isozymes, A, B1 and B2, by successive column chromatography runs on Sepharose 6B and DEAE-Sepharose CL-6B. At 49°C, isozyme B1 showed thermostability whereas B2 and A lost 20% and 76% of their activities after 30 min incubation. pH optima for A, B1 and B2 were 4.0—4.5, 3.5 and 3.5—4.0 respectively. The K_m values for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were 0.48 mM for A and 0.19 for B1 and B2. The XA animal liver extracts showed higher activities in all three isozymes than the M animal livers. Identical results, however, were obtained for liver isozymes from M and XA animals with regard to thermostability, pH vs. activity, elution profile on ion exchange column and affinity to *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

Introduction

Elevated urinary excretion [1] and abnormally high activity in serum [2,3] and tears [3] of *N*-acetyl-β-D-hexosaminidase (β-2-acetamido-2-deoxy-D-hexoside acetamidodeoxyhexohydrolase, EC 3.2.1.30) have been reported in human diabetic patients. In experimental diabetic rats induced by streptozotocin, this enzyme was reported to be increased in the serum, decreased in the kidney and remained normal in the intestine, liver and spleen, when the activity was measured in the 10 000 × *g* tissue extracts [4]. Fushimi and Taruri further postulated that the elevation of this enzyme activity in the serum was derived from kidney and that its abnormal level in tissues may play a role in the development of microangiopathy [4,5]. This study was designed to see if similar changes also occurred in hereditary diabetic Chinese hamsters.

In human tissues, *N*-acetyl-β-D-hexosaminidase exists as two isozymes, A being acidic and thermolabile and B more basic and relatively thermostable [6]. In certain human diseases such as Tay-Sachs and Sandhoff's, different isozymic forms of this enzyme are present in deficient states [6]. Therefore, this study was further carried out to determine if the abnormal levels of *N*-acetyl-β-D-hexosaminidase in some tissues of the diabetic animals were a result of variant isozymes in the highly inbred lines of Chinese hamsters.

Materials and Methods

The selection of animals, the preparations of kidney 12 000 × *g* supernatant and precipitate fractions and of 0.1% Triton X-100 extracts from various tissues, blood sugar determination and measurements of protein concentration were carried out as described previously [7]. Enzyme assay was performed by incubating at 37°C 0.01 ml appropriately diluted samples and 0.99 ml 5.26 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in a buffer made by adjusting 0.1 M Na_2HPO_4 with 0.05 M citric acid to pH 4.5 and activity was calculated from the initial rate of reaction. The reaction was terminated by adding 0.2 ml 30% trichloroacetic acid and 0.2 ml 5 M 2-amino-2-methylpropanediol was added to the supernatant obtained by centrifugation. The products were measured at 415 nm with *p*-nitrophenol as standards.

One (XA) or 0.6 (M) ml pooled plasma samples were chromatogrammed on DEAE-Sepharose CL-6B columns (0.9 × 15 cm) equilibrated with 0.01 M sodium phosphate, 0.1% NaN_3, pH 6.0 and eluted with a linear gradient made by mixing 50 ml each of 0 and 0.25 M NaCl in the same buffer. 1-ml fractions were collected and 0.1-ml aliquots were assayed for enzyme activity at 37°C for 2 h. Fractions containing *N*-acetyl-β-D-hexosaminidase activity were pooled and concentrated in Minicon®.

The 12 000 × *g* supernatant fractions of 0.1% Triton X-100 extracts of liver tissue were filtered through a Sepharose 6B column (2.6 × 40 cm), equilibrated with 0.01 M sodium phosphate, pH 6.0, 0.1% NaN_3 and 5 mM NaCl and 3.5 ml fractions were collected. 0.05 ml aliquots of each tube were assayed for 2 h at 37°C. Peaks containing *N*-acetyl-β-D-hexosaminidase activity were pooled and directly chromatogrammed on a column of DEAE-Sepharose CL-6B (0.9 × 15 cm) equilibrated with 0.01 M sodium phosphate, pH 6.0, 0.1% NaN_3 and

eluted with a linear gradient made by mixing 50 ml each of 0 and 0.25 M NaCl in the same buffer. 1.5-ml fractions were collected and 0.1-ml aliquots were used to assay enzyme activity. The peaks were pooled and concentrated in Minicon®.

Heat inactivation study was carried out by incubating 0.25-ml aliquots of partially purified isozyme fractions (pH 6.0) in a 49°C water bath for 30 min, cooling in an ice/water mixture, and assaying the heated and nonheated aliquots simultaneously.

Results

Activity of N-acetyl-β-D-hexosaminidase in plasma and renal 12 000 × g supernatant and particulate fractions of M and XA Chinese hamsters

Table I summarizes the study on a group of hyperglycemic (383 ± 30 mg/dl) XA line animals, male and 9—57 weeks of age, and their sex- and age-matched nondiabetic (117 ± 5 mg/dl) M line animals. The two groups of animals showed similar body and kidney weights and similar protein concentrations in kidney fractions and plasma. The activities of *N*-acetyl-β-D-hexosaminidase in the 12 000 × *g* supernatant and particulate fractions were also not significantly different between XA and M line animals whereas the XA animals showed twice as much activity of this enzyme in the plasma as the matched M line animals. In a study on six young (20—23 days) XA line males before the onset of hyperglycemia and their age-matched M line animals [7], similar levels of *N*-acetyl-β-D-hexosaminidase were also observed in the 12 000 × *g* precipitate fractions of kidney extracts (3180 ± 166 (M) vs. 3238 ± 77 (XA) unit/g, $P > 0.5$) and the XA supernatant fractions showed slightly higher activity than the M fractions (1092 ± 50 (XA) vs. 886 ± 77 (M) unit/g, $P < 0.05$). The plasma of these young animals was not assayed because the sample were used for other purposes.

Activity of N-acetyl-β-D-hexosaminidase in 0.1% Triton X-100 extracts of various tissue of M and XA Chinese hamsters

The animals used in the study presented in Table II were males and 11—23 months of age. The kidneys of these animals contained 5415 ± 152 (M line) and 5427 ± 202 (XA line) unit/g *N*-acetyl-β-D-hexosaminidase. The 0.1% Triton X-100 buffer was able to solubilize about 77% of the total activity in the kidney. The activity of *N*-acetyl-β-D-hexosaminidase was also measured in the 0.1% Triton X-100 extracts and no significant difference between XA and M animals could be detected in kidney, spleen, hind leg muscle, cheek pouch and spinal cord. The liver of XA animals, however, showed excessively high activity of this enzyme. The XA animals also showed excessively large kidney (0.94 ± 0.03 vs. 1.25 ± 0.07% body weight, $P < 0.05$) and spleen (0.10 ± 0.01 vs. 0.17 ± 0.01% body weight, $P < 0.001$) but their body and liver weights were normal as compared to the matched M animals. The activity of *N*-acetyl-β-D-hexosaminidase per unit weight was highest in the kidney and decreased in the following order: kidney > spleen > spinal cord > cheek pouch > liver > hind leg muscle and the order was the same for both M and XA lines.

Plasma N-acetyl-β-D-hexosaminidase of M and XA Chinese hamsters

The plasma samples from M or XA line animals studied in Table II were

TABLE I

KIDNEY AND PLASMA ACTIVITY OF *N*-ACETYL-β-D-HEXOSAMINIDASE IN AGE-MATCHED MALE CHINESE HAMSTERS OF NON-DIABETIC M AND DIABETIC XA LINES

One unit is defined as the amount of enzyme which liberated 1 nmol of *p*-nitrophenol per min. All values are mean ± S.E.M. (No. of animals). "N.S." denotes not significant, i.e. $P > 0.05$ according to Student's *t*-test.

Measurement	Unit	M-line	XA-line	*P*
Blood sugar	mg/dl	117 ± 5 (11)	383 ± 30 (11)	<0.001
Body weight	g	30.6 ± 1.5 (11)	33.6 ± 2.0 (11)	N.S.
Kidney				
weight	% body wt.	0.99 ± 0.02 (11)	1.06 ± 0.03 (11)	N.S.
12 000 × *g* supernatant				
protein concentration	mg/g	92.1 ± 2.0 (11)	87.7 ± 1.6 (11)	N.S.
N-Acetyl-β-D-hexosaminidase	unit/g	1652 ± 77 (11)	1478 ± 136 (11)	N.S.
12 000 × *g* precipitate				
protein concentration	mg/g	83.5 ± 3.6 (11)	89.3 ± 3.1 (11)	N.S.
N-Acetyl-β-D-hexosaminidase	unit/g	2422 ± 134 (11)	2383 ± 145 (11)	N.S.
Plasma				
protein concentration	mg/ml	64.2 ± 2.1 (11)	67.2 ± 1.2 (11)	N.S.
N-Acetyl-β-D-hexosaminidase	unit/ml	56.9 ± 1.8 (11)	107.7 ± 13.8 (11)	<0.001

TABLE II

ACTIVITY OF *N*-ACETYL-β-D-HEXOSAMINIDASE IN 0.1% TRITON X-100 EXTRACTS FROM VARIOUS TISSUES OF AGE-MATCHED MALE CHINESE HAMSTERS OF NONDIABETIC M AND DIABETIC XA LINES

For details see Table I.

Tissue	Unit/g fresh tissue		*P*
	M-line	XA-line	
Kidney	4219 ± 142 (7)	4187 ± 142 (7)	N.S.
Liver	420 ± 34 (7)	787 ± 109 (7)	<0.025
Spleen	2007 ± 36 (7)	2456 ± 171 (7)	N.S.
Hind leg muscle	190 ± 15 (7)	126 ± 24 (7)	N.S.
Cheek pouch	675 ± 100 (7)	769 ± 82 (7)	N.S.
Spinal cord	1089 ± 117 (7)	906 ± 66 (7)	N.S.

pooled and filtered on DEAE-Sepharose CL-6B columns (0.9 × 15 cm) and the elution profile is shown in Fig. 1; its activity was split into two major peaks, designated as isozymes B1 and B2, which were eluted off the column at 0 and 0.05 M NaCl respectively. A small peak was also detected at 0.09 M NaCl and it was not collected due to its relatively low activity. The pooled XA plasma (1 ml applied to column) showed about 4 times as much activity as the pooled M sample (0.6 ml on column) in both B1 and B2 peaks. The pooled and concentrated B1 and B2 isozymes were measured for their activities from pH 2.5 to

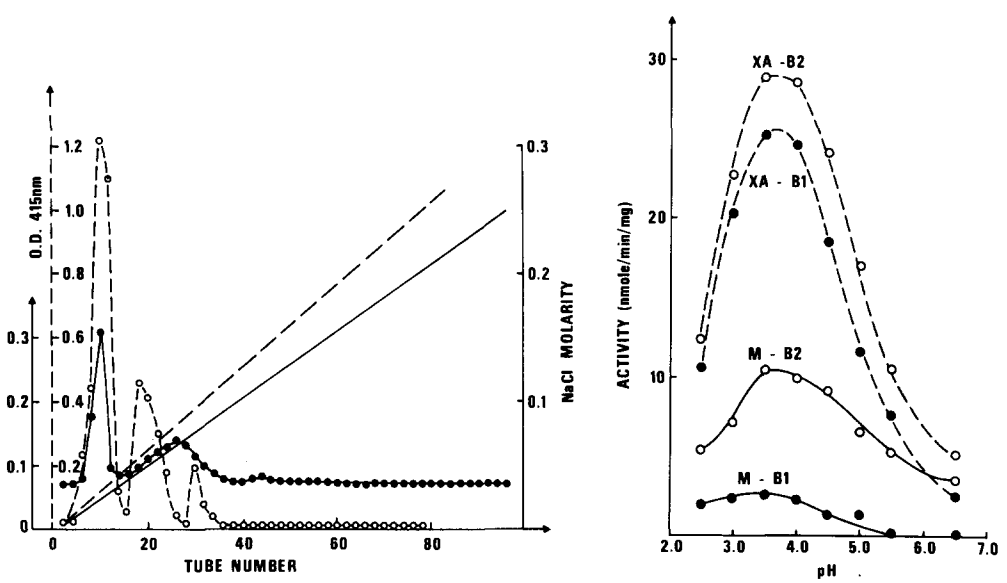

Fig. 1. Elution profiles of plasma *N*-acetyl-β-D-hexosaminidase of M and XA Chinese hamsters on DEAE-Sepharose CL-6B column. Column size was 0.9 × 15 cm. Each tube contained a 1.0-ml fraction. ●———●, M line; ○- - - - -○, XA line.

Fig. 2. Activity vs pH's of Chinese hamster plasma *N*-acetyl-β-D-hexosaminidase isozymes. Buffers were made by adjusting 0.1 M Na_2HPO_4 with 0.05 M citric acid to desired pH. Solid lines, M line; broken lines, XA line.

6.5 and the results are shown in Fig. 2. The optimal pH's for both B1 and B2 were between 3.5 and 4.0 and were the same for M and XA samples. Likewise, the Lineweaver-Burk plots of plasma *N*-acetyl-β-D-hexosaminidase B1 and B2 at pH 4.5 were the same (Fig. 3), yielding a K_m of 0.16 mM for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide.

Chromatography of liver 0.1% Triton X-100 extracts from M and XA Chinese hamsters on Sepharose 6B column

Preliminary studies with kidney and spleen extracts on DEAE-Sepharose CL-6B column revealed that Chinese hamster *N*-acetyl-β-D-hexosaminidase could be separated into 2 isozymes: A (acidic at pH 6.0 and eluted at 0.125 M NaCl) and B (basic at pH 6.0 and eluted near void volume). Isozyme B was coeluted with bulk proteins and, thus, poorly purified. Furthermore, when plasma samples were chromatographed on DEAE-Sepharose CL-6B, isozyme B was separated into two closely occurring peaks of activity, one at void volume and another at the initiation of salt gradient (Fig. 1). Therefore it was decided to filter through the liver extracts on a Sepharose 6B column in order to remove the bulk proteins first and then chromatogram the fractions with enzyme activity on ion exchange column to obtain the isozymes.

The liver extracts used were obtained from a 12-month old male XA animal (251 mg sugar/100 ml blood) with extremely high liver *N*-acetyl-β-D-hexosaminidase activity (1391 unit/g) and the age- and sex-matched M (60 mg/100 ml and 442 unit/g). This XA animal also had an enlarged liver (3.4 g vs. 2.0 g) but protein concentrations in the liver extracts were similar between the two. Fig. 4 shows the elution profiled of 12 000 × *g* supernatant of 0.1% Triton X-100

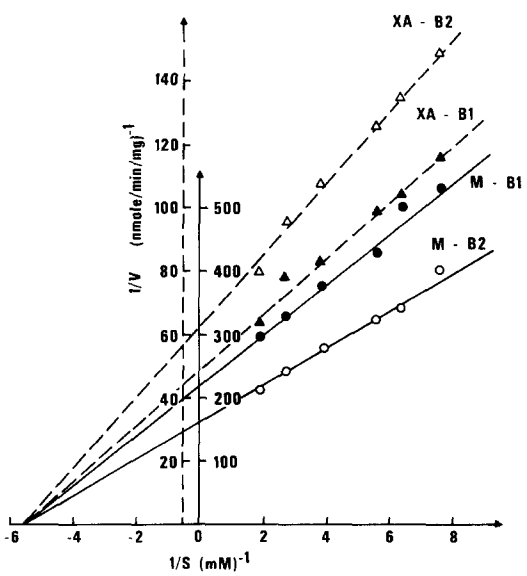

Fig. 3. Activity vs. substrate concentrations of Chinese hamster plasma *N*-acetyl-β-D-hexosaminidase isozymes. Activity was measured at pH 4.5 with *N*-acetyl-β-D-glucosaminide as substrate. ●———●, M line; ○- - - - -○, XA line.

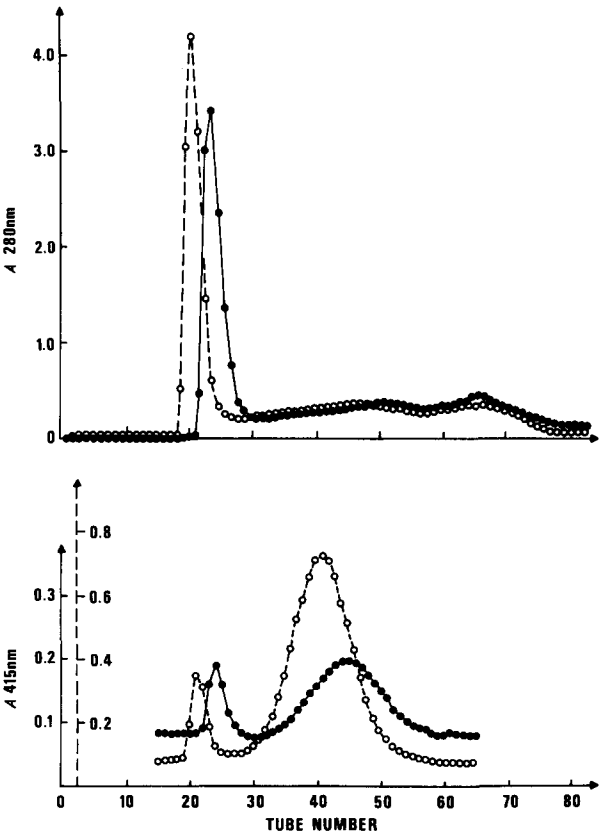

Fig. 4. Elution profile of Chinese hamster liver *N*-acetyl-β-D-hexosaminidase on a Sepharose 6B column. Column size was 2.6 × 40 cm. Each tube contained a 3.5 ml-fraction. ●———●, M line; ○- - - - -○, XA line.

extracts on Sepharose 6B column and two peaks were obtained for both M and XA samples. The slightly shorter retention times for both the bulk proteins and enzyme peaks of the XA sample on the same column were due to the fact that the column became more densely packed during the run of the XA sample which followed the M sample. In either case, peak I was coeluted with the bulk proteins and peak II contained the majority of enzyme activity and corresponded to the molecular size of $1-3 \cdot 10^5$ (Fig. 4).

Chromatography of enzyme peaks obtained from Sepharose 6B column on DEAE-Sepharose CL-6B columns

When peak I collected from Sepharose 6B column chromatography was eluted through DEAE-Sepharose CL-6B column, no *N*-acetyl-β-D-hexosaminidase activity was recovered with a linear salt gradient of 0—0.25 M NaCl from either M or XA sample. The result indicated that the enzyme activity detected in peak I was bound to particles and/or membranes which were solubilized in 0.1% Triton X-100 and could not be removed readily from the ion-exchange column. When peak II was eluted through DEAE-Sepharose CL-6B column with the same salt gradient, three peaks of enzyme activity were obtained for

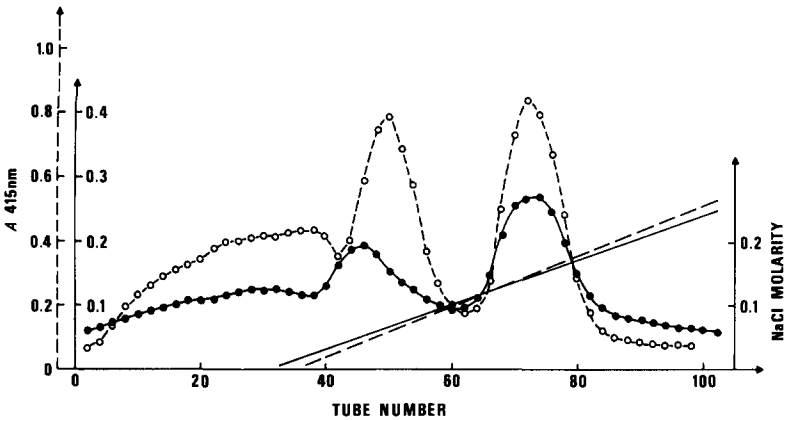

Fig. 5. Elution profile of peak II (tubes 35—50) in Fig. 4 on a DEAE-Sepharose CL-6B column. Column size was 0.9 X 15 cm. Each tube contained a 1.5-ml fraction. ●———●, M line; ○- - - - -○, XA line.

both M and XA samples, designated as isozyme B1 (void volume), B2 (after initiation of salt gradient) and A (0.125 M NaCl) and the profiles are shown in Fig. 5. Isozyme B1 from M sample was not pooled because of its low activity and the other fractions were pooled and concentrated for further studies.

Thermostability, pH dependence and Michaelis constants of liver N-acetyl-β-D-hexosaminidase isozymes from M and XA Chinese hamsters

Among the isozymes, *N*-acetyl-β-D-hexosaminidase B1 was least thermolabile and isozyme B1 from the XA animal retained 97% of its activity after incubating at 49°C for 30 min (Table II). Under identical condition, isozyme B2 lost 19—20% of its activity whereas isozyme A was most heat-sensitive and retained only 24% of its activity. Isozymes A and B2 from M and XA animals showed identical thermosensitivities.

Fig. 6 represents pH dependence of Chinese hamster liver *N*-acetyl-β-D-hexosaminidase. Isozyme A from either M or XA animals showed pH optimum at 4.0—4.5 and its activity fell sharply at either end of the pH curve; whereas M

TABLE III

THERMAL INACTIVATION OF LIVER *N*-ACETYL-β-D-HEXOSAMINIDASE ISOZYMES OF AGE-MATCHED MALE CHINESE HAMSTERS OF NONDIABETIC M AND DIABETIC XA LINES

For details see Table I.

Isozyme	Incubation at 49°C for 30 min	Specific activity (unit/mg protein)		% remaining	
		M-line	XA-line	M-line	XA-line
B1	—	—	26.9	—	
	+	—	26.0	—	97
B2	—	8.14	24.1		
	+	6.51	19.6	80	81
A	—	1.83	23.2		
	+	0.43	5.6	24	24

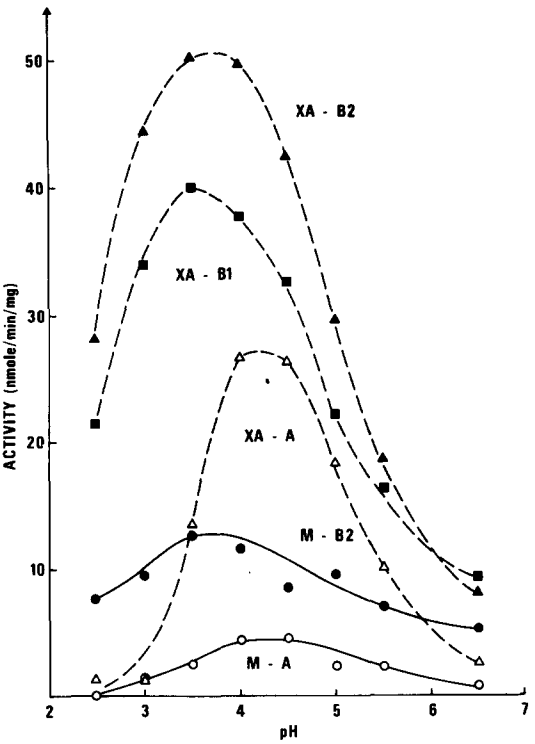

Fig. 6. Activity vs. pH of Chinese hamster liver *N*-acetyl-β-D-hexosaminidase isozymes. Buffer were made by adjusting 0.1 M Na_2HPO_4 with 0.05 M citric acid to desired pH. Solid lines, M line; broken lines, XA line.

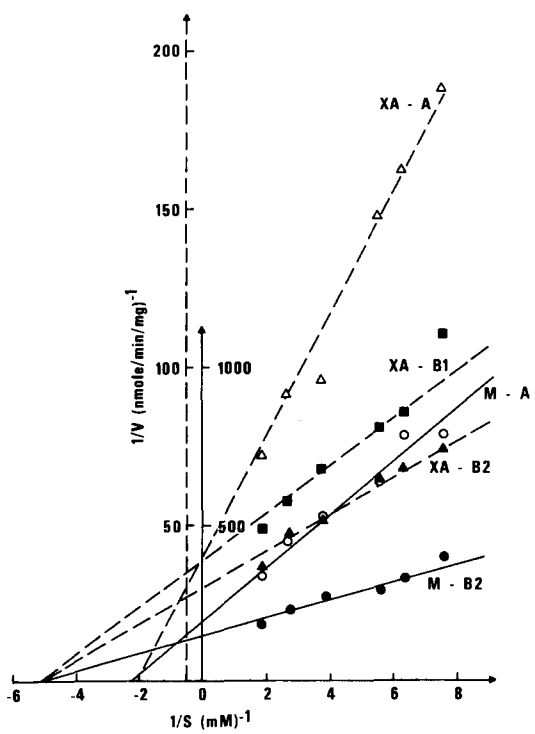

Fig. 7. Activity vs. substrate concentrations of Chinese hamster liver *N*-acetyl-β-D-hexosaminidase. Activity was measured at pH 4.5 with *N*-acetyl-β-D-glucosaminide as substrate. Solid lines, M line; broken lines, XA line.

and XA isozyme B2 showed an optimal pH between 3.5 and 4.0 and still retained considerable activity (50% of that at optimal pH) at the acidic pH extreme, i.e. 2.5. Isozyme B1 of XA line had pH optimum at 3.5 and also retained 50% activity at pH 2.5. The Lineweaver-Burk reciprocal plot produced the same Michaelis constants for isozyme B2 of M and XA and XA isozyme B1, i.e. 0.19 mM for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Fig. 7). The K_m values for isozyme A from M and XA samples were also identical, i.e. 0.48 mM.

Discussion

The data obtained in this study showed that, analogous to the elevated serum levels in human diabetic patients [2,3] and diabetic rats induced by streptozotocin [4], the hereditarily diabetic Chinese hamsters had elevated level of *N*-acetyl-β-D-hexosaminidase in the plasma. However, in contrast to what was reported in the streptozotocin-diabetic rats [4,5], the hereditary diabetic Chinese hamsters did not have depressed level of this enzyme in their kidneys but had increased activity in the liver. Furthermore the elevation of *N*-acetyl-β-D-hexosaminidase in the hereditary diabetic Chinese hamsters was limited only to the liver and not found in other tissues such as spleen, spinal cord, cheek pouch, hind leg muscle or kidney. The depression of renal *N*-acetyl-β-D-hexosaminidase reported in streptozotocin-diabetic rats [4,5] might arise from the direct effects of streptozotocin in the kidney, since in diabetic M-line Chinese hamsters induced by streptozotocin injection, depression of renal *N*-acetyl-β-D-hexosaminidase was observed two weeks after the injection of streptozotocin and its activity returned to normal at 6 weeks after injection [9].

The present study demonstrated that at least three isozymic forms of *N*-acetyl-β-D-hexosaminidase were present in the tissues of the Chinese hamster. The acidic isozyme, eluted at 0.125 M NaCl from DEAE-Sepharose CL-6B column at pH 6.0, was found to be the major form in the kidney (Chang, A.Y., unpublished data) and also present in the spleen and liver but essentially absent in the plasma. Based on its ionic character and thermolability, it appeared to correspond with isozyme A in human tissues [6] and was thus designated here. Two more basic isozymes were eluted in the earlier fractrions from DEAE-Sepharose CL-6B columns and were assigned as isozymes B1 and B2 in the order of their appearances. Their ionic characters and thermostability more mimicked human isozyme B [6].

Data from studies on pH dependence and substrate affinity suggest that isozyme A, B1 or B2 from various tissues and plasma were probably the same. The similarity between isozymes B1 and B2, evidence by studies on pH dependence and Km of isozymes obtained in different tissues, also led to the likelihood that these two isozymic forms were different versions of the same gene product. These two isozymes, however, showed different thermosensitivities. The mode of modification could involve cleavage of either a peptidic or a glycosidic bond and such changes would lead to minor difference in ionic character of the molecule which in turn could cause slight delay in the retention time on ion exchange column chromatography.

The absence of isozyme A in the plasma samples of both M and XA-line Chinese hamsters was surprising and in contrast to what had been reported in the sera of human [9] and rat [4]. It also raises the question as to why plasma contained certain isozymic forms but not others. The observation could arise from the preferred release of isozymes B1 and B2 from the tissues or, alternatively, the faster clearance of isozyme A from the circulation. In either case, structural differences in isozymes A and B1-B2 must play an important role in determining their concentrations in the plasma. A number of studies have been carried out on the clearance rates of proteins in the circulation and all data pointed to the pivotal role of carbohydrate structure in determining the survival time of protein in the plasma [10—13]. A good example is the study on the serum survival properties of ribonuclease; ribonuclease B, a mannose-terminated glycoprotein, was cleared rapidly from the serum whereas ribonuclease A, nonglycosylated, and C and D, containing sialic acid and fucose, showed a prolonged half-life in the serum [11]. Therefore, investigation on isozyme structural differences, in particular the terminating carbohydrate moieties, is required in order to delineate the cause for the predominance of *N*-acetyl-β-D-hexosaminidase isozymes B1 and B2 in the Chinese hamster plasma.

The studies reported here supported the notion that the different *N*-acetyl-β-hexosaminidase activities in the plasma and liver between M and XA line Chinese hamster were not a result of variant forms of enzyme since the isozymes from either line showed similar retention times on an ion-exchange column, pH vs. activity curves, K_m values for *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide and thermosensitivities. Furthermore, the young XA animals, studied before the onset of hyperglycemia showed similar activities of *N*-acetyl-β-D-hexosaminidase in the 100 000 × *g* liver supernatant fraction (668 ± 24 unit/g) as those in age- and sex-matched M line animals (618 ± 51 unit/g, $P > 0.05$). These lines of experimental evidence suggest that the elevation of liver *N*-acetyl-β-D-hexosaminidase levels in the hyperglycemic XA animals was a result of manifestation of diabetes. Although our data indicated the unlikelihood that the elevated level of *N*-acetyl-β-D-hexosaminidase in the diabetic XA animal plasma was largely derived from the kidney as postulated by Fushimi and Tarui [4], its source nevertheless stands unclear. A more important question, it appears, remains to be the consequences of such high levels of a degradative enzyme in the plasma and liver. What damage it would do to the tissues in relation to various forms of diabetic complications warrants further investigations.

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