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HUMAN URINARY *N*-ACETYL-β-HEXOSAMINIDASES

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SUMMARY

Analysis of urinary *N*-acetyl-β-hexosaminidases (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) by DEAE-cellulose chromatography revealed the presence of both the A and B isozymes and a new minor component designated *N*-acetyl-β-hexosaminidase M. This form, found only in male urine, was similar in its properties to the A form. The *N*-acetyl-β-hexosaminidase patterns were qualitatively similar between normal individuals and Tay–Sachs heterozygotes. *N*-Acetyl-β-hexosaminidase A was absent from urine of a Tay–Sachs patient.

INTRODUCTION

Multiple forms of *N*-acetyl-β-hexosaminidase (β-2-acetamido-2-deoxy-D-glucoside acetamidodeoxyglucohydrolase, EC 3.2.1.30) are known to exist in human tissues. At least two of these, designated A and B (ref. 1), have medical significance in that their deficiency has been associated with certain of the sphingolipidoses, namely Tay–Sachs disease[2], Sandhoff's disease[3], and juvenile G_{M_2} gangliosidosis[4]. Another form of the enzyme, *N*-acetyl-β-hexosaminidase P (ref. 5), has been isolated from human maternal serum, and other evidence suggests an even greater multiplicity of isozymic forms[6–9]. In this report we describe the characterization of yet another form of *N*-acetyl-β-hexosaminidase which has been observed in human urine. In addition, urine is shown to contain other of the known *N*-acetyl-β-hexosaminidases which makes it a convenient source of these enzymes for diagnosis and purification.

RESULTS

Chromatography of urinary *N*-acetyl-β-hexosaminidases on DEAE-cellulose produced a characteristic elution pattern with certain recognizable variations. Fig. 1 consists of two such elution profiles from adult male and female urine samples. The major component was eluted between tubes 50–80 and has been identified as *N*-acetyl-β-hexosaminidase A by its characteristic mobility upon DEAE-cellulose

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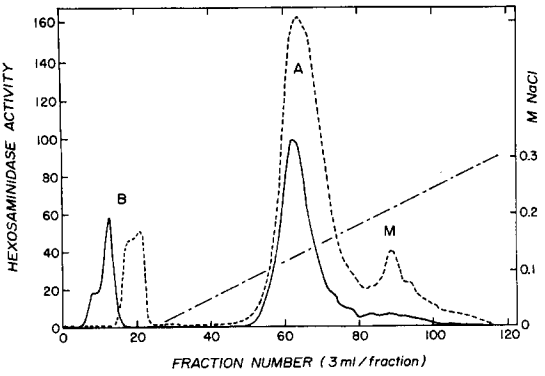

Fig. 1. DEAE-cellulose chromatography of urinary *N*-acetyl-β-hexosaminidases. Urinary proteins were precipitated with 80% $(NH_4)_2SO_4$, further fractionated to obtain the fraction which precipitated between 20 and 60% $(NH_4)_2SO_4$ concentration, and dialyzed against 0.01 M sodium phosphate buffer, pH 6.0. Proteins were applied to a 1.2 cm × 15 cm DEAE-cellulose column (Whatman DE-11) previously equilibrated with the same buffer. The DEAE-cellulose was washed prior to use, and between chromatographic runs, according to directions of Peterson and Sober[10]. The column was eluted with 60 ml of the same buffer followed by a linear salt gradient consisting of 300 ml buffer with 0 to 0.3 M NaCl. 50 μl of each fraction was assayed for *N*-acetyl-β-hexosaminidase activity in a reaction mixture which consisted of 20 μmoles citrate–phosphate buffer, pH 4.4, and 1 μmole *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Koch-Light) in a final volume of 250 μl. After incubation at 37 °C the reaction was stopped with 1.2 ml of 0.4 M glycine–NaOH buffer, pH 10.4, and absorbance read at 410 nm. Hexosaminidase activity is expressed as nmoles of substrate cleaved in 75 min. Enzymes from 625 ml of female urine (———), and from 950 ml of male urine (– – – –).

chromatography and on polyacrylamide gels, and by its heat lability (Figs 2 and 3). In urine samples from 12 individuals, its elution position on the gradient was invariable, the activity always appeared as a single peak, and it was always present as the major component.

A second *N*-acetyl-β-hexosaminidase component which eluted early has been identified as *N*-acetyl-β-hexosaminidase B using the same criteria. This component was always eluted before application of the gradient, but its precise location varied as did the relative amount compared to the A form. It generally comprised 15 to 25% of the total *N*-acetyl-β-hexosaminidase activity although in two of the twelve individuals tested (one male and one female), the B form was barely detectable. *N*-Acetyl-β-hexosaminidase B was usually eluted as a single sharp peak but occasionally it appeared as a double peak for reasons not known at present.

A third distinct *N*-acetyl-β-hexosaminidase peak was eluted after the A form on the gradient (dotted line in Fig. 1). This minor acidic peak, previously undescribed, appeared predominantly in male urine and accordingly has been designated as *N*-acetyl-β-hexosaminidase M. It was present in four of five males, and absent or present in only small amounts in seven females tested. The one male in which this M activity was absent was one of the individuals in which the B form was also virtually absent. The *N*-acetyl-β-hexosaminidases shown in Fig. 1 were partially purified by $(NH_4)_2SO_4$ fractionation prior to DEAE-cellulose chromatography as described in the legend. *N*-Acetyl-β-hexosaminidase M could also be detected by subjecting fresh urine directly to DEAE-cellulose chromatography with no prior treatment other than filtration and dialysis against 0.01 M phosphate buffer, pH 6.1.

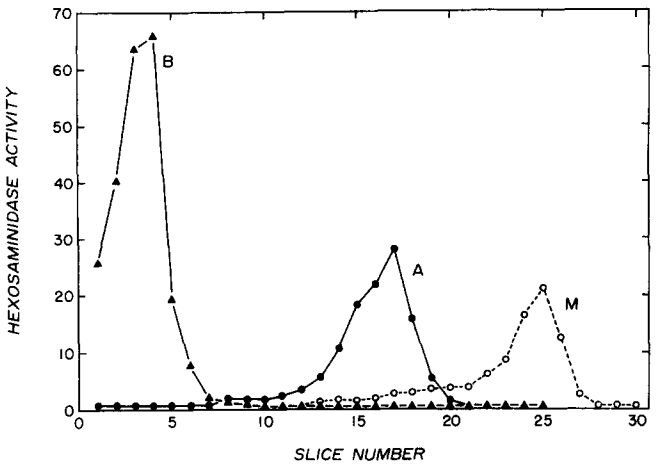

Fig. 2. Electrophoretic mobility of *N*-acetyl-β-hexosaminidase isozymes. Each of the enzyme forms obtained by DEAE-cellulose chromatography were run simultaneously on separate identical gels. The same separation resulted when all were mixed and run on the same gel. Anode is at right of figure. Gels were prepared by the method of Friedland *et al.*[11]. Proteins were applied to the gel in a mixture consisting of 50 μl of an appropriate chromatographic fraction which contained enzyme activity, 15 μl 0.01 M phosphate buffer, pH 7.0, containing 0.154 M NaCl, and 40 μl of a 40% sucrose solution. Gels were run at 2.5 mA/tube for 35 min at 4 °C. The enzymes were located by cutting gels into 1.1-mm slices and assaying the intact slices for enzyme activity as described in Fig. 1. Hexosaminidase activity is expressed as nmoles *p*-nitrophenol-*N*-acetyl-β-D-glucosaminide cleaved in 90 min.

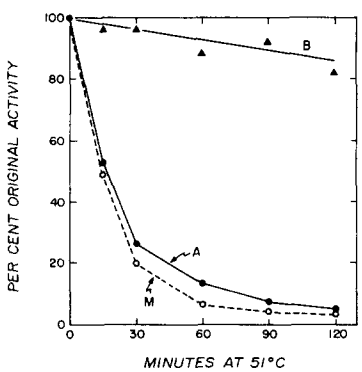

Fig. 3. Heat stability of the *N*-acetyl-β-hexosaminidase isozymes.

Each of the three enzyme fractions in male urine could also be separated electrophoretically on polyacrylamide gels (Fig. 2). The relative position of the M peak both on electrophoresis and DEAE chromatography showed it to be the most acidic of the three. The three peaks of *N*-acetyl-β-hexosaminidase activity were also obtained by electrophoresis of the 20–60% $(NH_4)_2SO_4$ fraction without prior DEAE fractionation or by electrophoresis of fresh untreated urine concentrated by ultrafiltration.

Properties of the enzymes

Heat stability. To compare the heat stability of *N*-acetyl-β-hexosaminidase M

with that of A and B, samples of each, obtained by DEAE-cellulose chromatography, were heated at 51 °C under conditions described by O'Brien *et al.*[12]. Like *N*-acetyl-β-hexosaminidase A, the M form is quite labile compared to the B form (Fig. 3).

pH optima. The activity of both the A and M forms over the pH range 2.8 to 6.4 (0.1 M citrate–phosphate buffer) was very similar, both showing a maximum at pH 4.0 to 4.4. The B form had the same pH optimum but retained more activity in the lower pH range than did the A and M forms.

Substrate specificity. All three enzyme forms were reactive with the 4-methylumbelliferyl derivatives of *N*-acetyl-β-D-glucosamine and *N*-acetyl-β-D-galactosamine, as well as with *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. Authenticity of the glycon moiety was ascertained by paper chromatography after enzymatic hydrolysis of the substrates[13]. The activity of the M form against 4-methylumbelliferyl-*N*-acetyl-β-D-galactosaminide was of interest since a third form of *N*-acetyl-β-hexosaminidase observed by Hooghwinkel *et al.*[7] was devoid of this activity.

Urinary N-acetyl-β-hexosaminidases in relation to Tay–Sachs disease

Since a deficiency of *N*-acetyl-β-hexosaminidase A appears to be the primary biochemical defect in Tay–Sachs disease[2], the urine of a Tay–Sachs patient and that of several heterozygous carriers of the Tay–Sachs gene was examined by DEAE chromatography for any variation in the normal enzyme pattern. Heterozygote urine showed no variations except the amount of B relative to A was greater than in normal urine. In five heterozygotes tested, the B form comprised between 30 and 40% of the total activity. In different urine samples of the same Tay–Sachs patient (3-year-old male), *N*-acetyl-β-hexosaminidase activity appeared either as a single peak corresponding in position to the normal B peak, or as two peaks, one corresponding to B, the other located intermediate between the normal positions of the A and B forms. Neither *N*-acetyl-β-hexosaminidase A nor M were ever present.

DISCUSSION

The *N*-acetyl-β-hexosaminidase M isozyme described here bears a strong resemblance to the A form. They are similar in each parameter measured, *i.e.* heat lability, substrate specificity, and pH optima, and differ only in their apparent charge. At present neither the *in vivo* role of the M form nor its distribution in other tissues is known. It is unlikely that the M form is an artifactual form arising from A because, (a) it is virtually absent in some individuals, and (b) following the initial separation by DEAE chromatography, neither form has ever been observed to undergo interconversion even after prolonged storage under various conditions, further purification, or rechromatography.

Two recent reports have described another form of *N*-acetyl-β-hexosaminidase (designated as C) in human tissue extracts as detected by electrophoresis on cellulose acetate[7,8]. This C isozyme, like the M form, was more anodic than A or B, suggesting that C and M may be identical. However, the available information does not support this possibility since the C form from brain extracts described by Hooghwinkel *et al.*[7] was unreactive with 4-methylumbelliferyl-*N*-acetyl-β-D-galactosaminide, while the isozyme from brain, liver, and lung described by Poenaru and Dreyfus[8] was specifically reported to be absent in urine.

Dance and coworkers[14–16] have examined the *N*-acetyl-β-hexosaminidases of kidney and urine by starch-gel electrophoresis for the purpose of using enzyme excretion levels as an indicator of kidney disease. They showed that normal kidney contained both the A and B isozymes while normal urine contained only the A, with little to none of the B form. The B form was present only in cases of kidney damage resulting from disease or surgery. By the procedures described here, we have found that in most cases the B form comprised from 15 to 25% of the total activity, although extremes ranging from 1 to 30% were observed in normal individuals. Despite reports that the A form on ageing may be converted to an apparent B form[9], it seems unlikely that the B form we have observed arose in this manner for the same reasons given above with respect to the M form. In addition, the expected higher ratio of B to A in heterozygotes suggests that the relative amounts of B observed represents the actual situation.

In studying the *N*-acetyl-β-hexosaminidases of serum, Price and Dance[9] have identified two new peaks of activity (I_1 and I_2) which upon DEAE chromatography were located between the A and B peaks. We have found neither of these forms in urine except for the occasional appearance of the intermediate form in Tay–Sachs urine which may correspond to the I_1 or I_2 form. The significance of this form is not known.

The complete absence of *N*-acetyl-β-hexosaminidase A from urine of Tay–Sachs patients makes this a simple and efficacious method for diagnosis of the disease in infants since it eliminates the need for a blood sample. Normal infant urine by contrast does contain the A form. In addition, the difference in the ratio of B to A between normal and heterozygous individuals observed here is in keeping with the report of Navon and Padeh[17] that urine may be a reliable source of the enzyme for determination of Tay–Sachs genotypes.

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