Assay of β-N-acetylgalactosaminidase isoenzymes in different biological specimens by means of determination of their activation energies

Luis F. Pérez and J. Carlos Tutor*

The activation energy ($E_a$) of β-N-acetylgalactosaminidase (Hex, EC 3.2.1.52) was determined with 3,3'-dichlorophenylsulfonphthaleinyl-N-acetyl-β-d-glucosaminide as substrate, with a much higher value being found for the Hex B isoenzyme ($E_a = 75.1$ kJ/mol) than for the Hex A isoenzyme ($E_a = 41.8$ kJ/mol). This fact allowed for the development of a fast and reliable thermodynamic method to determine the isoenzyme composition of Hex in different biological specimens (serum/plasma, saliva, cerebrospinal fluid, seminal plasma, urine, and leukocyte lysates). The results in serum given by the proposed method may be superimposed upon those obtained by the heat inactivation assay of O'Brien et al. (N Engl J Med 1970;273:15–20), and the catalytic activity calculated for Hex A offers a good correlation with that obtained by using the specific substrate 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide-6-sulfate ($n = 25, r = 0.953$).

The lysosomal β-N-acetylgalactosaminidase (Hex, EC 3.2.1.52) is a complex group of glycoprotein isoenzymes composed of two different polypeptide chains denoted α and β. The isoenzymes Hex A (αβ) and Hex B (ββ) are the two major forms [1–3], and Hex S (αα) has generally limited catalytic activity and is unstable [1,2]. The form Hex P, which increases in serum during pregnancy and liver disease, contains only β subunits [4,5] and is heat stable, as is the Hex B isoenzyme, whereas Hex A is heat labile [1–3]. There is present no method available to distinguish between Hex P and intermediate forms (Hex I).

Until a few years ago, the almost exclusive clinical application of the assay of Hex isoenzymes was the biochemical diagnosis of gangliosidoses GM2 (Tay–Sachs and Sandhoff diseases) and the detection of carriers [1–3]. However, more recently, numerous studies have examined these isoenzymes in other physiopathological situations [5–10].

For the determination of the Hex isoenzymes, various electrophoretic, chromatographic, immunochemical, or heat inactivation techniques have been put forward [1]. In the present work, we describe a fast, reliable, and economical spectrophotometric assay for Hex A and Hex B isoenzymes by determining their activation energies ($E_a$s) with 3,3'-dichlorophenylsulfonphthaleinyl-N-acetyl-β-d-glucosaminide (CPR-NAG) as a substrate.

Materials and Methods

Catalytic activities of Hex were measured with CPR-NAG as substrate by means of the commercially available reagent NAG Rate Test® from Shionogi and Co. Activity determinations were performed on a Cobas Bio centrifugal analyzer (Hoffmann-La Roche) programmed as follows: units U/L; calculation factor (see above); standard 1–3 conc. 0; limit 0; temperature (°C) 37.0; type of analysis 2; wavelength (nm) 575; sample volume (µL) 10; diluent volume (µL) 50; reagent volume (µL) 150; incubation time (s) 0; start reagent volume (µL) 0; time of first reading (s) 300; time interval (s) 30; numbers of readings 10; blanking mode 1; printout mode 1. For the preparation of reagent solution, 8.2 mL of distilled water was added to each bottle containing lyophilized synthetic substrate and buffer.

Bearing in mind the inhibitory effect of albumin on Hex activity with CPR-NAG as substrate [11], in the analysis of urine, saliva, cerebrospinal fluid (CSF), seminal plasma, and leukocyte lysates the analyzer was programmed with a calculation factor of 863, and for serum or plasma samples with a calculation factor of 1029. These factors were established with regard to the enzyme activity obtained for the Stand NAG® control (Shionogi and Co. 34-81-57 01 02. Nonstandard abbreviations: Hex, β-N-acetylgalactosaminidase; CPR-NAG, 3,3'-dichlorophenylsulfonphthaleinyl-N-acetyl-β-d-glucosaminide; $E_a$, activation energy; CSF, cerebrospinal fluid; BSA, bovine serum albumin; MN, mononuclear; PMN, polymorphonuclear; 4MU-NAG(-SO4), 4-methylumbelliferyl-N-acetyl-β-d-glucosaminide-6-sulfate; and ACD, acid–citrate–dextrose.

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of Hex activity, purchased from two manufacturers (Boehringer Mannheim and Behringwerke), was added to 100 μL of sample, and incubated at several temperatures for different periods of time.

Statistical analysis of the data was carried out with the SPSS package. The skewness and kurtosis coefficients or the Shapiro–Wilk test were performed to check for normality, depending on the size of the sample. The significance of differences between mean values was evaluated by the Wilcoxon sign rank test for matching data and by the Mann–Whitney U-test for unpaired data. Normally distributed data were analyzed statistically by using Pearson’s correlation coefficient; otherwise, Spearman’s coefficient was used. Linear regression analysis was performed with the Passing–Bablok method. Statistical significance was accepted at $P \leq 0.05$.

### Results

For Hex, linear Arrhenius plots between 25 °C and 37 °C were obtained, and the determination of the $E_a$ under these experimental conditions showed a high precision (Table 1). Storing the serum, urine, saliva, CSF, or leukocyte lysates frozen at −20 °C for at least 15 days did not significantly modify the activity of Hex or its $E_a$. However, when the $E_a$ of the enzyme was determined in distinct sera (n = 6) or urine (n = 6) samples from different subjects, a wide range of variability was obtained, with CVs of 12.3% and 15.4% respectively. Given the high precision with which the $E_a$ of Hex may be determined (Table 1), it is evident that this interindividual variability cannot only be caused by analytical imprecision. It therefore appears that it is more probably due to the enzyme heterogeneity of Hex. This hypothesis was confirmed when the $E_a$s of Hex A (ref. A8527) and Hex P (ref. A9175) purified human placental isoenzymes from Sigma Chemical Co. were determined and gave very different values, as shown in Table 2. The neuraminidase treatment of these commercial preparations of Hex A and Hex P, enriched with 7 g/L BSA, for 6 h at room

### Table 2. Arrhenius slope and some thermodynamic variables for Hex human placental isoenzymes.

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Arrhenius slope, 1/$K$</th>
<th>$E_a$, kJ/mol</th>
<th>$\Delta H_{37}^\circ$, kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hex A</td>
<td>14</td>
<td>$-6.537 \pm 0.068$</td>
<td>$54.4 \pm 0.57$</td>
<td>$51.8 \pm 0.57$</td>
</tr>
<tr>
<td>Hex A desialylated</td>
<td>4</td>
<td>$-6.483 \pm 0.065$</td>
<td>$53.9 \pm 0.54$</td>
<td>$51.3 \pm 0.54$</td>
</tr>
<tr>
<td>Hex P</td>
<td>17</td>
<td>$-9.032 \pm 0.108$</td>
<td>$75.1 \pm 0.90$</td>
<td>$72.5 \pm 0.90$</td>
</tr>
<tr>
<td>Hex P desialylated</td>
<td>4</td>
<td>$-9.030 \pm 0.050$</td>
<td>$75.1 \pm 0.39$</td>
<td>$72.5 \pm 0.39$</td>
</tr>
</tbody>
</table>

Values are expressed as mean $\pm$ 1 SD. $\Delta H_{37}^\circ$ apparent enthalpy changes.
temperature did not produce any significant modification of either the $E_a$ (Table 2) or the enzyme activity. Similarly, the addition of different quantities of BSA (7–70 g/L) did not significantly affect the value of these thermodynamic variables for Hex A and Hex P human placental isoenzymes, or for the Hex from bovine kidney (Precimat® $b$-NAG, Boehringer Mannheim). In agreement with previous results [11], the addition of albumin produced in all cases an analogous inhibition of the enzyme activity.

Thermal treatment at 52 °C of a pool of sera adjusted to pH ~5.8 led to a gradual inactivation of the Hex, with stabilization of the residual activity being reached after 8 h of incubation (Fig. 1), corresponding to the thermostable isoenzyme Hex B [11]. Determination of the $E_a$ of Hex in the different aliquots subjected to this heat inactivation revealed a progressive increase of this thermodynamic variable, becoming stable after 8 h of incubation in which a value of approximately 75.0 kJ/mol was reached (Fig. 1). This $E_a$, which corresponds to the thermostable isoenzyme Hex B, is practically the same as that obtained for purified human placental Hex P (Table 1). One may therefore expect that serum Hex B has a similar $E_a$ to that of the Hex P hypersialylate form.

Taking into account the great difference that exists between the $E_a$s of Hex A and Hex B, the possibility was put forward of elaborating a method for determining the isoenzyme composition of Hex by calculating this thermodynamic variable. To do this, in 131 serum samples from healthy control subjects, patients with liver diseases, and pregnant women, we determined the percentages of isoenzyme Hex B, by the previously described procedure of heat inactivation [11], as well as the $E_a$ of the serum Hex. As would be expected, bearing in mind the previously shown results, a highly significant correlation was obtained between both variables (Fig. 2), and an estimation may be made of the proportion of Hex B, and thus of Hex A, on the basis of the corresponding $E_a$ of Hex by means of the following equation: $\text{Hex B} (%) = 3.0 \times E_a - 125.3$.

Assigning to the percentage of Hex B variable values 0 and 100, we obtain an $E_a$ of 41.8 kJ/mol for Hex A and 75.1 kJ/mol for Hex B. The value thus calculated for Hex B is identical to that found for Hex P purified from human placenta, before or after treatment with neuraminidase (Table 1). Similarly, in 95 serum samples that were subjected to the process of heat inactivation of Hex A [11], an average $E_a$ of 75.2 kJ/mol (SEM = 0.23) was obtained for the thermostable fraction. However, the value calculated for Hex A is significantly lower than that found for Hex A purified from human placenta (41.8 kJ/mol vs 54.4 kJ/mol). This could be explained, at least in part, by considering the widely documented fact of the spontaneous conversion of Hex A to Hex B, by reordering of the subunits, in the purified tissue forms [1, 13], and we point out that the placental isoenzyme Hex A that we used had an electrophoretic pattern with an additional band with mobility similar to that of Hex B. Also, the thermostable fraction obtained after its incubation at 52 °C and pH ~5.8 for 8 h [11] had an $E_a$ of 75.5 kJ/mol, which would
indicate the presence of Hex B in this commercial preparation. It is also possible that in the process of isolating placental Hex A, a loss of catalytic quality of the isoenzyme may occur, with an increase in its $E_a$.

The neuraminidase treatment of urine and serum samples, adjusted to pH 5.8, was made at several temperatures (4, 25, and 37 °C) for different periods of time (5, 12, and 30 h). Significant modification of the catalytic activity of $E_a$ could not be seen in any case because of the effect of treating Hex with neuraminidase.

In 25 serum samples from healthy subjects and pregnant women, the proportion in percentage of Hex B was determined with regard to the enzyme $E_a$ and through the slightly modified heat inactivation assay of O’Brien et al. [14] with 4-methylumbelliferyl-$N$-acetyl-$\beta$-d-glucosaminide (4MU-NAG) as substrate. As shown in Fig. 3, a good correlation between the results was obtained, without a statistically significant difference between the means (43.2% vs 42.8%). Good correlation was also found in these 25 serum samples between the activity obtained for Hex A through the specific substrate for this isoenzyme, 4-methylumbelliferyl-$N$-acetyl-$\beta$-d-glucosaminide-6-sulfate (4MU-NAG-SO$_4$), and that calculated from the isoenzyme composition determined with regard to the $E_a$ with CPR-NAG as substrate: $4\text{MU-NAG-SO}_4 = 0.25 \times \text{CPR-NAG} + 0.29 (r = 0.953)$.

In a mixed-sex group of 70 clinically healthy adults an average activity at 37 °C of 9.1 U/L (interval: 6.3–13.5 U/L) was obtained for Hex in serum, with a percentage of Hex B calculated from its $E_a$ of 31.6% (interval: 21.1–40.3%).

In biological fluids that have a low enzyme activity, such as CSF, it may be advisable to increase the volume of sample programmed in the analyzer to 40 $\mu$L to achieve a more precise determination of the $E_a$ and accordingly of the proportions of Hex A and Hex B, without needing to concentrate the samples beforehand. The increase in volume of the sample did not produce a significant modification of $E_a$ obtained for different samples of serum, CSF, or leukocyte lysates. On the contrary, when the sample volume of the urine specimens was increased, a significant increase in the $E_a$ of Hex was obtained. Eliminating the urinary components of low molecular mass with a membrane of selective permeability with a cutoff of 7.5 kDa (Vivapore®, Vivascience) led to lower $E_a$s than in corresponding samples of native urine, with the differences being proportional to the concentration of urea (data not shown). The concentration of this endogenous metabolite in the reaction mixture appears to have a significant effect upon the $E_a$ of the Hex. Once the urinary components of low molecular mass have been eliminated by selective filtration, the value of the $E_a$ of Hex is not significantly modified when the volume of the sample is increased.

The inactivation of various enzymes has been described as due to the action of ultrasounds [15, 16]. When the applicability of the method proposed for determining the isoenzymes of Hex in leukocytes began to be studied, it became apparent that studying the possible effect of ultrasound on the catalytic properties of the Hex was necessary. To do this, a sera pool was subjected to ultrasound (48 ± 5 kHz) for different time periods (n = 9) up to 420 min, with no significant variation in the Hex activity (CV = 0.88%) or of the $E_a$ (CV = 0.35%) being found. In a pool of urine samples, analogous results for the enzyme activity (CV = 0.83%) and the $E_a$ (CV = 0.94%) were obtained.

The specific activity of Hex at 37 °C and its isoenzyme composition in MN and PMN leukocytes was determined in 18 healthy controls, isolated from whole-blood samples drawn into Vacutainer Tubes® (Becton Dickinson Vacutainer Systems) with EDTA or acid–citrate–dextrose (ACD) as an anticoagulant (Table 3). When ACD was used, the specific activity obtained in PMN was significantly higher than when EDTA was used ($P < 0.001$), without the relative proportions of the isoenzymes showing a significant difference.

In 17 healthy subjects an average activity of 17.0 U/L was obtained for Hex in saliva (interval: 5.7–28.8 U/L), with a Hex B percentage of 38.8% (interval: 29.4–48.0%). In CSF from nine control subjects without neurologic illness who had undergone a lumbar puncture for diagnostic purposes in our Hospital’s Emergency Service, an average activity for Hex of 1.8 U/L (interval: 1.1–2.8 U/L) was found, with a Hex B proportion of 45.2% (interval: 36.5–54.1%). In 10 samples of seminal plasma obtained

![Fig. 3. Correlation and regression between the percentages of Hex B in serum obtained by means of the determination of enzyme $E_a$ with CPR-NAG as substrate and heat inactivation assay with 4MU-NAG as substrate.](image-url)
from patients who had a postvasectomy checkup, an average activity of 1702.3 U/L was found (interval: 1045.2–2973.8 U/L), with a Hex B percentage of 66.2% (interval: 60.5–73.0%).

**Discussion**

In the experimental conditions used here, the $E_a$ for the reaction catalyzed by Hex may be precisely measured (CV $\leq$1.5%), and in most cases a correlation coefficient $r \geq 0.9990$ was obtained in Arrhenius plots.

The fact that Hex B has an $E_a$ that is much greater than that of Hex A (75.1 kJ/mol vs 41.8 kJ/mol) allowed for the development of a thermodynamic procedure to determine the isoenzyme composition of Hex. This difference between the $E_a$s is due to the different structures of the $\alpha$ and $\beta$ polypeptidic subunits responsible for the enzyme heterogeneity. The existence of two types of active sites has been described in Hex A (\(\alpha \beta\)) and of one type of active site in Hex B (\(\beta \beta\) \[17\]), which could explain a better catalytic quality of Hex A in the hydrolysis of CPR-NAG because of the presence of the $\alpha$ subunit in the Hex A molecule.

The intermediate forms and Hex P do not have activity towards 4MU-NAG-SO$_4$, indicating that they lack $\alpha$ subunits \[18\]. The only difference between serum Hex P and Hex B seems to be that the P form contains more sialic acid \[19\]. This implies that Hex P would show similar $E_a$s to Hex B, as the previously indicated results show that treatment with neuraminidase, and accordingly the sialylation degree of the enzyme molecule, do not affect the catalytic activity or $E_a$s of Hex.

The Hex isoenzymes could be divided into two groups with the thermodynamic method described: Hex A and Hex B. The latter thus includes Hex B, Hex P, and intermediate forms. The results given by this procedure for serum samples are similar to those obtained by the heat inactivation assay of O’Brien et al. \[14\], and the activity calculated for Hex A has a good correlation with that obtained with the specific substrate 4MU-NAG-SO$_4$.

Although albumin presents a noncompetitive inhibiting effect on Hex activity with CPR-NAG as a substrate \[11\], it does not affect the value of the enzyme $E_a$. This is an interesting point, as it allows for the use of the same regression equation obtained for serum samples (Fig. 2) in determining the isoenzyme composition of Hex with regard to its $E_a$ in other biological specimens such as saliva, CSF, seminal plasma, leukocyte lysates, urine, etc. Similarly, by not affecting the protein concentration in the reaction mixture in relation to the value of the $E_a$, the volume of the sample programmed in the analyzer may be increased to 40 $\mu$L, thus avoiding the necessity of concentrating biological fluids that have low enzyme activity, such as CSF, for a more precise estimation of the isoenzyme composition with this thermodynamic method. Recent studies have also shown that when heat inactivation tests are used for the determination of Hex isoenzymes in leukocytes, standardizing the cell concentration is necessary \[20, 21\]. Given the significant effect of the concentration of the sample on the apparent value of $E_a$, Prence et al. \[20\] recommend 8–12 $\mu$g of sample protein per tube. However, there is no “right” amount of sample and each testing laboratory should establish its own protocol and reference ranges to maximize test reliability \[20\]. In the thermodynamic method described, the quantity of sample that is used does not affect the results obtained for the relative proportions of Hex A and Hex B in leukocytes.

The relative proportions obtained for the Hex A and Hex B isoenzymes in PMN and MN leukocytes are in agreement with the data given by Ellis et al. \[22\], and show the great difference that exists for the isoenzyme composition of Hex between both leukocyte subpopulations. Similarly, the results obtained for the proportion of Hex A and Hex B in serum are similar to those obtained by other authors using different methods \[13, 23–26\].

When blood samples were collected with ACD, a significantly higher specific activity of Hex in PMN leukocytes was obtained than when EDTA was used as an anticoagulant (Table 3), although the relative proportions of Hex A and Hex B were not modified. This fact was not observed in the case of MN leukocytes, and has not been explained satisfactorily, as no type of analytical interference has been shown. Eicholtz et al. \[21\] found in total leukocyte lysates a higher total and thermostable activity of Hex in the samples collected with ACD than in those collected with heparin.

Some urinary metabolites with low molecular mass, at least urea, interfere positively in the determination of Hex $E_a$, and so for the isoenzyme assay one must eliminate these endogenous components of the urine samples. We are currently studying this aspect in detail to possibly eliminate this interference without selectively filtering urine specimens beforehand.

The method described allows for straightforward, precise, and economic spectrophotometric determination of the

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**Table 3. Specific activity and isoenzyme composition of Hex in MN and PMN leukocytes from healthy subjects (n = 18).**

<table>
<thead>
<tr>
<th></th>
<th>MN leukocytes</th>
<th>PMN leukocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EDTA</td>
<td>ACD</td>
</tr>
<tr>
<td>Hex, U/g protein</td>
<td>12.9 ± 2.39</td>
<td>12.4 ± 2.29</td>
</tr>
<tr>
<td>Hex B, %</td>
<td>47.3 ± 3.14</td>
<td>47.1 ± 2.71</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± 1 SD.
isoenzyme composition of Hex with regard to its $E_a$ by using CPR-NAG as a substrate. It is a valid alternative to the different established methods.

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References


