Automated Assay of Hexosaminidases in Serum

J. A. Lowden, M. A. Skomorowski, F. Henderson, and M. Kaback

In serum there are two classes of hexosaminidase. One of these is heat-labile and is inactive in children with Tay-Sachs disease. Serum from heterozygotes for Tay-Sachs disease has half the normal activity of the heat-labile enzyme. The heat-labile enzyme can be inactivated by heating serum diluted in buffer for 5 min at 60 °C. We have used this property to develop a reliable, fully automated assay for serum hexosaminidases, which can be used for mass carrier screening programs.

Additional Keyphrases: Tay-Sachs disease • screening technique • AutoAnalyzer • oral contraceptives • manual and automated methods compared

Tay-Sachs disease, G_{M2}-gangliosidosis, results from a defect in the activity of hexosaminidase A. Many manual methods have been described for assay of this enzyme (1-3). Carriers can be detected by demonstrating a decrease in hexosaminidase A. Screening for heterozygotes has been conducted not only in high-risk families but also in mass-screening programs in several North American communities (4, 5). About one year ago we began a screening program in Metropolitan Toronto. The Jewish population in the area numbers about 100,000 and probably includes about 25,000 people in the child-bearing age group. Because of the potentially large number of patients who could be studied, an automated system for measuring enzyme activity was essential.

This paper describes two systems, a semi-automated method, which we used in screening the first 6,000 patients in our study and a fully automated system, which we subsequently developed.

The conditions of hexosaminidase assays have been reviewed in detail by Kaback (6). Optimal conditions of pH, substrate concentration, time and temperature for heating, and the like are different in different laboratories, and some variations may change either the measured total activity or the percentage of activity that is destroyed by heating. Nevertheless, within reasonable limits, if all conditions are controlled in a reproducible fashion, the assay is reliable and can be used to detect heterozygotes in mass carrier screening programs. Automation provides the control necessary for reliability.

Normal serum contains at least two classes of hexosaminidases, which can be distinguished by their different response to heating. One is the heat-labile, "A" enzyme; the other is heat-stable (6). The heat-stable enzyme in serum differs from that in tissue both in electrophoretic migration and in heat stability (7). In the initial papers describing the heat-labile enzyme (1-3, 8), it was heat-denatured at 50 °C in a pH 4.1 buffer for 3 or 4 h. Recently Delvin et al. (9) demonstrated that the enzyme in serum can be denatured by heating for only 5 min at 60 °C. We used this property of serum hexosaminidases to establish a fully automated system.

Materials and Methods

Reagents and Samples

The substrate used was 4-methylumbelliferyl-β-D-N-acetyl-glucosaminide (Koch-Light). The buffers were citrate-phosphate (citrate, 40 mmol/liter, with phosphate added to give pH 4.10, 25°C) for incubation and 2-amino-2-methyl-propan-1-ol (MAP) (pH 10.4, 0.1 mol/liter, 25°C) to stop the reaction and increase the fluorescence. Most serum samples were collected from healthy young adults in

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large screening clinics; the blood was centrifuged within 6 h and stored at −20 °C. Standards of 4-
methylumbelliferone were prepared by dissolving 17.7 mg in 100 ml of glacial acetic acid and diluting
with buffer or saline (9 g/liter).

Procedures

Manual assay. Manual assay was performed by a
method modified from that of Okada and O’Brien
(1). To measure total activity, we diluted serum with
citrate-phosphate buffer (50 μl plus 1 ml of buffer)
and a 100-μl aliquot was incubated with 100 μl of
buffer containing substrate. To measure the heat-
stable activity the diluted serum was heated at ei-
ther 60 °C for 5 min (9) or at 50 °C for 3 h (1). The
heated enzyme was then also incubated for 30 min
(37.5 °C) with an equal volume of buffer containing
substrate. The reaction was stopped by adding 3 ml
of MAP rather than the usual (1, 8) glycine-carbo-
date buffer. MAP maintains a more stable pH, an
important factor in the automated assay, in which
the buffer must be exposed to the air for several hours.
Samples were read in a Farrand Fluorometer (inci-
dent wavelength, 365 nm; emission wavelength, 450
nm) vs. standards of 4-methylumbelliferone.

Semi-automated assay. The flow diagram for the
semi-automated assay is shown in Figure 1. The
equipment used is the “AutoAnalyzer II” (Technicon
Instruments Corp., Tarrytown, N. Y. 10591). Serum,
50 μl, was diluted to 1 ml with buffer and placed in
12 × 75 mm glass test tubes in a specially adapted
tray on the sampler. After every 10 to 12 tubes a
tube of water was inserted to wash the lines and to
clearly delineate the baseline. Before the AutoAna-
lyzer was set up, one aliquot of the diluted serum was
heat denatured at 50 °C for 4 h or at 60 °C for 5 min.
The samples were then placed in the sampler with
an unheated tube followed by a heated tube, so that
total and heat-stable enzyme activity could be calcu-
lated sequentially. All samples were run in duplicate,
and control samples of a known heterozygote and
known normal serum were assayed every 2 or 3 h
during the run.

The system could be standardized by using ali-
quots of 4-methylumbelliferone of appropriate con-
centration in the sampler. Because the peaks tail off,
it was essential to place a water tube between each
standard to allow the recording to return to baseline.
Tailing was not an important factor with serum
samples.

Fully automated system (Figure 2). Serum, which
was stored in polyethylene sample tubes with a tight
fitting cap (Eppendorf microtubes No. 3810), was
placed directly in the sampler in the storage contain-
ers. Use of alkaline MAP buffer in the wash cycle
decreased pump-line plugging. In this system (Figure
2), each serum sample is split into equal parts, one
of which passes through a heating coil before mixing
with the substrate, while the second passes through
a delay coil of equal diameter and length. The two
samples are then mixed with substrate, incubated at
37.5 °C in a 6.9-ml heating coil, mixed with MAP,
and read in separate fluorometers. The output from
the fluorometers is indicated on a dual-pen recorder.
By proper adjustment in the length of transit lines,
the peak for the total enzyme activity run and that
for the heat-denatured line can be caused to appear
in immediate juxtaposition to one another on the
chart paper (Figure 3). Control samples of normal
serum and a serum from a known heterozygote were
run every 2 to 3 h.

![Fig. 1. Semi-automated system for hexosaminidases](image1)

Figures on each pump line indicate delivery rates in ml/min. Technicon
connectors are indicated by catalog part number. Coils are standard
Technicon glass coils. Sampler contains diluted serum with alternating
total enzyme and heat denatured (50 °C, 3 h; or 60 °C, 5 min) speci-
mens in 12 × 75 mm glass tubes

![Fig. 2. Fully-automated system for hexosaminidases](image2)

See Figure 1 for details. Sampler contains undiluted serum in re-cappa-
bale Eppendorf microtubes

![Fig. 3. Tracing of chart recording from fully automated method](image3)

Heavy line: total enzyme activity. Light line: heat-stable activity. First
three peaks from sample run with a water wash between each serum
sample. Note tailing. Last five peaks are from consecutive serum sam-
ple. Note lack of cumulative effect

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With the extra tube length in the fully automated system, when we tried to establish a standard curve by using aliquots of 4-methylumbelliferone of known concentration, there was considerable distortion of the peaks. The distortion was not seen when methylumbelliferone was released from the substrate enzymatically but only when it had to pass through the entire transit line from the sampler. Methylumbelliferone is relatively insoluble in water and the distortion is probably attributable to adsorption onto the tubing walls. Control sera (both normal and heterozygotic) were therefore analyzed by the manual procedure and then used to standardize the instrument each day. To balance the two fluorometers before operation, samples were run through both lines without 60 °C heating. The heating coil was then brought to 60 °C and the control samples rerun. It is essential that peak heights for both total activity and heat-stable activity be reproducible on a day-to-day basis before unknown samples can be assayed.

**Results**

The manifolds depicted in Figures 1 and 2 were developed after much trial and error. Hexosaminidase activity in serum is easily measured with 4-methylumbelliferyl-N-acetylglucosaminide, and the major problem in establishing an automated system was in diluting the enzyme sufficiently to allow measurement of the released methylumbelliferone. We therefore used small sample tubes and large diluting volumes of buffer. A 1:2 sample-to-wash cam was chosen because carryover was apparent at a 1:1 ratio and peaks were indistinct when the sample was in greater proportion. The long transit time in the system and the relative insolubility of methylumbelliferone, usually resulted in some tailing of the peaks (Figure 3). We were therefore concerned that one peak might distort the height of the subsequent peak. We ran samples alternating each serum with a water wash and compared the results when sera were run consecutively (Table 1). With average or high-

**Table 1. Effect of a Peak on the Succeeding Peak**

<table>
<thead>
<tr>
<th>Total hexosaminidase activity*</th>
<th>Mean SD CV %</th>
<th>Mean SD CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Standard sample</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Successive tubes</td>
<td>451 3.8 0.8 52.6 0.4 0.8</td>
<td></td>
</tr>
<tr>
<td>Alternating with water wash</td>
<td>447 7.9 1.8 52.0 1.3 2.5</td>
<td></td>
</tr>
<tr>
<td><strong>High-activity sample</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Successive tubes</td>
<td>840 9.0 1.1 55.9 1.2 2.1</td>
<td></td>
</tr>
<tr>
<td>Alternating with water wash</td>
<td>845 14.0 1.7 55.9 1.1 2.0</td>
<td></td>
</tr>
</tbody>
</table>

* Enzyme activity is in nanomoles of 4-methylumbelliferone released per milliliter of serum per hour.

Figures represent means of 10 determinations on the same serum sample.

In Tables 1-3, "% A" is the percentage of heat-labile enzyme.

We also examined the precision of the method by using three sera with high, intermediate, and low activity. Twenty replicates of each sample were run successively as a group and were then mixed and run in no special order. The coefficient of variation was low at all three levels of enzyme activity (Table 2). Furthermore, mixing the samples did not alter the results. Precision remained high, confirming the observation that alternate wash cups were not required.

We tested and compared all three methods by using the same samples of normal and heterozygotic sera. Each sample was assayed 10 times. The results produced by the fully automated method are as reproducible as those produced by the other two methods. When adequate controls were used, all methods were reliable (Table 3). In this examination of the
method the observed values for mean total hexosaminidase activity differed from one another, although all were measured in the same serum. This difference is not real but was purposefully produced by altering recorder response to further accentuate the reliability of the automated procedure. Because standards of 4-methylumbelliferrone passed through the long tubing tend to give broad distorted peaks, we calibrated the instrument with control sera that were first assayed by the manual method. The fluorometer sensitivity was then adjusted to give an appropriate recorder response. When this adjustment was not made the values obtained by different methods varied. However, the purpose of the assay is to distinguish heterozygotes for Tay-Sachs disease. Even when the instrument has not been absolutely accurately calibrated, the calculated percentage of the total activity in the heat-labile fraction (%A) does not vary. Thus, in Table 3 the values for %A in all three methods do not differ. Again, the fully automated method yields more consistent results as judged by the lower coefficient of variation.

Because we used this method to standardize the recorder response the day-to-day precision for total activity cannot vary. The percentage activity remaining after heat denaturation (%B) in a "normal" control serum was 33.8 ± 1.6 during three months of assays. That for a heterozygote serum was 54.0 ± 2.1 over the same period.

The sera selected for the study of precision cover the range of normal serum hexosaminidase activities. In pregnancy (7) and in certain disease states (1) serum hexosaminidase activity increases markedly. Furthermore, for normal activity to be measured in serum, the sample must be diluted with buffer; therefore we tested the linearity of dilution over a range of 10-fold and found the serum could be diluted without affecting the absolute activity.

The manual method requires 30–60 min of incubation. Automation necessitated decreasing this to 5–6 min while retaining a linear response. Using the same sample-to-substrate and buffer ratios that we used in the automated system, we examined reaction rates manually at different times from 2 to 60 min of incubation, and found that the reaction proceeded linearly both for high- and low-activity samples.

The fully automated method has now been used to assay hexosaminidases in over 2000 sera. Values obtained at a screening clinic in Edmonton, Alberta, are shown as an example in Table 4. Results for 10 of those tested (2.2%) are in the "grey zone" between carriers and normals (47–52% A). Invariably, about 2% are in this range. Most of these could be clearly identified on retesting. Of normal, non-carrier women taking contraceptive pills, 18% tested positive as carriers. They could be properly categorized by measuring leucocyte hexosaminidases by the manual method. All women who tested as carriers and were taking oral contraceptives were routinely retested.

**Discussion**

Although the assays we describe were all performed on sera with moderate activity (300–850 nmol/ml per hour) the system can be used for any activity that we have found in human sera. By adjusting the fluorometer, enzyme activity below 50 nmol hydrolyzed per milliliter per hour can be measured and by diluting one can measure the high activity sera from women in the latter weeks of pregnancy (5 000 nmol/ml per hour). Total enzyme activity from column effluents of tissue extracts can also be measured on a single-channel system. The heat-stable hexosaminidase of tissues cannot be assayed by the fully automated system because it differs from the heat-stable enzyme in serum. The tissue enzyme is partly denatured by heating for 5 min at 60 °C, although it is unaffected by heating for 3–4 h at 47–50 °C. If tissue enzymes, including those from leukocytes, cultured fibroblasts, or cells from amniotic fluid are to be measured, the manual or semi-automated systems with 47 °C heat denaturation must be used.

Automated assays have certain distinct advantages over manual assays, all of which are accentuated when the assay is used for a large-scale screening program. Speed, cost, and reliability then become not only advantageous but essential. By the fully automated method, one can screen 60 specimens an hour at a cost of less than 50 cents per patient, which covers not only reagents, AutoAnalyzer supplies, and technician time, but also pays the cost of some data processing. It does not cover equipment cost. A good laboratory technologist can handle only 150 manually assayed samples per day, and usually manual tests become subject to human error after a few days because of boredom or fatigue. Manual testing costs more than twice as much as automated testing, and the results are less reliable.

As the demand grows for carrier screening programs of different types, more automation will be necessary to make testing possible. Our method, while

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**Table 4. Results of Testing Sera at a Typical Screening Clinic**

<table>
<thead>
<tr>
<th>No. patients tested</th>
<th>All</th>
<th>Normals</th>
<th>Carriers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>461</td>
<td>436</td>
<td>15</td>
</tr>
<tr>
<td>Total hexosaminidase nmol/ml/h</td>
<td>569 ± 143 (330–2144)</td>
<td>536 ± 134 (330–2144)</td>
<td>659 ± 287 (337–1336)</td>
</tr>
<tr>
<td>Heat-labile A enzyme, %</td>
<td>66.2 ± 5.4 (53.2 ± 85.6)</td>
<td>44.1 ± 2.6 (37.0 ± 46.3)</td>
<td></td>
</tr>
</tbody>
</table>

All figures for enzyme activity are means ±SD. Range shown in parentheses. Ten patients fell in a "grey zone" between carriers and normals.

* On the initial assay 10 patients could not be categorized. They included four women on oral contraceptives. These patients fall into a "grey zone," but definitive diagnosis can usually be made on retesting either serum or leukocytes.
developed for a specific two-channel system, can be easily adapted to a multichannel analyzer.

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References