An Error-Producing Interaction in an Automated Method for Measuring Cholinesterase Activity in Blood

Robert I. Ellin, William A. Groff, and Andris Kaminskas

Blood cholinesterase activity values obtained in our laboratory by the automated procedure of Levine et al. (Automat. Anal. Chem., Technicon Symp. 1965) are greater than those values obtained by a comparable manual method. This problem is caused by reaction of the standard (reduced glutathione) with the substrate (acetilthiocholine) to form acetylglutathione and thiocholine. Because thiocholine dialyzes about three times more rapidly than glutathione in the automated system and gives the same color reaction, proportionately higher activity values would be reported. Ways of correcting this problem are presented.

Additional Keyphrases  dialysis in automated methods  thin-layer chromatography  glutathione reaction with acetilthiocholine  AutoAnalyzer  dialysis in an automated system  thiocholine standard

The automated procedure of Levine et al. (1) for measuring cholinesterase activity in blood, either in red cells or plasma, is used in many clinical and industrial laboratories (2-4). Sample and acetilthiocholine substrate are dialyzed against a recipient solution containing Ellman reagent (5,5-dithio-bis-2-nitrobenzoic acid) (6). A major problem encountered with this method is caused by use of the secondary standard, reduced glutathione, to prepare calibration curves. We find that glutathione reacts rapidly with acetilthiocholine to form acetylglutathione and thiocholine. Because thiocholine dialyzes about three times as rapidly as glutathione, activity values as determined by automated systems based on this technique would be correspondingly higher than those obtained by the same assay done manually. Evidence for this reaction and means of resolving this problem are presented.

Material and Methods

Standard Calibration Curves for Glutathione and Thiocholine Iodide by the Levine Procedure

Solutions osh1 were prepared to give final concentrations ranging from 0.6 mmol/liter to 5 mmol/liter in water or Tris buffer (50 mmol/liter, pH 7.0). A stock concentration of acetylthiocholine iodide (Sigma Chemical Co., St. Louis, Mo. 63178), prepared in 0.2 mol/liter sodium hydroxide, hydrolyzed instantly to thiocholine. Thiocholine may also be prepared by treating acetilthiocholine with hemolyzed human red cells (5 g/100 ml, final concentration). Hydrolysis is complete within 20 min. Thiocholine was stable at pH values of 5 and 10 at 25°C for 24 h, not as stable at pH 7.0. Standard calibration curves for thiocholine, either in the presence or absence of substrate, and glutathione in the absence of substrate are shown in Figure 1A and C, respectively. The usually used calibration curve of glutathione in the presence of substrate is shown in Figure 1B. Incubation time was 5.9 min.

Dialysis Manifold

An automated system (Figure 2) was constructed to measure the separate dialysis rates of osh and thiocholine iodide. With it, one can measure the sulphydryl content of the sample stream before dialysis or of the recipient stream after dialysis. Volumes (and consequently flow rates) of samples entering the sample side of the dialyzer

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1 Nonstandard abbreviations used: osh, glutathione; Tris, tris(hydroxymethyl)aminomethane; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

1 The volunteers in these tests are enlisted U.S. Army personnel. These tests are governed by the principles, policies, and rules for medical volunteers as established in AR 70-25.

1 Thiocholine purchased from a commercial source was unacceptable as judged by elemental analysis (carbon, hydrogen, iodine, and sulfur), and failure to react with 5,5'-dithiobis-(2-nitrobenzoic acid) at pH values of 4.4 or 8.3.

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Received Dec. 29, 1971; accepted July 7, 1972.
Reaction of Glutathione with Acetylthiocholine Iodide

**Glutathione with excess acetylthiocholine iodide.**
Stock solutions of glutathione, 1 mmol/liter, and acetylthiocholine iodide, 10 mmol/liter, were prepared in distilled water. Initial concentrations to be assayed were prepared by diluting the stock solutions fivefold with Tris buffer, pH 8.2, and adjusting the final pH to 8.2, if necessary, with a 100 g/liter solution of sodium hydroxide. The buffered solutions were agitated for 15 min at 37°C before sampling and during the ensuing 5-min sampling period. Assays were performed with use of the manifold shown in Figure 2. Concentrations calculated from the absorbancies of reactants and solutions shown in Table 1 were investigated to determine whether or not a reaction had occurred. **Acetylthiocholine iodide with excess glutathione.**
Stock solutions of acetylthiocholine, 1 mmol/liter, and glutathione, 10 mmol/liter, were prepared in distilled water. Conditions and procedures for the reactions were identical to those described above.

**Reaction Rate Constant for Transesterification**

The automated system shown in Figure 2 was used to study the possible interaction between glutathione and acetylthiocholine. Glutathione was mixed with Tris buffer at pH 8.2; then with acetylthiocholine. After a 2.5-min incubation at 37°C the reaction mixture was dialyzed against a recipient stream of Ellman reagent in Tris buffer (0.05 mol/liter, pH 8.2). The rate constant for the reaction between glutathione and acetylthiocholine was found by maintaining the glutathione concentration constant at 0.18 mmol/liter and varying the acetylthiocholine concentration as shown in Table 2. The reaction could be followed in a 15-mm flow cell at 420 nm because of changes in absorbance.

**Table 1. Absorbance Values for Reaction Between Glutathione and Acetylthiocholine (at 420 nm)**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Initial concentration, mmol/liter</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylthiocholine iodide</td>
<td>10</td>
<td>0.197</td>
</tr>
<tr>
<td>Plus glutathione</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine iodide</td>
<td>10</td>
<td>0.027</td>
</tr>
<tr>
<td>Acetylthiocholine iodide</td>
<td>and red cells, 2 g/100 ml</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red blood cells, 2 g/100 ml</td>
<td></td>
</tr>
<tr>
<td>Acetylthiocholine iodide</td>
<td>1</td>
<td>0.620</td>
</tr>
<tr>
<td>Plus glutathione</td>
<td>10</td>
<td>0.540</td>
</tr>
<tr>
<td>Glutathione</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

* Reaction to yield thiocholine.
related to the formation of thiocholine. The remaining glutathione concentration was calculated and subsequently substituted into the bimolecular rate equation:

\[ k_2 = (2.3/A0) \times \log (a/a - x) \]

where \( A \) is the acetylthiocholine concentration, \( a \) is the initial concentration of glutathione, and \( x \) is the concentration of reacted glutathione after time, \( t \).

Results

If one assumes that the standard calibration curve for thiocholine (Figure 1) is a straight line passing through the origin (although it is in fact slightly curved), then the slope for the thiocholine calibration is about 2.5-fold greater than the calibration slope for glutathione. The difference in the slope values is directly proportional to differences in their respective rates of dialysis. About 10% of the total thiocholine present and 3.5% of glutathione dialyze across the membrane. The slope of the standard calibration curve for glutathione in the presence of acetylthiocholine is 1.9 times that for glutathione alone. Our results show conclusively that the latter phenomenon is the result of interaction between the two compounds to form thiocholine and acetylglutathione.

Reactions between glutathione and acetylthiocholine iodide were first carried out with an excess of acetylthiocholine, then with an excess of glutathione. In experiments in which excess acetylthiocholine was used, the absorbance of the products resulting from the reaction was compared with that resulting from the enzymatic hydrolysis of an equivalent concentration of acetylthiocholine. Table 1 shows absorbances for the following: (a) thiocholine iodide produced by the transesterification reaction and spontaneous hydrolysis of acetylthiocholine, (b) spontaneous hydrolysis of acetylthiocholine iodide at pH 8.2, (c) thiocholine produced by enzymatic hydrolysis, (d) red blood cells in a concentration of 2 ml of packed cells per 100 ml of water, (e) both thiocholine iodide by transesterification and the excess glutathione, and (f) glutathione, 10 mmol/liter. With these absorbance values, observed results agreed to within 6% of those calculated when excess acetylthiocholine was reacted with glutathione and to within 4% when excess glutathione was reacted with acetylthiocholine. After the latter reaction was complete, red blood cells were added; no additional thiocholine, which would form by the enzymatic hydrolysis of acetylthiocholine, was produced.

The course of the reaction between glutathione and acetylthiocholine was followed by thin-layer chromatography. Preliminary experiments showed that the developing solvent we used (the best of eight systems tested) could separate glutathione from S-acetylglutathione, but the small difference of 0.05 in Rf values was not considered sufficient for positive identification. The Ellman reagent, however, can be used to distinguish between the two: it produces a yellow color with glutathione, but no color with S-acetylglutathione. Ninhydrin gives a violet color with both glutathione and S-acetylglutathione, and iodine vapors give color with all initial reaction compounds as well as reaction products. Glutathione disappeared as S-acetylthiocholine appeared. The rate constant for the reaction between acetylthiocholine and glutathione at pH 8.2 and 37°C was calculated to be 370 liter/mole/min (Table 2). A comparison of red blood cell and plasma activity values obtained by Levine et al. and by our group using similar procedures is shown in Table 3. Our activity values prove to be about three times greater.

Discussion

The Levine method for determining blood cholinesterase has excellent precision and sensi-

<table>
<thead>
<tr>
<th>Source</th>
<th>No. samples</th>
<th>Activity values*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Levine et al. (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma*</td>
<td>19</td>
<td>3.6</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>20</td>
<td>10</td>
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<tr>
<td>Present report (type C membrane used)</td>
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<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>10</td>
<td>11.4</td>
</tr>
<tr>
<td>Red blood cells</td>
<td>10</td>
<td>28.3</td>
</tr>
</tbody>
</table>

* Activity values are in μmol/min per milliliter of sample. (Levine et al. (1) presents data for 3 min).
tivity and provides low blank values. However, the use of glutathione as a standard led to problems which were related to dialysis and an artificial transesterification interaction between glutathione and substrate. Depending on conditions used by an investigator, activity values could be reported that are about three times greater than valid values.

Why do the normal activity values reported by Levine et al. (1) apparently agree with the normal range reported in a manual method by Garry and Routh (6)? We do not find the values to be identical. Upon rerunning red cell activity, diluted 10-fold, or plasma activity, diluted four-fold, our absorbance values went off scale, while those obtained by the method of Levine et al. did not. When we changed from the more sensitive type C membrane to the less sensitive standard membrane, our data were in closer agreement with Levine et al. The value of the slope resulting from glutathione in the presence of 20 mmol of substrate with the standard membrane was identical with the slope resulting from that of glutathione alone with a type C membrane. If Levine et al. used glutathione in the presence of 20 mmol substrate with a standard membrane, the slope of the standard curve would be due partially to dialysis of glutathione, but mainly to the thiocholine that forms in the interaction. The value of the resulting slope would be about 70% that of the slope of thiocholine alone. Reported activity values would be high by about 30%. Since normal activity values of blood cholinesterase of a total population vary by a factor of 2 to 3, a 30% difference would not be noticeable.

Neither glutathione nor glutathione in the presence of substrate should be used to prepare a standard curve. The proper standard is thiocholine.

This problem can also be resolved by adding the DTNB color-forming reagent to the sample side of the dialyzing system (7). In this manner the sulfhydrol-containing products of the transesterification reaction, as well as unreacted material, would react with DTNB before dialysis. Dialysis rates of individual compounds would be of no consequence. We believe that the importance of dialysis in an automated system has been grossly underestimated and must be considered as thoroughly as the chemistry of the procedure (8). Additional problems in the Levine system can be caused by reaction with acetylthiocholine of either dialyzable or nondialyzable sulfhydryl substances in a biological sample.

References