Relative Stabilities of Purified Human Mitochondrial and Cytoplasmic Isoenzymes of Aspartate Aminotransferase in Lyophilized Materials

Eric J. Sampson, Sarah S. McKneally, Virginia S. Whitner, Carl A. Burtis, and David D. Bayse

Eight different pools of purified human mitochondrial and cytoplasmic isoenzymes of aspartate aminotransferase were prepared, to examine the effects of the following matrix variables: the matrix support material (bovine serum albumin and polyvinylpyrrolidone), endogenous pyridoxal concentration, and azide as an antimicrobial preservative. Storage temperatures of 25 and 37 °C were used as a rapid and convenient means of accelerating the degradation process. Activity of the enzyme was measured with and without pyridoxal in the reaction solution. We found that the mitochondrial isoenzyme was consistently more labile than the cytoplasmic isoenzyme under identical storage conditions. Both isoenzymes were more stable in matrices containing bovine serum albumin than in those containing polyvinylpyrrolidone. No apparent difference in the stability of either isoenzyme was observed at matrix pyridoxal concentrations of 15 μmol/L and 150 μmol/L. Only the mitochondrial isoenzyme in matrices containing bovine serum albumin and 15 μmol of pyridoxal per liter had increased activity (about 9%) when pyridoxal was added to the enzymatic reagent. The amount of activity in reconstituted specimens did not apparently change after 72 h at 4 °C.

Additional Keyphrases: enzyme stability • enzyme activity • variation, source of

Lyophilized materials containing enzymes are widely used by clinical laboratories, various proficiency testing programs, and methodology surveys to assess quality control and to gather interlaboratory data (1–9). Commercially produced lyophilized materials prepared from pooled human sera are commonly used for these purposes. Although activities of the various enzymes in human sera are suitable for values in the normal range, enzymes from nonhuman sources are usually added to attain values simulating pathologic activities. Dissimilar properties of enzymes from human and animal origin are well documented (6, 10, 11) and may compromise much of the data obtained in interlaboratory studies comparing different methodologies.

Stability of enzymes in lyophilized or reconstituted materials is another property that may dramatically influence analytical results. Although enzymes are particularly susceptible to deterioration under poor storage conditions, packing inserts accompanying most commercially prepared materials contain little information concerning this problem. Several individuals and organizations have published requirements for enzyme materials intended for various uses in the clinical laboratory (10, 12–15); however, the inherent difficulties of obtaining sufficient quantities of purified isoenzymes from human sources and the time and specialized equipment necessary to prepare and study freeze-dried materials have restricted research and development in this area.

Most of the developmental work on lyophilized materials containing enzymes has focused on the cytoplasmic isoenzyme of aspartate aminotransferase (AST, EC 2.6.1.1) (1, 6, 16, 17). The New York State Department of Health conducted the first interlaboratory experiment in which a lyophilized material containing purified AST from human erythrocytes in a matrix of polyvinylpyrrolidone (PVP) and several low-molecular-weight additives was used (6). More recently, we reported on a similar study (1) in which we used purified human cytoplasmic AST in a matrix containing bovine serum albumin (BSA). Although our materials were suitable for their intended use, their activity increased 20% when pyridoxal was added to our reagent. In addition, after the reconstituted specimens were stored for several days at 4 °C, they became visibly turbid, and AST activities gradually increased in measurements performed without cofactor in the reagent.

AST activity in human serum is derived from two major isoenzymes, of cytoplasmic and mitochondrial origin (18–20), which have been previously purified in our laboratory (1, 18). In this study we compared the relative stabilities of both isoenzymes in a variety of lyophilized matrices. The matrix variables we examined were: the matrix support (BSA vs. PVP), concentrations of pyridoxal, and an antimicrobial agent (azide). Specimens from each pool were maintained at 25 and 37 °C, to accelerate the effects of storage, and the stabilities were compared with that at 4 °C. AST was measured in paired runs with and without pyridoxal in the reagent, to detect any cofactor-related activation. Finally, the stability of the reconstituted specimens was examined by measuring the AST activity after 72 h at 4 °C.

Material and Methods

Chemicals. L-Aspartic acid (A grade), 2-oxoglutaric acid (A grade), and PVP were from Calbiochem, San Diego, CA 92112; dithiothreitol, tris(hydroxymethyl)aminomethane (Tris), and Tris hydrochloride were from Sigma Chemical Co., St. Louis, MO 63178; BSA Fraction V (Pentex) was from Miles

1 Nonstandard abbreviations used: AST, aspartate aminotransferase; PVP, polyvinylpyrrolidone; BSA, bovine serum albumin; MDH, malate dehydrogenase.
2 Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

Received Jan. 9, 1979; accepted Feb. 12, 1979.

CLINICAL CHEMISTRY, Vol. 25, No. 5, 1979 659
Table 1. Reaction Conditions for Measuring AST

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Volume, µL</th>
<th>Rotor well</th>
<th>Substance</th>
<th>Reaction concn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incubation solution</td>
<td>400</td>
<td>C</td>
<td>Tris-HCl, L-Aspartate, NADH, MDH, Pyridoxal</td>
<td>100 mmol/L, 125 mmol/L, 0.18 mmol/L, 0.60 U/L, 0 or 120 µmol/L</td>
</tr>
<tr>
<td>Sample</td>
<td>50</td>
<td>C</td>
<td>2-Oxoglutarate</td>
<td>6.7 mmol/L</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>50</td>
<td>B</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Reaction temperature 30 °C, pH 7.8. The loaded GEMSAEC rotor is placed in an incubator at 30 °C for 15 min before it is placed in the instrument and the reaction initiated. GEMSAEC settings: reading interval, 1 min; number of readings, 6; initial reading, 30 s.

Laboratories, Inc., Elkhart, IN 46515; sodium azide was from J. T. Baker Chemical Co., Phillipsburg, NJ 08865; malate dehydrogenase [MDH; L-malate: NAD+ oxidoreductase (EC 1.1.1.37)] from porcine heart in glycerol, β-nicotinamide adenine dinucleotide (reduced form, disodium salt, NADH), and pyridoxyl 5-phosphate (pyridoxal) were from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

AST measurements. The method used for measuring AST was a modification of the procedure of Henry et al. (27) and was performed on a GEMSAEC Centrifugal Analyzer (Electro-Nucleonics Inc., Fairfield, NJ 07006). Reaction conditions and reagent concentrations are listed in Table 1.

AST was routinely measured in single determinations on two different specimens from the same pool and storage history, with and without pyridoxal in the reaction mixture. Lyophilized specimens were reconstituted with distilled water, and measurements were performed within 1 to 8 h unless otherwise stated.

Isoenzyme preparations. Cytoplasmic AST was purified from human erythrocytes by the procedure of Rej et al. (16) as adapted in our laboratory (1, 17). Mitochondrial AST was purified from human liver as detailed previously (18). The final specific activities of the preparations containing the cytoplasmic and mitochondrial isoenzymes were 81 and 138 U/L, respectively. Each preparation was subjected to polyacrylamide disc gel electrophoresis and developed for AST activity and protein according to techniques described previously (1, 17). Only one band of AST formed for each preparation, with the cytoplasmic isoenzyme migrating substantially further towards the anode than the mitochondrial isoenzyme. Similarly, gels developed for protein showed only one band for the mitochondrial isoenzyme and one major band and two minor bands for the cytoplasmic isoenzyme.

Pool preparation. Table 2 lists variables included in the experimental design for preparing the 16 pools examined in this study. A given pool was prepared by combining one variable from each category in a common solution containing, per liter, 50 mmol of Tris-HCl buffer (pH 7.5 at 25 °C), 50 µmol of 2-oxoglutarate, and 50 µmol of dithiothreitol. The total volume of each pool was 150 mL.

The 16 pools were filtered individually through Falcon 0.22-µm disposable filters (no. 7103; Falcon, Oxnard, CA 93030) and stored at 4 °C for 18 h. One-milliliter portions were then dispensed with automatic pipets (Micromedics Systems, Inc., Horsham, PA 19044) into 2.5-mL amber-colored vials (no. S 883 B; Wheaton Scientific, Millville, NJ 80332).

Lyophilization. Specimens were lyophilized in a Stokes PV-60 lyophilizer in which the shelf temperature was adjusted to -50 °C. After 18 h the vacuum was applied, the shelf-temperature control was adjusted to 0 °C, and during the primary drying phase a vacuum of 2.65 x 10^-9 Pa was maintained for 16 h. The specimens were then sealed under reduced pressure. All 16 pools were lyophilized simultaneously.

Stability studies. Thirty vials from each of the 16 lyophilized pools were stored in incubators at 25 and 37 °C (Model 352700; Hotpack Corporation, Philadelphia, PA 19114), and the remaining vials, about 90, were stored at 4 °C. On storage days 1, 7, 14, and 39, duplicate vials from each pool at each temperature were reconstituted with 1.00 mL of distilled water and paired AST measurements were made with and without pyridoxal added to the reagent. Specimens reconstituted and analyzed 39 days after lyophilization were recapped and stored at 4 °C for 72 h. The AST activity was then measured again, with and without pyridoxal in the reaction solution.

Matrix blanks. In addition to the 16 pools containing the purified isoenzymes, eight pools containing just the matrix blank were also dispensed, lyophilized, and stored with the other pools. The day after they were lyophilized, mean values for these matrix blanks, measured without and with pyridoxal in the reagent, were 4.0 U/L (range 3.1–4.9 U/L) and 3.1 U/L (range 1.9–5.2 U/L), respectively, for the four pools containing BSA, and 6.3 U/L (range 2.2–9.4 U/L) and 8.7 U/L (range 4.4–10.4 U/L), respectively, for the four pools containing PVP. Storage as described led to essentially no changes in AST activity, although there was a visible change in the physical structure of the matrixes containing PVP, (see below).

Results and Discussion

Criteria used in selecting the matrix variables and concentrations listed in Table 2 were largely based on the composition of several materials used in two previously reported interlaboratory studies of AST (1, 6). The materials prepared for both studies contained only the purified cytoplasmic isoenzyme of AST. In one study (1), we reported using AST from either human erythrocytes or porcine heart in a matrix of BSA (30 g/L) and pyridoxal (15 µmol/L). Similarly, Rej et al. (6) used a matrix containing, per liter, 10 g of PVP and 16 µmol of pyridoxal to stabilize the human cytoplasmic isoenzyme. In this study, we examined pyridoxal concentrations of 15 and 150 µmol/L in matrixes containing BSA (30 g/L) and PVP (10 g/L). These concentrations of pyridoxal bracket the cofactor concentration of 100 µmol/L used in the AST reagent recently proposed by the International Federation of Clinical Chemistry (22). Other matrix additives common to the materials reported on in references 1 and 6 and incorporated in our materials were Tris-HCl buffer (50 mmol/L), 2-oxoglutarate, and a reduced thiol. Azide was examined as an antimicrobial preservative at a concentration of 7.7 mmol/L in eight of the matrixes prepared for this study. Rej and Vanderlinde (23)
evaluated the efficacy of azide in various reagents for measuring AST activity and showed that a concentration of 7.7 mmol/L adequately suppressed the growth of microorganisms but did not inhibit the measurement of either the mitochondrial or cytoplasmic isoenzyme. Finally, specimens were maintained at 25 and 37 °C as well as at 4 °C, to accelerate the effects of storage, a technique the World Health Organization recommends in examining biological materials (24).

Figure 1 shows the stability of the cytoplasmic isoenzyme of AST in four different matrices with pyridoxal concentration of 15 μmol/L. All AST measurements shown were made with pyridoxal in the reagent. At a storage temperature of 37 °C the isoenzyme appeared to be inactivated more rapidly in matrices containing PVP than in those containing BSA. The accelerated inactivation in PVP appeared to coincide with a visible loss of structural integrity and the eventual collapse of the lyophilized matrix, which occurred in the individual vials after several weeks at 37 °C. After this time, matrices containing PVP were considerably more difficult to reconstitute than were the corresponding BSA matrices, which showed no visible changes in structure. Adding azide to the PVP matrix appeared further to accelerate the loss of AST activity at 25 and 37 °C, whereas no appreciable effect was observed in the BSA matrices. At a storage temperature of 4 °C, the pools shown in Figure 1 showed no appreciable change in AST activity after 39 days. Rej et al. (6) reported that lyophilized specimens stored at 4 °C in a PVP matrix analogous to one shown in Plot 4 of Figure 1 retained their original activity after three years.

To depict the changes in AST activity as a consequence of storage in the different matrices, Table 3 lists the analytical values measured at 39 days for each of the eight pools containing the cytoplasmic isoenzyme, stored at 4 or 37 °C. Values are shown for specimens freshly reconstituted and again 72 h after reconstitution, measured with and without pyridoxal in the AST reagent. For comparison, the mean values of all eight pools measured the day after the specimens were removed from the lyophilizer were 52.6 U/L (SD, 2.1 U/L; n = 16) without pyridoxal in the reagent and 51.1 U/L (SD, 1.9 U/L; n = 16) with pyridoxal in the reagent. The mean values (and SD) shown in Table 3 for all eight pools stored at 4 °C for 39 days and measured without and with pyridoxal in the reagent were 51.2 (±1.34) U/L and 50.4 (±1.68) U/L, respectively, which were not significantly different by Student's t-test (α = 0.05) from mean values the day after lyophilization. There are only minor differences, which we attribute to analytical variability, between individual values in Table 3 measured with and without pyridoxal in the reagent.

A comparison of matrices which differ only with respect to the concentration of pyridoxal (e.g., PVP, no azide, storage temperature 37 °C) indicates that 150 μmol of cofactor per liter offers no greater protection than does 15 μmol/L. Of the matrices tested, those containing azide in PVP appeared to be the most detrimental for the isoenzyme, as verified in Figure 1. Storing the reconstituted specimens for three days appeared to affect negligibly the measured activity and was independent of azide, pyridoxal concentration in the matrix, and previous storage conditions. Mean values (and SD) for all eight pools stored at 4 °C for 39 days and then reconstituted and stored for three days were 50.6 (±2.33) U/L without pyridoxal and 51.8 (±2.08) U/L with pyridoxal in the reagent. These values were not significantly different by Student's t-test (α = 0.05) from either the means of the freshly reconstituted pools stored at 4 °C for 39 days or the means of the pools measured the day after they were lyophilized. In addition, on inspecting specimens from each of the pools three days after reconstitution, we saw no detectable turbidity.

These results suggest that the cytoplasmic isoenzyme (or that portion that retains its activity) is saturated with pyridoxal in the lyophilized and reconstituted states in each of the matrices examined. However, it does not exclude the possibility that structural changes in the cofactor bound covalently or in the proximity of the enzyme in the lyophilized material may cause the loss of enzyme activity when the specimens are stored.

The characteristics of the cytoplasmic isoenzyme in the materials described above differ markedly from those we described in a previous report (1). More specifically, we previously observed a 20% increase in activity when pyridoxal (120 μmol/L) was added to the AST reagent in freshly re-
Table 3. Cytoplasmic AST Activity Measured in Specimens Reconstituted after 39 Days of Storage

<table>
<thead>
<tr>
<th>Matrix support</th>
<th>Pyridoxal, mmol/L</th>
<th>Azide, mmol/L</th>
<th>Storage temp., °C</th>
<th>Freshly reconstituted</th>
<th>Reconstituted 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No pyridoxal b</td>
<td>Pyridoxal b</td>
<td></td>
<td>No pyridoxal b</td>
<td>Pyridoxal b</td>
</tr>
<tr>
<td>BSA</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>49.7</td>
<td>48.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>42.9</td>
<td>44.6</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td></td>
<td>4</td>
<td>51.6</td>
<td>47.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>41.2</td>
<td>49.7</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0</td>
<td>4</td>
<td>51.1</td>
<td>51.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>44.5</td>
<td>45.0</td>
</tr>
<tr>
<td>PVP</td>
<td>15</td>
<td>0</td>
<td>4</td>
<td>51.6</td>
<td>49.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>39.6</td>
<td>41.4</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td></td>
<td>4</td>
<td>53.2</td>
<td>51.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>25.8</td>
<td>28.0</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>0</td>
<td>4</td>
<td>50.2</td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>41.5</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>7.7</td>
<td></td>
<td>4</td>
<td>52.8</td>
<td>54.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>37</td>
<td>32.9</td>
<td>34.6</td>
</tr>
</tbody>
</table>

* Means of single determinations on two different specimens from each pool with and without pyridoxal in the reagent. After AST activity was measured on the freshly reconstituted specimens, they were placed at 4 °C for approximately 72 h and then retested.

b In the AST reagent.

constituted specimens. Reconstituted specimens measured without cofactor in the reagent were relatively stable for several hours, but gradually increased in activity to values approaching those measured with cofactor in the reagent. Finally, the previously described materials were clear after they were reconstituted, but slowly developed turbidity after several days of storage. Although the source of the enzyme and the composition of the matrix were essentially identical to those in the first pool listed in Table 3 (cytoplasmic AST from human erythrocytes, in 30 g/L BSA, 50 mmol/L 2-oxoglutarate, 15 mmol/L pyridoxal, 50 mmol/L Tris-HCl buffer, pH 7.5, and 50 mmol/L dithiothreitol), a small amount of glycerol (5 g/L) present in the other specimens was not added to the materials prepared for the study reported on here. Although we suspect that the glycerol is responsible for the anomalous effects, its role has not been formally established.

Figure 2 shows the effects of storage on the human mitochondrial isoenzyme in matrixes, under conditions analogous to those for the cytoplasmic isoenzyme. When the effects of the large-molecular-weight components of the matrixes were compared, those containing BSA again protected the enzyme substantially better than did those containing PVP; however, with the former there were apparent losses of AST activity even at a storage temperature of 4 °C. In matrixes containing PVP, the isoenzyme lost most of its activity within a week of storage at 25 or 37 °C, and then activity appeared to plateau.

![Fig. 2. Temperature-accelerated stability studies with lyophilized specimens containing the mitochondrial isoenzyme of AST](image)

662 CLINICAL CHEMISTRY, Vol. 25, No. 5, 1979
at approximately 5 U/L. We believe that a substantial portion of this residual activity is accounted for in the matrix blank, which contained no added AST activity (see Materials and Methods), and that it does not represent an artifact of the mitochondrial isoenzyme. As mentioned previously, the matrices containing PVP began to collapse structurally at the higher temperatures. Finally, azide appeared to accelerate the loss of activity further, particularly in PVP matrices.

Data in Table 4, for the mitochondrial isoenzyme, are analogous to those in Table 3. For comparison, the mean values of all eight pools measured the day after they were lyophilized were not significantly different by Student’s t-test ($\alpha = 0.05$) at 121.5 U/L (SD, 2.06 U/L; n = 16) without pyridoxal in the reagent and 121.4 U/L (SD, 3.01 U/L; n = 16) with pyridoxal in the reagent. Although Figure 2 shows that all of the matrices containing pyridoxal gradually lost activity when stored at 4 °C, the two pools containing BSA (15 μmol of pyridoxal per liter, with and without azide) showed a greater loss of AST activity when measured without pyridoxal in the reagent. After 39 days at 4 °C (Table 4), both pools had a difference in activity of 11 U/L between the values measured with and without pyridoxal in the reagent. These results indicate that the mitochondrial isoenzyme did not remain fully saturated with cofactor in these two matrices. In contrast, the same isoenzyme stored at 4 °C in the matrix containing PVP and 15 μmol of pyridoxal per liter, without azide, lost essentially the same amount of activity as the comparable matrix containing BSA but showed no increase in activity when pyridoxal was added to the reagent. Furthermore, matrices stored at 4 °C and containing 150 μmol of pyridoxal per liter in BSA (with and without azide) and in PVP (without azide) lost only minor amounts of AST activity and did not increase in activity with pyridoxal in the reagent. However, the increased matrix pyridoxal concentration did not appear to confer any added stability at increased storage temperatures or to deter the deleterious effects of azide in PVP. When the activity in these materials was measured three days after they were reconstituted, essentially the same values were obtained as those for freshly reconstituted specimens. As was true for the cytoplasmic specimens, the reconstituted specimens were not visibly turbid after three days.

In summary, the human cytoplasmic isoenzyme appears to be substantially more stable than the mitochondrial isoenzyme in lyophilized materials of comparable composition. The large-molecular-weight component of the matrix apparently exerted the largest single effect on the stability of both isoenzymes, with BSA stabilizing the activity more than did PVP. Because none of the specimens reconstituted for three days exhibited visible signs (turbidity) of microbial growth, adding azide to the matrix was not only nonessential but also accelerated the decay of both isoenzymes in PVP. AST activity lost by the lyophilized specimens as a result of storage in the different matrices could not be recovered, except that the mitochondrial isoenzyme in a matrix of BSA and 15 μmol of pyridoxal per liter showed a small increase in activity with pyridoxal added to the reagent. Increasing the concentration of pyridoxal in the matrix from 15 to 150 μmol/L did not substantially increase the stability of either isoenzyme in terms of loss of activity at a storage temperature of 37 °C.

On the basis of both the results of this study and of our previous interlaboratory AST study (1), we have prepared additional lyophilized materials containing the cytoplasmic isoenzyme of AST purified from human erythrocytes in a matrix of, per liter: 15 μmol of pyridoxal, 50 μmol of 2-oxoglutarate, 50 μmol of dithiothreitol, 50 mmol of Tris-HCl buffer (pH 7.5), and no preservative. These materials have been extensively characterized in terms of the purified isoenzyme, stability in the lyophilized and reconstituted states, contaminating enzymes, among-vial variability, and assigned target values with the recently published IFCC method for measuring AST. Because we prepared only a few vials of each pool, these materials will be used primarily to assess the transferability of nationally or internationally endorsed methods for quantitating AST. More specifically, we are currently examining the interlaboratory performance of the recently proposed IFCC reference method for measuring AST (22).

We gratefully thank Dr. James Barbaree for his assistance in the lyophilization of the materials used in this study.
References


