A Discussion of Enzyme Reference Materials: Applications and Specifications

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Clinical laboratories estimating enzyme activity in serum are using commercial lyophilized sera for four major purposes. These uses—as a standard and for intermethod, intramethod, and precision control—are segregated, and specifications for each deployment are examined in terms of requirements for the enzyme material: freedom from interfering or indicator enzymes and chromogens; high specific activity; inclusion of optimal cofactor concentrations; commutability, human properties and source; the presence of a single isoenzyme; and stability. The effects of serum matrix and variable assay conditions on the utility of enzyme materials are analyzed. Specifications differ for each enzyme material application. The compatibility of commercial lyophilized sera containing aspartate aminotransferase activity with several cited specifications is assessed.

Additional Keyphrases: commercial lyophilized reference sera, enzyme activity units, aspartate aminotransferase, assessment of interlaboratory accuracy and precision, origin of reference material

A primary function of the Clinical Chemistry Section, Division of Laboratories and Research, New York State Department of Health, is evaluation and improvement of performance in clinical chemistry under the clinical laboratory licensure program (I), involving submission of mailed and investigator-carried test specimens for analysis by more than 450 clinical laboratories outside of New York City. Enzyme assays surveyed under this program since 1965 are acid and alkaline phosphatases; amylase; LD; and AST. Both LD and AST assays have been studied extensively since 1968. In addition to assessing laboratory performance, we have noted the actual methods routinely used in the State and the manner in which they are standardized. Table 1 lists a summary of methods and standardization procedures for AST and LD activity determinations in New York State (excluding New York City) in January 1972. Differences noted between kinetic methods and absorbance change standardization are attributable to a small number of laboratories that “standardize” the rate reactions by using control sera!

In our program we have noted a steady increase in the use of kinetic reactions to determine enzyme activity. Only 5.8% of the laboratories used kinetic LD methods in 1968; this increased to 11.4% in 1969 and 19.8% in 1972. A doubling in the use of kinetic AST assays was noted: from 7.6% of the laboratories in 1969 to 14.9% in 1972. In spite of this increased popularity in kinetic methods, “two-point” assays requiring external standardization still comprise the majority of AST and LD assays used in the State. Most of the laboratories performing these enzyme assays

2Nonstandard abbreviations and trivial names used: Acid phosphatase, EC 3.1.3.2, orthophosphoric monoester phosphohydrolase; alkaline phosphatase, EC 3.1.3.1, orthophosphoric monoester phosphohydrolase; amylase, EC 3.2.1.1, α-1,4-glucan 4-glucanohydrolase; lactate dehydrogenase (LD), EC 1.1.1.27, l-lactate:NAD oxidoreductase; aspartate aminotransferase (AST), EC 2.6.1.1, l-aspartate:2-oxoglutarate aminotransferase, formerly known as glutamic-oxaloacetic transaminase (GOT); glutamate dehydrogenase (GD), EC 1.4.1.3, l-glutamate:NAD(P) oxidoreductase (deminating); malate dehydrogenase (MD), EC 1.1.1.37, l-malate:NAD oxidoreductase.
Table 1. Enzyme Methods and Standardizations Used in Clinical Laboratories in New York State as of January 1972.  

<table>
<thead>
<tr>
<th>Method</th>
<th>No. laboratories</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>AutoAnalyzer colorimetric</td>
<td>51</td>
<td>14.9</td>
</tr>
<tr>
<td>AutoAnalyzer “340 nm”</td>
<td>37</td>
<td>10.8</td>
</tr>
<tr>
<td>Retman-Frankel</td>
<td>169</td>
<td>49.4</td>
</tr>
<tr>
<td>Babson</td>
<td>28</td>
<td>8.2</td>
</tr>
<tr>
<td>Kinetic</td>
<td>51</td>
<td>14.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Standardization</th>
<th>Method</th>
<th>No. laboratories</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyruvate</td>
<td>AutoAnalyzer colorimetric</td>
<td>41</td>
<td>13.5</td>
</tr>
<tr>
<td>Serum</td>
<td>AutoAnalyzer “340 nm”</td>
<td>40</td>
<td>13.2</td>
</tr>
<tr>
<td>Absorbance change (340 nm)</td>
<td>Cabaud-Wroblewski</td>
<td>121</td>
<td>39.9</td>
</tr>
<tr>
<td>Absorbance change (366 nm)</td>
<td>Babson-Phillips</td>
<td>36</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>Kinetic</td>
<td>60</td>
<td>19.8</td>
</tr>
</tbody>
</table>

Methods and types of standardization used by fewer than 1.5% of the laboratories are not listed. (New York City not included).

have elected to standardize with commercial lyophilized control sera (Table 1). While the deficiencies of these materials as enzyme standards have been documented (2, 3) only six (2%) laboratories performing the LD determination and three (0.9%) laboratories performing the AST assay restandardize the serum “standard.” Control sera are also used by nearly all of these laboratories for quality control. Widespread use of serum-based enzymes has prompted our examination of these materials and their applications in clinical enzyme determinations.

There are four purposes for which enzyme materials are used: Standard, to calibrate assays, compensate for assay variables, and quantitate recovery; Intermethod control, to assess accuracy of a method by comparison to reference method value; Precision control, to establish day-to-day variability found in the assay of patient sera; and Intramethod control, to detect when assay condition alterations have caused or are about to cause error in determined patient values. Materials to be used for these purposes need not meet identical specifications. In fact, it is often preferable that enzyme materials used for different purposes possess dissimilar properties. Specifications that may be required of enzyme materials and the applicability of these specifications to the four potential uses are shown in Table 2.

Specifications for Enzyme Material Application

Freedom from interfering enzymes. Because the activity of a standard should reflect any changes in the assay system to a degree equal to changes in patient serum activity, interfering enzymes should be absent from an enzyme material used as a standard. Interfering enzymes will have optima different from those of the enzyme assayed, and their activities will respond differently to deviations in assay conditions. Interlaboratory controls should also be free from interfering enzyme activity. These materials—whether enabling a user to assess his own accuracy or a surveyor to assess the user’s accuracy—will be used in diverse assays. Thus the ratio of interfering enzyme activity to enzyme activity being monitored will not be constant. It is impossible to assess accuracy under these conditions.

An interfering enzyme having properties such that its activity will change more than that of the enzyme assayed, as reagents or temperature vary, is valuable in an intramethod control but undesirable in a precision control. Such a material could indicate a change in assay conditions prior to a change in patient values, but it will not correctly assess routine precision.

GD, an interfering enzyme in the AST assay when NH₄⁺ is present, was measured in 10 commercial lyophilized control sera, and none were found to possess greater than 1.5 U of GD activity per liter at 30°C. This is in contrast to our findings five years ago, where GD activity was as high as 7.5 U/liter in commercial control materials (4).

Freedom from indicator enzymes. Indicator enzymes should be absent from standards, intermethod controls, and intramethod controls. In each case, data should reflect the viability of the reagents used. Indicator enzyme activity in an enzyme material would prevent detection of a deficiency of this coupling enzyme. Precision controls, however, are used to ascertain repeatability under defined conditions, so it is unnecessary to ensure that they are free from indicator enzymes. Previous work (4) indicated that significant amounts of MD, the indicator enzyme for the kinetic AST assay, were present in commercial materials.

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3"Precipath E" (Boehringer-Mannheim Corp., New York, N.Y. 10006); "Monitrol II" and "Enzatrol" (Dade Division, American Hospital Supply Corp., Miami, Fla. 33152); "Technicon SMA 12 Reference Serum" (Technicon Instruments Corp., Tarrytown, N.Y. 10591); "Metrix Normal" (Metrix Clinical and Diagnostic Division, Armour Pharmaceutical Co., Chicago, Ill. 60690); "Leder-Zyme E" (Lederle Laboratories, American Cyanamid Co., Pearl River, N.Y. 10965); "Hyland Abnormal" and "Hyland QII" (Hyland Division, Travenol Laboratories, Costa Mesa, Calif. 92628); "Versatol E" (General Diagnostic Div., Warner-Chilcott Laboratories, Morris Plains, N.J. 07950); and "Chemtrol" (Clinton Laboratories, Los Angeles, Calif. 90019).
Table 2. Specifications for Use of Enzyme Materials

<table>
<thead>
<tr>
<th>Specification</th>
<th>Standard</th>
<th>Inter-method control</th>
<th>Precission control</th>
<th>Intra-method control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freedom from interfering enzymes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Freedom from indicator enzymes</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Freedom from chromogens</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>High specific activity</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cofactor optimum</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Commutability</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Human enzyme properties</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Human source</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Homoisoenzymic</td>
<td></td>
<td>-</td>
<td>-</td>
<td>0</td>
</tr>
<tr>
<td>Matrix mimic</td>
<td></td>
<td>+</td>
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<td>+</td>
</tr>
</tbody>
</table>

+ Specification necessary.
- Specification potentially undesirable.

Freedom from chromogens. Chromogens that interfere in colorimetric assays should be absent from an enzyme material used as a standard, precision control, or intermethod control, because chromogen interassay reactivity is likely to be inconsistent. An exception is interlaboratory control material used by the persons doing the survey, when information obtained is used to assess laboratory competency for legal purposes.

Lack of chromogen, on the other hand, may be an undesirable attribute of an intramethod control. Interfering chromogens are an advantage in the control of colorimetric assays, for without chromogens and a concurrent serum blank one is assuming that exogenous chromogen–color reagent reactivity is constant, or at least influenced in the same direction and to the same extent by changes as is product–reagent activity.

High specific activity. Bowers and McComb (5) require that reference enzyme materials possess “molecular activity . . . comparable to highest literature values.” High purity minimizes the possibility of interfering enzyme, indicator enzyme, or chromogen contaminations; precludes the presence of proteins that affect enzyme catalysis; and enables determination of enzyme activity optima uninfluenced by matrix interference. If a standard enzyme material is to be used in recovery experiments, it must be of high specific activity. But high specific activity does not improve the other functions of an enzyme material. For example, stability is compromised when a high-purity AST enzyme material is prepared (6).

Cofactor optimum. Need sufficient cofactor be present in the enzyme material for optimum activity? In this instance, “cofactors” refers to materials present in the final assay system in cofactor amounts; therefore NAD, NADH, and the like, if they are present in co-substrate amounts, are not included.

Generally cofactors such as pyridoxal phosphate and metal ions are not deliberately added in clinical assays; they are usually presumed to be already present in serum in sufficient quantity for optimum activity. When an enzyme material deficient in a certain cofactor is recovered from serum, its measured activity will be increased due to cofactor present in the serum. An enzyme material saturated with cofactor is, however, an undesirable intramethod control in assays in which reagents are cofactor-supplemented. Because pyridoxal phosphate, for example, is unstable in solution, a control material deficient in pyridoxal phosphate should be used to monitor the reagent cofactor. Although no commonly used assay for AST prescribes that pyridoxal phosphate be added to the reagent system, results described by Hamfelt (7) warrant its inclusion. Amador and Salvatore (8) have shown that one commercial material is deficient in pyridoxal phosphate for measurement of AST activity. When pyridoxal phosphate (20 μmol/liter) was added to 10 commercial lyophilized sera, we were able to demonstrate more than twice as much AST activity in three materials and an increase from 13 to 64% in six others. Only one material was demonstrably saturated with pyridoxal phosphate.

Commutability. “Commutability” refers to the ability of an enzyme material to show interassay activity changes comparable to those of the same enzyme in human serum. While commutability is a consequence of other effects noted in Table 2, its significance warrants separate discussion.

The plethora of assays and units associated with each enzyme is a major dilemma in clinical enzymology. Our testing program reveals that 47 and 48 different units, methods, and temperature combinations are being used for the LD and AST assays, respectively. While development of a uniform interlaboratory methodology is at present impractical, utilization of an enzyme standard—rated by a reference assay—for calibration of various determinations is feasible. The prime property of such a standard is predictability of enzyme catalytic activity as measured by various methods, based on its activity in a reference procedure. This prediction must be based on the same numerical factor observed for patient sera. It is therefore objectionable to rate a material in several different units, so that every assay may continue promulgating eponymous units.

If the enzyme activity of an intermethod control is commutable, the material can be used to ascertain the validity of results from one method by a reference method. Commutability is not needed for assessing precision or day-to-day accuracy when a single method is being monitored.

There is reason to question the commutability of available materials. The standardization of AST activity by the Reitman–Frankel assay (9) was empirically derived, and units by this method should be identical with units described by Karmen (10). Yet commercial control-sera package inserts indicate
that there may be a 50% difference in rated values by these two procedures. Many other discrepancies are noted not only from the expected activity ratios but even between manufacturers and between lots by one manufacturer. Transmogrified assays and unit definitions by manufacturers also contribute to these discrepancies.

**Human enzyme properties.** Enzymes used as standards or intermethod controls must respond to assay conditions as does the enzyme of human serum. Standards must compensate for assay variations; their optima must coincide with those of enzymes in serum. With deviations from optimum conditions, the rate of change of standard activity must equal that seen for patient sera. Temperature responses must also be identical. An intermethod control material must also possess the same enzymatic properties as samples from humans if it is to be of use in evaluation of nonreference methods.

If enzyme material activity does not vary with random assay changes as does the enzyme in human serum, it is not useful in assessing average precision. Precision controls must therefore possess enzymatic responses equal to those of human serum enzymes. Changes in enzyme activity for every variation in assay condition must be at least as great in intra- method controls as in sera from patients. Changes greater than those seen in human enzyme response are acceptable, even preferable, in an intramethod control material.

**Human source.** It is unlikely that enzymes from nonhuman sources will meet the requirements of human properties and commutability. If immunologic enzyme assays become popular, human control material would invariably be required. However, use of a human source does not ensure that the enzyme isolated will have properties that are the same as those of the enzyme in untreated serum. Purified materials must be completely evaluated by comparison with native serum enzymes. Even enzymes obtained from serum should be investigated to identify artifactual enzyme alterations.

**Homoioenzymic character.** Need the enzyme material consist of a single isoenzyme? For certain enzymes this may be undesirable. Data by Gay et al. (11) for LD isoenzymes (lactate substrate) show that pH variation from 8.75 to 8.85 produces little change in the activity of LD I and nearly 20% change in LD V activity. The ratio of LD I activity to LD V activity is about 1.0 at pH 8.5; this ratio becomes 1.4 at pH 8.85. Thus both isoenzymes have deficiencies when used as a standard, intermethod control, or precision control for the clinical estimation of total LD activity at 30°C. From the same data (11) LD V can be seen as a sensitive intramethod indicator of pH changes between pH 8.5 and pH 8.8.

**Matrix mimic.** It is necessary that serum constituents that influence the enzyme activity or the color-development process be present in control materials used to assess accuracy or precision. Conversely, ad- ditives that can mask effects (such as chelators, which may remove inhibitory metal ions) must be absent.

We (6) have demonstrated significant effects of serum protein in the AST assay of Babson et al. (12) and in the Technicon "340 nm method" (13, 14). We have also observed matrix effects on tetrazolium dye-coupled LD assays, where serum proteins are required to stabilize the formazan produced. With use of a purified LD I from human erythrocytes (5 g of protein per liter), the Warner-Chilcott "Lac-Dehy- strate," Dade "Teta-Form" and Coulter "Accu- zyme" kits give very low results on evaluation samples. We also observed absorption artifacts in kinetic assays involving NADH oxidation, in the case of some commercial control sera (4): turbid materials artifactually increased absorbance change at 340 nm in the kinetic AST assay of Henry et al. (15).

Because standard or reference enzyme materials are used in recovery experiments, matrix effects already present will compromise this utility. Where matrix effects may be necessary, inactivated human serum can be easily added to the standard. We find that lowering the pH of pooled serum to 3.0 for 48 h at room temperature, neutralizing with base, and dialyzing produces a matrix free from LD and AST activity.

**Stability.** For normal use, an enzyme material must be adequately stable. Although it is difficult to fix stability specifications, a two-month minimum is necessary for statistical purposes. In general, utility increases with stability. From human erythrocytes we have produced AST and LD materials, both serum-based and in purified form, that are satisfactorily stable for more than two years.

**Manufacturer's rated values.** It is necessary that rated (labeled) values of commercial materials be accurate, for, in our survey, more than 97% of the laboratories using these materials as standards accept the rated values. Information on the rating process must also be completely and clearly stated. Details—including final substrate concentrations, buffer, pH, and temperature of the reference assay—must be included. Unfortunately, deficiencies in both accuracy and completeness of data are well documented (2, 3, 16, 17). Ideally, the material should be evaluated by one or two reference methods, at two or more temperatures.

**Envol**

Enzyme materials may be prepared to achieve either of two results: a single material to satisfy all needs, or separate materials suited for specific applications. A practical solution is a single material for each enzyme, which meets the desired specifications of a standard (Table 2). These purified materials, with defined properties similar to those of human serum enzymes, will be of great assistance in the standardization, control, and comparison of enzyme material activity.
methods. Although difficulty may arise in the application of a highly purified material, provision can be made for reinstatement of a serum matrix. Such a material can be acceptably used for all purposes, even though better materials for specific purposes may be produced.

References