

Standardization of clinical enzyme assays

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During 1969, discussions between the Standards Subcommittee of the Laboratory Equipment and Methods Advisory Group of the Department of Health and Social Security and representatives of professional societies concerned with the assay of enzymes for diagnostic purposes led to the establishment of a working party to consider the current state of accuracy and reliability of diagnostic enzymology and to make recommendations for their improvement. The working party was based on one which was then in process of formation by the Association of Clinical Biochemists and its composition was chosen so that the Association of Clinical Pathologists and the Royal College of Pathologists would also be represented. This first report discusses the general principles of standardization of enzyme assays, and deals in detail with alkaline phosphatase, the commonest serum enzyme assay performed by a fixed time procedure. The second report will deal with aspartate and alanine transaminases, the commonest serum enzyme assays performed by a kinetic procedure.

General Principles

Measurements of enzyme activity involve the comparison of rates of reaction, with and without the active enzyme in the reaction mixture. Units of enzyme activity are thus the amount of chemical change taking place in unit time, while the activity of an enzyme in a given sample is expressed as the number of units in a stated volume or mass of the sample. The significance of a particular enzyme unit depends on the values chosen for a number of variables: the concentration of substrate and its nature (where this can be varied), the pH and constitution of the buffer, temperature, presence of activators or inhibitors, the reaction product selected for estimation and the method by which this is done, the time interval to which change is referred, eg, per minute, per hour, etc. Enzyme units are thus functions of the methods used to determine them, and any deviation from the prescribed conditions

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will alter the meaning of the results of an enzyme assay which purports to provide results in terms of specified units.

The first recommendation of the Working Party is therefore that, for enzyme units in common clinical usage, acceptable tolerances should be defined for the variables in the assay methods which specify these units: modifications exceeding these tolerances should be considered to alter the value of the units so that they can no longer be regarded as equivalent to those defined by the original assay method.

This is not intended to imply that no improvement to existing methods can be contemplated. It is desirable that enzyme assays should be carried out as far as possible under optimal conditions, and when older-established methods are re-examined this is often found to be far from the case, eg, with respect to substrate or cofactor concentrations, pH etc. Furthermore, many older methods are based on fixed-time or 'two-point' assay principles, which are more open to error in comparison with kinetic methods especially when a wide range of enzyme activity is likely to be encountered and when factors such as accumulation of inhibitory products fall in substrate concentration, enzyme inactivation etc, make the maintenance of linearity during the reaction period uncertain. For these reasons therefore, the increasing availability of suitable recording spectrophotometers will favour the replacement of fixed-time assays by kinetic procedures. Changes in an enzyme assay method which involve modifications of the original conditions, even if these stop short of alterations in the principles of assay, should be regarded as equivalent to the introduction of a new method with new units, normal ranges, etc. The name of the old units should not be applied to those derived from the modified procedure, since much of the present lack of comparability of results of enzyme analyses from different laboratories is probably caused by deviations from published methods which are assumed not to alter the value of the resulting enzyme units.

Expert panels organized by the International Federation for Clinical Chemistry are now con-

sidering problems of standardization in clinical enzymology. It is expected that their recommendations will include the definition of reference methods for selected enzymes which will be based on the most recent advances in knowledge and technique and will therefore define new units.

In this and subsequent reports the Working Party will consider the application of these principles to the assay of some enzymes in extensive use in clinical enzymology.

Alkaline Phosphatase

At least 20 methods or modifications of methods, each with its own unit, have been proposed for the estimation of this enzyme in serum. However, three alkaline phosphatase units are in general use. The King-Armstrong unit (substrate, phenyl phosphate) is widely used in Europe, while the Bodansky unit (substrate, β -glycerophosphate) and the Bessey-Lowry-Brock unit (substrate, *p*-nitrophenyl phosphate) are often adopted in America. The use of AutoAnalyzer methods for alkaline phosphatase which depends on the hydrolysis of phenyl phosphate and which are designed to provide results in King-Armstrong units has extended the currency of this particular phosphatase unit, and it is therefore the definition of the King-Armstrong unit which is considered in this report.

THE KING-ARMSTRONG UNIT

The original method of King and Armstrong (1934) employed 0.005 M-disodium phenyl phosphate in veronal (barbitone) buffer at pH 9.0; incubation was for 30 min at 37°C, and one unit corresponded, to liberation of 1 mg phenol in this time. Later modifications (King, Haslewood, and Delory, 1937; King, Haslewood, Delory, and Beall, 1942) preserved the same concentration of substrate but raised the pH of incubation to approximately 10 by use of carbonate-bicarbonate buffer mixtures (Delory and King, 1945). King and Armstrong (1934) preferred the Folin-Ciocalteu reagent to diazotization as the means of estimating phenol released by phosphatase. Later variations have made use of 4-amino antipyrine (4-aminophenazone) (King and King, 1954). The final version of the King-Armstrong method using the Folin-Ciocalteu reagent can be found in King and Wootton (1956) and the King-King modification in Wootton (1964).

The modifications of the original King-Armstrong method were reported to produce little change in the value of the unit. However, quantitative data on this point are now hard to elicit since the earlier method has fallen into almost total disuse and the King-Armstrong method is today regarded as essentially

synonymous with its later modifications. It is therefore recommended that the procedure which defines the King-Armstrong Unit of alkaline phosphatase should be that described in King and Wootton (1956, p. 83). The alternative method of measuring phenol released, ie, the amino antipyrine reaction, is given in Wootton (1964, p. 102). Both methods use identical incubation conditions, and although there is an impression that slightly different values are obtained at high enzyme levels when the two methods are compared or that the precision of the amino antipyrine method is somewhat lower, published data do not warrant the exclusion of the later modification as an alternative reference method. Since the third edition of King and Wootton's book (1956) is out of print, the suggested reference method is set out below:

REFERENCE METHOD

Test

Two ml buffer and 2 ml substrate solution: mix and warm to 37°C in an accurate thermostatic water bath, then add exactly 0.2 ml enzyme solution and mix. After exactly 15 minutes' incubation, add 1.8 ml Folin-Ciocalteu reagent (diluted 1 + 2 v/v with distilled water). Mix and centrifuge or filter.

Control

Two ml buffer and 2 ml substrate solution: mix and warm to 37°C. Add 0.2 ml enzyme solution followed immediately by 1.8 ml dilute Folin-Ciocalteu reagent, mix, and centrifuge or filter.

Measure 4 ml of the clear supernatant or filtrate from each of the 'test' and 'control' tubes and to each add 2 ml sodium carbonate solution (150 g anhydrous Na₂CO₃ per litre), mix, and warm to 37°C for 15 minutes. Read absorbances at 700 nm in a spectrophotometer. A phenol standard and reagent blank are prepared as follows:

Standard

A stock phenol standard solution (1 g AR phenol per litre of 0.1 M-HCl) is diluted 1 in 100 by adding to 5 ml of it 100 ml dilute Folin-Ciocalteu reagent and diluting to 500 ml with water. Four ml of this working phenol standard is mixed with 2 ml of the 15% sodium carbonate and warmed to 37°C for 15 min before measuring absorbance. (A stock phenol solution is now commercially available from BDH Ltd, but the absorbance of a new batch of this reagent, or of a laboratory-made standard solution, should be checked before use against a phenol solution of accurately-known composition.) The diluted phenol standard is stable for several months at 4°C.

Blank

Water, 3.2 ml, and 0.8 ml dilute Folin-Ciocalteu reagent are treated with sodium carbonate as above.

Calculation

King Armstrong units/100 ml =

$$\frac{\text{Reading of (test - control)}}{\text{Reading of (standard - blank)}} \times 30$$

NOTES ON THE REFERENCE METHOD

The substrate solution consists of 2.18 g disodium phenyl phosphate or 2.54 g of the dihydrate dissolved in a final volume of 1 litre of distilled water. The solution is brought quickly to the boil and cooled. Four ml chloroform per litre is added as a preservative and the solution is stored at 4°C.

The buffer (0.1 moles/litre sodium carbonate-bicarbonate) is prepared by dissolving 6.36 g anhydrous Na₂CO₃(AR) and 3.36 g NaHCO₃ (AR) in CO₂-free distilled water (1 litre). The pH of this mixture is 10.14 at 20°C, corresponding to pH 9.90 at 37°C. At the final substrate concentration of 4.75 millimoles per litre the pH activity curve of alkaline phosphatase has a fairly sharp optimum. To avoid errors in enzyme activity measurement of greater than ± 5% arising from pH variation, the pH of the buffer should lie within ± 0.1 pH unit of its nominal value.

The carbonate-bicarbonate buffer specified by the King-Armstrong method should not be replaced by one of different chemical constitution, even if the pH remains the same, since changes in ionic strength can alter phosphatase activity while certain buffer ions (eg, tris) accelerate the reaction by acting as phosphate acceptors.

The pH optimum of alkaline phosphatase is also markedly dependent on substrate concentration: for this reason, and also because the substrate concentration used in the method is possibly not fully saturating for all alkaline phosphatase isoenzymes, the substrate solution should be accurately prepared and dispensed.

No addition of magnesium ions is specified in the King-Armstrong method and therefore none should be made since the activation produced thereby would alter the value of the enzyme units.

The method described above suggests that the enzyme solution and Folin-Ciocalteu reagent should be added to the 'control' tube in the reverse order to that set out in the published procedure: this is because it has been observed that, for some enzyme samples, addition of the Folin-Ciocalteu reagent before the enzyme solution gives a falsely low control reading. For the most accurate work the period elapsing between these two additions (usually not

more than 20 seconds) should be timed and the incubation period of the 'test' extended by a corresponding interval. (The amino antipyrine method is free from this anomaly.)

When high phosphatase activities are to be measured the incubation time should be reduced until it is certain that the progress of the reaction is linear over the chosen interval; this may involve repeating the estimation with progressively shorter incubation times. The calculation is then adjusted. Dilution of phosphatase samples should be avoided since activation, ie, a non-linear response to dilution, has been shown to occur.

For a reaction which is linear with respect to time an error of 1 min in timing an incubation period of 15 min corresponds to an error of 6.7%, while a deviation of 1°C from 37°C may introduce an error of nearly 10% in many enzyme estimations.

CALIBRATION OF AUTOMATED ALKALINE PHOSPHATASE METHODS

Probably the majority of serum alkaline phosphatase estimations are now carried out by an adaptation of the King-Armstrong procedures to the Auto-Analyzer. Two methods of calibration are possible. In the first, the phenol liberated from phenyl phosphate is measured by comparison with phenol standards and the incubation time is estimated: the milligrams of phenol liberated in 15 min are then calculated to give results in King-Armstrong units. In the second, enzyme-containing samples which have been calibrated by the manual reference method are analysed together with the unknown samples which are thus assigned values in King-Armstrong units by comparison. Since the conditions of incubation in the automated procedure are not identical with those prevailing in the manual method with regard to such variables as period of incubation, the second alternative is to be preferred. Its use implies that the manual reference method must be available to allow calibration specimens to be prepared. The calibrated samples which form the calibration curve, eg, five samples covering the range 5-50 King-Armstrong units per 100 ml, are introduced into each AutoAnalyzer run. Serum pools which have been enriched with appropriate amounts of alkaline phosphatase, divided into 1-2 ml portions and stored at -20°C after manual assay, provide useful calibration standards¹. It is preferable to avoid the use of pools of human serum because of the risk of contamination with hepatitis virus, but

¹ The alkaline phosphatase activity of enriched frozen-serum pools increases slowly after thawing, and a similar effect follows reconstitution of lyophilized preparations (Brojer and Moss, 1971). When these materials are used for standardization of phosphatase assays, therefore, an adequate period, eg, overnight at room temperature, should be allowed for the enzyme activity to stabilize.

horse serum may be substituted. Enrichment may be by addition of an extract of bovine or human liver. If the latter is used the absence of Australia antigen (hepatitis-associated antigen) should be confirmed by immunological tests. The phosphatase activities of the pools should be re-checked every one or two weeks.

The recognition that alkaline phosphatase exists in tissue-specific isoenzyme forms and that phosphatases from tissues other than bone and liver can enter the circulation, eg, from the placenta in pregnancy or from the small intestine in several conditions, places limits on the validity of comparisons that may be drawn between methods which use different substrates. The relative rates at which various substrates are hydrolysed by alkaline phosphatase isoenzymes are not identical: while liver and bone phosphatases are similar in respect to this they differ from the intestinal and placental isoenzymes. Thus, while a particular conversion factor valid for liver phosphatase may be established between methods using different substrates (eg, phenyl phosphate and *p*-nitrophenyl phosphate) the same factor will not correctly express the relationship between the methods if samples containing the placental or intestinal isoenzymes are analysed. It is therefore recommended that only automated methods in which the substrate is phenyl phosphate

should be calibrated in terms of King-Armstrong units. Furthermore, the calibration samples which are analysed by the two methods, automated and manual, should have similar isoenzyme compositions. Since the majority of clinical samples contain liver or bone phosphatase, these enzymes should be present in the calibration samples. It should be noted that some commercial enzyme-containing control sera contain human placental alkaline phosphatase or bovine intestinal phosphatase and ideally should not be used for calibration.

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