Factors Affecting Measurement of Total Alkaline Phosphatase Activity in Human Serum, Especially Wavelength Accuracy

John A. Lott, Kathie Turner, and Jacqueline Scott

Serum alkaline phosphatase estimations by the Selected Method [Clin. Chem. 21, 1988 (1975)] are very sensitive to small errors in the wavelength setting of the spectrophotometer. With a setting error as small as 3 nm, results can be too low by as much as 23%. The product of the reaction, 4-nitrophenol, can be prepared in very pure form and can be used to check both the wavelength and absorbance accuracy of a spectrophotometer. The molar absorptivity for 4-nitrophenol obtained in the instrument being used should be used in calculating alkaline phosphatase activities.

Additional Keyphrases: molar absorptivity measurements • variation, source of • centrifugal analyzer

We would like to call attention to one aspect of a recently published Selected Method for serum alkaline phosphatase [orthophosphoric monoester phosphohydrolase (alkaline optimum); EC 3.1.3.1] (1) that may limit its reproducibility from instrument to instrument and laboratory to laboratory.

The two GEMSAEC centrifugal analyzers (Electronucleonics, Fairfield, N.J. 07006) in our laboratory are used exclusively for serum enzyme analyses. We follow the Selected Method procedure for alkaline phosphatase except that we initiate the reaction with the sample rather than the substrate. Recently, we noticed that results for alkaline phosphatase with use of the same samples on the two instruments did not agree, one instrument consistently giving results 6–8% higher than the other. For the NADH-based enzymes routinely assayed in serum we did not observe a difference between the two instruments for analyses done on the same specimens, but rather a good agreement. For this reason, we ruled out a temperature difference as the cause of the problem. We evaluated the wavelength accuracy of the two instruments with a diethylamine filter and found a slight difference in the absorbance at 573 nm (2). We concluded that one of the monochromators was slightly misaligned. On replacing the suspect monochromator, we found good agreement between the two instruments for both the diethylamine filter and for alkaline phosphatase determinations with duplicate specimens.

We were puzzled as to why a small wavelength misalignment should make such a large difference in the measured activity of alkaline phosphatase. From Figure 1 of the Selected Method it appears that 4-nitrophenol (4NP) has a broad absorbance band with a peak absorbance at 402.5 nm. It might erroneously be assumed from this figure that the substrate, 4-nitrophenyl phosphate (4NPP) does not absorb at 402.5 nm. These considerations and the fact that our NADH-based enzyme results were unaffected when the defective monochromator was replaced led us to look further into this problem.

Evaluation of 4NPP

Preliminary studies were carried out with a Model 222A (Gilford Instrument Lab, Oberlin, Ohio 44074) spectrophotometer (a converted Beckman Model DU), fitted with a cuvet chamber thermostated at 25 ± 0.2 °C. The 486.1-nm emission line of the deuterium lamp was used to check the wavelength calibration of the instrument. The maximum galvanometer response to the line occurred exactly at a wavelength setting of 486 nm. The absorbance accuracy of the instrument was checked with National Bureau of Standards SRM 930 glass filters at 440, 465, 590, and 635 nm, and the values found were within 1% of the values given by the Bureau (3).

We determined the molar absorptivity of a 90.0 μmol/liter solution of disodium 4-nitrophenyl phosphate, assumed to be the hexahydrate (No. 104-0, lot no. 47C50391, Sigma Chemical Co., St. Louis, Mo. 63178), molecular weight 371, in 10 mmol/liter NaOH at 311 nm, exactly as described by Bowers and McComb (1). We found it to be 8042, which is 91.8% of the reported molar absorptivity of 8650 (1). This lower value was probably attributable to moisture content of the 4NPP. In further studies we used 404.1 (371/0.918) as the apparent molecular weight of the 4NPP we were using. The inorganic phosphorus content of a 225 mmol/liter solution of 4NPP was found to be 0.354 mmol/liter, or 0.15 mmol/100 mol of the compound, well within the 1 mol percent limit of acceptability. To determine the 4NP content of the 4NPP, we measured the absorbance at 415 nm of a 112.5 mmol/liter solution in 10 mmol/liter NaOH at 25 °C. It was 0.614, and from the molar absorptivity of 15.900 we calculated the 4NP content of the 4NPP to be 0.343 mmol of 4NP per mole of 4NPP, which is acceptable (1). Culbreth et al. (4) examined 4NP for the presence of 4NP by high-performance liquid chromatography and they considered a 4NP with less than 1 mmol of 4NP per mole of 4NPP to be acceptable. Clearly, our substrate was acceptable by this standard also.

4NP reportedly absorbs at 404 nm (4). We found it also absorbs at 402.5 nm in 0.89 mol/liter, pH 10.33 (30 °C) 2-amino-2-methyl-1-propanol buffer as used in the Selected Method (1). An 18 mmol/liter solution of 4NPP in the AMP buffer had an absorbance of 0.660 at 402.5 nm. From the 4NP content of the 4NPP and the molar absorptivity of the 4NP at 402.5 nm of 18 478 that we determined (see below), we calculated that 0.114 of the measured absorbance of 0.660 was due to 4NP, the rest to 4NPP. Thus the molar absorptivity

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of the 4NPP is 30.3 at 402.5 nm in 2-amino-2-methyl-1-propanol buffer. With a 15 mmol/liter solution of pure 4NPP and under the conditions of the Selected Method, the starting absorbance at 402.5 nm would be expected to be 0.455.

Examination of 4NP in a Gilford Spectrophotometer and in the Centrifugal Analyzers

We measured the absorbance of 4NP solutions in the spectrophotometer and in both centrifugal analyzers, to see if the peak absorbance was observed at the correct wavelength and if we could obtain the published value for the molar absorptivity. 4NP (Eastman no. 192) was recrystallized from hot water, yielding long, slightly yellow needles. These were filtered off and dried over silica gel in a desiccator for two weeks. A 40 µmol/liter solution of 4NP in 10 mmol/liter NaOH had an absorbance of 0.724 at 401 nm in the spectrophotometer at 25°C, which corresponds to a molar absorptivity of 181,000, or 98.4% of the published value, 18,400 (1). For the same concentration of 4NP in 2-amino-2-methyl-1-propanol buffer (0.89 mol/liter, pH 10.33), we found an absorbance of 0.739 at 402.5 nm in the spectrophotometer at 25°C, corresponding to a molar absorptivity of 18,478, or 98.3% of the published value of 18,800. For the latter solution, we found molar absorbptivities of 18,668 and 18,800 with the two centrifugal analyzers. The wavelength of maximum absorption for 4NP in the spectrophotometer was 403 nm, differing by only 0.001 Å from the absorbance at 402.5 nm. In the two analyzers, the wavelengths of maximum absorbance were both exactly at 402.5 nm. We also checked the adherence to Beer's law of 4NP in 2-amino-2-methyl-1-propanol in the analyzers and found excellent linearity in both instruments, with all points on a straight line and a zero-zero origin for 4NP solutions over the absorbance range from 0 to about 1.0.

Studies with a Scanning Spectrophotometer

A Model 551 (Perkin-Elmer, Norwalk, Conn. 06852) scanning spectrophotometer was used to follow the change in the spectrum between 380 and 500 nm during the course of the action of alkaline phosphatase on 4NPP according to the Selected Method. The wavelength calibration of the instrument was checked with the deuterium lamp at 486 nm. The spectrum of the 4NPP in 2-amino-2-methyl-1-propanol was obtained first, then the reaction was initiated with a control serum having about 300 U of activity per liter. The spectrum was obtained at 2-min intervals during the reaction (Figure 1). As there was no discernible peak at 402.5 nm, the substrate was diluted 250-fold with 2-amino-2-methyl-1-propanol plus magnesium chloride to bring the absorbance at 311 within the range of the instrument. The spectrum of the substrate alone was recorded, serum was added, and the spectrum recorded again at the time intervals shown in Figure 2. The conditions here were far from the optimum substrate concentration and the reaction was very slow and nonlinear with time. Never-
Table 1. Apparent Alkaline Phosphatase Activity of Control Sera at Different Wavelengths, U/liter (Means of Four Values)

<table>
<thead>
<tr>
<th>Wavelength, nm</th>
<th>Sample A</th>
<th>Sample B</th>
<th>Approx. starting absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>396</td>
<td>45</td>
<td>186</td>
<td>1.7</td>
</tr>
<tr>
<td>399</td>
<td>86</td>
<td>214</td>
<td>1.3</td>
</tr>
<tr>
<td>402</td>
<td>112</td>
<td>276</td>
<td>0.7</td>
</tr>
<tr>
<td>405</td>
<td>98</td>
<td>259</td>
<td>0.5</td>
</tr>
<tr>
<td>408</td>
<td>100</td>
<td>259</td>
<td>0.3</td>
</tr>
</tbody>
</table>

theless, we did see an increase in the absorbance at 402.5 nm, a fairly flat shoulder of absorbance at this wavelength, and a decrease in the absorbance at 311 nm. An additional peak at 285 nm was seen after serum was added to the substrate.

Reaction Rates at Various Wavelengths

We estimated the alkaline phosphatase activity of two control sera with supranormal activities in one of the analyzers according to the Selected Method, except that the reaction was initiated with serum rather than the substrate. Table 1 gives the observed activities at five different wavelengths. The change in activity with wavelength was particularly significant at shorter wavelengths. Penton et al. (5) reported similar results: at wavelengths +10 and +20 nm away from the peak absorbance, the apparent mean activity was 93 and 74% of the activity measured at the peak wavelength.

At the peak wavelength of 402.5 nm we observed strict proportionality between measured activity and amount of enzyme present in a series of serum dilutions.

Discussion

We judge that the 4NPP (from Sigma Chemical Co.) that we used was satisfactory by the criteria of low inorganic phosphorus and 4NP content. Also, the analyzers were well calibrated, both for wavelength and absorbance accuracy. Contrary to what might be expected, there was no observable absorbance maximum at 402.5 nm during the course of the reaction (Figure 1). Before the reaction was started, part of the absorbance at 402.5 nm was attributable to the absorbance of contaminating 4NP in the 4NPP, but most of it was attributable to the substrate itself. Because the absorbance is measured on the shoulder of a very large absorbance peak of the substrate, the band-pass of the instrument at 402.5 nm must be quite narrow, probably less than 10 nm, if the observed rate of the reaction is to be proportional to the amount of enzyme activity taken. And for accurate estimations of alkaline phosphatase activities, Beer's law must be followed. Strictly speaking, Beer's law does not hold for measurements made on the shoulder of a peak (6); however, we found that with the spectrophotometer and the analyzers the rate was proportional to the enzyme activity.

Moreover, the wavelength accuracy of the instrument must be within 1 nm of the true value if the observed results are to be within 5% of the expected results; an approximation calculated from the data in Table 1. A wavelength setting of 3 nm below 402.5 nm can lead to values that are 23% too low. The effect of wavelength accuracy is predicted to be more critical for instruments that have a very narrow band-pass, e.g., 2 nm. It is probably wise, for optimum precision, that the wavelength setting for prism or grating instruments always be approached from the same direction, because there may be some lag in the gear train.

With a satisfactory substrate, an indication of a mis-set monochromator, where the true value is greater than the dial value, is a starting absorbance exceeding about 0.75. This would be true for a substrate with 1 mmol of 4NP per mole of 4NPP. A starting absorbance of less than about 0.5 indicates that the actual wavelength is less than that indicated on the dial.

Another factor affecting starting absorbance is temperature. Burtis et al. (7) showed that 4NPP is thermodichromic, i.e., absorbance depends on temperature. If the temperature of the solution being examined at 402.5 nm changes during the course of the reaction, the contribution of 4NPP to the total absorbance will change and erroneous values for enzyme activities will result. The effect of this error is especially large if the measuring intervals are short, and the change in temperature significant. For a 1-min time interval and a 0.5 °C change in temperature during this interval, a 10% error is introduced (7).

In the calculation of alkaline phosphatase activities by kinetic methods, the molar absorptivity of 4NP is used. If the published value is used and if the instrument does not produce the same molar absorptivity, then comparisons with peer labs using the same method will be poor. It is more appropriate to determine the molar absorptivity on the instrument being used rather than uncritically to use the published value. 4NP, readily recrystallized from hot water, produces a very pure product. It has a distinct wavelength of maximum absorbance of 402.5 nm in 2-amino-2-methyl-1-propanol buffer, and a properly calibrated narrow band-pass instrument should produce the expected molar absorptivity value. It is therefore a very useful material for checking wavelength and absorbance accuracy of spectrophotometers, and it is particularly useful for improving the precision of alkaline phosphatase estimations. A rather minor consideration in the Selected Method is that the absorbance of the substrate decreases as the absorbance of the product increases—but the molar absorptivities are so different, 30.3 vs. 18 900, respectively, that the error introduced is miniscule.

Grannis and Masson found in a recent College of American Pathologists enzyme survey that alkaline phosphatase determinations done in different laboratories by kinetic methods with 4NPP as substrate and 2-amino-2-methyl-1-propanol as buffer on the same kind of instrument were precise (8). However, alkaline phosphatase determinations carried out with the same substrate and buffer on different instruments showed a great deal of variability. Also, the variability observed for alkaline phosphatase estimated on different instruments was greater than the variability that was observed for aspartate- or alanine aminotransferase or lactate dehydrogenase when the latter four enzymes were estimated by kinetic methods on different instruments. The cause of this greater variability for alkaline phosphatase estimations is most likely subtle inaccuracies in the wavelength settings of the instruments or the use of different or inappropriate wavelengths, or the use of an inappropriate molar absorptivity value for 4NP.

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References

During the past four years, we secured six batches of purified human prolactin for iodination by the same lactoperoxidase technique. Two of these batches gave us more satisfactory results in terms of assay sensitivity and consistency than did the others, regardless of the antisem used. Gel filtration of $^{125}$I-labeled hormone revealed that these two batches were composed of at least 55% "small" prolactin while the other four batches contained from 20 to 68% "big" prolactin. This qualitative difference was the most important contribution to assay discrepancies.

**Additional Keyphrases:** variation, source of • analytical error • "big" and "small" prolactin

Homologous radioimmunoassay for human prolactin (hPRL) has been commonly used in the assessment of hypothalamus-pituitary function (1), diagnosis of pituitary tumors (2, 3), drug therapy for psychoses (4), and management of amenorrhea associated with galactorrhea (5). Under ordinary circumstances, a routine radioimmunoassay for documentation of hyperprolactinemia does not require great accuracy or precision. However, there are cases for instance, patients with asymptomatic pituitary microadenoma in which precise measurement of serum hPRL becomes necessary. Furthermore, in research relating to physiological concentrations of circulating prolactin, high measurement precision is desired.

In our experience, a significant interassay variation of hPRL radioimmunoassay might result from different tracers being used in the system, even if the same batch of an extremely specific antisem is used. We believe that quality difference in purified prolactin is so important that a short note is worthwhile.

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