An Automated p-Nitrophenylphosphate Serum Alkaline Phosphatase Procedure for the AutoAnalyzer

Stanley Morgenstern, Gerald Kessler,* Joseph Auerbach, Richard V. Flor and Bernard Klein

A new and simplified procedure for the automated determination of serum alkaline phosphatase uses the AutoAnalyzer. The substrate p-nitrophenylphosphate in 2-amino-2-methyl-1-propanol offers the prime advantage of providing directly its own chromogen, p-nitrophenol, following enzyme action. The procedure also permits use of small sample volumes, provides linear reaction rates, and has a simple manifold design. Correlations are presented between the manual procedure and the automated system.

This paper reports the development of a simplified automated procedure for the determination of serum alkaline phosphatase. The method, developed for use with the AutoAnalyzer,† utilizes a p-nitrophenylphosphate substrate which, following enzyme action, produces its own chromogen, p-nitrophenol.

One existing automated serum alkaline phosphatase procedure (1) using a phenylphosphate substrate is based on the procedures of Kind and King (2) and Powell and Smith (3). Recently, this method has been modified to permit simultaneous determination of both the enzyme reaction and control (4). The product of enzyme action, phenol, must be further treated with aminoantipyrine and alkaline ferricyanide to produce a pink or reddish color whose absorbance is measured. Both automated methods express the results in King-Armstrong units (5).

From the Automation Research Laboratory, Veterans Administration Hospital, Bronx, N. Y. 10468, and the Division of Neoplastic Medicine, Montefiore Hospital, Bronx, N. Y.

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*Present address: Bio-Science Laboratories, Los Angeles, Calif. 90025.
†Technicon Instruments Corporation, Chauncey, N. Y.
Reports of two additional automated procedures, each using a β-glycerolphosphate substrate have recently been published (6, 7). Here, too, the phosphate produced must be determined independently.

While the present procedure was being developed from another approach, Sterling et al. (8a) published a report of an automated glycine buffered p-nitrophenylphosphate substrate procedure wherein the need for control analyses was eliminated. This was accomplished by dialyzing the post-incubation reaction stream. The procedure described in this paper is based generally on the method of Bessey et al. (8b), augmented by the use of a more capable buffer, 2-amino-2-methyl-1-propanol, also suggested by these investigators in a later paper (9).

Experimental

Reagents and Apparatus

Buffer, 0.5 M (pH 10.25 ± 0.05; 25°) For convenience, a stock 50% (w/v) solution of 2-amino-2-methyl-1-propanol* is prepared. To 95 ml. stock buffer solution, in about 700 ml. distilled water, 5 N hydrochloric acid is added, with mechanical or magnetic-bar stirring, until the pH of the solution, monitored with a reliable meter, is brought to 10.25. About 22 ml. is usually required. The solution is diluted to 1 L. and stored under refrigeration.

Buffered substrate Immediately before use, sufficient disodium p-nitrophenolphosphate† (2.0 mg./ml. buffer) is weighed to prepare enough solution for analysis of all specimens and dissolved in the calculated volume of buffer. Sufficient 1 M magnesium chloride is added to make the concentration equivalent to 0.001 M. The pH is rechecked and, if necessary, readjusted to 10.25 ± 0.05 (25°).

Magnesium chloride, 1 M. To 9.52 gm. magnesium chloride (dried overnight at 110°) dissolved in about 80 ml. water, 2 drops of concentrated hydrochloric acid are added and the solution is diluted to 100 ml.

Enzyme standards Serum of high enzyme activity—e.g., from a carefully analyzed refrigerated serum pool—appropriately diluted with inactivated serum is used. Alternatively, inactivated serum to which known amounts of purified alkaline phosphatase have been added‡ may also be used.

p-Nitrophenol standards

1. Stock solution (10 mM/L.): This may be obtained commercially

*Distillation Products Industries (Eastman) No. P-4780 was usually used in this study. Redistilled product or material obtained from other suppliers were also used to prepare buffered substrates. All showed identical activity.

†Satisfactory analyses have been obtained with substrate material purchased from both Mann Research Laboratories, New York, N. Y., and Sigma Chemical Co., St. Louis, Mo.

‡See Sterling et al. (8a) for details.
(Sigma Chemical Co., St. Louis, Mo., No. 104-1) or prepared by dissolving 139.1 mg. p-nitrophenol (Sigma No. 104-8) in 100 ml. buffer solution. Keep refrigerated.

2. Dilute standards: 100-ml. quantities are prepared by dilution of the indicated volumes of stock solution with buffer, as follows.

<table>
<thead>
<tr>
<th>Stock solution (ml.)</th>
<th>p-Nitrophenol (mmoles/L.)</th>
<th>( \mu \text{moles/L.} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>0.25</td>
<td>25</td>
</tr>
<tr>
<td>5.0</td>
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<td>5.0</td>
</tr>
<tr>
<td>10.0</td>
<td>1.00</td>
<td>1.0</td>
</tr>
<tr>
<td>15.0</td>
<td>1.50</td>
<td>1.5</td>
</tr>
<tr>
<td>20.0</td>
<td>2.00</td>
<td>2.0</td>
</tr>
</tbody>
</table>

All standard solutions should be refrigerated.

**Enzyme controls**  As a quality control technic, the procedure should also be monitored daily with serum of high enzyme activity—e.g., from the carefully analyzed, refrigerated serum pool, appropriately diluted with 0.9% saline before use. Alternately, a commercial lyophilized serum enzyme control, such as Versatol E,* reconstituted according to the manufacturer’s directions and suitably diluted with 0.9% saline may be used. A plot relating calculated enzyme activity to absorbance is linear up to about 20 mmoles p-nitrophenol per liter per hour.

The manifold and flow diagram is shown in Fig. 1. Recordings should be made on absorbance (optical density) chart paper. In the work reported here the chart speed was 18 in./hr. Filters transmitting at 400 m\( \mu \) are used.

*General Diagnostics Division, Warner-Chilecott Laboratories, Morris Plains, N. J.
Operating Procedure

Buffered substrate is pumped through the manifold. When the substrate stream reaches the colorimeter, the baseline is set at 0.01 absorbance. Standards and specimens are aspirated at a rate of 40 samples per hour. The specimens and substrate are incubated at 37° and, following dialysis, the absorbance of the reaction mixture passing through a 15-mm. flow cell cuvet is measured at 400 m\(\mu\) and recorded.

Calculation of Enzyme Activity

A plot of absorbance of the serum standards against units will produce a calibration curve. Enzyme activity of specimens can be obtained by location of the absorbance given by the specimen.

Results

A typical calibration curve with p-nitrophenol standards and the strip chart recording from which this was constructed is illustrated in Fig. 2.

![Fig. 2. Calibration curve, p-nitrophenol (AutoAnalyzer). Strip chart recording from which curve was constructed is at right.](image-url)
The plot is linear to approximately 20 Bessey-Lowry-Brock units. Also illustrated are calibrations obtained with dilutions from reconstituted Versatol E (Fig. 3). These are plotted as 100, 75, 50, and 25% concentrations since Bessey-Lowry-Brock values based on 2-amino-2-methyl-1-propanol buffer are not specified (see below under Discussion).

**Discussion**

The most important advantage of this procedure is the use of the substrate p-nitrophenylphosphate which, following enzyme action, produces its own chromogen. The substrate had been studied by King and Delory (10) and others (11a, b) but Bessey et al. (8b) described the results obtained with the substrate in 0.05 M glycine buffer at pH 10.0–10.1 with human serum. A footnote in a later paper (9) mentioned the advantages of 2-amino-2-methyl-1-propanol as a buffer system.

In these laboratories, using a manifold without dialysis, procedures

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*Fig. 3. Calibration curve, Versatol E (AutoAnalyzer). Strain chart recording from which curve was constructed is at right.*
had been developed for use with glycine, ethanolamine/HCl (12), as well as the preferred 2-amino-2-methyl-1-propanol buffer described in this paper. In these studies, optimum conditions of substrate concentration, pH, and buffer concentration were determined. These are shown in Fig. 4–6. The system finally chosen and described in this paper contains 2.0 mg. disodium p-nitrophenylphosphate per milliliter 0.5 M 2-amino-2-methyl-1-propanol, at pH 10.25, 0.001 M in magnesium.

**Fig. 4.** Determination of optimum substrate concentration.

**Fig. 5.** Determination of optimum pH.
chloride. This is very similar to the substrate system used by Amador et al. in their study of urinary alkaline phosphatase activity (13).

The standardization procedure is simple and direct. Alternate use of p-nitrophenol solutions has the advantage of utilizing a fundamental molecular property, the spectral absorption maximum (400 m\(\mu\)) and its molar extinction coefficient (\(\varepsilon = 18,200\) at pH 9 or higher). Highly purified p-nitrophenol is available for preparation of standard solutions, and the enzyme reaction product concentration can be determined with remarkable accuracy merely by measurement of the absorbance at that wavelength. Additional chemical reaction to form colored products is not required in this procedure. This permits reduction of the manifold design to the utmost simplicity.

The Sterling et al. flow diagram (8a) indicates the addition of 0.1 N alkali following dialysis. In the present procedure, the reaction product is dialyzed against the exceedingly capable 2-amino-2-methyl-1-propanol buffer, completely eliminating the need for additional alkali, since the chromogen is produced immediately.

No attempt has been made to determine the stability of the buffered substrate solution. There is a slow hydrolysis of the substrate in alkaline solution with the production of the yellow p-nitrophenolate. However, refrigerated buffered substrate solution can be used provided the absorbance of the yellow solution against a water blank does not exceed
0.1. In these laboratories only enough substrate solution is prepared for daily use. Refrigerated buffer solutions without magnesium chloride will keep indefinitely. Solutions containing magnesium chloride at this pH will slowly deposit magnesium hydroxide, which is difficult to redisolve and, if used, will produce noisy recordings and baseline drift. It is, therefore, advisable to add the magnesium chloride solution to the buffer just before use—i.e., before addition of substrate.

After preparation of a daily calibration curve, and during the analysis, it is good practice to monitor possible baseline drift with a 1.0-mmole/L. p-nitrophenol standard every tenth specimen. In these laboratories, it is also customary to monitor the analyses with three levels of serum enzyme activity (see "Dilute Standards" under Reagents and Apparatus); within normal limits, moderately elevated, and markedly elevated, but still within the range of recorder.

Although dilution of reconstituted Versatol E with 0.9% saline is not recommended by the manufacturer for alkaline phosphatase determinations, linear reaction rates are obtained by the present procedure using either glycine or the improved buffer with up to 10-fold saline dilution.

Sterling et al. (8a) introduced the use of dialysis to eliminate the need for a control analysis to determine the errors and the interferences introduced by hemolysis (8b) and bilirubin (14). Dialysis has been continued in the present procedure although the user must be mindful that an additional operational sequence has been introduced which requires careful and watchful maintenance. For those who do not have or cannot spare a dialyzer module, flow diagrams are available from this laboratory whereby serum enzyme activity can be obtained either by sequential assay and control analysis (0.2 ml. serum) or by simultaneous assay and control determination on 0.1 ml. serum, without dialysis, and with equal accuracy.

The use of the p-nitrophenolate absorption peak at 400 mµ increases the sensitivity of the method. The interference filters used in the present investigation had these specifications: Schott-Genussen DAL, band double, maximum absorbance at 400 ± 5 mµ; half band width 17.5 ± 2.5 mµ; maximum transmission 27.5 ± 2.5%. The ratio of extraneous to total light of these filters is 0.2% and the spectral response curve shows extremely sharp skirts. Other 400-mµ filters were found unsuitable. If these are not available from the supplier, 410- to 415-mµ filters with the same spectral characteristics are also satisfactory but give an approximate 15% reduction from the peak absorption of p-nitrophenol. Increased sensitivity is also achieved by using the standard 15-mm. flow cell.
Table 1. Values (Bessey-Lowry-Brock Units) Obtained on Identical Specimens by Manual and Automated Procedures

<table>
<thead>
<tr>
<th>Specimen</th>
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<td>12.8</td>
<td>26.3</td>
<td>2.03</td>
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</table>

AV. 3.99 3.91 17.5 1.93

Versatol E (No. 5341063) 7.8‡ 9.3 (AV.) 17.5 1.88

*Bessey-Lowry-Brock procedure (¶†).
†System contained 1.25 mg. substrate per milliliter 0.025 M glycine buffer, pH 10.35, 0.001 M in magnesium chloride.
‡Manufacturer's stated value.

The use of glycine buffer, though continued traditionally in many laboratories, is undesirable because of its well-known inhibitory action on alkaline phosphatase. Since most clinical biochemistry laboratories reporting serum alkaline phosphatase in Bessey-Lowry-Brock units, still use the glycine buffer originally studied by these investigators and continued in a commercial kit (Kit 104*), a comparison between the values obtained on identical specimens by the manual and automated glycine buffered substrate procedures is listed in Table 1. The agreement is excellent. Also listed are the values obtained on the same specimens with the 2-amino-2-methyl-1-propanol buffer. In the present work the relationship between the Bessey-Lowry-Brock unit based on glycine buffer and the unit based on 2-amino-2-methyl-1-propanol was deter-

*Sigma Chemical Co.
mined to be 1.93 ± 0.04 (Table 1). This value is identical with the Lowry et al. (9) finding that, in the manual procedure, phosphatase activity in the presently used buffer is approximately twice that in glycine buffer. A similar comparison with Versatol E showed the factor to be 1.88.

Thiers and Oglesby (19) observed that specimen interaction is one important factor among several which can contribute a significant error in a flowing analytical system. They suggested a daily determination of the percentage correction to be applied for each analytical system to reduce this error. Development of a new, or modification of an existing, automated procedure would surely require the determination of the degree of sample interaction.

We have evaluated sample interaction (Fig. 7). Samples of Versatol E reconstituted according to directions (called 100% for this purpose) and also diluted to 25% concentration were analyzed at varied sampling rates in the sequence: 25%, 100%, 25%. The calculated interaction and relation to continuous sampling are given in Table 2. With the Sampler II, least interaction (less than 1%) was obtained at a rate of 50 specimens per hour, 2:1 sample-wash ratio, although sampling at 70 specimens per hour (2:1 sample-wash ratio) showed less than 3% interaction. Results of replicate analyses on specimens showing different enzyme activity levels by the present method (see "Dilute standards un-
Table 2. Interaction of Specimens

<table>
<thead>
<tr>
<th>Samples/hr.*</th>
<th>% Interaction</th>
<th>25%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
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<td>40</td>
<td>4.78</td>
<td>93.8</td>
<td>97.3</td>
</tr>
<tr>
<td>50</td>
<td>0.84</td>
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<td>96.2</td>
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<td>60</td>
<td>3.88</td>
<td>94.5</td>
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</tr>
<tr>
<td>70</td>
<td>2.59</td>
<td>94.5</td>
<td>93.0</td>
</tr>
</tbody>
</table>

*Sample-wash ratio 2:1.

Fig. 8. Typical recordings of enzyme activity.

der Reagents and Apparatus) were consistent with results of similar studies presented by Sterling et al. (8a).

Figure 8 shows typical recordings obtained by assay of unselected serum samples submitted to the clinical biochemistry laboratory.

Conversion to Other Alkaline Phosphatase Units

The procedure described above reports enzyme activity in Bessey-Lowry-Brock units. However, the present work indicates that normal
values with the improved buffer would be approximately twice the values obtained with glycine buffer given by Bessey et al. (adults, 0.8–2.3; children, 2.8–6.7) and continued by use of the commercial kit (15).

It is recognized that many laboratories (including the Veterans Administration Hospital, Bronx, N. Y.) report serum alkaline phosphatase activity in other units obtained with other substrates and by well-known procedures. Bessey et al. (8b) demonstrated that their unit was 1.79 ± 0.10 times the Bodansky unit. In recent years several studies have been reported in which conversion factors among the most frequently used serum alkaline phosphatase procedures have been determined (16–18). These have been critically re-examined by Deren et al. (20), who conclude that, although linear relationships do exist among the Bodansky, King-Armstrong, and Bessey-Lowry-Brock procedures and they may be interconverted by an appropriate factor, local laboratory experimental modifications require these conversion factors to be individually re-determined. At the Veterans Administration Hospital (Bronx, N. Y.) no change in reporting (Shinowara units) has taken place. Serum pools at several levels of activity were carefully analyzed in replicate by the Shinowara method on several succeeding days and re-analyzed similarly by the automated method. A calibration curve relating p-nitrophenol formation and Shinowara units was constructed and is used for reporting serum alkaline phosphatase activity to the clinical services. A similar procedure is suggested to the using analyst to permit the laboratory to report values in locally familiar, staff-oriented units.

References


