An Automated Alkaline Phosphatase Assay with Phenolphthalein Monophosphate as Substrate

Kirsten Hviid

The manual procedure of Babson et al. (1) for the determination of serum alkaline phosphatase has been automated. The assay is based on the colorimetric determination of phenolphthalein formed on hydrolysis of phenolphthalein monophosphate. The procedure utilizes 0.16 ml. of serum without dialysis. Blanks are required only for turbid sera. Results are compared with those obtained by the manual procedure, and data relating to sample interaction, precision, blank values, and normal values are presented.

Several manual methods for serum alkaline phosphatase have now been automated. Among them, the methods by Keay et al. (2) and Tietz et al. (3), adapted from the Bodansky (4) assay, require pre- and post-hydrolysis phosphate determinations, while the method by Marsh et al. (5) requires both an assay and a serum blank run. Morgenstern et al. (6) and Sterling et al. (7) in their adaptations of the Bessey, Lowry, and Brock (8) procedure avoided the blank run by dialyzing the hydrolysate. Two recent methods (9, 10), employing as substrates thymolphthalein monophosphate and pentasodium phenolphthalein diphosphate, respectively, have been developed especially for automation, and do not require blank runs or dialysis.

Ideally an automated procedure should involve only one reaction, and dialysis, which is known to generate difficulties in other systems, should be avoided. The method of Babson et al. (1) seemed to satisfy both of these criteria and hence was adopted for the present investigation. This method employs as a substrate phenolphthalein monophosphate, which is converted to the chromogen, phenolphthalein, by the action of the enzyme.

From the Clinical Chemistry Laboratory, Peter Bent Brigham Hospital, Boston, Mass. 02115. Received for publication, Sept. 30, 1966; accepted for publication Oct. 17, 1966.
Materials and Methods

Equipment

The glassware used in preparation of the reagents and standards consisted of volumetric flasks and pipets, respectively. Serum was pipetted with 0.1-ml. TC pipets while volumetric pipets were used for the reagents. A 37° water bath was used for incubation, and the absorbance of each assay was read against water at 550 m\(\mu\) in a Coleman Junior spectrophotometer equipped with a 10-mm. vacuum-pump cuvet. For the automated procedure, a standard AutoAnalyzer was used, consisting of a Sampler II, pump, dialyzer bath, photocell colorimeter equipped with a 15-mm. tubular flowcell and interference filters 550-18-28, and recorder with absorbance paper, set to run at 18 in./hr.

Reagents

These reagents are supplied as kits by Warner-Chilcott Laboratories, Morris Plains, N. J.

Substrate concentrate: 65 mM phenolphthalein monophosphate in 7.8 M 2-amino-2 methyl-1-propanol at pH 10.15 This is supplied in a small plastic vial which should be kept under refrigeration but brought to room temperature before use. The vial is made to allow either dropwise expulsion or complete outpouring of the substrate concentrate. To each drop, 2 ml. distilled water is added, or the whole content of a vial may be quantitatively transferred to a 200-ml. volumetric flask and then diluted to volume with distilled water. This makes the working substrate, which is stable for only 1 day.

Color stabilizer: 0.1 M phosphate buffer at pH 11.2 This is supplied in crystalline form. Dissolved in distilled water, it will make 500 ml. of color stabilizer.

Standards* Mixtures of Versatol-E and -EN and of Versatol-E and 0.9% NaCl were used.

Procedure

Manual

To 1 ml. of substrate, heated to 37°, is added 0.1 ml. of serum, and incubation is allowed to proceed at 37° for exactly 20 min. Following incubation, 5 ml. of color stabilizer is added, the contents of the tube mixed, and the absorbance read at 550 m\(\mu\) against water within half an hour after the addition of the color stabilizer. A reagent blank is determined by incubating 1 ml. substrate for 20 min. at 37°, then adding 5 ml. color stabilizer and reading as for the assay.

*Supplied by Warner-Chilcott, Versatol-E and -EN are lyophilized enzyme standards in serum (known abnormal and normal activities, respectively).
A standard calibration curve is constructed by plotting absorbance, corrected for the blank, against enzyme activity of the standard samples. Unknown enzyme activities are read from this curve.

**Automated**

The flow diagram for the determination at a rate of 40 samples/hr. (2:1 ratio) is shown in Fig. 1.

Substrate and color stabilizer are pumped through their respective lines while the sample line is aspirating water. The reagent baseline appears on the recorder after about 8 min., and is adjusted to zero absorbance prior to assay.

Samples are mixed and incubated with substrate at 37° in two double-mixing coils for about 5 min. After incubation the reaction is stopped by the addition of the color stabilizer. Mixing takes place in a double-mixing coil, and then the absorbance is measured at 550 μm in a 15-mm. tubular flow-cell.

The absorbances of the standards are plotted against enzyme activities on a plainly lined plastic overlay where each division represents 1 U. The calibration curve is constructed from these points, and the activities of the unknowns read from this curve.

**Manual**

The linearity of the assay was assessed by taking a high-activity serum (143 U.), diluting it with saline to represent 0.005, 0.010, 0.025, 0.050, and 0.075 ml. of serum, respectively, and running each dilution as
an individual assay, according to the method of Babson et al. (1). The resulting absorbances were plotted against the corresponding amounts of serum in each dilution, and are shown in Fig. 2.

Another high-activity serum (112 U.) was incubated for 4, 8, 12, 16, and 20 min., respectively, and the absorbances plotted against the corresponding incubation time (Fig. 3).

The reaction is not quite linear with respect to either concentration or time (Fig. 2 and 3). This may be due to the type of spectrophotometer used. Babson et al. (1) have indicated that "the absorbance deviates somewhat from linearity on most filter colorimeters owing to the narrow absorption peak of phenolphthalein." Perhaps the same holds true for spectrophotometers with rather wide fixed monochromator slits.

**Serum Blanks**

One of the advantages of the present method is that readings are made at 550 m\(\mu\), the absorption maximum of alkaline phenolphthalein. At this wavelength the contribution of bilirubin to the total absorptivity is negligible, and hence the color of the serum should be unimportant. This aspect of the procedure was examined by running

---

**Fig. 2 (left).** Assessment of linearity of assay. One high-activity serum was assayed at several different dilutions. Absorbance readings, corrected for reagent blank, are plotted against corresponding serum concentrations. **Fig. 3 (right).** Effect of varying incubation times. Absorbance readings, corrected for reagent blank, are plotted against corresponding incubation times.
saline blanks on some sera with high bilirubin content (Table 1). The blanks consisted of 6 ml. saline to which 0.1 ml. serum was added. The mixture was subsequently read against water at 550 m\(\mu\) in a Coleman Junior spectrophotometer equipped with a vacuum-pump cuvet with a 10-mm. light path.

The results clearly demonstrate negligible interference from bilirubin, and consequently the assay does not require that serum blanks be run unless a turbid serum sample must be analyzed.

**Manual Versus Automated Assays**

With the manifold shown in Fig. 1 and the same reagents and standards used for the manual assay, 50 automated assays were run for comparison. With the manual method, values were an average of 3 U. higher than with the automated.

To find the cause of the difference between the two methods, blanks were run on the commercial standards and samples by both methods. In the automated system saline was pumped through substrate and color stabilizer lines, the baseline set at zero absorbance, and the samples aspirated in the usual way.

It was found that the blanks of the commercial standards corresponded to 4 U. on the AutoAnalyzer standard curve but only 1.5 U. on the manual standard curve, while the difference in the patients' serum blanks was negligible. The blanks from the commercial standards had higher values on the AutoAnalyzer assay than on the Coleman spectrophotometer assay probably because of the turbidity present in the reconstituted commercial preparation—the interference filters are more sensitive to such effects than is the Coleman spectrophotometer.

Part of the effect of the standards' turbidity can be eliminated by doing comparisons using standard-saline mixtures as the working standards. Such a series of 15 samples yielded an average assay of 36 U. by the manual procedure and 34.5 U. by the automated procedure, a difference of 1.5 U. rather than the 3 U. obtained with mixtures of the standards themselves.

<table>
<thead>
<tr>
<th>Serum blank (No.)</th>
<th>Absorbance (550 m(\mu))</th>
<th>Corresponding bilirubin (mg/100 ml.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>357</td>
<td>0.005</td>
<td>1.9</td>
</tr>
<tr>
<td>410</td>
<td>0.005</td>
<td>2.4</td>
</tr>
<tr>
<td>432</td>
<td>0.003</td>
<td>3.5</td>
</tr>
<tr>
<td>493</td>
<td>0.008</td>
<td>7.8</td>
</tr>
<tr>
<td>422</td>
<td>0.005</td>
<td>8.9</td>
</tr>
<tr>
<td>469</td>
<td>0.012</td>
<td>31.0</td>
</tr>
</tbody>
</table>
Automated Blanks

To determine different blank values with the automated assay, saline blanks were run on commercial standards and standard-saline mixtures, on icteric, turbid, and normal serum (Fig. 4).

Fig. 4. At bottom, recording of serum and standard blanks; at top, corresponding assays (manifold and flow diagram as in Fig. 1). In blanks, saline was substituted for substrate and color-stabilizer. Peaks represent (left to right): peak 1, commercial standard (18.7 U.); peaks 2–5, mixtures of commercial standards (37.3, 55.9, 73.5, and 93.1 U., respectively); peak 6, commercial standard (111.7 U.); peak 7, normal serum; peaks 8–13, saline mixtures of commercial standard as in 6 (16.8, 33.5, 50.3, 67.0, 83.4, and 100.5 U., respectively); peaks 14–17, serum with bilirubin concentrations of 1.6, 2.5, 4.0, and 7.8 mg./100 ml., respectively; peak 18, slightly turbid serum; peak 19, turbid serum; peaks 20–25, clear serum with normal bilirubin concentrations.

Fig. 5. Calibration curves plotted from recordings in Fig. 4; no correction for blank values.
Aside from the 16.8-U. standard-saline mixture, such mixtures in general have blank values which correspond more closely to those of normal serum than do the commercial standards. It also seems that the influence of bilirubin is negligible. Indeed, a series of clear normal sera, subsequently assayed and also run for saline blanks, gave as extreme blank values absorbances of 0.006 and 0.022, the latter being higher than that of any of the icteric sera analyzed in the present series. A turbid serum, however, definitely increases the assay reading, and it is therefore recommended that saline blanks be run for all turbid sera. The difference between the absorbance of the turbid serum and the mean absorbance found for normal serum should be subtracted from the absorbance of the assay. A series of 56 clear serum samples taken from a normal population and determined for saline blank gave a mean value of 0.011 absorbance with an S.D. of ± 0.004.

The standard curves for the commercial standards and standard-saline mixtures are plotted in Fig. 5. The discrepancy between the curves illustrates the difference in the blank values obtained for the two mixtures.

Sample Interaction

Thiers and Oglesby (12) showed that sample interaction is an inherent source of error in a continuous flow system, while Skeggs (13) stated that a common fault in automated methods is the use of too high a rate of analysis. The rate should yield a consistently good wash-out between samples, and the height of the peaks should, at least momentarily, reach the steady state value at the recorder.

Employing the manifold described in Fig. 1, sample interaction was evaluated using the commercial standard reconstituted according to directions and called 100% ; part of this standard was diluted fourfold with saline to give a sample of 25% of the original concentration. These samples were assayed at different sampling rates (Fig. 6) in the sequence—25%, 100%, and 25%—and continuously for about 5 min. each to yield a steady state recording.

The calculated interaction and relation to continuous sampling is shown in Table 2. The interaction is quite low at all speeds, but only at speeds of 40 and 50/hr. do the peaks closely approach the steady state for both low- and high-activity samples. The speed of 40 samples/hr. was chosen because it gave the best wash-out between samples.

Precision

Pooled serum mixed with high-activity commercial standard was poured into 27 sample cups and assayed. The activity ranged from 58.0 to 58.5 U. with a mean of 58.2 U. The coefficient of variation was 0.5%.
Normal Range

The normal value for the assay will depend on how the commercial standards are diluted (Fig. 5). Since the standard-saline blanks are like serum blanks, and the assay is to be run without blanks, it would seem appropriate to use standard-saline as working standards.

In the present work, all normal values were calculated on the basis of the commercial standards as well as a standard-saline curve. The same lot number of the commercial standards were used, and each standard-saline curve was made from a standard activity of 130 U. by dilutions yielding 15, 30, and 50% activity, respectively.

Clear serum samples from 56 normal people were assayed. With the commercial standards, the extreme values were 9 and 44 U. The mean and S.D. were 21 ± 7 U., giving a normal range (mean ± 2 S.D.) of

\[
\begin{align*}
\text{Table 2. Sample Interaction (Sample: Wash Ratio 2:1)}
\end{align*}
\]

<table>
<thead>
<tr>
<th>Samples/hr.</th>
<th>Interaction (%)</th>
<th>Continuous sampling (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25%</td>
<td>100%</td>
</tr>
<tr>
<td>40</td>
<td>1.2</td>
<td>100.2</td>
</tr>
<tr>
<td>50</td>
<td>0.6</td>
<td>99.6</td>
</tr>
<tr>
<td>60</td>
<td>0.0</td>
<td>98.3</td>
</tr>
<tr>
<td>70</td>
<td>0.6</td>
<td>96.1</td>
</tr>
</tbody>
</table>

Fig. 6. Recording of sample interaction and wash-out between samples (manifold and flow diagram as in Fig. 1). Samples were assayed in the order of 25%, 100%, and 25% at speeds shown, with sample:wash ratio of 2:1.
7–35 U. With standard-saline as working standards, the extreme values were 12 and 49 U. The mean and S.D. were 25 ± 7.8 U., giving a normal range (mean ± 2 S.D.) of 9–41 U. The normal value for the manual assay reported by Babson et al. (1) was 9–35 U. (mean ± 2 S.D.).

References