Kinetic Determination of Granulocyte Alkaline Phosphatase by the GEMSAEC

LYNN CROOK, M.D., PH.D.,* NICOLAS M. SARJI, M.S.,*
PAUL I. LIU, M.D., PH.D.,* RICHARD H. GADSDEN, PH.D.†
and THOMAS R. WILLIAMS, B.S.‡

Hematology* and Clinical Chemistry † Divisions,
Department of Laboratory Medicine,
Medical University of South Carolina,
Charleston, SC 29403

Abstract

Granulocyte alkaline phosphatase (ALP) activity is measured kinetically using the GEMSAEC centrifugal analyzer and p-nitrophenylphosphate as substrate. Measurements of enzyme activity are made at pH 9.8 utilizing 1.0 M diethanolamine as a buffer containing 0.5 mM magnesium chloride.

Granulocytes are separated from heparinized blood using dextran sedimentation, followed with ficoll-hypaque separation. The separated cells are suspended in saline, and the red cells are lysed. Manual counting of stained smears of the cells isolated showed that 95 percent of these cells were granulocytes. After lysing the red cells, the granulocyte suspension is rinsed in 12.5 percent heat inactivated serum in saline and separated by centrifugation. After 200 µl of the diluent solution (12.5 percent heat inactivated serum in saline) is added to the cells, they are sonicated for seven sec at 0° in an ice bath. The extracts are then assayed kinetically for ALP activity.

Granulocyte ALP activity varies from day to day in normal individuals. The daily variation of ALP activity observed correlates closely with and is confirmed by the Sigma Histochemical Procedure based on Kaplow’s Scoring Method. The coefficient of variation of the ALP method within assay performed on two consecutive days on a pooled sample is 0.75 percent and 1.38 percent, respectively, and between assays 4.82 percent.

Introduction

Knowledge of alkaline phosphatase (ALP) activity of granulocytes is a helpful diagnostic tool, especially in differentiating between chronic granulocytic leukemia where the ALP activity is low and leukemoid reaction where the ALP activity of the granulocytes is high, with some exceptions in both cases.9

Principle

The substrate, p-nitrophenylphosphate, in the presence of Mg²⁺ ions and at a pH of 9.8 is hydrolysed by ALP from granulocytes to p-nitrophenol and phosphate. The p-nitrophenol liberated is proportional to the ALP activity and is determined by measuring the ΔA/t at 405 nm. ALP activity is first expressed in mU per
ml, where $U$ is defined as the ALP activity which converts one $\mu$mol of substrate in one min at standard conditions. From the ALP activity measured, the ALP activity in mU/10^6 granulocytes is calculated.

**Methods and Materials**

**REAGENTS**

* Sodium chloride, 0.9 percent. This is prepared by dissolving 9 g of reagent grade sodium chloride in one liter of distilled water.

* Dextran, 6.0 percent. This is prepared by dissolving 6 g of clinical grade dextran (average Mol. wt. 170,000) in 100 ml of 0.9 percent NaCl.

* Ficoll, 9.0 percent. This is prepared by dissolving 9 g of ficoll (Mol. wt., approximately 400,000) in 100 ml of distilled water.

* Hypaque, 33.3 percent. This is prepared by diluting 20 ml of 75 percent hypaque-M with 25 ml of distilled water.

* Ficoll-Hypaque solution. This is prepared by mixing 100 ml of 9.0 percent ficoll with 41.7 ml of the 33.3 percent hypaque solution.

* Heat inactivated serum. This is prepared by heating pooled serum from normal donors at 65° for one hour. No ALP activity is detected after heating.

* Heat inactivated serum, 12.5 percent. This is prepared by diluting 12.5 ml of the heat inactivated serum to 100 ml with 0.9 percent NaCl. This solution is referred to as the rinsing diluent.

* Saponin, 0.5 percent. This is prepared in the rinsing diluent by dissolving 0.25 g saponin in 50 ml of 12.5 percent heat inactivated serum. This solution is referred to as the extraction diluent.

* Saponin Solution. One gram of saponin is dissolved in 100 ml 0.9 percent NaCl to prepare 1 percent saponin in saline.

All solutions are refrigerated until used.

---

**TABLE I**

<table>
<thead>
<tr>
<th>Rotataloader IV Instructions</th>
<th>Volume</th>
<th>Pump</th>
<th>%</th>
<th>Well</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>10 µl</td>
<td>50 µl</td>
<td>20</td>
<td>B</td>
</tr>
<tr>
<td>Flush</td>
<td>50 µl</td>
<td>200 µl</td>
<td>25</td>
<td>B</td>
</tr>
<tr>
<td>Reagent</td>
<td>500 µl</td>
<td>1000 µl</td>
<td>50</td>
<td>C</td>
</tr>
<tr>
<td>Sample Tip: Stainless steel</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyser/Control Module Instructions</th>
<th>Reaction temperature:</th>
<th>30°</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength</td>
<td>405 nm</td>
<td></td>
</tr>
<tr>
<td>Filter position:</td>
<td>385 - 420 nm</td>
<td></td>
</tr>
<tr>
<td>Reaction mode:</td>
<td>Auto/Rate</td>
<td></td>
</tr>
<tr>
<td>First reading:</td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>Reading interval:</td>
<td>20 sec</td>
<td></td>
</tr>
<tr>
<td>Number of readings:</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Computer Instructions</th>
<th>Header: ALK</th>
<th>IR: 1565</th>
<th>Hi: 150</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 TF: 1</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>20 TC: 2</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>6 AD: 4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>0 CD: 0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

**STANDARD SOLUTIONS**

The substrate, p-nitrophenylphosphate, and the buffer, diethanolamine containing 0.5 mM magnesium chloride, pH 9.8, are from a commercial kit.* These are the same reagents used for alkaline phosphatase determination in serum.

**SPECIAL APPARATUS**

In table I are indicated the exact reaction conditions used on the GEMSAEC centrifugal analyzer† for determining the ALP activity. The directions and centrifugal analyzer settings for alkaline phosphatase determinations are included with the test kit and adhere very closely to those outlined by Hausamen et al.³ and as confirmed in our laboratory.

Upon investigating the stability of the granulocytes total ALP activity in different extraction media, heat inactivated normal human serum is found to be the most suitable extractant. In figure 1 are illustrated differences in ALP activity

---

* Marked (Catalog #15990) by Bio-Dynamics Division of Boehringer Mannheim Corporation.
† Electronucleonics Inc., Model #400255.
after storage at $-20^\circ$ for 24 hrs using three extractants. When 0.9 percent NaCl was used as the extraction diluent, ALP activity dropped to 58.9 percent of the original activity on repeated analysis of the same extracts after a 24 hr period. Similar results were found when Tris buffer, pH 8.8, was substituted for saline. When 12.5 percent normal pooled human serum in saline was used as the extracting diluent, ALP activity increased to 139 percent after 24 hrs. This confirms the findings of Wiltshaw and Moloney.\textsuperscript{10} The purpose of using human serum was to provide a more natural environment for the protection of the granulocyte alkaline phosphatase activity. Since some factor in serum enhances the ALP activity, the serum is heated at 65$^\circ$ in a dry hot air bath for one hour. Repeated determinations on the same extract using the heat inactivated serum in saline showed the ALP activity was stabilized yielding 103.1 percent. However, the activity dropped to 75.5 percent after three months and 53.3 percent after six months in 12.5 percent heat inactivated serum in saline when stored frozen at $-20^\circ$.

**Procedure**

Venous blood samples (10 ml) were collected from heparinized vacutainer tubes from American Red Cross male blood donors between the ages of 21 and 52 years old.\textsuperscript{†} After thorough mixing of the heparinized blood, two ml of 6.0 percent dextran solution\textsuperscript{6} were added slowly with gentle mixing. The mixture is allowed to stand at room temperature for 45 min to allow most of the red cells to settle. The plasma supernatant, rich in leukocytes with some contaminating red cells, is transferred with a plastic pipet and carefully layered on top of three ml of ficoll-hypaque solution.\textsuperscript{2} This is centrifuged at 1200 rpm in a Damon centrifuge for 10 min at 25$^\circ$ (240 g RCF). The granulocytes and red cells settle to the bottom. The liquid supernatant is discarded and the sedimented cells are resuspended in five ml of saline. After thorough mixing, the granulocytes in the cell suspension are counted on a Coulter counter, Model ZBI. Manual differentials of stained smears of these isolated cells showed that 94.8 ± 0.67 percent (± SEM, N = 20) were granulocytes. To the remainder of the cell suspension 50 $\mu$l of 1.0 percent saponin in saline is added, mixed for 15 to 30 sec and centrifuged at 2800 rpm for five minutes at room temperature. The supernatant is discarded. The pellet of granulocytes is resuspended in five ml of the rinsing diluent and centrifuged. The granulocytes are rinsed twice with five ml of rinsing diluent, centrifuged and the supernatant discarded. After the second decantation, the sides of the tube are wiped dry and 0.2 ml of the extraction diluent is added. After mixing, the granulocytes are sonicated for seven sec at 0$^\circ$ using an ice bath. The homogenates are centrifuged for five minutes at 2800 rpm.

\textsuperscript{†} Permission for performance of this testing was secured from each donor on forms approved by the Human Research Committee at the Medical University of South Carolina.
The supernatant is removed and assayed for ALP activity. The results are reported as mU ALP activity per $10^8$ granulocytes per min at 30°C.

Results and Discussion

Granulocyte ALP activity is usually high in leukemoid reaction and low in chronic granulocytic leukemia, with some exceptions.9 Generally, clinical evaluations of the ALP activity of the granulocytes are done histochemically using Kaplow's Scoring Method.4,5 While this is fast and reproducible, it is not a suitable method for very low granulocyte ALP activity and the results are interpreted subjectively. A kinetic analytical method is more objective and leaves little chance for subjective interpretation. It offers higher sensitivity and precision as well as the possibility to repeat assays. For this purpose, the GEMSAEC kinetic method for determining ALP activity in serum is adapted. Only healthy Red Cross male blood donors between the ages of 21 and 52 years old were investigated since females in that age group exhibit higher ALP activity.7

Further studies of ALP extraction from the granulocytes indicated that incorporation of 0.5 percent saponin into the 12.5 percent heat inactivated serum in saline released most of the ALP activity from the cells in the first extraction. This is demonstrated by comparing the ALP activity obtained by using 12.5 percent heat inactivated serum in saline with and without saponin. It is illustrated in table II that with saponin, most ALP activity is extracted in the first extraction and only 3.2 percent of the activity is left for the second extraction, whereas without saponin, the yield of ALP activity in the first extraction is only 22 percent of the first saponin extraction and only an additional 7.5 percent activity during the second extraction without saponin.

These data confirm the findings of Rosner et al8 that saponin increases the yield of granulocyte ALP, but in contrast to his findings, no further significant release of ALP is found after retreatment with saponin.

Initial studies on a healthy male subject showed diurnal variations in agreement with Anstey et al.1 In order to verify this, three healthy male subjects were tested for four consecutive days with Sigma's histochemical procedure using Kaplow's scoring method and with the proposed kinetic enzyme assay. Both methods showed diurnal variations with similar trends as illustrated in figure 2. A comparison of these data indicate that a Kaplow score of 1 is equivalent to approximately 6 mU/10^8 granulocytes per minute at 30°C.

The reproducibility of the ALP method was checked by assaying 27 samples in duplicate beginning with whole blood and performing the total extraction procedure. A correlation coefficient of 0.989 ($P < 0.001$) between the duplicates and Students' paired t test ($t = 0.3223$) indicated that no significant difference exists between the sample results. The precision of the kinetic method is demonstrated by assaying pooled granulocyte extracts in duplicate for two successive days. The coefficient of variation within assay is 0.75 per-

---

### Table II

<table>
<thead>
<tr>
<th>ALP Activity</th>
<th>1st Extract</th>
<th>2nd Extract</th>
<th>Additional % Activity in 2nd Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>With 0.5 mU/ml ± SE</td>
<td>mU/ml ± SE</td>
<td>mU/ml ± SE</td>
<td>percent</td>
</tr>
<tr>
<td>31.3 ± 7.0</td>
<td>4.0 ± 1.5</td>
<td>3.2 ± 0.5</td>
<td>59</td>
</tr>
<tr>
<td>Without 0.5 percent saponin*</td>
<td>68.8 ± 19.9</td>
<td>23.5 ± 8.2</td>
<td>30.0 ± 5.3%</td>
</tr>
</tbody>
</table>

*N = 17 Range = 0 - 8.2 percent
†N = 11 Range = 0 - 67 percent
cent and 1.38 percent, respectively, and between assay 4.82 percent.

Sources of Error

The red blood cells must be completely lysed in order to avoid high background absorbance during the assay. The sonication time in an ice bath must be kept constant. Plastic laboratory ware should be used throughout the procedure. Granulocyte counts must be done within 30 min after lysing the red blood cells to avoid granulocyte agglutination. Saponin gives an almost complete extraction of ALP activity with a single extraction.

Reference Range

Although diurnal variations exist, the reference range was established on 88 healthy Red Cross male donors between

![Figure 2](image-url)  
**Figure 2.** Diurnal variation of normal granulocyte alkaline phosphatase activity in three normal males.

![Figure 3](image-url)  
**Figure 3.** Cumulative frequency distribution of log granulocyte alkaline phosphatase activity of 88 normal males plotted on normal probability paper.
the ages of 21 and 52 years old. A logarithmic normal distribution is demonstrated in figure 3. The calculated reference range extended from 95 to 750 mU/10⁸ granulocytes per minute at 30°. Females between the ages of 21 and 36 years old were tested and appeared to have a higher reference range; however, an adequate number of donors was not available to calculate the reference range for females.

Summary

Total ALP activity of granulocytes is determined kinetically on the GEMSAEC centrifugal analyzer. The method adapted for this purpose is similar to that used for the determination of ALP in serum. The extraction procedure is simple, but requires a sonicator as a special piece of equipment which may not be available in all laboratories. With this method, diurnal variations have been shown by us in granulocyte ALP activity and the normal range applicable to the method has been determined for adult males.

References