THE COLORIMETRIC DETERMINATION OF PHOSPHATASES IN HUMAN SERUM*

BY ARNOLD M. SELIGMAN, HOWARD H. CHAUNCEY, MARVIN M. NACHLAS,† LEON H. MANHEIMER, AND HERBERT A. RAVIN†

(From the Kirstein and Yamine Laboratories for Surgical Research, Beth Israel Hospital, and the Department of Surgery, Harvard Medical School, Boston, Massachusetts)

(Received for publication, November 16, 1956)

There are a number of methods currently available for the quantitative estimation of acid and alkaline phosphatase activity in biologic materials (1–5). In all the techniques, a phosphoric acid ester serves as a substrate and colorimetric determinations are made either of the inorganic phosphate or of the organic moiety of the substrate released by enzymatic hydrolysis.

A description of a new method for the quantitative estimation of acid and alkaline phosphatase activity of human serum forms the basis of this report. The substrate consists of sodium β-naphthyl phosphate (6). The calcium salt of this ester was used in a method for the histochemical localization of alkaline phosphatase (7, 8). The α isomer was used for the histochemical localization of acid phosphatase (9), and, based on this work, the α isomer was used in methods for demonstrating semen on clothing in cases of suspected rape (10), and for estimating urinary acid phosphatase activity (11).

The new method is in general similar to the method described for estimating esterase and lipase activity in serum and in urine with β-naphthyl laurate as the substrate (12–14). The β isomer, rather than the α isomer, is used for acid phosphatase in the present method, because the azo dye formed from β-naphthol is, in the presence of protein, much more readily extracted into ethyl acetate for measurement of color density than is the case with the azo dye formed from α-naphthol. The method is applicable to any source of the enzyme such as serum, plasma, whole blood, tissue homogenates, tissue sections, semen, gastric juice, and urine.

In essence, the method consists of incubation of the enzyme source with a solution of sodium β-naphthyl phosphate buffered to an appropriate pH (4.8 for acid phosphatase and 9.1 for alkaline phosphatase) at 37.5° for a standard period of time (2 hours and 1 hour respectively). 2 molecules of

* This investigation was supported by a research grant from the National Cancer Institute, the National Institutes of Health, United States Public Health Service, in part by a grant from the Massachusetts Division of the American Cancer Society, and in part by an institutional grant to Harvard University from the American Cancer Society.
† Research Fellow of the National Cancer Institute.
DETERMINATION OF SERUM PHOSPHATASES

\[ \beta \text{-naphthol released in the course of the reaction are coupled with tetra-} \]
\[ \text{zotized diorthoanisidine to yield an insoluble, purple azo dye, which is then} \]
\[ \text{extracted with ethyl acetate for measurement of the color density in a} \]
\[ \text{photoelectric colorimeter (Klett).} \]

Method

Reagents—

1. Substrate. A stock solution of sodium \( \beta \)-naphthyl phosphate (6) is
made in distilled water in a concentration of 0.2 mg. per cc. (0.0008 M).
This solution may be stored at 4\(^\circ\) for a month with no significant sponta-
neous hydrolysis.

2. Veronal buffer (pH 9.1, 0.1 M) is prepared by mixing 950 cc. of 0.1
M sodium diethyl barbiturate with 50 cc. of 0.1 M hydrochloric acid.

3. Acetate buffer (pH 4.8, 0.2 M) is prepared by mixing 120 cc. of 0.2
M sodium acetate with 80 cc. of 0.2 M acetic acid.

4. Tetrazotized diorthoanisidine.\(^2\) The powder (4 mg. per cc.) is dis-
solved in cool water immediately before use. In solution, the diazonium
compound decomposed extensively on standing at room temperature for
20 to 30 minutes.

5. Sodium carbonate (1.0 M).

6. Trichloroacetic acid (40 per cent).

7. Anhydrous ethyl acetate.

Just prior to use, the desired amount of stock solution (1) of the sub-
strate is mixed with an equal volume of either stock buffer solution (2)
or buffer solution (3). The appropriately buffered substrate solution is
then added to the enzyme preparation as described below.

Procedure

Serum was obtained by centrifugation of freshly clotted blood. The
serum (1 cc.) was removed with a pipette and diluted with 19 cc. of distilled
water. 1 cc. of this diluted serum was placed in a 20 cc. test-tube and 5
cc. of the appropriately buffered substrate solution were added. In a
separate test-tube, buffered substrate solution alone served as a control
for non-enzymatic hydrolysis. The tubes were then incubated at 37.5\(^\circ\)
for 1 hour in the determination of alkaline phosphatase, and for 2 hours
in the determination of acid phosphatase. In the latter determination 4
drops of 1 M sodium carbonate solution were added after the period of in-

\(^1\) All the reagents used in this test may be obtained from the Dajac Laborato-
ries, Monomer-Polymer, Inc., 3430 West Henderson Street, Chicago 18, Illinois.

\(^2\) Available in powder form containing 20 per cent tetrazotized diorthoanisidine,
5 per cent zinc chloride, and 20 per cent aluminum sulfate. Trade name, DuPont
naphthanil diazo blue B.
cubation, in order to raise the pH to the optimal level for coupling. 1 cc. of the solution of tetrazotized diorthoanisidine was added to each tube, and the tubes were agitated vigorously or inverted several times to insure thorough mixing. 3 minutes were allowed to elapse for completion of coupling. To each tube 1 cc. of 40 per cent trichloroacetic acid was added to precipitate protein and favor release of dye from its protein complex. 10 cc. of ethyl acetate were added from a burette and the tubes were shaken vigorously until an even emulsion was produced. The tubes were then centrifuged for 10 minutes at 2500 r.p.m., and 5 cc. of the organic layer were transferred with a pipette to a Klett tube. The color did not fade on standing, but evaporation of ethyl acetate in over 1 hour resulted in increased color density. This was avoided by stoppering the tubes when such delay was unavoidable. The color density was measured in a photoelectric colorimeter through a green filter (540 mp). The readings were converted to mg. of naphthol with a calibration curve prepared from β-naphthol in the presence of serum according to the procedure described above. The visible absorption spectrum of this dye and the linear calibration curve obtained with β-naphthol have been published earlier (14). A calibration curve falling within the range of the colorimeter may be obtained with 0.01 to 0.08 mg. of β-naphthol.

Definition of Unit—1 unit of phosphatase activity is defined as that amount of enzyme which liberates the color equivalent of 10 mg. of β-naphthol per hour at 37.5°.

Calculation—The number of units of alkaline phosphatase per 100 cc. of serum was obtained by multiplying by 200 the number of mg. of β-naphthol released in 1 hour when 0.05 cc. of serum was used. The number of units of acid phosphatase per 100 cc. of serum was obtained by multiplying by 100 the number of mg. of β-naphthol released in 2 hours when 0.05 cc. of serum was used.

Results

The sera of 89 normal adults contained an average of 1.8 units of alkaline phosphatase per 100 cc., with a range of 1.9 to 3.0 units per 100 cc. and a peak distribution between 1.6 and 2.0 units per cc. The sera of 62 of the same normal subjects contained an average of 1.0 unit of acid phosphatase per 100 cc. with a range of 0.7 to 1.6 units per 100 cc. (Fig. 1).

Several sera containing abnormally high amounts of alkaline phosphatase, as measured by the Bodansky method (1), were analyzed simultaneously by the method described here (Table I). In no case did serum exhibiting abnormal phosphatase activity by the Bodansky method fall into the range of normal by this method. There was a rough correlation between the results obtained by both methods. The ratio of activity in
Bodansky units to activity in units of \( \beta \)-naphthol varied from 1.3 to 2.4 over a range of concentration of phosphatase from 6.4 to 43.1 units per 100 cc. (Bodansky method).

**Fig. 1.** Distribution of acid and alkaline phosphatase concentrations obtained in normal human sera with the substrate sodium \( \beta \)-naphthyl phosphate.

**TABLE I**

*Comparison of Abnormal Sera by Present Method and by Bodansky Method*

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Bodansky (A)</th>
<th>Seligman et al. (B)</th>
<th>Ratio ( \frac{A}{B} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units</td>
<td>units</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6.4</td>
<td>4.9</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>7.1</td>
<td>4.0</td>
<td>1.8</td>
</tr>
<tr>
<td>3</td>
<td>7.4</td>
<td>3.6</td>
<td>2.1</td>
</tr>
<tr>
<td>4</td>
<td>9.9</td>
<td>6.8</td>
<td>1.5</td>
</tr>
<tr>
<td>5</td>
<td>13.9</td>
<td>13.4</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>15.5</td>
<td>6.6</td>
<td>2.4</td>
</tr>
<tr>
<td>7</td>
<td>16.9</td>
<td>11.1</td>
<td>1.5</td>
</tr>
<tr>
<td>8</td>
<td>22.1</td>
<td>11.1</td>
<td>2.0</td>
</tr>
<tr>
<td>9</td>
<td>43.1</td>
<td>25.2</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Sera with abnormally high phosphatase activity were tested at several dilutions, the amount of serum in each test ranging from 0.01 to 0.05 cc. The enzyme activity per unit volume of serum was unaltered by the greater dilution of the serum. Experiments were carried out to determine the course of enzymatic hydrolysis. The rate was constant for both enzymes during the first 2 hours. Therefore, different periods of incubation, up
to 2 hours, may be used without introducing correction factors other than simple proportionality. With very short periods of incubation (less than 30 minutes) the 3 minutes allowed for coupling were included in the timing in the case of alkaline phosphatase. Conversely, in sera with very low alkaline phosphatase activity, the time of incubation was limited to 2 hours, because of significant hydrolysis of the substrate at pH 9.1 and 37.5°. In such cases it was found better to use more serum (0.1 cc.).

Since the azo dye is bound to proteins present at the time of its formation, the effect of differences in protein concentration on the extraction of dye with ethyl acetate, after addition of trichloroacetic acid, was determined with varying amounts of serum. The amount of serum was varied from 0.01 to 1.0 cc., and the amount of β-naphthol from 0.01 to 0.06 mg. The final volume of the solutions in all cases was brought to 7 cc. before extraction into ethyl acetate. Less dye was extracted when more protein was present. At both extremes of the series of dilutions, which differed in the concentration of serum proteins by a factor of 100, the difference in the amount of dye extracted was only 8 per cent. With very low concentrations of β-naphthol the amount of dye extracted was independent of protein concentration. Over the range of protein concentration encountered in most determinations this source of error was not significant.

Coupling of tetrazotized diorthoanisidine with β-naphthol proceeded rapidly and quantitatively at a range of pH 7.4 to 9.1. At pH 9.2 or higher, or if the period of coupling was unduly prolonged, the tetrazonium salt decomposed and the aqueous phase became orange-brown in color. This colored material had a markedly different absorption band from that of the azo dye, and was, in addition, not significantly extractable with ethyl acetate. The control tube acted as a suitable check against any slight error from this cause.

Completely hemolyzed blood showed an increase in alkaline phosphatase activity of 0.3 to 0.5 unit per 100 cc. over that for serum. The acid phosphatase activity of hemolyzed blood was 3.5 to 3.8 units per 100 cc. higher than for serum. Serum stored in the solid frozen state did not lose more than 5 per cent of its original alkaline or acid phosphatase activity, and could be stored indefinitely. Serum stored at 4° showed no significant decline in phosphatase activity in 24 hours, but fell 10 per cent below its original level within 3 to 5 days (1). Duplicate determinations of sera did not vary by more than 10 per cent. The average variation encountered in duplicate determinations was 3 to 7 per cent.

**DISCUSSION**

A comparison with other methods for measuring acid and alkaline phosphatase activity is given in Table II, in which may be found the substrates
<table>
<thead>
<tr>
<th>Method</th>
<th>Substrate*</th>
<th>Color determinant</th>
<th>Temperature</th>
<th>Time</th>
<th>pH</th>
<th>Unit of activity per hr.</th>
<th>Normal values†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Acid</td>
<td>Alkaline</td>
</tr>
<tr>
<td>Bodansky (1), 1933</td>
<td>β-Glycerophosphate PO₄</td>
<td></td>
<td>37°C</td>
<td>1</td>
<td>8.7</td>
<td>1 mg. P</td>
<td></td>
</tr>
<tr>
<td>King and Armstrong (2), 1934</td>
<td>Phenyl phosphate Phenol</td>
<td>37.5°C</td>
<td>1</td>
<td>5.0</td>
<td>9.0</td>
<td>1“ phenol</td>
<td>2.4</td>
</tr>
<tr>
<td>Huggins and Talalay (3), 1945</td>
<td>Phenolphthalein phosphate Phenolphthalein</td>
<td>38°C</td>
<td>1</td>
<td>5.5</td>
<td>10.3</td>
<td>0.1“ phenolphthalein</td>
<td>3 -10</td>
</tr>
<tr>
<td>Bessey et al. (4), 1946</td>
<td>p-Nitrophenyl phosphate p-Nitrophenol</td>
<td>38°C</td>
<td>0.5</td>
<td>10.3</td>
<td>1 mm p-nitrophenol</td>
<td>2.4–5.7‡</td>
<td></td>
</tr>
<tr>
<td>Hudson et al. (5), 1947</td>
<td>“ “</td>
<td>38°C</td>
<td>0.5</td>
<td>5.4</td>
<td>1.0–2.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seligman et al., present paper</td>
<td>β-Naphthyl phosphate β-Naphthol</td>
<td>37.5°C</td>
<td>1-2</td>
<td>4.8</td>
<td>9.1</td>
<td>10 mg. β-naphthol</td>
<td>0.7–1.6</td>
</tr>
</tbody>
</table>

* All substrates used in the form of the sodium salt.
† Normal values are all expressed in terms of units per 100 cc. of serum, except in the case of the methods of Bessey et al. and of Hudson et al., for which they are reported in terms of mm units per liter of serum.
‡ These values for alkaline phosphatase, taken from the paper by Bessey et al., were obtained in twelve normal young children, whose alkaline phosphatase as determined by the Bodansky method was 6 to 10 units per 100 cc., or approximately 2 to 3 times normal adult levels.
used, definition of unit activity, and values obtained in the serum of normal subjects.

Although several of these methods, notably those of Bodansky and of King and Armstrong, have been used widely, certain disadvantages in each have stimulated the quest for new ones. Some of the disadvantages will be considered here in order to point out to what extent they are overcome in the present method.

The method of Bodansky (1) has the disadvantage of requiring two controls for each determination, in order to account for the spontaneous hydrolysis of the substrate and the serum inorganic phosphate present before incubation. All the other methods noted, since they do not depend upon the measurement of an ion which is normally present in the serum, eliminate the need for a preincubation control. Stock solutions of the substrate, even though preserved under the prescribed conditions in the cold, deteriorate significantly within 1 month. If the period of incubation is increased or decreased from the standard period of 1 hour, in order to obtain readily measurable amounts of free phosphate ion with sera of very low or very high phosphatase activity, conversion factors determined empirically must be used to obtain results in standard units.

The method of King and Armstrong (2) has the theoretical disadvantage that the specific activity of the enzyme toward the substrate in high enzyme concentrations is decreased. Empirically determined correction factors must be introduced for accurate estimation of concentrations of more than 60 units of alkaline phosphatase per 100 cc. of serum. In clinical practice, however, a fairly large error is permissible in this range of activity.

The method of Huggins and Talalay (3) presents disadvantages in that the substrate is not chemically pure, and hence new batches must be completely restandardized. In addition, the color increment produced by variations in enzyme concentration does not bear a simple linear relationship to the amount of enzyme present. The color of phenolphthalein is not stable and fades appreciably within 1 hour.

The use of p-nitrophenyl phosphate, applied to the measurement of alkaline phosphatase by Bessey et al. (4), and modified for the measurement of acid phosphatase by Hudson et al. (5), provides a rapid and sensitive test for phosphatase estimation. The absorption band of the colored product, however, is so close to that of chromogens that are frequently encountered in abnormal serum (as in icterus or hemolysis) that the blank control often has high color values, with the consequence of an increased error in the determination. Furthermore, the very limited phase of the reaction during which color density is proportional to enzyme concentration (30 minutes) suggests that one of the products of the reaction is inhibitory to the enzyme. Falsely low values may be obtained with
high enzyme concentrations. As in the case of the method of King and Armstrong, diminished accuracy in the range of high phosphatase activity is not of paramount importance in clinical practice, but detracts from the general applicability of the method in other work.

The new method reported here avoids many of the errors attendant on dilution of the enzyme and provides, over a much broader range of enzyme concentration, a degree of accuracy and reproducibility equal to any of the previous methods. It also presents advantages in the stability of the reagents and in the need for only a single control tube for a large number of determinations run simultaneously. The substrate solution will remain intact for at least 1 month when stored in the refrigerator, eliminating the need for periodic check analyses. The stock buffer solutions are also quite stable and readily prepared, and no secondary adjustment of the pH of the buffered substrate solutions is required, since the molar strength used is sufficient to maintain a constant pH throughout the period of incubation. Since the ethyl acetate does not extract chromogens usually encountered in serum, little or no error is introduced into the determination by the presence of icterus or slight hemolysis. The solution of azo dye in ethyl acetate is stable and fading does not occur.

The method outlined for measurement of phosphatases in serum may be applied equally well to the quantitative estimation of enzymatic activity in homogenates or sections of tissue. This makes possible simultaneous study with the histochemical techniques (8, 9).

The method reported here is a simple one, based on the same fundamental principles on which methods have been developed for other hydrolytic enzyme systems, viz., esterase and lipase (14), β-glucuronidase,3 sulfatase,3 glucosidase,4 carboxypeptidase (15), and chymotrypsin.4

SUMMARY

A new method for the determination of acid and alkaline phosphatase activity of serum, utilizing sodium β-naphthyl phosphate, is described. This method presents several advantages over prevailing techniques in simplicity, in stability of reagents, in lack of interference by chromogens of serum, and in providing a high degree of accuracy over a much wider range of phosphatase concentrations.

This method also provides a means of correlating quantitative estimations of phosphatase activities in homogenates of tissue with the histochemical demonstration of phosphatases in tissue sections.

The mean value for serum acid phosphatase obtained by this method in

3 To be published by R. B. Cohen and A. M. Seligman.
4 To be published by H. A. Ravin and A. M. Seligman.
62 normal sera was 1.0 unit per 100 cc. The mean value for serum alka-
line phosphatase obtained in 89 normal sera was 1.8 unit per 100 cc.

BIBLIOGRAPHY

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