Improved Determination of Prostatic Acid Phosphatase (Sodium Thymolphthalein Monophosphate Substrate)

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We have modified a previously described method for determining acid phosphatase, with thymolphthalein monophosphate as substrate, to increase its sensitivity. We assessed effects of serum on variables influencing acid phosphatase activity as measured by this method. The method is shown to be not completely specific for prostatic acid phosphatase. The importance of standardizing methodology in measurement of enzyme activities is demonstrated.

A method for measuring acid phosphatase [orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2] activity in serum, with thymolphthalein monophosphate as substrate, was described by Roy et al. (1) and is the basis of a “kit” method (Worthington Biochemical Corp., Freehold, N.J. 07728) and the method used in the “SMAC” (Technicon Instruments Corp., Tarrytown, N. Y. 10591) system. The method is described as having “good sensitivity over a wide range of activity” and almost complete specificity for prostatic acid phosphatase.

Daily use of the method in our laboratory has shown the method to be extremely insensitive, whether we used kit reagents or reagents that we prepared as described by Roy et al. (1). Activities at the upper limit of the normal range gave changes in absorbance under the conditions of assay of only 0.02 with a reagent blank of 0.07 and a serum-in-reagent blank that may exceed 0.10 with turbid sera. Patients whose acid phosphatase activity in serum gave a change in absorbance under the conditions of the method only 0.005 above the upper limit of the normal range were occasionally found to have cancer of the prostate gland. Such small absorbance differences between normal and abnormal activities fall almost within the error tolerance of commonly used laboratory spectrophotometers such as the Gilford-300-N instrument, and decrease confidence in the reliability of results.

We examined factors influencing acid phosphatase activity, in an attempt to increase the sensitivity of the method. Here, we describe modifications that improve the sensitivity while at the same time decreasing the volume of specimen required, and we also describe the effects of the serum/final reaction-mixture volume ratio on results for prostatic acid phosphatase determined by this method. Nonspecificity of the method for prostatic acid phosphatase is noted and the importance of reagent and methodology standardization is illustrated. The modified method we describe has been in routine use in our laboratory for two years.

Materials and Methods

Reagents

Sources. Thymolphthalein monophosphate, disodium salt, was obtained from Regis Chemical Co., Morton Grove, Ill. 60053; from Sigma Chemical Co., St. Louis, Mo. 63178; and from Worthington Biochemical Corp. Formula weights for the product from the three sources were given as: Regis, 671 (17.7% H2O); Sigma, 625.5 (4H2O); and Worthington, 606 (4H2O). Reagents in kit form were also obtained from Worthington Biochemical Corp. The surfactant Brij-35 (300 g/liter solution) was obtained from BDH Chemicals, Montreal Canada. All other chemicals were of the highest purity available.

Acetate buffer, 5 mol/liter, pH 5.4 (25 °C). Dissolve 68.0 g of sodium acetate trihydrate in distilled water and dilute to 100 ml with distilled water. Measure 28.87 ml of glacial acetic acid into a 100-ml volumetric flask and dilute to the mark with distilled water. To this solution add the sodium acetate solution until the pH of the mixture is 5.4 at 25 °C.

Substrate/buffer reagent. Dilute the Brij-35 solution with distilled water to a concentration of 3.24 g/liter, and dissolve 74.7 mg of disodium thymolphthalein monophosphate (17.7% H2O) in 50 ml of it. Add 1.92 g of sodium acetate trihydrate to the solution, mix until dissolved, and adjust the pH of the solution to 5.4 (25
°C) with 0.1 mol/liter HCl. Bring the total volume to 100 ml with distilled water. Store refrigerated and discard after one month or when blank values become unacceptable.

Alkaline solution for color development (Na₂CO₃, 1.0 mol/liter in NaOH, 1.0 mol/liter). Dissolve 53 g of anhydrous Na₂CO₃ and 20 g of NaOH in distilled water and dilute to 500 ml. Dilute this solution 10-fold before use (working solution).

Thymolphthalein stock standard solution, 3 mmol/liter. Dissolve 129.0 mg of thymolphthalein (mol wt, 431) in sufficient n-propanol/water (70/30 by vol) to make a total volume of 100 ml.

Procedures

Preparation of prostatic extract. Fresh prostatic tissue was finely chopped and ground in acetate buffer (0.25 mol/liter, pH 5.4) with a pestle and mortar. The resulting slurry was centrifuged to sediment debris and the clear supernatant fluid was further diluted with acetate buffer as required, for assay of prostatic acid phosphatase activity.

Preparation of tubes for serum preservation. Pipette 25 µl of the acetate buffer (5 mol/liter, pH 5.4) into tubes. Evaporate in an oven at 60 °C. Stopper and store until required.

Preservation of serum. Immediately after the blood samples are centrifuged, add 0.5 ml of the serum to tubes containing the dry acetate buffer. Agitate gently to dissolve the acetate salts, then store the specimens frozen if they are not to be assayed on the same day.

Measurement of acid phosphatase activity in serum. Concentrations of reagents in the final reaction mixture are: acetate buffer, 0.15 mol/liter; thymolphthalein monophosphate, 1.0 mmol/liter; and Brij-35, 1.5 g/liter.

For each specimen to be assayed two tubes are used (control and test). Pipette into each 550 µl of buffered substrate. Bring the “test” solutions to 37 °C in a water bath and at convenient intervals (e.g., 30 s) add 50 µl of each specimen to the appropriate “test” tube, mix gently and allow to incubate at 37 °C for exactly 30 min. Add 1.0 ml of the working alkaline solution to each “test” tube and mix.

While the “test” samples are incubating, add 1.0 ml of the working alkaline solution to each of the “control” tubes, followed by 50 µl of the appropriate serum, and mix.

Read the absorbances of test and control solutions at 590 nm, subtract control from test absorbances and determine acid phosphatase activity from a standard curve prepared as described below.

For specimens with acid phosphatase activity yielding a color exceeding the limits of the spectrophotometer (up to an absorbance of 3.0) either dilute the specimen and re-assay or repeat the assay, with use of a 5-min incubation, read the absorbance at 590 nm, subtract the absorbance of the control, read the activity from the standard curve and multiply the answer by six.

Preparation of standard curve; standardization to 80 U/liter. Dilute stock thymolphthalein solution by adding enough of the propanol/water mixture to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, and 0.8 ml of thymolphthalein stock standard solution to make 1.0 ml in each case. Into a set of nine tubes dispense 550 µl of buffered substrate reagent and 1.0 ml of the working alkaline solution. Pipette 50 µl of distilled water into the first of these tubes and 50 µl of each diluted standard solution into subsequent tubes. Mix well and read the absorbances of these solutions at 590 nm, using the first of the tubes as blank.

The absorbances obtained will be equivalent to those obtained by the action of 10, 20, 30, 40, 50, 60, 70, and 80 U of acid phosphatase per liter of serum. Plot absorbances vs. U/liter on linear graph paper.

Results

Effect of Source of Substrate

Roy et al. (I) found that under the conditions of their method, prostatic acid phosphatase activity was maximal with a substrate concentration of 2.2–2.4 mmol/liter. Morin (2), using ammonium thymolphthalein monophosphate, also found the optimal substrate concentration to be 2–3 mmol/liter. Using thymolphthalein monophosphate supplied by Regis Chemical Co. and the method described by Roy et al. (I), we found that a substrate concentration half that obtained by these workers gave maximal activity. When we compared substrate concentration optima for thymolphthalein monophosphate from the three sources listed above, we found that optimal concentration for maximum prostatic acid phosphatase activity varied with the source of thymolphthalein used, as did also the values for activities of prostatic acid phosphatase—the activity was greatest when substrate from Regis Chemical Co. was used, followed by those from Sigma and Worthington (Figure 1).

Two different lot numbers of thymolphthalein monophosphate from Regis Chemical Co., one purchased
a year earlier and stored at room temperature and the second purchased three months earlier and stored at 
\(-20\) °C, gave identical acid phosphatase activities, although blank values were about 40% smaller with the 
product stored at \(-20\) °C. We used thymolphthalein monophosphate from Regis for the rest of the work 
reported here, except as specifically noted.

Effect of S/R Volume Ratio

Roy et al. (1) used an s/r ratio of 1/6 and Morin (2) used a ratio of 1/3.5. We found that optimum substrate 
concentration was influenced by this ratio, decreasing as the ratio was decreased; for prostatic extract alone 
the optimum substrate concentration was as low as 0.15 
mmol/liter, when we used the buffer conditions of Roy 
et al. (1)—i.e., citrate buffer, 0.1 mol/liter, pH 6.0 
(Figure 2a). The ratio also influenced substrate con-
centration giving maximal activity in the modified 
method described herein, although less markedly in this 
acetate buffer medium than in the citrate buffer of Roy 
et al. (1). The optimum substrate concentration by our 
method (s/r ratio, 1/12) was 1.0–1.2 mmol/liter. With 
a ratio of 1/6, similar to that of Roy et al. (1), activity was 
greatest with a substrate concentration of 1.2–1.4 
mmol/liter, and with prostatic extract alone optimum 
substrate concentration was 0.6 mmol/liter (Figure 2b).

Effect of Buffer

Citrate buffer. Roy et al. (1) states that prostatic acid 
phosphatase activity was maximal with a 0.1 mol/liter 
concentration of citrate buffer (80 mmol/liter when 
dilution by sample is considered). We observed no peak 
activity for acid phosphatase in citrate buffer, pH 6.0 
in concentrations ranging from 10 mmol/liter to 200 
mmol/liter as long as the pH of the serum or extract 
used was preadjusted to reaction pH by addition of ac-
etate buffer; increasing citrate concentrations resulted in 
decreasing acid phosphatase activities. We confirmed 
these results by use of thymolphthalein monophosphate 
from the three suppliers. When serum was not acidified 
buffer concentrations with substrate from all three 
sources, but under these conditions the pH of the re-
action medium deviated from the original pH. Data il-
dustrating this are shown in Figure 3 for substrate from 
Regis Chemical Co.

Acetate buffer. With serum containing prostatic 
extract and an s/r ratio of 1/12, the optimal acetate

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\(^1\) The “s/r ratio” is the ratio of the volume of serum sample to the 
volume of the final reaction mixture.
buffer concentration was 150–200 mmol/liter at pH 5.4. In the absence of serum a buffer concentration of 350–400 mmol/liter was required for maximal prostatic acid phosphatase activity (Figure 4). The optimal pH with acetate buffer 150 mmol/liter was 5.2–5.4 with an s/r ratio of 1/12, and 5.8–6.0 for prostatic extract alone (Figure 5).

Effect of Surfactant

The concentration of Brij-35 optimal for hydrolysis of substrate by prostatic acid phosphatase in our method was determined. In the presence of serum (s/r ratio, 1/12) a concentration in the final reaction mixture of 1.5 g/liter was found to be optimal. In the absence of serum a concentration of 5 g of Brij-35 per liter was required to give maximal activity, and in the absence of both serum and Brij-35, the activity of prostatic acid phosphatase was negligible (Figure 6). Roy et al. (1) used Brij-35 (5 g/liter) in their reaction mixture to stabilize the substrate in the buffer solution. In the absence of the surfactant we, too, noticed that precipitation occurred in the substrate/buffer reagent on refrigeration, but inclusion of Brij-35 at the concentration giving maximal activity under our conditions (1.5 g/liter) prevented this.

Linearity

Prostatic acid phosphatase activity is linearly related to time of incubation for at least 30 min in the presence of a constant concentration of serum and of saturating substrate conditions. Linearity with concentration extends to an absorbance of 6.0 in the kit method and to an absorbance of 10.0 in the method described herein (determined in each case by dilution of the final colored solutions with working alkali color developer solution to bring the absorbance into a readable range) in the absence of serum but addition of normal serum decreased the range of linearity in both the kit and modified methods (Figure 7). Activities first increased and then decreased with increasing s/r ratio when the medium contained a constant concentration of prostatic extract (Figure 7). With an s/r ratio of 1/12 linearity with concentration of prostatic acid phosphatase extended to an absorbance of 3.5. When the s/r ratio exceeded 1/12, activity became nonlinear with concentration of enzyme (Figure 8).

Termination of Reaction, and Color Development

Roy et al. (1) used 5 ml of Na₂CO₃ (50 mmol/liter) and NaOH (50 mmol/liter) solution per 1.2 ml of reac-
**Figure 7. Effect of serum on linearity of acid phosphatase activity in a prostatic extract**

(a) o — o, activities found by use of the kit method (half specified volumes, 0.6 ml total reaction mixture). (b) o — o, activities found by our modified method (0.6 ml total reaction mixture). Absorbance of 3.5 equivalent to 100 U/liter.

**Figure 8. Linearity of acid phosphatase with increasing concentrations of serum containing prostatic acid phosphatase**

Reaction mixture: that of the present method, 0.6 ml. Absorbance of 1.75 equivalent to 50 U/liter.

tion mixture (a final concentration of 40 mmol/liter) to terminate acid phosphatase activity and develop the color. This resulted in more than fivefold dilution of thymolphthalein liberated by enzyme activity. We reduced the volume of alkali to 1.0 ml per 0.6 ml of reaction mixture, so that liberated thymolphthalein was diluted only half as much and final absorbances were thus doubled. A solution of Na$_2$CO$_3$, 0.1 mol/liter, and NaOH, 0.1 mol/liter, (equivalent to a final concentration of 60 mmol/liter) was found to be adequate for color development and pH change.

**Serum Preservative**

Roy et al. (1) recommended use of 20 μl of acetate buffer (5 mol/liter, pH 5.0) per milliliter of serum as preservative. Because acetate buffer, 0.15 mol/liter, pH 5.4 (25 °C), is used in the final reaction medium of the modified method, we used 50 μl of acetate buffer, 5 mol/liter, pH 5.4, per milliliter of serum to bring the pH to that required and adjusted the concentration of acetate in the buffer substrate reagent to allow for this.

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<th>Activity (U/liter)</th>
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Table 1. Activities of Prostatic Acid Phosphatase as Measured by Our Modified Method (I), the Method of Roy et al. (II) and the Worthington Kit Method (III)

*But containing Brij-35 surfactant, 1.5 g/liter.

First three data are for prostatic extract; remaining 16 are for serum samples containing prostatic extract.

**Comparison of Acid Phosphatase Activities by Our Modified Method, the Method of Roy et al. (1), and the Worthington Kit Method**

Table 1 compares acid phosphatase activities obtained by the modified method described herein; the method of Roy et al. (1), but using thymolphthalein monophosphate from Regis Chemical Co.; and the Worthington kit method. An about threefold increased sensitivity is apparent in the modified method when prostatic extract is added to normal serum. Absence of serum from the reaction medium, shown above to affect optimum substrate and buffer concentration, results in greater increase in activity of prostatic acid phosphatase under the conditions of the modified method compared with the other procedures.

**Acid Phosphatase Activity in Normal Serum**

By either the unmodified kit method or our modified method described above, acid phosphatase activities were similar in sera of both male and female hospital patients. The normal range of activities in individuals without prostatic disease ranged from 0.5 to 1.9 U/liter (X ± 2 SD) by our modified method as compared to a normal range of up to 0.53 U/liter for the kit method.

**Acid Phosphatase Activity in Urine**

Acid phosphatase activities in undialyzed urines of female children ranged up to 20-fold the activities in sera of normal men.
Discussion

The modifications we have introduced into the method of Roy et al. (1) include reduction in substrate concentration; change in buffer type, ionic strength and pH; reduction in s/r ratio; reduction in concentration of Brij-35; reduction in total reaction volume and in volume of alkaline color developer; and a change in the pH and final concentration of acetate buffer used as acid phosphatase preservative in serum. These modifications increase the sensitivity of the method by a factor of three, enabling use of one-quarter the volume of serum (50 μl) used previously (0.2 ml). Linearity of the reaction under the modified conditions extends to an absorbance of about 3.5, 100 U/liter. Variations in the optimum concentration of buffer and surfactant that occur on addition of serum to the reaction medium and on changing the s/r ratio could be related to an effect of the protein or Brij-35 in complexing with the thymolphthalein monophosphate and providing a more readily available substrate for the enzyme.

The work reemphasizes the already well known but often neglected importance of standardization in clinical enzyme methodology. The observed effects of the s/r ratio on prostatic acid phosphatase activity, together with the differences found on using thymolphthalein monophosphate from different manufacturers, illustrates the necessity for absolute definition of methods, even to source of reagents. The complexity of phosphatase reactions, well-documented with respect to alkaline phosphatase, where changing buffer ion markedly alters enzyme activity, applies also to acid phosphatases. Discrepant results and much confusion can arise as a result of even the most minor modification in methodology. Our point of view about the importance of standardization has previously been expressed (3).

While Roy et al. (1) have shown that thymolphthalein monophosphate provides a substrate with greater specificity for prostatic acid phosphatase than some other substrates commonly used, it is apparent from the acid phosphatase activities found in urines of female children that the substrate is not completely specific for the prostatic form of the enzyme. This is confirmed by the similarities in the ranges of acid phosphatase activities found in the serum of males and females. The latter observation agrees with the inference of other workers (4, 5) that the acid phosphatase normally found in serum is not of prostatic origin.

A method involving an alternative substrate, α-naphthyl phosphate, shown previously to have relatively high specificity for prostatic acid phosphatase (6), was subsequently criticized when it was shown that platelet and urinary acid phosphatases have some affinity for this substrate (4, 7). Erythrocyte acid phosphatase apparently has a lower affinity for thymolphthalein monophosphate than other substrates considered to be relatively specific for prostatic acid phosphatase, such as α-naphthyl phosphate, but the relative activity of platelet to prostatic acid phosphatase with thymolphthalein monophosphate as determined by Roy et al. (1) indicates only a small increase in specificity—a ratio of 1.5 for α-naphthyl phosphate as compared with 1.0 for thymolphthalein monophosphate (1, Table 3). During routine use of our method here we have observed that acid phosphatase activities more than twice the upper limit of the normal range may be found in patients with lymphoproliferative disorders and markedly elevated platelet counts. Platelets can be differentiated from prostate as a source of acid phosphatase in specimens from such patients by comparing the acid phosphatase activity in platelet-free heparinized plasma with that of serum; if the elevated activity in serum is of blood-cell origin, the activity falls within the normal range in the platelet-free plasma.

In summary, we have increased approximately threefold the sensitivity of the method for determination of acid phosphatase with thymolphthalein monophosphate as substrate, and have shown that serum affects critical variables for determination of prostatic acid phosphatase activity, and that the source of substrate affects the activities obtained. The work reemphasizes the importance of standardization of methodology for determination of enzyme activities in clinical laboratories.

References