Sodium Thymolphthalein Monophosphate: A New Acid Phosphatase Substrate with Greater Specificity for the Prostatic Enzyme in Serum

A. V. Roy, Mary E. Brower, and Jean E. Hayden

We describe the use of sodium thymolphthalein monophosphate as a substrate for measuring acid phosphatase activity in serum. Thymolphthalein is liberated in the reaction and is conveniently measured by increasing the pH of the medium, which produces a color and also stops the hydrolysis. Optimal conditions for enzymatic activity were defined for use in the final assay system. Purified acid phosphatase isoenzymes from various human tissues and sera from patients with nonprostatic diseases most likely to affect serum acid phosphatase activity were used to show that the new method is more specific for the prostatic enzyme than other commonly used procedures for acid phosphatase. Other advantages include simplicity, good sensitivity over a wide range of activity, high precision, zero-order kinetics to about 200 times the upper limit of normal, easy standardization by a pure standard, and reagent stability.

Additional Keyphrases specificity of various substrates compared • optimal conditions • inhibitors • clinical studies • relative activities of enzyme from various (human) sources

Ideally, serum acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) determination should be simple, precise, not susceptible to interference from other serum constituents, and specific for prostatic acid phosphatase.

Many substrates have been proposed for prostatic acid phosphatase determination, but they are too insensitive to detect small increases in prostatic acid phosphatase activity; either the blank is large (e.g., β-glycerophosphate) or the substrates are undesirably sensitive to nonprostatic acid phosphatase present in the serum (e.g., phenol-phosphate and p-nitrophenol-phosphate) (1).

α-Naphthyl-phosphate, when introduced as a substrate for acid phosphatase determination, was reported to be specific for prostatic acid phosphatase (1, 2); however, recent work has shown that it is hydrolyzed by platelet acid phosphatase (3), and its clinical usefulness is diminished by the large group of falsely positive results obtained with patients without prostatic carcinoma (4).

Sodium thymolphthalein monophosphate, which yields its own chromogen on enzymatic hydrolysis has been used recently to determine serum alkaline phosphatase (5). Therefore, we investigated its usefulness for acid phosphatase assay. We report here the method developed with this substrate, the tissue specificity of this and five other commonly used substrates, and the relative inhibitory effect of L-tartrate and formaldehyde on the activities of acid phosphatase isoenzymes from various human tissues, when measured by use of these substrates.

Materials and Methods

All batches of sodium thymolphthalein monophosphate (Worthington Biochemical Corp., Freehold, N.J. 07728) contained less than 1% free thymolphthalein, an amount easy to blank out and which did not inhibit the enzymatic reaction. They were standardized as previously reported (5). All other chemicals were reagent grade or of the highest purity available.

Analytical Variables

Buffer pH. We used citrate buffer because it is widely used in other procedures for acid phosha-
tase, and because it reportedly enhances the prostatic acid phosphatase activity (6). Figure 1 shows the total and L-tartrate-refractory activities obtained with a serum from a patient with prostatic cancer, as a function of the buffer pH. Maximum total activity is obtained at a pH 6.0-6.2.1 Similar results were obtained with a prostatic extract. Our results disagree with the previously reported pH optima, which range between 4.7 and 5.9 (1, 4, 6-9), but which were determined with different substrates; it has been shown (10) that optimum pH varies with the substrate and buffer used.

A pH of 6.0 (5.98 at 37°C) was routinely used. Interference by alkaline phosphatase at this pH was ruled out as follows: a serum with normal acid phosphatase activity and an extremely elevated alkaline phosphatase activity was left standing for four days at room temperature, thus inactivating its acid phosphatase. Acid phosphatase activity of this serum was then measured with the new buffered substrate (see below) at several pH values, from 6.0 to 6.5. Even at pH 6.5, no hydrolysis was detected, but there was, as expected (11), no appreciable decrease in alkaline phosphatase activity.

Buffer concentration. Figure 2 shows the effects of citrate concentration on prostatic acid phosphatase activity at pH 6.0 with 2.2 mmol of sodium thymolphthalein monophosphate per liter. Activity was maximum with both a prostatic extract and a serum from a patient with prostatic cancer when the citrate concentration was 0.1 mol/liter. This finding disagrees with previously reported optima determined with other substrates, which range between 0.045 and 0.2 mol/liter (4, 9). Apparently, optimum citrate concentration also depends on the particular substrate used. We chose a citrate concentration of 0.1 mol/liter for routine use.

Substrate concentration. In citrate buffer (0.1 mol/liter, pH 6.0) maximum activity was obtained—with both a prostatic extract and a serum from a patient with prostatic cancer—at 2.2-2.4 mmol of sodium thymolphthalein monophosphate per liter (Figure 3). For routine use, we chose a concentration of 2.2 mmol/liter. At this concentration no spontaneous, nonenzymatic hydrolysis was noted after a 1-h incubation at 37°C under the final test conditions.

Substrate stability. Refrigerated, dry sodium thymolphthalein monophosphate is stable for at least 9 months. Solutions of sodium thymolphthalein monophosphate refrigerated in our citrate buffer became hazy after a few days. Also if 0.2 ml of serum was added to 1.0 ml of these solutions, some of the substrate was pre-

---

1 pH throughout, unless otherwise specified, was determined at 25°C.

---

Fig. 1. Effect of citrate buffer pH, 0.1 mol/liter, on the total and tartrate-refractory activities of a serum from a prostatic cancer patient

In all Figures, except as noted, assay conditions were as described in the recommended procedure.

---

Fig. 2. Effect of citrate concentration, pH6.0, on the acid phosphatase activities of a prostatic extract and serum from a prostatic cancer patient

In all Figures, except as noted, the prostatic extract was dissolved in citrate buffer, 10 mmol/liter, pH6.0
Prostatic acid phosphatase activities were measurably increased, and NaOH, we decided on a solution containing 50 mmol each of Na2CO3 and NaOH per liter for stopping the enzymatic reaction and obtaining full color development. The color did not fade measurably in 2 h.

Serum acidification. All blood specimens were centrifuged within 2 h after venipuncture. Because a pH near 6 is needed to preserve the serum acid phosphatase activity (12, 13), we immediately added 20 μl of acetate buffer (5 mol/liter, pH 5 at 37°C) per ml of serum, which resulted in a pH between 5.1 and 5.8; this sometimes increased, but by no more than 0.3 pH units, after 4-week storage at 4°–8°C. Prostatic acid phosphatase activity was unaffected by such storage.

Temperature. Although 30°C is a generally recommended temperature for enzyme measurements (14), we used a temperature of 37°C here because the prostatic acid phosphatase was only about half as active at 30°C.

Prostatic acid phosphatase is stable at 37°C under our final test conditions: When a 0.2-ml aliquot of a human serum with moderately enhanced prostatic acid phosphatase activity was pre-incubated for 2 h at 37°C with 1.0 ml of our citrate buffer and subsequently assayed (by adding 0.1 ml of sodium thymolphthalein solution, 22 mmol/liter), the result differed only negligibly from that for a sample not pre-incubated.

Reagent blank vs. serum blank. Because absorbance readings are made at 590 nm, little interference by bilirubin or hemoglobin was expected. At first, readings were made vs. a reagent blank, prepared by substituting serum with water. However, turbid, lipemic, highly icteric, or heavily hemolyzed sera have, with the new method, a serum blank high enough to bring the low activities of normal sera up to the abnormal range; consequently, we decided to use a serum blank routinely.

Reagents

Brij-35, 200 g/liter. Dissolve 20 g of Brij-35 in approximately 60 ml of water by gently warming. Cool and dilute to 100 ml. Stable for at least 6 months at room temperature.

Citrate buffer, 0.1 mol/liter, pH 5.95. Solution A: Dissolve 29.41 g of trisodium citrate dihydrate in about 950 ml of water, add 25 ml of Brij-35 (200 g/liter) and dilute to 1000 ml.

Solution B: Dissolve 2.1 g of citric acid monohydrate in about 95 ml of water, add 2.5 ml of Brij-35 (200 g/liter) and dilute to 100 ml.

To about 900 ml of solution A, add a sufficient amount of solution B to bring the pH (at 25°C) to 5.95. Stable, at 4°–8°C for at least six months.

Buffered substrate. Dissolve 0.185 g of sodium thymolphthalein monophosphate in 100 ml of buffer. The alkalinity of sodium thymolphthalein monophosphate changes the pH of the buffer to 6.0. Stable at 4°–8°C for at least 2 months.

Color developer. Dissolve 2 g of NaOH and 5.3 g of anhydrous Na2CO3 in water, and dilute to 1 liter. Stable at room temperature for at least six months.

Thymolphthalein stock standard solution, 22.5 mmol/liter. Dissolve 968.7 mg of thymolphthalein (Sigma Chemical Co., St. Louis, Mo. 63178) in sufficient ethanol to make 100 ml of solution. Stable at 4°–8°C for at least a year.

Serum preservative, acetate buffer, 5 mol/liter, pH 5.0 (at 37°C). To 43.5 g of sodium acetate trihydrate in a 100-ml volumetric flask, add 50 ml
of water, 13 ml of glacial acetic acid, and water until the volume is about 95 ml. Adjust pH (at 37°C) to 5.0 by dropwise addition of glacial acetic acid, and dilute to 100 ml.

Procedure

Pipet 1 ml of buffered substrate into test tubes or cuvets and incubate them for 5 min in a water bath at 37 ± 0.1°C.

Add 0.20 ml of serum with a pipet ("to contain") and rinse the pipet at least twice. (If more than one acid phosphatase is to be determined, add other sera at 30-s intervals.)

Add 5.0 ml of color developer exactly 30 min after the serum was added. Stopper, and invert to mix.

Read the absorbances at 590 nm against a serum blank prepared by mixing 1.0 ml of buffered substrate, 5.0 ml of color developer, and 0.2 ml of serum, added in that order. Acid phosphatase activities are read from the standard curve.

If the absorbance for any test mixture is greater than that for the highest standard, dilute the test solution with water and read it against a blank diluted the same. If the activity is greater than 110 U/liter, repeat the assay with serum diluted 10-fold with saline (9 g NaCl/liter).

Standardization. freshly prepare a dilute stock standard solution by diluting 1 ml of stock standard to 50 ml with ethanol.

Prepare the working standards as follows: label six tubes and add 1.0, 2.0, 4.0, 6.0, 8.0, and 10.0 ml of dilute stock standard, respectively, then 9.0, 8.0, 6.0, 4.0, 2.0, and 0.0 ml of ethanol, respectively.

Pipet 1.0 ml of buffered substrate into cuvets (or test tubes). From a pipet ("to contain") add 0.20 ml of working standards to the respective cuvets. Add 5.0 ml of color developer to each cuvet, and invert to mix. Read at 590 nm against a blank prepared by substituting ethanol for the standard.

The acid phosphatase activity is expressed in International Units, i.e., as micromoles of thymolphthalein formed per minute per liter of serum at 37°C. The above working standards are thus respectively equivalent to 1.5, 3, 6, 9, 12, and 15 U/liter.

With most filter colorimeters and some broadband spectrophotometers (e.g., the Coleman Jr., Model C), the absorbance of thymolphthalein deviates somewhat from linearity, but accurate results may be obtained by interpolation.

Results and Discussion

Normal Range and Correlation with Other Procedures

Acid phosphatase activity, as determined by the new method in sera from 85 apparently healthy fasting men, ages 26 to 84, ranged from 0.11 to 0.60 U/liter (mean ± sd, 0.28 ± 0.09 U/liter; 95% limits calculated by Normal Equivalent Deviate method (15) after the logarithmic conversion of the data, 0.13 to 0.56 U/liter).

Acid phosphatase determinations on those sera were also carried out by a p-nitrophenol-phosphate method (16). Activities obtained by the new procedure correlated poorly with either the total or l-tartrate inhibited activities measured with p-nitrophenol-phosphate (r, 0.416 and 0.553, respectively). However, a better correlation (r, 0.889) was obtained between total thymolphthalein monophosphatase activity and l-tartrate-inhibited p-nitrophenol-phosphatase activity of 21 sera that had moderately or greatly supernormal prostatic acid phosphatase activity. These results suggest that some nonprostatic acid phosphatase, which hydrolyzes the new substrate and p-nitrophenol-phosphate at different rates, is normally present in serum.

Linearity of Enzymatic Reaction

For the new method, the linearity with respect to enzyme concentration and incubation time is excellent (Figures 4 and 5). Kinetics are zero order up to about 110 U/liter, at which activity about 28% of substrate depletion has occurred.

We also determined the linearity of various other acid phosphatase procedures. Table 1 shows that our procedure was the most widely linear.

Reproducibility

Within-run precision of the new procedure was tested with 21 replicate assays of a 10-fold dilution of a human serum that had an acid phosphatase activity of about 30 U/liter. Values ranged from 2.98 to 3.17 U/liter (mean ± sd, 3.09 ± 0.06). Day-to-day variation was tested with the same diluted serum, stored at 4°–8°C for three weeks. Acid phosphatase was measured on 21 successive days. Values ranged from 2.87 to 3.27 U/liter (mean ± sd, 3.07 ± 0.10 U/liter).

| Table 1. Upper Limit of Total Normal Activity and Linearity of Various Acid Phosphatase Methods |
|:---|:---|:---|:---|
| Substrate | Ref. no | Upper limit, U/liter | Linearity to, U/liter |
| Sodium thymolphthalein monophosphatase (STM)* | here | 0.56 | 110 |
| α-Naphthyl-phosphate (α-NP) | (19, 34) | 2.0 | 20 |
| Phenol-phosphate (PP) | (17, 18) | 7.0 | 124 |
| Phenolphthalein monophosphate (PMP) | | 5.0 | 25 |
| p-Nitrophenol-phosphate (PNPP) (16) | | 11.0 | 22 |
| β-Glycerophosphate (β-GP) (80) | | 7.0 | 110 |
| * Abbreviations used here are those used in Tables 2-4, Figure 3. |

1096 CLINICAL CHEMISTRY, Vol. 17, No. 11, 1971
Inhibition by L-Tartrate

Hydrolysis of sodium thymolphthalein monophosphate by prostatic acid phosphatase was inhibited by L-tartrate. With both a prostatic extract and serum from an individual with prostatic cancer, inhibition was greater than 95% when the L-tartrate concentration was 40 mmol/liter or greater (Figure 6).

Tissue Specificity of Various Acid Phosphatase Substrates

The tissue specificity of the new and of various other substrates was established by using several acid phosphatase preparations and selected sera.

Substrates. The substrates investigated were: sodium thymolphthalein monophosphate, as described in this paper; phenol-phosphate, with the Hansen modification (17) of the Kind and King method (18); α-naphthyl-phosphate, as described by Babson and Phillips (19), but corrected with a serum blank (4); p-nitrophenolphosphate, as described by Berger and Rudolph (16); β-glycerophosphate, as described by Kaser and Baker (20); and thymolphthalein monophosphate, as described by C. M. Coleman [CLIN. CHEM. 12, 529 (abstract) and personal communications]. The last procedure is as follows: 0.1 ml of sample is added to 0.5 ml of buffered substrate (calcium phenolphthalein monophosphate, 1 g/liter, and citrate, 0.4 mol/liter, pH 6.0). After 30-min incubation at 37°C, the enzymatic reaction is stopped and the phenolphthalein color fully developed by adding 2.5 ml of alkalinizing reagent (Na₂CO₃, 20 g/liter, and Na₄EDTA, 5 mmol/liter). Absorbance readings against a serum blank are taken at 550 nm, and compared to those obtained with phenolphthalein standard solutions. Enzymatic activities in all methods are

![Graph](image-url)
expressed in International Units, that is, as micro-
moles of substrate converted per min, either per
liter (U/liter) or per gram of dried extract (U/g)
(14).

Prostatic tissue. An extract of prostatic tissue
was prepared, essentially according to Smith and
Whitby (21). After the first chromatography,
column effluents with acid phosphatase activity
were pooled and lyophilized without dialysis. The
product thus obtained contained 27% protein,
and showed an acid phosphatase activity of
9000 U/g when tested with sodium thymolphtha-
lein monophosphate. This extract, as well as
others mentioned below, was dissolved in serum
pools having very low acid phosphatase activities.
The pools were prepared with normal sera that
had been standing at room temperature for 3 days.
Immediately before use, the pools were acidified
with acetate buffer as described for fresh sera.
Residual acid phosphatase activity in the serum
pool was subtracted from the results obtained
when the enzymes were in solution. Purified
enzymes were dissolved in citrate buffer, pH 6.0,
at first, but we discovered that the enzymatic
activities measured with the various substrates
were affected differently by the buffer concen-
tration. Enzyme solutions in serum eliminated
this variable and concomitantly imitated hypo-
thetical clinical conditions.

Erythrocytes. We also isolated the three erythro-
cytic acid phosphatase isoenzymes reported by
Fenton and Richardson (22), by essentially their
chromatographic method. However, after the
effluents corresponding to each peak were pooled,
the NaCl present in the eluted fluid was dialyzed
away overnight against citrate buffer (10 mmol/
liter, pH 6.0, with 20 mg of reduced glutathione
added per liter). The dialysands were lyophilized
and, in order of elution, labeled “Erythrocytic
Acid Phosphatase I, II, and III.” Their activities,
measured with p-nitrophenol-phosphate as the
substrate (16), were respectively 18.3, 23.3, and
7.2 U/g.

Bone, kidney, and liver. Acid phosphatases
from bone, kidney, and liver were prepared by
the same procedure: several fetal bones, a normal
kidney, and about 200 g of a normal liver, obtained
separately during three different autopsies, were
individually homogenized and the acid phospha-
tases isolated by passing each extract through a
chromatographic column of DEAE-cellulose,
especially as described by Smith and Whitby (21)
for the separation of the prostatic isoenzymes.
Kidney and liver extracts each gave three sharp
peaks of acid phosphatase activity; bone extract
gave only one small peak. In each case, the NaCl
effluents corresponding to the same peak were
pooled, the NaCl dialyzed away (16 h against
several changes of citrate buffer, pH 6.0, 10
mmol/liter, with 0.2 ml of Triton X-100 added
per liter), and the dialysands were lyophilized.
The enzymes from kidney and liver were assigned
numbers according to their order in elution. Their
acid phosphatase activities measured with p-
nitrophenol-phosphate as the substrate (16) were:
for kidney fractions I, II, and III, 9.08, 2.06,
and 2.25 U/g, respectively; for liver fractions
I, II, and III, 6.04, 0.85, and 2.56 U/g, respec-
tively, and for the bone preparation, 0.21 U/g.

Platelets. Acid phosphatase extracts from plate-
lets were prepared as follows: 9 ml of blood from
healthy adults was drawn into siliconized test
bottles containing 1 ml of trisodium citrate, 0.1
mol/liter. Platelets were separated by differential
centrifugation (23) and then suspended in acidi-
cified serum pool. To increase the yield of enzyme,
the platelet suspension was repeatedly frozen
and thawed (4, 24). Cellular debris was eliminated
by a 10-min centrifugation at 6000 g. When tested
with p-nitrophenol-phosphate (16), all the plate-
let extracts thus prepared showed acid phospha-
tase activities several times higher (15 to 25 U/
liter) than the original activities of the serum
pools (<2 U/liter). Platelet acid phosphatase
was therefore effective released.

Urine. Urinary acid phosphatases was deter-
mined in freshly voided urine from unmarried
females. The urines were free from protein, hemog-
lin, and erythrocytes. Urinary pH was adjusted
to about 6 with either trisodium citrate or citric
acid, 1 mol/liter solutions, and the urines were
then dialyzed for 7 h against frequently changed
saline, 9 g/liter. The pH of such dialysands was
between 5 and 6. They were utilized the day of
their preparation. Urinary acid phosphatase activ-
ity, as measured with sodium thymolphthalein
monophosphate, increased 25-45% after dialysis.
Amador et al. (4), who used α-naphthyl-phosphate,
and Fernandez et al. (26), who used phenol-
phosphate, did not observe this phenomenon.
The action of this inhibitor apparently depends
on the kind of substrate being used.

Inhibitors

Most substrates being used with acid phos-
phatase are insufficiently specific for the prostatic
enzyme; the usefulness of serum acid phosphatase
measurements is, therefore, enhanced by the
use of inhibitors, such as L-tartrate and formalde-
hyde. Thus, with phenol-phosphate as the sub-
strate, the formaldehyde-refractory (26) or L-
tartrate-inhibited (27) activities reportedly cor-
relate better with prostatic acid phosphatase
activity. Inhibition of the various acid phospha-
tase isoenzymes by L-tartrate and formaldehyde
was, therefore, measured for each of the substrates
under consideration. The amount of inhibitor
recommended for each method was used; if no
amount was indicated, then 40 μmol of L-tartrate
and 0.1 ml of formaldehyde (50 g/liter) were used.

Interferences by the nonprostatic enzymes cannot be completely eliminated by the use of L-tartrate or formaldehyde inhibition, with any of the substrates studied (Table 2). Although prostatic acid phosphatase can be distinguished fairly well from erythrocytic acid phosphatase by the use of either inhibitor with all but β-glycerophosphate as substrate, the same is not true for other nonprostatic enzymes: they are generally inhibited too strongly by L-tartrate and too weakly by formaldehyde, thus mimicking the prostatic acid phosphatase. If these enzymes reach the circulation, in most cases neither L-tartrate nor formaldehyde will serve to distinguish them from the prostatic enzyme. Consequently, the hydrolysis rates by the nonprostatic enzymes will bear importantly on the specificity of any substrate for prostatic acid phosphatase.

Relative hydrolysis rates for the various substrates with acid phosphatases from various human tissues are given in Table 3. The results for each isoenzyme are expressed, in this table, as the ratio between the activities (in International Units) measured with the various substrates and that (also in International Units) measured with thymolphthalein monophosphate as the substrate, arbitrarily taken as 1.0 for all the enzymes. The results show that other substrates are hydrolyzed by the prostatic acid phosphatase more rapidly than thymolphthalein monophosphate. Comparison of hydrolysis rates by prostatic and nonprostatic acid phosphatases show, however, that relative to thymolphthalein monophosphate, the other substrates are hydrolyzed by most of the nonprostatic acid phosphatases more rapidly than by the prostatic enzyme. Consequently, thymolphthalein monophosphate is the substrate less affected by the nonprostatic acid phosphatases.

Acid phosphatases from platelets and erythrocytes are of special importance: acid phosphatases from liver, kidney, bone, and urine may or may not be present in serum, but platelet acid phosphatase, released during blood clotting (9, 24), is present in all sera, as are erythrocytic acid phosphatases in any hemolyzed specimen.

Since the amount of acid phosphatase activity derived from platelets varies with the technique used to obtain the serum, the time interval between clot formation and testing, and the treatment of the clot (9, 24), different sera from the same individual may contain different amounts of platelet acid phosphatase. This variability in platelet acid phosphatase activity may impair the precision and sensitivity of methods based

---

**Table 2. Inhibition (in Per Cent) by L-Tartrate and Formaldehyde of the Activities of Various Human Acid Phosphatase Isoenzymes on Several Substrates**

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Inhibition by</th>
<th>STM</th>
<th>PP</th>
<th>PMP</th>
<th>α-NP</th>
<th>PNPP</th>
<th>β-GP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostate</td>
<td>L-Tart.</td>
<td>98.6</td>
<td>90.2</td>
<td>98.4</td>
<td>98.1</td>
<td>87.6</td>
<td>87.2</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>2.2</td>
<td>...</td>
<td>12.2</td>
<td>5.6</td>
<td>5.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Erythrocyte I</td>
<td>L-Tart.</td>
<td>0</td>
<td>16.0</td>
<td>1.7</td>
<td>25.9</td>
<td>0</td>
<td>76.2</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>100</td>
<td>...</td>
<td>96.7</td>
<td>70.4</td>
<td>96.4</td>
<td>58.3</td>
</tr>
<tr>
<td>Erythrocyte II</td>
<td>L-Tart.</td>
<td>0</td>
<td>12.9</td>
<td>0.9</td>
<td>18.8</td>
<td>6.1</td>
<td>93.0</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>75.7</td>
<td>...</td>
<td>99.1</td>
<td>86.6</td>
<td>96.1</td>
<td>23.0</td>
</tr>
<tr>
<td>Erythrocyte III</td>
<td>L-Tart.</td>
<td>0</td>
<td>26.6</td>
<td>0.8</td>
<td>35.5</td>
<td>3.1</td>
<td>21.5</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>75.0</td>
<td>...</td>
<td>98.0</td>
<td>67.5</td>
<td>70.1</td>
<td>37.0</td>
</tr>
<tr>
<td>Liver I</td>
<td>L-Tart.</td>
<td>97.9</td>
<td>96.1</td>
<td>92.9</td>
<td>97.5</td>
<td>95.6</td>
<td>98.4</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>10.0</td>
<td>...</td>
<td>19.8</td>
<td>22.1</td>
<td>6.3</td>
<td>4.7</td>
</tr>
<tr>
<td>Liver II</td>
<td>L-Tart.</td>
<td>97.2</td>
<td>96.0</td>
<td>43.5</td>
<td>94.2</td>
<td>84.5</td>
<td>70.2</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>18.2</td>
<td>...</td>
<td>58.5</td>
<td>38.4</td>
<td>22.4</td>
<td>16.8</td>
</tr>
<tr>
<td>Liver III</td>
<td>L-Tart.</td>
<td>87.0</td>
<td>94.7</td>
<td>39.6</td>
<td>73.6</td>
<td>85.3</td>
<td>98.1</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>20.6</td>
<td>...</td>
<td>53.9</td>
<td>32.5</td>
<td>6.9</td>
<td>19.4</td>
</tr>
<tr>
<td>Bone</td>
<td>L-Tart.</td>
<td>50.0</td>
<td>97.8</td>
<td>55.9</td>
<td>48.6</td>
<td>56.8</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>22.2</td>
<td>...</td>
<td>100.0</td>
<td>2.7</td>
<td>8.8</td>
<td>3.4</td>
</tr>
<tr>
<td>Kidney I</td>
<td>L-Tart.</td>
<td>99.0</td>
<td>97.1</td>
<td>97.4</td>
<td>97.1</td>
<td>86.5</td>
<td>94.2</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>14.3</td>
<td>...</td>
<td>2.5</td>
<td>42.4</td>
<td>7.3</td>
<td>39.0</td>
</tr>
<tr>
<td>Kidney II</td>
<td>L-Tart.</td>
<td>98.0</td>
<td>82.6</td>
<td>89.1</td>
<td>97.5</td>
<td>82.2</td>
<td>78.5</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>11.1</td>
<td>...</td>
<td>22.5</td>
<td>25.9</td>
<td>8.2</td>
<td>46.5</td>
</tr>
<tr>
<td>Kidney III</td>
<td>L-Tart.</td>
<td>92.8</td>
<td>80.7</td>
<td>67.3</td>
<td>92.3</td>
<td>76.9</td>
<td>48.3</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>12.5</td>
<td>...</td>
<td>37.2</td>
<td>43.6</td>
<td>14.9</td>
<td>22.8</td>
</tr>
<tr>
<td>Urine (av of 5 spec.)</td>
<td>L-Tart.</td>
<td>97.3</td>
<td>86.8</td>
<td>91.7</td>
<td>97.7</td>
<td>84.3</td>
<td>91.4</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>7.5</td>
<td>...</td>
<td>8.2</td>
<td>5.4</td>
<td>7.1</td>
<td>5.2</td>
</tr>
<tr>
<td>Platelets (av of 4 prep.)</td>
<td>L-Tart.</td>
<td>58.7</td>
<td>50.8</td>
<td>3.5</td>
<td>58.0</td>
<td>55.0</td>
<td>23.3</td>
</tr>
<tr>
<td></td>
<td>Form.</td>
<td>8.7</td>
<td>...</td>
<td>81.2</td>
<td>45.3</td>
<td>32.7</td>
<td>42.0</td>
</tr>
</tbody>
</table>

Formaldehyde inhibition with PP could not be carried out because of turbidity development.
on the L-tartrate inhibited activity measured with α-naphthyl-phosphate, phenol-phosphate or p-nitrophenol-phosphate as substrates, because with these three substrates the platelet acid phosphatase shows a relatively large activity, which is partially inhibited by L-tartrate; sera with normal or slightly supranormal prostatic acid phosphatase activities will be more affected. Interferences by platelet acid phosphatase will be minimized with methods based on the L-tartrate inhibited activity measured with phenolphthalein monophosphate as substrate, because the activity of platelet acid phosphatase is little inhibited by L-tartrate when this substrate is used; it will also be minimized with the new method because thymolphthalein monophosphate is the substrate with the lowest turnover rate for platelet acid phosphatase.

Phenol-phosphate, phenolphthalein monophosphate, p-nitrophenol-phosphate, or β-glycerophosphate are hydrolyzed by erythrocytic acid phosphatases thousands of times more rapidly than the new substrate. Consequently, the former substrates should be used in conjunction with either L-tartrate or formaldehyde to avoid falsely positive results, which might occur even with imperceptible hemolysis. If a relatively large amount of erythrocytic acid phosphatases are present, however, the large “enzymatic blank” thus obtained may impair the sensitivity of the method, especially when sera have normal or slightly supranormal prostatic acid phosphatase activities. Even α-naphthyl-phosphate, a substrate insensitive to erythrocytic acid phosphatases (2, 3), is hydrolyzed by these enzymes many times more rapidly than the new substrate. Therefore, we conclude that only a negligible fraction of the total acid phosphatase activity measured with thymolphthalein monophosphate can be attributed to the erythrocytic enzymes. Consequently, neither L-tartrate nor formaldehyde is included in our assay system.

### Clinical Studies

Increases in serum acid phosphatase activity reported in patients with nonprostatic diseases (28–33) indicate that nonprostatic acid phosphatases appear occasionally in the blood. Abnormal results will be more frequent, however, when substrates relatively more sensitive to these enzymes are used. We determined the relative incidence of falsely positive results by using 87 selected patients: 21 women with breast cancer (Group I), 23 patients with liver or biliary tract diseases (Group II), 7 patients with kidney diseases (Group III), and 36 patients with cancer of nonprostatic, nonmammary, nonhepatic and nonrenal origin (Group IV). Total acid phosphatase activity measured with thymolphthalein monophosphate and α-naphthyl-phosphate as substrates (upper limit of normal: 0.56 and 2.0 U/liter, respectively) (34) were compared to the L-tartrate-inhibited activities measured with phenol-phosphate, phenolphthalein monophosphate and p-nitrophenol-phosphate as substrates (upper limit of normal: 1.05, 0.6, and 2.0 U/liter, respectively) (9, 27). The large amount of serum needed for its determination made us exclude β-glycerophosphate. Incidence of falsely positive results for sera of group I, II, III, and IV was: with p-nitrophenol-phosphate as substrate, 2, 3, 2, and 3, respectively; with α-naphthyl-phosphate as the substrate, 1, 9, 0, and 5, respectively; with phenol-phosphate as the substrate, 9, 1, 1, and 14, respectively; and with phenolphthalein monophosphate as the substrate, 0, 2, 0, and 1.

---

**Table 3. Relative Activities of Various Human Acid Phosphatase Isoenzymes as Measured with Six Substrates***

<table>
<thead>
<tr>
<th>Source of enzyme</th>
<th>Substrate</th>
<th>Substrate</th>
<th>Substrate</th>
<th>Substrate</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PP</td>
<td>PMP</td>
<td>α-NP</td>
<td>PNPP</td>
<td>β-GP</td>
</tr>
<tr>
<td>Prostate</td>
<td>2.7</td>
<td>1.8</td>
<td>3.7</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Erythrocyte I</td>
<td>10000</td>
<td>20700</td>
<td>13.5</td>
<td>41600</td>
<td>14200</td>
</tr>
<tr>
<td>Erythrocyte II</td>
<td>17300</td>
<td>25300</td>
<td>275</td>
<td>30400</td>
<td>12200</td>
</tr>
<tr>
<td>Erythrocyte III</td>
<td>7250</td>
<td>9670</td>
<td>211</td>
<td>12300</td>
<td>10500</td>
</tr>
<tr>
<td>Liver I</td>
<td>8.9</td>
<td>1.3</td>
<td>4.5</td>
<td>11.8</td>
<td>14.6</td>
</tr>
<tr>
<td>Liver II</td>
<td>4.7</td>
<td>3.2</td>
<td>8.1</td>
<td>9.7</td>
<td>11.0</td>
</tr>
<tr>
<td>Liver III</td>
<td>7.0</td>
<td>2.8</td>
<td>3.5</td>
<td>10.1</td>
<td>12.6</td>
</tr>
<tr>
<td>Bone</td>
<td>10.2</td>
<td>1.9</td>
<td>4.0</td>
<td>15.2</td>
<td>68.2</td>
</tr>
<tr>
<td>Kidney I</td>
<td>8.7</td>
<td>1.6</td>
<td>2.5</td>
<td>12.7</td>
<td>16.4</td>
</tr>
<tr>
<td>Kidney II</td>
<td>2.9</td>
<td>2.1</td>
<td>4.1</td>
<td>4.0</td>
<td>4.4</td>
</tr>
<tr>
<td>Kidney III</td>
<td>3.6</td>
<td>2.4</td>
<td>4.3</td>
<td>4.3</td>
<td>5.2</td>
</tr>
<tr>
<td>Urine (av of 5 spec.)</td>
<td>3.4</td>
<td>1.9</td>
<td>4.6</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>Platelets (av of 4 prep.)</td>
<td>16.1</td>
<td>5.2</td>
<td>5.5</td>
<td>54.3</td>
<td>19.0</td>
</tr>
</tbody>
</table>

* For purposes of comparison, the turnover rates for STM are taken as unity throughout this table.
respectively. With thymolphthalein monophosphate as the substrate, no abnormal results were obtained with any of the 87 sera. These findings confirm the results obtained when we worked with the purified isoenzymes: thymolphthalein monophosphate is the substrate least affected by the nonprostatic acid phosphatases.

The increased serum acid phosphatase activities reported after rectal palpation (35) and in certain benign prostatic conditions, such as prostatic infarct (36, 37), have been easily confirmed with the new method, because of its greater specificity for the prostatic enzyme. Thus, the acid phosphatase activities measured immediately before and 2, 14, 26, and 38 h after a rectal examination in the serum from a 74-year-old patient with benign prostatic hypertrophy (surgically confirmed) were, respectively, 0.35, 0.89, 0.71, 0.53, and 0.27 U/liter. Serum from a 28-year-old patient with prostatitis showed, on three different occasions, activities of 0.71, 0.63, and 0.85 U/liter. However, repeated testing failed to show any increase in the serum acid phosphatase activity in three other patients with prostatitis.

The serum acid phosphatase activities determined by the use of the various substrates, before and five days after surgical intervention, in a patient with a prostatic infarct, served to compare the sensitivity of the various substrates when the serum prostatic acid phosphatase activity was increased only slightly. Results with all the substrates were slightly supranormal in presurgical and normal in postsurgical specimens. Greatest increase in the presurgical specimen, in relation to the upper limit of normal, and greatest differences of activities between pre- and postsurgical specimens were observed, however, with the new substrate (Table 4). These results indicate that the new substrate has a greater sensitivity when the prostatic acid phosphatase activity is increased only slightly, probably because of its lesser hydrolysis by the nonprostatic acid phosphatases present normally in the serum.

With higher enzyme activities the sensitivity of the new procedure is as good or better than with other methods, because thymolphthalein is the chromogen with the highest absorptivity (Table 5). This compensates for the lower hydrolysis rate that thymolphthalein monophosphate has with the prostatic acid phosphatase. For example, the prostatic acid phosphatase hydrolyzes phenolphosphate 2.7 times more rapidly than thymolphthalein monophosphate. The overall sensitivity still favors the new substrate, however, because the absorptivity of thymolphthalein is about five times greater than that of phenol.

Serum acid phosphatase activities were determined by the new method in 23 patients with prostatic carcinoma. The two lowest activities obtained were 0.66 and 1.2 U/liter; all others fell between 6.2 and 423 U/liter. We have yet to encounter a falsely negative result, but this risk should be borne in mind, especially in cases of anaplastic or undifferentiated prostatic carcinoma (38, 39), or when the enzyme from the carcinoma does not enter the blood stream. Furthermore, the two lowest values obtained in prostatic carcinoma were within or near the range of values obtained in benign prostatic conditions. Consequently, slightly increased activities should be evaluated in conjunction with other clinical methods: rectal and radiological examinations, cystoscopy, needle biopsy, and cytology.

The increased acid phosphatase activities determined by any method in benign prostatic conditions should not be considered falsely positive results. As shown by Howard and Fraley (37), they are caused by an actual leakage of the enzyme into the blood. In fact, the incidence of slightly high results in benign prostatic conditions may increase with the present method because of better specificity for the prostatic enzyme and greater sensitivity when the prostatic acid phosphatase activity is slightly supranormal. We also hope, for these same reasons, that it may be helpful in the early diagnosis of prostatic carcinoma, the most important factor for a good prognosis, as recently reported by Gilbersten (40) after a 20-year study.

Our thanks to Drs. Celestino Corral and James Zangrilli, from the Urology Department, South Side Hospital, for their valuable clinical evaluation of some of the cases here presented. Dr. Charles M. Coleman kindly supplied the Ca phenolphthalein monophosphate.

Table 4. Pre- and Postsurgical Serum Acid Phosphatase Activities in a Case of Prostatic Infarct, Determined with Various Methods

<table>
<thead>
<tr>
<th>Specimen before prostatectomy</th>
<th>Total STM activity</th>
<th>Tartrate Inhibited PP activity</th>
<th>Tartrate Inhibited PNPP activity</th>
<th>Total activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>U/liter</td>
<td>1.15</td>
<td>1.47</td>
<td>1.25</td>
<td>2.27</td>
</tr>
<tr>
<td>Percentage over upper normal limit</td>
<td>105%</td>
<td>38.7%</td>
<td>108%</td>
<td>11.9%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Specimen after prostatectomy</th>
<th>U/liter</th>
<th>0.35</th>
<th>0.95</th>
<th>0.50</th>
<th>1.68</th>
<th>1.41</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percentage decrease</td>
<td>70%</td>
<td>35.4%</td>
<td>60%</td>
<td>30%</td>
<td>54.7%</td>
<td></td>
</tr>
</tbody>
</table>

CLINICAL CHEMISTRY, Vol. 17, No. 11, 1971
Table 5. Absorptivities (a)* of Chromogens of Various Acid Phosphatase Methods

<table>
<thead>
<tr>
<th>Chromogen and method</th>
<th>Ref. no.</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thymolphthalein</td>
<td>here</td>
<td>0.0379</td>
</tr>
<tr>
<td>Phenolphthalein</td>
<td>pers. commun.</td>
<td>0.0292</td>
</tr>
<tr>
<td>α-Naphthol</td>
<td>(19)</td>
<td>0.0256</td>
</tr>
<tr>
<td>p-Nitrophenol</td>
<td>(16)</td>
<td>0.0168</td>
</tr>
<tr>
<td>Phosphate, measured at 345 nm</td>
<td>(80, 41)</td>
<td>0.0121</td>
</tr>
<tr>
<td>Phenol</td>
<td>(18)</td>
<td>0.0077</td>
</tr>
<tr>
<td>Phosphate, measured at 700 nm</td>
<td>(80)</td>
<td>0.0041</td>
</tr>
</tbody>
</table>

* a = (A/ebc), where A: Absorbance; b: cell length, cm; and c: activity, U/liter.

As determined by measuring their absorbancies at an activity equivalent to 10 U/liter in a Gilford Spectrophotometer, Model 300 N, at the wavelength specified in each method.

References


