Automated Determination of Acid Phosphatase

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A procedure is presented for the automated determination of acid phosphatase activity in biological materials from either plant or animal origin. The Technicon N-7 flow diagram has been simplified, and the reagents for the measurement of enzymatically produced phenolic substances have been modified without loss of range or sensitivity. Both phenylphosphate and α-naphthylphosphate, introduced by Babson et al. (2, 3) for serum acid phosphatase, may be used as substrates. The Emerson reaction (alkaline aminoantipyrine and ferricyanide) with α-naphthol forms a more stable and reproducible color than the coupled product with tetrazotized o-dianisidine (Babson). Supporting data for these modifications are included.

This paper presents a simplified procedure for the automated determination of acid phosphatase, which is applicable to material of either plant or animal origin. The modified flow diagram and reagents for both the substrate and the colorimetric reactions can be used interchangeably for the expression of enzyme activity in terms of either phenol (phenylphosphate substrate) or α-naphthol (α-naphthylphosphate substrate) equivalents.

Materials and Methods

Phenylphosphate Procedure

Reagents and Apparatus

Control buffer (0.1 M citrate solution, pH 4.8) This solution contains 21 gm. citric acid and about 200 ml. 1 N sodium hydroxide in 1 L. of solution. The pH of the solution is checked with a reliable meter and adjusted if necessary.

Buffered substrate Disodium phenylphosphate, 3.0 gm., is dissolved in 250 ml. 0.1 M citrate buffer, pH 4.8. The pH of the solution is rechecked with a reliable meter and adjusted if necessary. This solution

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The authors thank Mr. Morris Oklander, who conducted the dialysis experiments and suggested several flow diagram improvements. Mrs. Jean Meyer's clerical contributions are also gratefully acknowledged.

Received for publication May 27, 1965; accepted for publication July 19, 1965.
is prepared daily to avoid complications arising from reagent decomposition.

Buffered aminoantipyrine Aminoantipyrine (Distillation Products Industries No. 6902)*, 11.25 gm., is dissolved in 1 L. of solution containing 30 gm. each of sodium bicarbonate and sodium carbonate.

Potassium ferricyanide Potassium ferricyanide, 40 gm., is dissolved in 800 ml. distilled water and diluted to 1 L.

Phenol stock standard 1 ml. = 1.0 mg. in 0.1 N hydrochloric acid.

Dilute phenol standards Working phenol standards are diluted with 0.005 N hydrochloric acid (1). When phenol standards are used, the incubation time must be determined daily to accommodate manifold changes. Phenol equivalents are converted to enzyme units (1).

Enzyme standards

1. Wheat germ acid phosphatase (Worthington Biochemical Corp.) A stock solution is prepared by dissolving 10 mg. dry material in 5 ml. pooled, clear, unhemolyzed serum. Dilutions are prepared with the pooled serum to give solutions containing 0.05-0.5 mg./ml. wheat germ phosphatase. These solutions are analyzed and the phenol equivalents obtained are converted to clinical units by the equation given in the Technicon N-7 procedure (1).

2. High-activity serum A high-activity serum preserved with sodium citrate (General Diagnostics Division, or Sigma Chemical Co., Item 104-9) is carefully analyzed in replicate by a reliable manual procedure and diluted with clear, pooled serum (preferred) or with 0.9% saline.

When these 2 solutions are used as enzyme standards, the daily incubation-time determination required to accommodate flow system changes is avoided. A plot relating corrected absorbance to enzyme activity in units is constructed and used in the calculations.

Enzyme control A commercial enzyme control, such as Versatol E (General Diagnostics Division), properly reconstituted and suitably diluted may be used to monitor the assays and check base-line drift.

Chart paper recording in absorbance (optical density) is used. The chart speed is 18 in./hr. Filters absorbing at 505 m$\mu$ are used.

Method

The modified flow diagram for use with the Technicon AutoAnalyzer (Technicon Instruments Corporation) is shown in Fig. 1.

With control buffer in the diluent line, the sample tube aspirating water, and all other reagents properly connected, the base line is ad-

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*One lot of J. T. Baker No. A630 gave a high control absorbance and was recrystallized from benzene.
justed to 0.01 absorbance. First the standard enzyme solutions and then the specimens are sampled at a rate of 40 specimens per hour. This gives the control absorbance.

The diluent line is transferred to a solution of buffered substrate, and the base line is readjusted to 0.01 absorbance. The standard enzyme solutions and specimens are resampled. The values now recorded are the total absorbance. The difference in absorbance gives the acid phosphatase activity, which is determined by reference to the calibration plot. This may be expressed in any of the clinical unit systems.

**α-Naphthylphosphate Procedure**

**Reagents**

*Control buffer* 0.1 M citrate pH 5.2.

*Buffered substrate* Sufficient substrate is prepared for a day’s analysis to contain 0.67 mg. sodium α-naphthylphosphate (Mann Research Laboratories, Inc., or Sigma Chemical Co.) per ml. 0.1 M citrate buffer, pH 5.2. The pH is checked and adjusted if necessary.

*Buffered aminoantipyrine* The same as in the phenylphosphate procedure.

*Potassium ferricyanide* The same as in the phenylphosphate procedure.

*α-Naphthol standards*

1. Stock standard, 10 mmole/L. 0.144 gm. pure recrystallized α-
naphthol is dissolved in 20 ml. absolute ethanol and diluted to 100 ml. with distilled water.

2. Dilute standards The stock solution is diluted 1.0–5.0 ml. to 100 ml. with pooled, clear, unhemolyzed serum. This gives \( \alpha \)-naphthol concentrations of \( 1 \times 10^{-4} \) to \( 5 \times 10^{-4} \) mmole/ml. When \( \alpha \)-naphthol standards are used, the incubation time must be determined daily also to accommodate manifold changes. Naphthol equivalents are converted to enzyme units by analyzing serums with known activity.

**Enzyme standards and control** The same as in the phenylphosphate procedure.

**Method**

The flow diagram (Fig. 1) and technics are those used in the phenylphosphate procedure.

**Results**

Figures 2 and 3 show typical calibration curves based on standard phenol and \( \alpha \)-naphthol solutions, respectively, and the strip chart recordings from which they were constructed. Figure 4 shows an acid phosphatase activity calibration curve based on Versatol E and the strip chart recording from which it was constructed. Similar data were obtained with solutions of wheat germ acid phosphatase and with dilutions of high activity serums.

**Discussion**

An evaluation of the existing automated procedure for the determination of serum acid phosphatase (1) was undertaken following discussions with the Ad Hoc Committee on Automation, Veterans Administration Central Office, when it was agreed that this constituent surely would be included as one of the parameters in any contemplated long-range study on the aging process in the American male. Another consideration was the anticipated examination of \( \alpha \)-naphthylphosphate as a substrate material for this determination by automated analysis, since Babson et al. (2, 3) presented evidence that this substrate was highly specific for prostatic acid phosphatase.

The Technicon N-7 flow diagram and procedure is rather formidable. A portion of the divided buffer line is used to dilute the sample (actually to reduce the adsorption of the phenol standard solutions onto the sample manifold tube). Following enzyme incubation, the reaction stream is made alkaline with approximately 0.4 N alkali, and both segments of the Emerson reaction (4) are conducted in carbonate-bicarbonate buffer.

Initial studies were aimed at simplification of the flow system. Accumulated experience in this laboratory with comparatively unstable
substrates (5) and pilot experimental trials clearly indicated that buffer and acid phosphatase substrate could be combined in one solution, if prepared daily in quantities sufficient for immediate use. Substitution of the Sampler II with its convenient intermediate wash cycle further simplifies the procedural mechanism and helps reduce both sample interaction (see below) and uneven phenol adsorption-desorption on the tygon tubing.

**Fig. 2.** Calibration curve, phenol standards, and strip chart recording, showing interaction patterns and continuous sampling.

**Fig. 3.** Calibration curve, α-naphthol standards and strip chart recordings, showing interaction patterns and continuous sampling.
The automated Emerson reaction characteristics then were examined. An alkali line is used in the Technicon N-7 procedure to bring the pH of the incubated reaction stream to about 10.2 (6), thereby halting further acid enzyme reaction (but not necessarily the alkaline phosphatase action). Figure 5 shows a plot relating absorbance of the dye produced with $5 \times 10^{-4}$ mmole/ml. naphthol and buffered aminoantipyrine and ferricyanide, with changes in sodium hydroxide concentration. Maximum absorbance is obtained with no alkali at all. The same result is obtained with the phenol standards. The alkali line, therefore, is eliminated.

Similarly, optimum aminoantipyrine and potassium ferricyanide reagent concentrations were determined (Fig. 6 and 7), and the compositions of these reagents are altered accordingly. It was observed that the required alkalinity for the coupling of the phenol (or naphthol) with aminoantipyrine, and suppression of "antipyrine red" formation (6, 7)

Fig. 4. Acid phosphatase activity calibration curve prepared from analysis of Versatol E.
Fig. 5. Effect of alkali on the Emerson reaction.

Fig. 6. Determination of optimum aminoadipyrine concentration in carbonate-bicarbonate buffer.

Fig. 7. Determination of optimum aqueous ferricyanide concentration.
are adequately maintained by dissolving only the aminoantipyrine in the carbonate-bicarbonate buffer. The ferricyanide oxidation does not require additional alkaline buffer, which is eliminated also. A double mixing coil is substituted for a single coil to insure complete oxidation by increasing the reaction time, although a single mixing coil would probably suffice.

In an examination of the automated enzymatic reaction conditions, it was determined that optimum substrate concentration is almost double the 3.0 gm./250 ml. substrate concentration used in the N-7 procedure, but in the present flow system, 95% of maximum enzyme activity is attained. Similarly, Babson and Read (3) used their \( \alpha \)-naphthylphosphate substrate at 1.34 mg./ml. 0.1 M citrate buffer (pH 5.2). A patent issued to Dr. Babson (8) describing the preparation of the substrate tablet used in the serum acid phosphatase kit,* indicates that the 21-mg. tablet contains 0.77 mg. sodium \( \alpha \)-naphthylphosphate. This means an actual concentration of 1.54 mg./ml. In the present experiments, 95% of maximum activity is obtained at the concentration used. These differences may be ascribed to differing experimental conditions between those cited and those used in the present study. The substrate concentrations are left unchanged (Fig. 8).

In agreement with Ettinger et al. (7), who used a different experimental technic, we observed that the phenol Emerson reaction product had an appreciably higher aqueous molar absorbance at 505 \( \mu \mu \) \( \log \epsilon = 4.12 \) than the comparable naphthol Emerson reaction product \( \log \epsilon = 3.99 \). This difference allows determination of increased amounts of acid phosphatase activity with the \( \alpha \)-naphthylphosphate substrate—e.g., about a threefold increase in range of elevated enzyme activity without sample dilution—yet with good sensitivity in the critical borderline area.

Babson and Read (3) indicated that protein (e.g., serum) is required when \( \alpha \)-naphthol standards are used, to keep the unstable tetrazotized \( o \)-dianisidine coupling product in solution. In this laboratory, attempts to automate this azo coupling reaction were unsuccessful. Unpublished observations in this laboratory indicate that azo dye coupling reactions are usually difficult to process and frequently are unsuitable for automation because of marked dye adsorption onto and subsequent uneven desorption from the Tygon transmission tubing. This produces "noisy" records, as well as erratic and nonreproducible results. Moreover, the dye becomes deposited on both the tubing and the flow cell. In the present instance, this problem could not be eliminated by substituting glass tubing in the appropriate segments of the flow system, by using various

*General Diagnostics Division, Warner-Chilcott Div., Morris Plains, N. J.
wetting agents, or by resorting to any of the established and recommended devices used in automated analytical technology.

In the present reaction system, standard α-naphthol solutions must also contain protein (e.g., serum) for an uncomplicated Emerson reaction although the colored product is more stable than the dye obtained by coupling with o-anisidine. The troublesome plastic tubing adsorption-desorption phenomenon, though still present, could be completely eliminated by substituting glass tubing in the places indicated in the flow diagram.

Difficulties related to the use of phenol and α-naphthol standards and the requirement for daily incubation time determinations are completely
eliminated by the use of carefully analyzed high activity human serum, which is frequently difficult to obtain and store. Alternatively, wheat germ acid phosphatase preparations or commercial abnormal serum controls containing increased amounts of enzyme can be used. The commercial controls are more convenient and are recommended.

In the development of automated analytical methodology, close attention must be paid to the possibility of specimen interaction. This phenomenon was recently studied and discussed by Thiers and Oglesby (9). In this laboratory, all methods under development are similarly evaluated.

With the Sampler I operating at the rate of 40 specimens per hour, when a phenol solution containing $1.0 \times 10^{-4}$ mmole/ml. immediately follows a solution containing $5.0 \times 10^{-4}$ mmole/ml., a 22–25% increased absorbance of the more dilute specimen reaction product is observed when compared to the same solution sampled under noninteraction conditions. With the Sampler II, an identical experiment at the same rate (2:1 wash ratio) showed 7.5% interaction. Sampling at a rate of 60/hr. (2:1 wash ratio), in a comparable experiment, showed extensive interaction with shoulder formation (Fig. 2). The absorbance given by the more concentrated phenol solution analyzed at this rate gives only 83.6% of the continuous sampling absorbance and is, therefore, not recommended, although it is at borderline precision.

Similar experiments with $\alpha$-naphthol (Fig. 3) show that with the Sampler II at the rate of 40 samples per hour (2:1), there is a 4.5% interaction when a specimen containing $1 \times 10^{-4}$ mmole/ml follows a specimen containing $5 \times 10^{-4}$ mmole/ml. At the rate of 60 samples per hour (2:1), this interaction is increased to 12.5%. The absorbance of the color produced by the higher naphthol concentration at this sampling rate is only 79% of the absorbance by continuous sampling and, therefore, is not recommended.

Experiments designed to eliminate control determinations, thereby doubling the analysis rate, by subjecting the postincubation reaction stream to dialysis (5, 10) were unsuccessful. Only 7.7% of $\alpha$-naphthol solution (in serum) at a concentration of $5 \times 10^{-4}$ mmole/ml. is recovered by dialysis at 37°. Increasing the $\alpha$-naphthol concentration tenfold to produce greater absorbance ratios on the recorder strip chart introduced additional complications which signified appreciable $\alpha$-naphthol absorption onto the membrane (most probable) or onto the Tygon tubing connections, with subsequent erratic desorption, or possibly both. Furthermore, the strip chart recording obtained in these experiments demonstrated extensive specimen interaction. This investigative approach was abandoned.
Recordings of 10 specimen analyses using 2-naphthylphosphate are shown in Fig. 9. First are the assays followed by the control analyses. The ninth assay is run in triplicate showing the identical response obtained even at 1.4 absorbance. The tenth analysis is shown undiluted (absorbance, 0.84) and diluted 1:1 (absorbance, 0.43). Differences in the control absorbance bring the net absorbance into linear agreement.

References