Rapid Method for the Quantitative Determination of Serum Alkaline Phosphatase

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Serum alkaline phosphatase activity is the enzyme analysis most frequently made in clinical laboratories. It has long been a diagnostic tool and therapeutic index in both liver and osseous disease. Its usefulness could be expanded by a method which would measure alkaline phosphatase activity rapidly, accurately, and with a minimum of technical manipulation. Ideally, the substrate for such a method should be a substance which, following enzyme action, provides its own chromogen directly or by the addition of a simple reagent.

This report presents the results obtained with a method using an improved buffered phenolphthalein phosphate substrate. A semiquantitative procedure, in which this substrate is used in tableted form, has been available for some time as a rapid test for serum alkaline phosphatase activity (1). The substrate solution, which is prepared by dissolving a reagent tablet in water, remains colorless until decomposed by the enzyme. The amount of phenolphthalein liberated under controlled conditions is determined photometrically and is a measure of alkaline phosphatase activity.

REAGENTS
1. Buffered alkaline phosphatase substrate, pH 9.9: To 0.5 ml. distilled water, one substrate tablet, containing 0.3 mg. sodium

*Available as PHOSPHATABS (alkaline)—patent pending—from General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N. J.

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phenolphthalein phosphate, is added and allowed to dissolve. This provides substrate for a single determination. For convenience when many sera are to be assayed at the same time, a volume of substrate solution for 1 day's use can be prepared by dissolving substrate tablets in distilled water in the same proportion.

2. 0.5 N sodium hydroxide.

3. Phenolphthalein standard: (a) Stock solution:* One hundred milligrams of reagent grade phenolphthalein is dissolved in 95% alcohol and diluted to 100 ml. One milliliter of this solution contains 1 mg. (b) Working standard: One milliliter of stock standard is diluted to 50 ml. with water. One ml. of the diluted standard contains 0.02 mg.

PROCEDURE

PREPARATION OF CALIBRATION CURVE

To 0.5, 1.0, 2.0, and 3.0 ml. of the diluted standard solution, add 0.1 ml. of 0.5 N NaOH and dilute to 5.8 ml. with distilled water. These solutions contain the equivalent of 10, 20, 40 and 60 µg. of phenolphthalein, respectively. The optical density of each solution is read in a photometer at 550 mµ against a distilled-water blank. A graph relating micrograms of phenolphthalein or units of alkaline phosphatase to optical density is prepared. This should be checked at regular intervals. Typical calibration curves are shown in Fig. 1.

ASSAY

To 0.5 ml. of buffered substrate at 37°, add exactly 0.2 ml. of clear, unhemolyzed serum, holding the tip of the pipet just above the surface of the solution. If a 0.2-ml. pipet calibrated "to contain" is used, it is rinsed several times with the reaction mixture. Following incubation for exactly 30 minutes, 0.1 ml. of 0.5 N NaOH is added and the reaction mixture is diluted with 5.0 ml. of distilled water. The solution is mixed and the optical density is measured at 550 mµ against a reagent blank. The blank contains 0.5 ml. of buffered substrate solution, 0.1 ml. of 0.5 N NaOH, and 5.2 ml. of distilled water. The amount of phenolphthalein released is obtained from the calibration curve. One unit of alkaline phosphatase activity is defined as the amount of enzyme that will liberate 1.0 mg. of phenolphthalein in 30 minutes at 37°. Since 0.2 ml. of serum is used in the assay, the activity, expressed as units per 100 ml. of serum, is numerically equal

* Available as a service from General Diagnostics Division, Warner-Chilcott Laboratories.
to half the amount (in micrograms) of phenolphthalein released. This can be converted to Bodansky units per 100 ml. by means of the curve shown in Fig. 2 (see Discussion).

RESULTS

The serums of 63 hospitalized patients with no evidence of liver dysfunction or bone disease were assayed for alkaline phosphatase activity. The values ranged from 0.6 to 4.0 phenolphthalein units per 100 ml.; the distribution is shown in Fig. 3. Of the values obtained in this series, 92.1 per cent lie between 1 and 3.5 U.

Forty-seven serum samples representing a broad range in alkaline phosphatase activity were assayed by the present method and by a modification (2) of the Bodansky (3) procedure. The correlation between these two methods is shown in Fig. 2.
DISCUSSION

One of the principal advantages of the present procedure is the stability of the substrate. Huggins and Talalay (4) prepared a form of sodium phenolphthalein phosphate which was rather unstable. The buffered substrate tablets used in the present procedure are stable and can be kept at room temperature indefinitely. Substrate solutions have been incubated at 37.5° for as long as 24 hours without measurable release of free phenolphthalein.

A second advantage is simplicity. Deproteinization is eliminated, and a single easily prepared reagent produces the color necessary for photometric measurement. A glycine-sodium hydroxide buffer containing sodium chloride and the specific alkaline phosphate inhibitor, sodium pyrophosphate, may be used to terminate enzyme action and convert the quinoid form of phenolphthalein to the red dye form (4). However, 0.5 N sodium hydroxide was found to serve equally well since there is negligible alkaline phosphatase activity at a pH above 11.

The concentration of the phenolphthalein phosphate in the substrate tablet and the conditions of assay have been designed to give
Fig. 3. The distribution of normal levels of serum alkaline phosphatase expressed as phenolphthalein units.

...an S-shaped curve, relating activity to phenolphthalein released, in the clinical range of alkaline phosphatase activity. The S-shaped curve has the disadvantage of making it difficult to convert phenolphthalein units to other units by a simple factor. It has, however, a definite practical advantage over other curves in that the accuracy of the assay increases where there is an increase in slope (change of optical density per unit of activity), while the effective range of the assay is increased where there is a decrease in slope. Thus, in the range where the greatest accuracy is desired (4–16 Bodansky units) the slope is greatest. The slope is less in the normal or markedly elevated ranges, thereby increasing the effective range of the assay.

Certain technical points are of interest: The 30-minute incubation should be used with most serums. When several samples are assayed, the individual determinations should be started at regular intervals—for example, 1 minute apart—so that each sample is incubated for the same time. The color is developed and the final readings are made at the same time intervals. With serums containing more
than 30 phenolphthalein units, the incubation time can be decreased to 10 minutes, at which time the reaction mixture will be noticeably pink. The reaction can be terminated at this point by adding 0.1 ml. of 0.5 N NaOH and proceeding as with 30-minute incubation. The phosphatase activity at 10 minutes cannot be converted directly to phenolphthalein activity at 30 minutes by multiplication of the chromogen value by three. However, the conversion can be made by multiplying by three the activity in Bodansky units obtained from the conversion curve (Fig. 2).

In the procedure described here, the final volume of the assay has been set at 5.8 ml. This is a convenient volume for use in many clinical photoelectric colorimeters. Obviously, the final dilution can be adjusted by the analyst to suit the requirements of the particular instrument in use, provided the optical density of the final solution is kept between 0.1 and 0.8. Alkaline phosphatase activity to about 30 Bodansky units (equivalent to approximately 30 phenolphthalein units) can be determined in the 30-minute incubation procedure, or to about 75 Bodansky units in the 10-minute incubation.

Separate serum controls are not necessary ordinarily unless the serum is turbid or very hemolyzed; in these cases a serum blank should be included. This is prepared before the end of the incubation period by diluting 0.2 ml. of the serum with 0.1 ml. 0.5 N NaOH and diluting with 5.5 ml. water. The optical density of the serum blank is subtracted from the optical density of the incubated sample, and the alkaline phosphatase activity determined from the corrected optical density. Bilirubin does not interfere except at concentrations above 10 mg./100 ml. In these cases, a serum blank should be prepared.

Either serum or plasma may be used for the assay. In the collection of blood for plasma, the usual anticoagulants may be used, with the exception of ethylenediaminetetraacetic acid, which interferes by binding magnesium. Serum containing more than 75 Bodansky units should be diluted, preferably with normal serum, when the assay is repeated.

SUMMARY

A new procedure is described for the simple, rapid, quantitative measurement of serum alkaline phosphatase. The procedure is based on the direct photometric measurement of phenolphthalein released from sodium phenolphthalein phosphate. The substrate is available in a convenient, stabilized form as a tablet containing buffer and
substrate in optimum amounts for a single analysis. As many as 30 analyses can be performed in an hour.

REFERENCES