To distinguish them from the better known "alkaline" phosphatases, phosphomonoesterases with optima on the acid side of neutrality are referred to as "acid" phosphatases (1). The quantitative determination of these enzymes in serum is of interest chiefly because (a) the acid phosphatase activity of normal blood serum (2–4) is attributed to distinct phosphomonoesterases of uncertain physiological significance (5), and (b) the blood serum of subjects with metastasizing carcinoma of the prostate gland contains an acid phosphatase (6) with properties corresponding to the enzyme found by Kutscher and Wolbergs (7) in normal prostate tissue and in seminal fluid. Invasion of the circulating fluids by carcinomatous prostate tissue liberates prostate acid phosphatase into the blood where the enzyme can be identified and the source of the primary tumor so determined.

The general principles underlying the determination of serum "alkaline" phosphatases apply also to the determination of serum "acid" phosphatases. We have adapted the King and Armstrong method for alkaline phosphatase (8) to the estimation of serum acid phosphatase activity (5, 6). Barringer and Woodard (9) have suggested a variant of the Bodansky method (10); Lundsteen and Vermehren (11) have modified their own method (12) for the same purpose. We wish to consider here certain specific conditions which must be satisfied in adaptations of this kind: (a) Since serum contains both alkaline and acid phosphatases, the former usually in great excess, hydrolysis must be conducted under conditions which are optimal for acid but completely inhibit alkaline enzymes. This involves a study of pH-activity relations,
particularly of pathological sera containing varying proportions of both enzymes. (b) The acid phosphatase activity of normal sera is extremely small. A number of substrate-buffer combinations in varying concentrations were investigated in order to obtain satisfactory colorimetric readings without having to hydrolyze too long. After prolonged hydrolysis significant deviations from linear time-activity relations occur.

Selection of pH—Fig. 1 illustrates the pH-activity relations of the serum acid phosphatases of principal interest, that normally present (Curve 1) and that occurring in the serum of patients with metastasizing prostatic carcinoma (Curve 2). Under the stated conditions of hydrolysis, there is a rapid decline in activity outside of the approximate limits of pH 4.0 to 6.0, the pH range suitable for estimating serum acid phosphatases as defined by optimal zones of activity.

The curves in Fig. 1 are discontinuous in the region of neutrality, where alkaline phosphatase activity, though only a fraction of that at the pH optimum, is nevertheless sufficient to be significant in relation to the acid phosphatase activity of most sera. In experiments such as those summarized in Table I, we have at-
tempted to determine how far on the acid side of neutrality inactivation of serum alkaline phosphatase is incomplete, particularly in sera extremely rich in alkaline phosphatase (Paget’s disease). The hydrolyses were conducted in the presence of 0.02 M NaF, which inhibited serum acid phosphatase to minimal activity (0.1 to 0.2 unit at pH 5.0). The results with monophenyl phosphate substrate (Table I) show measurable activity of serum alkaline phosphatase as far as pH 6.0. Similar experiments with β-glycerophosphate substrate reveal measurable alkaline phosphatase activity at least as far as pH 6.0.

Barringer and Woodard (9), using β-glycerophosphate substrate, likewise noted appreciable serum alkaline phosphatase activity at pH 6.4.

In view of the results indicated, we have elected to buffer at pH 4.9, which is within the zone of optimum activity of serum acid phosphatases and beyond the range of significant activity of serum alkaline phosphatases under the conditions of hydrolysis specified.

1 The substrate used affects the results of such experiments in two ways: As pointed out by King and Delory (13), and in accord with the Michaelis-Menten equation, the optimum pH for hydrolysis of phosphoric esters by alkaline phosphatase increases with increasing acidity of the ester; and the rate of decline in activity exhibited by alkaline phosphatase when buffered at increasingly acid levels decreases with increasing acidity of the ester.
Selection of Substrate—Within the same period of time, 2 to 3 times as much phenol is split off from monophenyl phosphate by serum acid phosphatase at pH 5.0 as phosphate (expressed as inorganic phosphorus) from β-glycerophosphate (Table II).2 With α-glycerophosphate substrate, the proportion in favor of monophenyl phosphate is even greater (5, 6, 11). Because of the low range of activity of most sera and the theoretical and practical advantages of short periods of hydrolysis, monophenyl phosphate would seem, therefore, to be particularly suitable for the estimation of serum acid phosphatases. Phenol can be quantitatively estimated in the hydrolysate with an accuracy at least as great as is possible with phosphate.

The concentration of phosphoric ester substrate in the hydrolysate This ratio of about 2.5:1 is considerably less than that observed with phosphatases in alkaline medium, because at pH 5.0 the rate of hydrolysis of monophenyl phosphate is slower than that of β-glycerophosphate (cf. (13)).
sis mixture is another factor determining the rate of scission, which increases with increasing molarity of the substrate. With \( \beta \)-glycerophosphate, comparatively small increases in substrate concentration effect large differences in the amount of phosphate liberated. The rate of hydrolysis of monophenyl phosphate in acid medium, however, is much less susceptible to changes in substrate concentration; between 0.005 \( \text{M} \) and 0.01 \( \text{M} \) the differences in phenol split off are small (6).

Impurities in the disodium monophenyl phosphate employed as substrate affect the rate of liberation of phenol by alkaline (14, 15) and acid phosphatases. With sera of normal or moderately increased acid phosphatase activity, the use of pure disodium monophenyl phosphate (for which we are indebted to Mr. H. Scharer) gave values up to 15 per cent higher than those obtained with commercial products. In sera exhibiting marked acid phosphatase activity, however, the increase did not exceed 4 per cent.

Selection of Buffer—In estimating serum acid phosphatases with monophenyl phosphate substrate, it was found that acetate-acetic acid, citrate-NaOH, succinic acid-borax, veronal-acetic acid, or glycine-HCl could be employed to buffer at \( \text{pH} \) 4.9. With \( \beta \)-glycerophosphate substrate, a variety of buffers could also be used but difficulties arise, as for example with citrate buffer; in 0.1 \( \text{M} \) concentration, the development of color in determining liberated phosphate with molybdcic acid is inhibited (16) (0.04 \( \text{M} \) citrate, however, does not inhibit color development and is an adequate buffer (5)).

With monophenyl phosphate substrate, acetate-acetic acid buffer (suggested by Lundsteen (16)) consistently gave higher values for serum acid phosphatase activity than did citrate (Table II) and was our buffer of choice for many purposes. In subjects with metastasizing prostatic carcinoma the differences in values obtained with these two buffers was small \((b)/(c)\), Table II), whereas in patients with other bone conditions and in normal subjects the differences were large; \( i.e. \), 0.1 \( \text{M} \) citrate appreciably inhibits the activity of serum acid phosphatases other than the prostate acid phosphatase which appears in the serum in association with metastasizing prostatic carcinoma. This observation is of interest in two connections: In doubtful cases, it permits the differentiation of prostate acid phosphatase from other acid phos-
phatases in the serum; and by means of citrate buffer it is possible to establish a sharper critical level for diagnostic purposes (3.0 units of acid phosphatase activity per 100 cc. of serum). For example, with acetate buffer the serum of a patient with Paget's disease (Serum 6, Table II) gave a value of 3.0 units, within the range of values obtained with metastasizing prostatic carcinoma, but with citrate buffer gave a value of only 1.9 units. The serum of a patient with metastasizing prostatic carcinoma (Serum 5, Table II) gave a value of 3.3 units with acetate buffer, 3.1 units with citrate buffer.

Selection of Method—It appeared from the foregoing experiments that the principles of the King and Armstrong method for determining alkaline phosphatase activity (8) could be applied advantageously to the estimation of serum acid phosphatases. The results of recent critical studies and suggested variants of the King and Armstrong method (17-19, 15) were taken into account in our adaptation.

Reagents—
1. Buffer-substrate (0.005 M monophenyl phosphate, 0.1 M citrate at pH 4.9). Mix equal parts of Solutions A and B as needed; check pH.
   Solution A. Dissolve 1.09 gm. of disodium phenyl phosphate (obtainable from Eimer and Amend, New York) in 500 cc. of water.
   Solution B. Dissolve 42.0 gm. of crystalline citric acid in water, add 376 cc. of 1 N NaOH, make up to 1 liter. Adjust the pH to 4.9 with NaOH or HCl as needed (when colorimetric methods are used, nitrazine (Squibb) is a convenient indicator). Preserve in well stoppered bottles in a refrigerator.
2. Phenol reagent of Folin and Ciocalteu (20). The stock reagent is prepared as described by Folin and Ciocalteu, kept in a well stoppered, amber bottle, and for use diluted 1:3.
3. Sodium carbonate (20 per cent solution).
4. Standard phenol. For the stock solution, which keeps indefinitely, dissolve 1 gm. of crystalline phenol in 0.1 N HCl and make up to 1 liter with 0.1 N HCl. Standardize by the convenient method of Koppeschaar (21). From this stock phenol solution, a diluted phenol solution containing exactly 10 mg. of phenol per 100 cc. is made up; it remains stable for months in the refrigerator.
5. Standard phenol solution and reagent. To 1 cc. of diluted phenol solution in a test-tube, add 6 cc. of distilled water and 3 cc. of diluted phenol reagent. Prepare shortly before use.

Procedure

Two test-tubes, each containing 10 cc. of buffer-substrate solution, are kept in a water bath at 37° for about 5 minutes. Pipette exactly 0.5 cc. of the serum to be tested into each tube, stopper, mix, and incubate at 37° for 3 hours. Then remove the tubes from the water bath, at once add 4.5 cc. of diluted phenol reagent, mix, and filter. To two control tubes each containing 10 cc. of buffer-substrate solution add 0.5 cc. of the serum and at once add 4.5 cc. of diluted phenol reagent, mix, and filter.

Pipette 6 cc. of each of the test and control filtrates into test-tubes, add 1.5 cc. of 20 per cent sodium carbonate solution, and mix. To the standard phenol solution and phenol reagent (No. 5) prepared shortly before, add 2.5 cc. of 20 per cent sodium carbonate solution and mix. Place test, control, and standard tubes together in the water bath for 5 minutes to develop the color, cool about 20 minutes, and compare in the colorimeter.3

(Although the color is not maximally developed, the standard, test, and control tubes are all made up at the same time and read within a few minutes of each other.) The unknown is placed in the left cup of the colorimeter and set at 30. The standard is placed in the right cup, which is adjusted to match the unknown.

Calculation

The results are expressed in units of acid phosphatase activity per 100 cc. of serum. A unit is defined as that degree of acid phosphatase activity which at 37° will liberate from the specified buffer-substrate solution (pH 4.9) 1 mg. of phenol in 1 hour. The units of phosphatase activity in 100 cc. of serum = mg. of phenol present in 100 cc. of serum after hydrolysis minus mg. of "phenol" in 100 cc. of the non-incubated control serum divided by the number

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3 The Evelyn photoelectric colorimeter can be used advantageously. Proceed as indicated, except allow the color to develop 1 hour for stabilization. The readings are approximately 10 per cent higher than with the ordinary colorimeter.
of hours of hydrolysis. To calculate the mg. of phenol in 100 cc. of serum before and after hydrolysis,

\[
\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{strength of standard} \times \frac{\text{final volume of unknown}}{\text{final volume of standard}} \times \frac{100}{\text{cc. serum used}}
\]

With the unknown set at 30, the standard containing 0.1 mg. of phenol and the reagents used in the proportions indicated, this equation becomes

\[
\frac{\text{Reading of standard}}{30} \times 0.1 \times \frac{7.5}{12.5} \times \frac{15}{6} \times \frac{0.5}{100}
\]

which cancels out to "reading of standard." The final result, units of acid phosphatase activity per 100 cc. of serum, is obtained as follows: reading of standard against incubated serum minus reading of standard against control serum divided by the number of hours of hydrolysis.

With sera of increased acid phosphatase activity it may be more convenient to set the unknown at 20, 15, or 10, and use the appropriate factors 3/2, 2, or 3. In the case of sera with very high acid phosphatase activity (when the reading of standard is over 60), there is marked inhibition of hydrolysis by the products of scission and to obtain optimal values it is necessary to reduce the time of hydrolysis. When 1/4 hour's hydrolysis still gives too high readings, the serum must be diluted appropriately with physiological saline solution and the determination repeated, including controls with the diluted serum. Over periods of hydrolysis from 1/4 to 3 hours, time-activity curves are linear except with sera very high in acid phosphatase activity, and these are linear if diluted as indicated. Significant deviations from Beer's law were not observed.

Results

In the past 2 years, we have employed the method described in over 500 determinations of serum acid phosphatase activity in a variety of conditions. Agreement between duplicates is usually within 0.2 unit in the normal range and better than 5 per cent in sera with elevated values. The normal range was found to be
0.5 to 2.0 unit per 100 cc. of serum, with increases to several hundredfold in subjects with metastasizing prostatic carcinoma (22, 23).

SUMMARY

Optimal conditions of hydrolysis in the estimation of serum "acid" phosphatases were determined. The King and Armstrong method for "alkaline" phosphatases was adapted to the estimation of serum "acid" phosphatases.

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ESTIMATION OF "ACID" PHOSPHATASE ACTIVITY OF BLOOD SERUM
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