A METHOD FOR THE RAPID DETERMINATION OF ALKALINE PHOSPHATASE WITH FIVE CUBIC MILLIMETERS OF SERUM

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The alkaline phosphatase of the serum increases early and markedly in rickets and returns completely to normal only after healing is complete. Because of this fact, serum phosphatase is the most satisfactory index now known for the detection of this deficiency. The phosphatase activity of serum is not strictly specific in this respect and has also proved clinically useful in a number of other pathological states; *e.g.*, Paget's disease, hyperparathyroidism, liver disease, etc.

In connection with nutritional studies on large groups of population, it became necessary to have a rapid method for the determination of this enzyme on small amounts of serum. By the use of a new substrate (*p*-nitrophenyl phosphate) a method has been devised which requires only 5 c.mm. of serum (0.005 ml.) and which permits 50 to 100 analyses to be made in 2 hours. The simplicity and speed of the method recommend it for macro- as well as microdeterminations and for either alkaline or acid phosphatase.

A number of methods have been described for the determination of the phosphatase content of serum and other biological materials, all of which depend upon the principle of measuring the rate of hydrolysis of various phosphate esters under specified conditions of temperature and pH. The two most widely used methods are those of Bodansky (1) and King and Armstrong (2) in which glycerol phosphate and phenyl phosphate respectively are employed as substrates. While these methods are satisfactory for many uses, they are rather time-consuming when large numbers of determinations are needed; furthermore, they require larger samples of serum than is convenient for the purpose of dietary surveys.

The substrate, *p*-nitrophenyl phosphate, was studied by King and Delory (3) and has been used for phosphatase estimations by Ohmori (4) and by Fujita (5). The compound is colorless, but upon splitting off the phosphate group, the yellow salt of *p*-nitrophenol is liberated (absorption maximum, 400 mμ). Hence the substrate is itself an indicator of the amount of splitting and thus a measure of phosphatase activity. It is only necessary to incubate serum with the buffered reagent, stop the reaction

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by dilution with alkali, and measure the amount of color developed. Since serum itself makes a small contribution to the color, the first colorimetric reading is followed by the addition of acid to the sample (converting the yellow sodium salt into colorless free nitrophenol) and a second colorimetric reading which furnishes a blank correction. This procedure is considerably simpler than those now in use. Furthermore, p-nitrophenyl phosphate is split by alkaline phosphatase 15 per cent faster than phenyl phosphate (3), 2 or 3 times more rapidly than glycerol phosphate, and 25 or 30 times faster than phenolphthalein phosphate (6). Because of this rapid splitting, and the high chromogenicity of the salts of p-nitrophenol, the reagent is well suited for adaptation to microprocedures.

Materials and Procedure

Reagents and Standards—

Reagent A. Dissolve 7.50 gm. (0.1 mole) of glycine and 95 mg. (0.001 mole) of $MgCl_2$ in 700 to 800 ml. of H_2O, add 0.085 mole (*e.g.* 85 ml., 1 N) of NaOH, and dilute to 1 liter.

Reagent B. Prepare 0.4 per cent disodium p-nitrophenyl phosphate (Eastman) in 0.001 N HCl. (At present the Eastman product contains about 50 per cent inert material; hence a double amount of this preparation should be used. If desired, the compound may be purified by recrystallization from hot 87 per cent alcohol.) If the pH is not 6.5 to 8.0, adjust with acid or base. To test for free nitrophenol, dilute 1 ml. with 10 ml. of 0.02 N NaOH and measure the light absorption at 415 mμ. If the extinction is greater than 0.08 (*i.e.* light transmission less than 83 per cent for a 1 cm. light path or 70 per cent for a 2 cm. light path), remove free phenol by extracting Reagent B two or three times with equal volumes of water-saturated butyl alcohol, and once with water-saturated ether, finally aerating off traces of ether. Store in the ice box. Reextract when Reagent B fails to pass the above test.

Reagent C (complete reagent). Mix equal parts of Reagents A and B. If necessary, adjust the pH to 10.3 and 10.4 with a little strong NaOH or HCl. Store in the refrigerator, or, better, store frozen. When 2 ml. plus 10 ml. of 0.02 N NaOH have an extinction (1 cm.) greater than 0.1, either discard or extract with butyl alcohol and ether as above and readjust the pH.

Standards. Prepare solutions containing 1, 2, 4, and 6 mM per liter of p-nitrophenol (Eastman), mol. wt. 139.1.

Apparatus—For 5 c.mm. serum volumes, (*a*) 5 and 50 c.mm. Lang-Levy constriction pipettes (Fig. 1); (*b*) 6 × 50 mm. serological tubes, Kimble No. 45060; (*c*) any spectrophotometer or photoelectric colorimeter adapted to 0.5 ml. volume measurements; *e.g.*, Beckman spectrophotometer or

Junior Coleman spectrophotometer (model 6) (Adapters for the Coleman instrument may be obtained from Samuel Ash, 3044 Third Avenue, New York 56); (d) wire rack to hold 100 tubes; this may be made conveniently from $\frac{3}{8}$ inch mesh wire screen.

Principle and Use of Lang-Levy Constriction Pipettes—These pipettes were originally described by Levy (7) working in the laboratory of Dr. Linderstrøm-Lang. They are particularly useful for the easy, rapid, and precise measurement of volumes of 1 to 200 c.mm. (0.001 to 0.2 ml.), although larger pipettes are occasionally of value. With 10 to 200 c.mm. pipettes the percentage accuracy of delivery compares favorably with conventional pipettes delivering 2 to 10 ml.; *i.e.*, approximately 0.1 per cent. Below 10 c.mm. the precision falls off somewhat, but even with 1 c.mm. volumes a precision of at least 1 per cent is obtained and may be much better with a well made pipette properly used. These pipettes are easy to construct for any one familiar with the rudiments of glass blowing,[1] or they may be obtained from the Arthur H. Thomas Company, Philadelphia.

Drawings of Lang-Levy pipettes are shown in Fig. 1. They are filled and emptied by the use of a small rubber tube such as is used with blood-diluting pipettes. The pipette is dipped not more than 1 or 2 mm. into

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[1] The following directions are for making an ordinary 50 c.mm. constriction pipette. A 20 to 25 cm. length of either soft glass or Pyrex tubing, 4 or 5 mm. outer diameter, is heated in the middle and drawn down to a diameter of 1.5 to 2 mm. This furnishes material for two pipettes. 2 cm. from that point where the tube is narrowed (Fig. 1, *a*) the slender portion is further drawn down to a diameter of 0.5 to 0.8 mm. This narrowest portion is bent 1 cm. below the 2 mm. portion (*b*) at an angle of about 45° from a straight line and is cut off with a diamond point 3 or 4 mm. below the bend (*c*). The upper large end is fire-polished. A 0.1 or 0.2 ml. graduated pipette is partially filled with water and placed horizontally on the table, a rubber tube is attached to the new pipette, its tip is touched to the tip of the graduated pipette, and 50 c.mm. of water are sucked into the new pipette. A mark is made with the diamond point at the meniscus and just above this point the pipette is narrowed, for a distance of about 1 cm. or so (*d-d*), to an *inner* diameter of perhaps 1 to 1.5 mm., without thinning the wall. Once again 50 c.mm. of water are drawn in, and the meniscus should now fall in this narrow portion. If it does, a new mark is made with a diamond point and the actual constriction is made by heating just above this mark with as slender a flame as possible. Without pulling or pushing, the glass will thicken where the flame strikes it, and heating is continued until the bore is 0.1 to 0.2 mm. at its narrowed point (*e*). The constriction should be small enough to stop the meniscus from going by when moderate pressure is applied, but large enough so that undue pressure is not required to force the meniscus by. The opening at the tip (*c*) should be a trifle smaller than the upper constriction so that the pressure which pushes the meniscus past the upper constriction will not cause the pipette to deliver too rapidly. The delivery time for a 50 c.mm. pipette should be 2 to 5 seconds. With a little practice, pipettes within 1 to 2 per cent of the desired volume can easily be made. However, each pipette is subsequently calibrated by delivery of water into a weighing bottle containing moisture and weighing on a micro balance.

the liquid, and by sucking, liquid is pulled to just above the constriction. With gentle pressure the liquid level is blown down to the constriction, where surface tension stops the meniscus automatically. The pressure is not released until the tip of the pipette is removed from the remaining liquid. To deliver, the pressure is increased sufficiently to drive the meniscus past the constriction and this pressure is maintained until delivery is complete (2 to 5 seconds). With smaller pipettes the tip should be sufficiently constricted (by fire polishing) so that surface tension prevents air from following the liquid after it is delivered, since otherwise part of the liquid might be spattered. During delivery, the bend in the tip is used to keep the lower shaft of the pipette away from the wall of the tube into which the sample is being delivered. This is very important, since otherwise surface tension will cause the sample to run up the shaft of the pipette, and only part of the sample will be delivered into the tube, the rest clinging to the pipette. Similarly, the tip of the pipette must always touch a

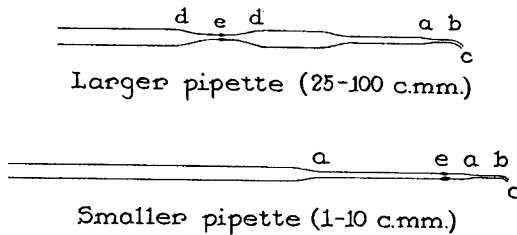Larger pipette (25-100 c.mm.)

Smaller pipette (1-10 c.mm.)

Fig. 1. Lang-Levy pipettes. See foot-note 1

surface during delivery, since if the tip is free in the air, much of the sample adheres to the outside of the pipette.

Procedure with 5 C.mm. of Serum—Serum samples (5 c.mm.) are transferred to the bottom of 6 × 50 mm. tubes in a wire rack. (A simple method for collecting small samples of serum has been previously described (8).) The rack is immersed in a shallow pan of ice water and to each tube are rapidly added 50 c.mm. of ice-cold Reagent C, with a constriction pipette. Each tube is mixed by tapping with the finger. Care is taken not to warm the tube by so doing.

The whole rack of tubes is now immersed in a water bath at 38° at a depth sufficient to cover the bottom half of the tubes. After exactly 30 minutes the rack is again placed in the pan of ice water and 0.5 ml. of 0.02 N NaOH is added to each tube with sufficient force to mix the sample. (A syringe pipette is very convenient for this purpose.) This addition stops the reaction and dilutes the samples which are now transferred to colorimeter tubes and read at 400 to 420 mμ = R_1.

After the initial reading, 2 to 4 c.mm. of concentrated HCl are added with a 0.1 ml. graduated pipette (drawn out tip) and a second reading, R_2, is made.

R_1 and R_2 are converted into optical densities ($-$ log transmission or $2 -$ log per cent transmission) $= D_1$ and D_2. Then $D_1 - D_2 = D_c$, the corrected density.

Standards and blanks are provided by treating 5 c.mm. volumes of the standards and of distilled water exactly as though they were serum samples. The corrected densities (D_c) are used to construct a standard curve from which the serum values are calculated. Since sera and standards undergo the same dilution, it is unnecessary to take into account the exact volumes of the various pipettes. It is to be noted that D_c is the density corrected for possible residual absorption after acid addition but is not corrected for the D_c of the blank analysis. This second necessary correction is automatically provided by the standard curve. A "millimole unit" is defined as the phosphatase activity which will liberate 1 mM of nitrophenol per liter of serum per hour. Therefore, since the standard incubation time is only 30 minutes, the 1, 2, 4, and 6 mM standards are equivalent to sera with activities of 2, 4, 8, and 12 mM units. 1 such unit is approximately equal to 1.8 Bodansky units (see below). For adult sera, which have low phosphatase activity, the volumes of serum and reagent may be doubled without increasing the volume of alkali; this will nearly double the amount of color.

Procedure with 20 C.mm. of Serum—Volumes of serum, reagent, and 0.02 N NaOH are increased to 20 c.mm., 200 c.mm., and 2 ml., respectively. Otherwise the procedure is nearly identical with that described for 5 c.mm. of serum. The sample may be conveniently incubated directly in $\frac{3}{8}$ inch photocolorimeter tubes. 1 drop of 5 N HCl is added before the second reading.

Procedure with 0.1 Ml. of Serum—Use 0.1 ml. of serum, 1 ml. of reagent, and 20 ml. of 0.02 N NaOH. Because of the larger volumes, the tubes containing 1 ml. of reagent are placed in the water bath and allowed to come to temperature before the addition of serum. As each serum sample is added, the time is noted, and exactly 30 minutes later 20 ml. of NaOH are added.

DISCUSSION

Fig. 2 shows the differences in spectral absorption between p-nitrophenyl phosphate, p-nitrophenolate, and free p-nitrophenol. It is on these differences in absorption that the proposed method depends. Upon removing the phosphate group from p-nitrophenyl phosphate to form p-nitrophenolate, the absorption maximum is shifted from 310 to 400 mμ and

is nearly doubled in height.   On converting the liberated nitrophenylate into free nitrophenol, by acidification, the absorption maximum is shifted back to 318 mμ and the absorption at 400 mμ is abolished.   This latter reaction makes it possible to correct for the color contributed by the serum itself.

King and Delory (3) observed that the pH optimum of phosphatase with p-nitrophenyl phosphate as the substrate is more alkaline than with glycerol phosphate.   The pH optimum with human serum was found to be 10.0 to 10.1, under the conditions described here (uncorrected glass electrode pH).   Since alkaline phosphatase has a sharp pH optimum, devi-
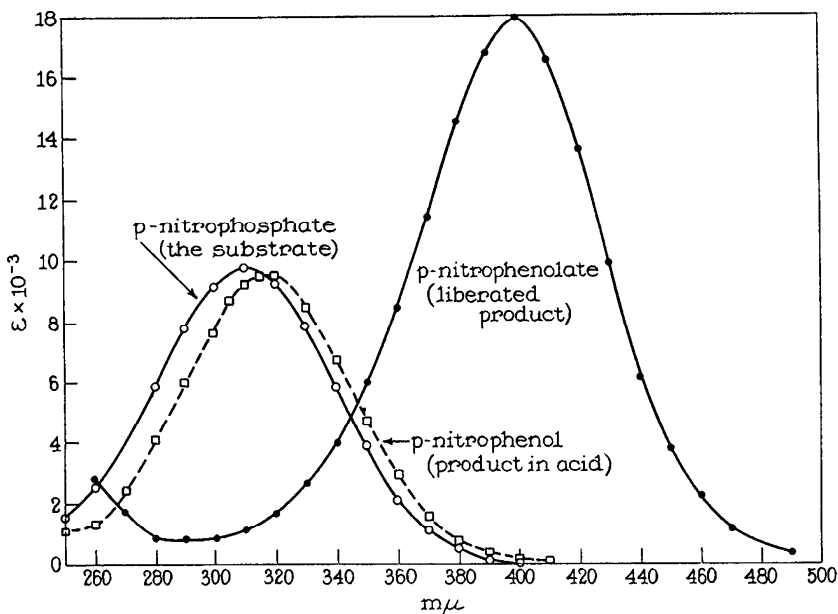

FIG. 2. Absorption curves of p-nitrophenol and p-nitrophenyl phosphate

ations of more than 0.1 pH unit will affect the readings significantly.   For rat serum the optimum pH range is from 9.1 to 9.7.   It is advisable to check the pH of the reagent against a standard buffer in the same pH range, such as 0.1 M sodium borate, pH 9.2.   The reagent is well buffered; hence there is no danger of CO_2 from the air affecting the activity during the incubation, but the pH of the reagent itself should be rechecked occasionally.   Since the buffer capacity is large, it is permissible in the case of sera having very high phosphatase to reduce the volume of serum to one-half or one-quarter without changing the other volumes.   The phosphatase reagent of Bodansky (1) is less well buffered and considerably more care

must be exercised with it to prevent pH changes from affecting phospha-
tase activity. The p-nitrophenyl phosphate reagent contains added mag-
nesium, which, however, has less effect on the activity of the phosphatase
than in the case of glycerol phosphate.

The degree of splitting in 30 minutes has been found to be proportional
to the concentration of enzyme, but has not been found to be strictly linear
with time for more than about 30 minutes. Therefore, it is desirable not
to increase the period of incubation.

The blank correction for contribution of color from the serum is based on
the finding that serum has very nearly the same absorption at 415 mμ in
acid as in alkaline solution. Hemoglobin, if it is present as the result of

TABLE I

Comparative Results with Bodansky and Nitrophenyl Phosphate Procedures

Serum No.	Bodansky units	p-Nitrophenyl phosphate units	Ratio, p-nitrophenyl phosphate to Bodansky units
1	8.36	4.68	1.79
2	8.80	4.98	1.77
3	10.7	5.68	1.88
4	8.30	4.81	1.73
5	9.30	4.76	1.95
6	9.27	4.76	1.95
7	5.60	3.27	1.71
8	7.42	4.22	1.76
9	7.14	4.32	1.66
10	8.36	4.99	1.67
11	4.48	2.41	1.86
12	6.54	3.70	1.77

Average... 1.79
Standard deviation.. 0.10

hemolysis, absorbs considerably less light at 415 than at 400 mμ, which is
one reason for preferring the longer wave-length. Hemoglobin does not
absorb quite the same amount of light in acid and alkaline solution; hence
excessive hemolysis should be avoided.

Comparison with Other Methods—In Table I are shown comparative
results of analyses of twelve children's sera made by the Bodansky proce-
dure (1) on 0.1 ml. of serum and by the method described here on 5 c.mm. of
serum. It would appear that the Bodansky units (mg. of P liberated per
100 ml. of serum per hour) bear a ratio of 1.79 to the mM unit of the present
procedure (mM of substrate hydrolyzed per liter of serum per hour). We
have found the same ratio for adult sera, but the activities were so low that

accurate measurements by the Bodansky procedure were difficult to make, and hence a less regular correlation was found.   In a personal communication, Dr. S. H. Jackson, Children's Hospital, Toronto, reported that the ratio of King-Armstrong units (mg. of phenol split from phenyl phosphate per 100 ml. of serum per hour) (2) to mM units is 7.3, with a variance of 8 per cent.   With rat sera we have found in agreement with Fujita (5) that a much lower ratio is obtained.   This observation deserves further investigation.   The standard deviation of replicate determinations on the same serum by the proposed procedure with 5 c.mm. is 0.15 mM unit.

Huggins and Talalay (6) have recently introduced phenolphthalein phosphate as a phosphatase substrate.   Like nitrophenyl phosphate it
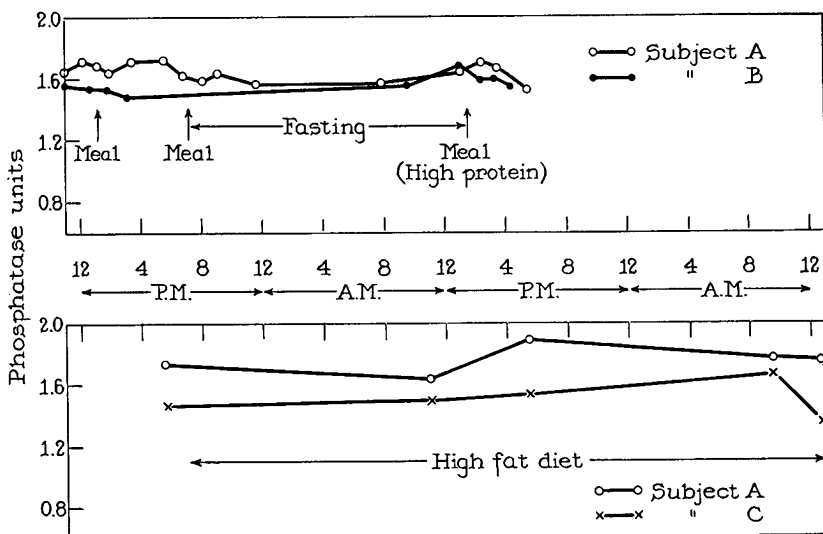

FIG. 3. Blood serum alkaline phosphatase under various dietary conditions

can function as its own indicator.   It is, however, split only 3 or 4 per cent as fast as the latter and the color produced is stated not to be proportional to the enzyme concentration, presumably because there are two phosphate groups attached which may both have to be removed before color develops.

Illustrative Data—Fig. 3 gives the results of experiments to determine whether the alkaline phosphatase of human serum is influenced by the diet. Weil and Russell (9) observed that in the rat fasting for as little as 8 hours caused a marked decrease in serum alkaline phosphatase, confirming similar findings of Bodansky (10) for the dog.   Gould (11) observed that a high fat diet produced an increase of 400 per cent in the alkaline phosphatase of rat serum.   It is apparent (Fig. 3) that in the human subjects tested

there were no significant changes in the serum alkaline phosphatase attributable to an 18 hour fast, a high protein meal, or a 40 hour period of very high fat intake. The blood samples were all obtained by finger puncture. Duplicate 10 c.mm. serum samples were analyzed. It seems possible to conclude that for human phosphatase studies it is unnecessary to consider the immediate dietary history.

SUMMARY

A method is described for the determination of serum alkaline phosphatase, which permits analysis of 5 c.mm. (0.005 ml.) samples of serum at the rate of 50 to 100 per 2 hours. The simplicity and speed of the method also recommend it for macro- as well as microdeterminations, and for either alkaline or acid phosphatase.

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