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## An Ultramicro Phosphorimetric Assay for Alkaline and Acid Phosphatase Activities

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A sensitive phosphorimetric method for the assay of alkaline and acid phosphatases in biological materials is described. *p*-Nitrophenol, formed enzymatically from the substrate *p*-nitrophenyl phosphate, is extracted with ether and then determined phosphorimetrically in a mixture of ether and ethanolic potassium hydroxide. The method is rapid and very sensitive, and thus requires as little as 0.5–5  $\mu$ l of human serum or 0.1–5  $\mu$ g of protein of rat tissue with a detection limit for *p*-nitrophenol formed of as little as 10 pmol.

**Keywords**—alkaline phosphatase; acid phosphatase; phosphorimetry; ultramicro assay; serum; tissue; *p*-nitrophenylphosphate; *p*-nitrophenol

Alkaline (ALP)<sup>2a)</sup> and acid phosphatases (AcP)<sup>2b)</sup> have usually been assayed by colorimetric and fluorimetric methods. Colorimetric methods include those using chromogenic substrates, such as monophosphates of thymolphthalein,<sup>3)</sup> phenolphthalein<sup>4)</sup> and *p*-nitrophenol.<sup>5)</sup> Sensitive fluorimetric methods have been developed with the use of monophosphates of 1- and 2-naphthol,<sup>6)</sup> flavone,<sup>7)</sup> 7-hydroxycoumarin,<sup>8)</sup> 4-methylumbelliferone<sup>9)</sup> and naphthol AS derivatives<sup>10)</sup> as substrates.

Recently, a phosphorimetric method has been successfully applied to the microdetermination of biological materials in our laboratory.<sup>11,12)</sup> The phosphorimetric characteristics of *p*-nitrophenol in ethanol have been described previously.<sup>13)</sup> During studies of the microassay of ALP and AcP, we found that the phosphorescence of *p*-nitrophenol in ethanol was greatly enhanced by the addition of an alkali and could be measured even at subpicomol/ml concentrations. This observation was successfully applied to the ultramicro assay of ALP and AcP activities in serum and tissue homogenates, based on the determination of *p*-nitrophenol formed enzymatically from the substrate *p*-nitrophenyl phosphate.

### Experimental

**Chemicals and Apparatus**—All chemicals were of reagent grade, unless otherwise specified. Double-distilled water and solvents were used. *p*-Nitrophenol and sodium *p*-nitrophenyl phosphate (Sigma) were purified by recrystallization.

The phosphorescence spectra and intensities were measured with the sample solution in the form of a clear solid frozen at liquid nitrogen temperature (77 °K), using a Hitachi MPF-3 spectrofluorimeter equipped with a Hitachi phosphoscope attachment using quartz sample tubes (4.0 mm i.d., 5.0 mm o.d., 200 mm

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- 2) Orthophosphoric monoester phosphohydrolase; a) EC 3.1.3.1; b) EC 3.1.3.2.
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long; sample volume required, 150  $\mu$ l). The slit widths in the exciter and analyzer in terms of wavelength were both set at 10 nm. The phosphorescence spectra are uncorrected. The phosphorescence lifetime was measured in the usual manner by recording the decay of the phosphorescence at 525 nm as a function of time on a Hitachi V-051 synchroscope after the exciting radiation (375 nm) had been stopped by closing the excitation shutter in the fluorimeter. The lifetime corresponds to the time required for the phosphorescence intensity to drop to  $1/e$  of its initial value. pH was measured with a Hitachi-Horiba M-7 pH meter at 25°.

**Biological Sample Solutions**—Serum: Normal and pathological human sera were used.

**Rat Tissue Homogenate:** A rat was killed by decapitation, and portions (30–50 mg) of the liver, spleen, lung, adrenal and kidney were rapidly removed, blotted and placed in 4.0–5.0 ml of ice-cold 0.25 M sucrose. The tissue was homogenized in a Potter-Elvehjem homogenizer and centrifuged at  $1000 \times g$  for 20 min to remove unbroken cells, nuclei and debris. The supernatant was diluted with 0.25 M sucrose to contain 1–2.5  $\mu$ g of tissue in 5  $\mu$ l. Protein concentration was determined by the procedure of Lowry *et al.*<sup>14)</sup>

**Procedure for A1P Assay**—The substrate solution was 0.1 M glycine buffer (pH 10.5) containing 5.4 mM sodium *p*-nitrophenyl phosphate and 1.0 mM  $MgCl_2$ . The solution (50  $\mu$ l) was pre-incubated at 37° for 2 min, then incubated for exactly 15 min after addition of 5  $\mu$ l of biological sample solution at 37°. The reaction was stopped by the addition of 5  $\mu$ l of 3 M trichloroacetic acid. The mixture was cooled in ice-water. *p*-Nitrophenol in the mixture was extracted into 1.0 ml of ether, and 0.7 ml of the ether layer was diluted with 0.2 ml of ethanolic 0.5 M KOH.

For the blank, the same procedure was carried out except that the order of addition of the sample solution and trichloroacetic acid was reversed to avoid the enzyme reaction and that incubation was omitted. To prepare a standard curve, 50  $\mu$ l of the substrate solution in the procedure was replaced with 50  $\mu$ l of a solution of *p*-nitrophenol (0.25–50 nmol) dissolved in substrate solution containing no *p*-nitrophenyl phosphate. The standard curve was linear up to at least 50 nmol of *p*-nitrophenol and passed through the origin. The phosphorescence intensities were measured at 520 nm with excitation at 375 nm.

**Procedure for AcP Assay**—For the assay of total AcP activity, the same procedure as for A1P activity was used, except that 50  $\mu$ l of 90 mM citrate buffer (pH 4.9) containing 5.5 mmol of *p*-nitrophenyl phosphate was used as the substrate solution. Prostatic AcP (tartrate-labile AcP) activity was calculated by subtracting the activity obtained in the presence of 40 mM sodium tartrate in the substrate solution from total AcP activity.

## Results and Discussion

### A1P Assay

A1P in human serum and rat tissue homogenates was generally most active at pH 10–11; pH 10.5 was selected for the assay. A maximum and constant activity was obtained in the presence of 0.7–10.0 mM *p*-nitrophenyl phosphate with an observed  $K_m$  value of 0.27 mM. Thus, 5.0 mM *p*-nitrophenyl phosphate was used as a saturating concentration. Magnesium chloride stimulated the enzyme activity 1.3 times in the concentration range of 0.5–3 mM; 1.0 mM was selected for the standard procedure.

The enzyme activity was linear with time up to at least 120 min when incubated at 37°. The amount of *p*-nitrophenol formed at an incubation time of 15 min was proportional to the human serum sample size up to at least 80  $\mu$ l, and to the amount of protein in rat tissue homogenates up to at least 40  $\mu$ g.

The *p*-nitrophenol was effectively extracted from the acidified assay mixture with ether. The ether extract (0.7 ml) readily forms a clear solid at 77°K when mixed with 0.1–0.3 ml of ethanol. In our final procedure, 0.2 ml of ethanol was used. Dissolving potassium hydroxide in the ethanol increased the phosphorescence obtained from the *p*-nitrophenol. Ethanol which was 0.5 M or greater in potassium hydroxide gave the maximum phosphorescence from the extracted *p*-nitrophenol (Fig. 1).

The phosphorescence excitation (maximum, 375 nm) and emission (maximum, 525 nm) spectra and the lifetime (0.2 sec) for the final solution in the assay were identical with those for *p*-nitrophenol dissolved in an alkaline medium with the same solvent composition as the final solution (Fig. 2).

Comparison with the Bessey-Lowry method<sup>5)</sup> (substrate, *p*-nitrophenyl phosphate),

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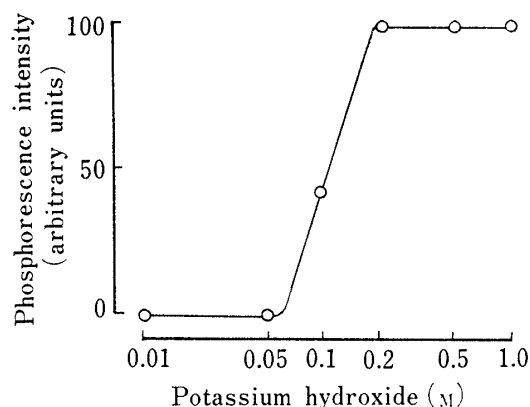


Fig. 1. Effect of Potassium Hydroxide Concentration on the Phosphorescence Intensity of the Final Solution

Portions (0.7 ml) of the ether layer obtained in the procedure were diluted with 0.2 ml of ethanolic potassium hydroxide of various concentrations.

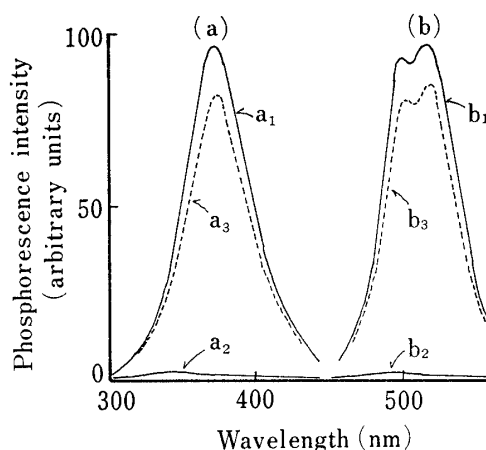


Fig. 2. Excitation and Emission Spectra of the Phosphorescence of the Final Solution in the Procedure and of *p*-Nitrophenol

a: excitation spectra; b: emission spectra.  
 a<sub>1</sub> and b<sub>1</sub>: portions (5 μl) of serum with an AIP activity of 1.24 mmol/hr/l was treated by the standard procedure.  
 a<sub>2</sub> and b<sub>2</sub>: blank corresponding to a<sub>1</sub> and b<sub>1</sub>.  
 a<sub>3</sub> and b<sub>3</sub>: *p*-nitrophenol (1.0 nmol/ml) was dissolved in ether-ethanolic 0.5 M potassium hydroxide (7/2, v/v).

which has been widely employed, using normal and pathological sera, gave a correlation coefficient of 0.969 ( $n=53$ ), and the regression equation for the present method ( $x$ ) against the Bessey-Lowry method ( $y$ ) was  $y=1.01x-0.24$ . The within-day precision was examined using sera with mean AIP activities of 1.02 and 4.40 mmol/hr/l. The coefficients of variation were 3.9 and 2.9% ( $n=20$  each), respectively.

AIP activity in normal serum assayed by the present method was  $1.39 \pm 0.41$  mmol/hr/l (mean  $\pm$  SD,  $n=28$ ). The activity in sera of patients with Basedow's disease was  $3.06 \pm 0.85$  mmol/hr/l (mean  $\pm$  SD,  $n=20$ ). The values are in agreement with the data obtained by other workers.<sup>15)</sup>

AIP activities found in rat tissue homogenates are shown in Table I. These values are similar to those in human tissue homogenates obtained by the colorimetric method using phenyl phosphate as a substrate.<sup>16)</sup>

TABLE I. Alp and AcP Activities in Rat<sup>a)</sup> Tissue Homogenates

Tissue	AIP activity <sup>b)</sup>	AcP activity <sup>b)</sup>
Liver	2.06	1.43
Spleen	2.11	0.98
Lung	1.50	0.71
Adrenal	3.46	1.70
Kidney	2.45	6.94

a) Donryu male rat, 9 weeks of age.

b) *p*-Nitrophenol formed, nmol/hr/μg protein.

### AcP Assay

Maximum activity of AcP in human serum and rat tissue homogenates was observed in the range of pH 4.8–6.0; pH 5.0 was selected for the assay. AcP activity did not change

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over the concentration range of 3.0–20.0 mM sodium *p*-nitrophenyl phosphate, with an observed  $K_m$  value of 1.0 mM; 5.0 mM was employed as a saturating concentration.

The enzyme activity was linear with time up to at least 35 min when incubated at 37°. The amount of *p*-nitrophenol formed at an incubation time of 15 min was proportional to the human serum sample size up to at least 30  $\mu$ l, and to the amount of protein in rat tissue homogenates up to at least 50  $\mu$ g.

The phosphorescence spectra and the lifetime for the final solution in the assay were identical with those observed for AIP.

Parallel tests using the Bessey-Lowry method,<sup>5)</sup> using sera with total AcP activities ranging from 0.10 to 0.62 mmol/hr/l, gave a correlation coefficient of 0.958 ( $n=30$ ), and the regression equation for the present method ( $x$ ) against the Bessey-Lowry method ( $y$ ) was  $y=0.99x-0.02$ . The within-day precision was established using sera with mean total AcP activities of 0.10 and 0.42 mmol/hr/l. The coefficients of variation were 4.0 and 2.5% ( $n=20$  each), respectively.

Total and prostatic AcP activities in normal serum ( $n=20$ ) obtained by the present method were  $0.35\pm 0.15$  (mean $\pm$ SD) and  $0.04\pm 0.03$  (mean $\pm$ SD) mmol/hr/l, respectively. These values are in good agreement with those described by other workers.<sup>17)</sup>

AcP activities in rat tissue homogenates are shown in Table I. These values are identical with those in human tissue homogenates obtained by the colorimetric method using phenyl phosphate as a substrate.<sup>16)</sup>

#### Features of the method

The present method is not affected by bilirubin present in the biological sample, though the Bessey-Lowry method is interfered with this compound, which shows light absorption in the same wavelength region as *p*-nitrophenol.

The lower limit of detection for *p*-nitrophenol enzymatically formed is 10 pmol in both AIP and AcP assays; this gives a phosphorescence intensity of twice the blank value. This sensitivity is much higher than those of the fluorimetric methods,<sup>4-7)</sup> and may permit the assay of AIP and AcP in only 0.5  $\mu$ l of serum or 0.1  $\mu$ g protein of rat tissue homogenate. The method is precise and rapid, and should be useful in cases where only an extremely small amount of serum or tissue (such as a biopsy sample) is obtainable.

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