Pyranine Phosphate as a New Fluorogenic Substrate for Acidic and Alkaline Phosphatases[1)]

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8-Hydroxypyrene-1,3,6-trisulfonic acid (HPTS, pyranine) is highly fluorescent not only in the alkaline range but also at an acidic pH, and in a water-soluble fluorescent compound. By phosphorylation of a pyranine, its fluorescence intensity was quenched markedly. A pyranine phosphate was shown to be a potential fluorogenic substrate for the assay of acid and alkaline phosphatases, and also for phosphatase activities in human serum.

Keywords pyranine; quenching; fluorogenic substrate; acid phosphatase; alkaline phosphatase; fluorometric enzyme assay

Alkaline phosphatase (EC 3.1.3.1) is a broad term associated with non-specific phosphomonoesterases (orthophosphoric monoester phosphohydrolase) with activity optima at an alkaline pH. It is a glycoprotein and a dimer comprising two subunits and a zinc matallo-enzyme.[3)] Elevation of alkaline phosphatase activity is known to occur in a variety of hepatic diseases. Abnormal electrophoretic varients of alkaline phosphatase are found in the serum and tissue of patients with hepatocellular cancer.[4)] Acid phosphatase (EC 3.1.3.2) is also a non-specific phosphomonoesterase with an optimal pH below 7, and catalyzes the hydrolysis of orthophosphoric monoester to alcohol and orthophosphoric acid. It is widespread throughout nature, and hydrolysis of a variety of orthophosphate esters is catalyzed by enzymes from many sources.[5)] Acid phosphatase determinations using synthetic substrates play an important role in the diagnosis of prostate cancer and bone tumors.

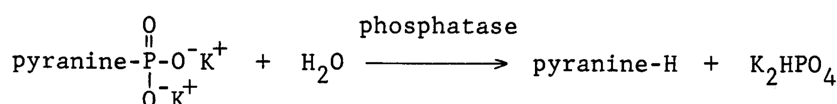
Several methods are available for the assay of phosphatase activity. The most frequently used is the *p*-nitrophenyl phosphate method.[6)] On the other hand, synthetic fluorogenic substrates have been widely used to detect and quantify hydrolytic enzymes. For those purposes, aminocoumarin derivatives[7)] and bimane substrates[8)] have been developed. The use of fluorogenic substrates for the determination of proteases continues to be of increased interest. A 4-methylumbelliferyl phosphate was reported as a fluorogenic substrate for alkaline phosphatase,[9)] but fluorescence intensity of 4-methylumbelliferone is small at an acidic pH for continuous fluorometric assay though it is highly fluorescent at an alkaline pH. 8-Hydroxypyrene-1,3,6-trisulfonic acid (pyranine, **2**) has been reported as a fluorescent pH-indicator and it has very interesting fluorescent characteristics.[10)] Compound **2** exhibits both longwave excitation and emission maxima, and is intensely fluorescent in both alkaline and acidic solutions.[10)] This fluorophor has already been applied to the fluorogenic substrate for esterases.[11)]

In this paper, we wish to present a new substrate with **2** for the kinetic study and quantitative assay of phosphatases such as alkaline phosphatase from bovine intestinal mucosa and acid phosphatase from sweet potato to examine that pyranine phosphate (**1**) is susceptable to hydrolysis by the substrate.

The substrate (**1**) was synthesized by phosphorylation of **2** with phosphorous pentachloride in dry pyridine in the presence of 18-Crown-6. Relative fluorescence intensities (RFI) of **1** are 0.021 at pH 8.8, and 0.045 at pH 5.8 *versus* a fluorescence intensity of **2**. The fluorescence intensity of **1** was markedly quenched by phosphorylation. Therefore, phosphorylated pyranine can be expected to be the fluorogenic substrate for phosphatases. Kinetic parameters of **1** for both acid and alkaline phosphatases were obtained by direct continuous spectrofluorometric assay and the results are listed in Table I. In order to compare the kinetic parameters of the pyranine substrate (**1**) with those of the previously reported spectrophotometric substrate of *p*-nitrophenyl phosphate (**3**), the kinetic parameters of **3** were also measured with the same lot of phosphatase and under the same assay conditions of pH, buffer and temperature. For alkaline phosphatase, although the K_m value of **1** is a little smaller than that of **3**, the k_{cat} value of **1** is very slightly low. Therefore, the k_{cat}/K_m value of **1** is almost comparable with that of **3**. For acid phosphatase, both values of K_m and k_{cat} of **1** are comparable with those of **3**, so the k_{cat}/K_m of **1** is almost comparable with that of **3**. The linearities of the plots of fluorescence intensity *vs.* enzyme concentration

TABLE I. Kinetic Parameters of the Substrate for Phosphatases

Enzyme	Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)
Alkaline phosphatase				
	1	8.3×10^{-5}	1.2×10	1.4×10^{5}
	3	2.1×10^{-4}	2.2×10	1.0×10^{5}
Acid phosphatase				
	1	3.3×10^{-5}	2.4×10	7.3×10^{5}
	3	7.7×10^{-5}	7.3×10	9.5×10^{5}



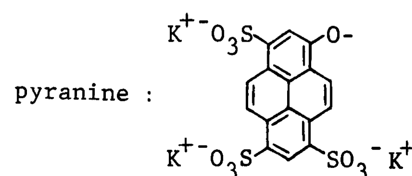


Chart 1

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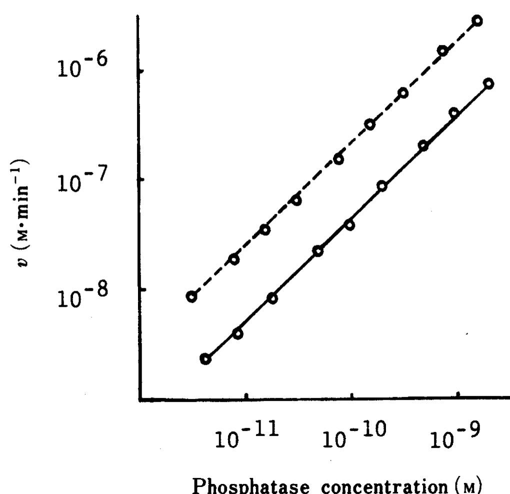

Fig. 1. Linearity with Respect to Phosphatase Concentration of the Velocity of **2** Formation Catalyzed by Phosphatases Using **1** as Substrate

Assays were carried out as described under Experimental procedure. —○—, alkaline phosphatase; --○--, acid phosphatase.

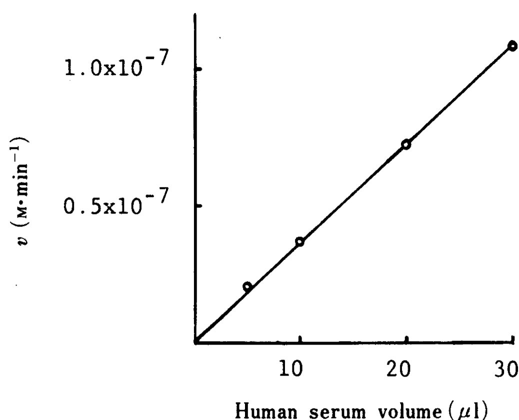

Fig. 2. Linearity with Respect to Human Serum Volume of the Velocity of **2** Formation Catalyzed by Human Serum Using **1** as a Substrate

Assays were performed as described under Experimental procedure.

were satisfactory with the detection limit of 4.73×10^{-12} M for alkaline phosphatase at a substrate (**1**) concentration of 9.82×10^{-7} M, 2.98×10^{-12} M for acid phosphatase with a substrate (**1**) concentration of 8.42×10^{-7} M (Fig. 1).

As a further application of **1**, **1** was used for an assay of phosphatase activity in human serum. In this work, total phosphatase activity was measured in order to confirm the applicability of **1** to the phosphatase activity determination in serum, though there are many isoenzymes in an alkaline phosphatase.[4b] The phosphatase activities with **1** are proportional to the serum volume over the range of 5—30 μl of human serum as shown in Fig. 2, and the value of unit was 1.1 (substrate concentration: 1.0×10^{-4} M). For the comparison of **1** with the known substrate of **3** by the Bessey–Lowry method,[6] the unit value of **3** was estimated as 0.84 (substrate concentration: 5.0×10^{-3} M) with the same lot of human serum under the same assay conditions of buffer, pH and temperature. This value is in the range of normal unit value of 0.8—2.9.[12]

In the present work, it has thus been demonstrated that **1** can be a potent fluorogenic substrate for the assay of phosphatases. Compared with previously reported substrates for phosphatase, the pyranine system has the following advantages. 1) The pyranine system can provide a continuous and rapid fluorometric assay in both acidic pH and in alkaline pH, though known procedures with **3** and umbelliferyl phosphate are time-consuming end-point assays in acidic pH. 2) The pyranine system has a longer wavelength excitation maximum (460 nm at alkaline pH, 400 nm at acid pH)[10] than the umbelliferone system (325 nm),[13] so that interference from most biological compounds is low.

Because of these advantages of the pyranine system, the use of **2** is promising for the syntheses of substrates for other enzymes like sulfatases or glucosidases. An alkaline phosphatase has many isoenzymes on the basis of an organ, and has different electrophoretic mobility.[4b,12] Therefore, analyses of each variant of alkaline phosphatase are important in the clinical field. Further applications of this **1** to the clinical analyses of each variant of phosphatase or to *in vivo* assays of intracellular phosphatase activities are expected.

Experimental

The melting point was determined on a YAMATO MP-21 apparatus and was uncorrected. The pH was measured with a Horiba M-12 pH meter. Ultraviolet and visible absorption spectra were obtained with a Hitachi 210-10 spectrophotometer. Fluorescence spectra were recorded on a Hitachi 650-10 fluorescence spectrophotometer.

Alkaline phosphatase (E.C. 3.1.3.1. Type VII-S: from bovine intestinal mucosa) and acid phosphatase (EC 3.1.3.2. type X: from sweet potato) were purchased from Sigma Chemical Company. Human serum was obtained from the Hokkaido Red Cross Blood Center.

Synthesis of Substrate (1) To an ice-water cooled, stirred solution of 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt (105 mg, 0.2 mmol) and 18-Crown-6 (159 mg, 0.6 mmol) in dry pyridine (2 ml), phosphorous pentachloride (125 mg, 0.6 mmol) was added portionwise, and stirring was continued for 2 h at the same temperature. After the addition of ice, the solvent was removed under reduced pressure and the residue was treated with water followed by evaporation three times. The residual oil was dissolved in 20 ml of water, and extracted with ethyl acetate 5 times, and then the aqueous layer was condensed to 2 ml followed by the addition of potassium carbonate (69 mg, 0.5 mmol). After evolving gas ceased, ethanol (50 ml) was added. Precipitates were collected by suction and washed with ethanol, and then dried *in vacuo*. 128 mg (88%) of pale yellow powders, mp 255—260 °C (dec.). *Anal.* Calcd for $C_{16}H_6K_5O_{13}PS_3 \cdot 4H_2O$: C, 23.99; H, 1.76; S, 12.01. Found: C, 23.85; H, 1.89; S, 11.72.

Fluorometric Measurement and Enzyme Assays a) Kinetic Parameters (K_m, k_{cat}) Measurement: Fluorometric measurements were conducted with the following buffer and pH; alkaline phosphatase: 0.2 M glycine buffer, pH 8.8 with 0.05 M $MgCl_2$ at 25 °C. Acid phosphatase: 0.2 M sodium acetate buffer, pH 5.8 at 35 °C. Enzyme concentrations were determined from molecular weight and absorbance measurement at 278 and 280 nm for alkaline phosphatase (14000, $E_{278} = 7.6$),[3a] and acid phosphatase (110000, $E_{280} = 25.2$),[14] respectively.

RFI were determined at 5.0×10^{-6} M of substrate concentration in the same buffer above at the following pH and fluorescence wavelength, pH 8.8 (excitation at 460 nm and emission at 515 nm) and 5.8 (excitation at 400 nm and emission at 515 nm).

For the enzyme assays, fluorescence excitation and emission wavelengths were 460 nm (alkaline phophatase), 400 nm (acid phosphatase) and 515 nm, respectively. The increase of fluorescence caused by hydrolysis of the substrate (**1**) was automatically recorded *vs.* time, usually for 6 min. From the slope of this plot, the activity of the enzyme was calculated in comparison with the fluorescence intensity of the reference compound (**2**). The K_m and k_{cat} values were obtained from Lineweaver–Burk plots with the following substrate and enzyme concentration. **1** (for alkaline phosphatase): 0.635×10^{-4}—2.12×10^{-4} M, alkaline phosphatase: 1.46×10^{-9} M. **1** (for acid phosphatase): 0.417×10^{-4}—1.39×10^{-4} M, acid phosphatase: 1.49×10^{-9} M.

b) Linear Relation of the Fluorescence Intensity *vs.* Enzyme Concentration: Fluorometric assays were carried out in the manner described in a) with the following final concentrations of substrates and

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enzymes. **1** (for alkaline phosphatase): 9.82×10^{-7} M, alkaline phosphatase: 4.73×10^{-12}—1.89×10^{-9} M. **1** (for acid phosphatase): 8.42×10^{-7} M, acid phosphatase: 2.98×10^{-12}—1.49×10^{-9} M.

c) Kinetic Parameters with **3**: Assay for alkaline phosphatase was carried out by the continuous assay procedure in the literature.[3a] Assay for acid phosphatase was carried out by the same procedure of an end-point assay as reported.[15]

Assays of Phosphatase Activities in Human Serum a) Phosphatase Activity with **1** in Human Serum: Serum (30 μl) was added to the substrate solution (3.3 ml) of **1** (final substrate concentration 1.0×10^{-4} M) in 50 mM glycine buffer (pH 10.3 containing 0.5 mM $MgCl_2$) at 38 °C,[6] and the increase in emission at 515 nm (appearance of **2**) was measured (excitation at 460 nm). Time of incubation was 30 min. The rate of hydrolysis was established from the rate of increase in fluorescence intensity based on the fluorescence intensity of **2**. A unit is defined as the phosphatase activity which will liberate 1 mmol of *p*-nitrophenol per liter of serum per hour.[6]

b) Linear Relation of the Initial Velocity *vs.* Serum Volume with **1** (Fig. 2): Measurements were carried out in the manner described in a).

c) Phosphatase Activity with **3** in Human Serum: Activity was determined by the Bessey–Lowry method with slight modification.[6] Serum (30 μl) was added to the substrate solution (3.3 ml) of **3** (final substrate concentration 5.0×10^{-3} M) in the same buffer and at the same pH and temperature described in a), and the increase of optical density at 410 nm (appearance of *p*-nitrophenol) was recorded for 30 min. The rate of hydrolysis was estimated from the optical density at 410 nm and a molar extinction coefficient of 17000 M^{-1} cm^{-1}.[15] The unit values were defined as *p*-nitrophenol liberated mmol per liter of serum per hour.[6]

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