FLUOROMETRIC MEASUREMENT OF ALKALINE PHOSPHATASE AND AMINOPEPTIDASE ACTIVITIES IN THE ORDER OF 10^{-14} MOLE*

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Fluorometric techniques for assaying enzyme activity of single tissue cells and of individual enzyme molecules has been instituted by the work of Lowry et al. (1961), who measured pyridine nucleotides by enzymatic cycling, and of Rotman (1961), who demonstrated the measurement of β-D-galactosidase activity of single enzyme molecules. The latter technique employs the substrate, 6 hydroxyfluoran-β-D-galactopyranoside, a compound of low fluorescence that upon enzymic hydrolysis gives rise to 6 hydroxyfluoran which can show a linear relationship between fluorescence and concentration. Although this fluorescent nucleus could be used to tailor substrates for other enzymes, it presents many problems of synthesis inherent in phthalein chemistry. For this reason, other fluorescent nuclei were examined. In 1944 Menten et al. demonstrated alkaline phosphatase activity histochemically by employing calcium β-naphthylphosphate as a substrate. This approach was modified by Seligman and Manheimer (1949), who used the more soluble sodium salt of α-naphthyl acid phosphate to demonstrate acid phosphatase. This same

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substrate was employed to measure serum alkaline phosphatase (Moss, 1960) and acid phosphatase (Campbell and Moss, 1961) fluorometrically. Similarly, β-naphthylamine conjugated in amide linkage with various amino acids has been employed for the histochemical assay of aminopeptidase activity (Gomori, 1954; Folk and Burstone, 1955; Green et al., 1955). This communication deals with naphthyl acid phosphate and phenylalanyl β-naphthylamide as fluorogenic substrates.

Samples of the sodium salts of both α and β naphthyl acid phosphate (Sigma Chemical Co.) in 0.1 M Tris buffer, pH 10.4, were examined in an Aminco Bowman spectrophotofluorometer and found to have activation maxima at 290 (α), and 275 and 315 mμ (β), and fluorescence maxima at 360 and 345 mμ, respectively. The corresponding phenols had activation maxima at 335 and 350 mμ, respectively, and fluorescence maxima at 455 and 410 mμ. Equimolar concentrations of the sodium salts of both α and β naphthyl acid phosphate, in 0.1 M Tris buffer, pH 10.4, were compared as substrates for alkaline phosphatase. It can be seen (Fig. 1) that following enzymic hydrolysis, a threefold greater fluorescence intensity results when the β ester is used as a substrate than with the α ester. In both cases linearity was maintained for 3 min. Consequently, the β ester was selected for more extensive investigation. When dissolved in 0.1 M Tris buffer, sodium β-naphthyl acid phosphate showed negligible change in fluorescence intensity over a pH range from 6.8 - 12, whereas a maximum fluorescence occurred when the pH of the hydrolysis mixture was 10.4, which was the pH maximum for the enzyme reaction and also the pH at which β naphthol displayed maximum fluorescence intensity.

A plot of the change in fluorescence intensity/2 min. against varying substrate concentrations (Fig. 2) revealed typical Michalis

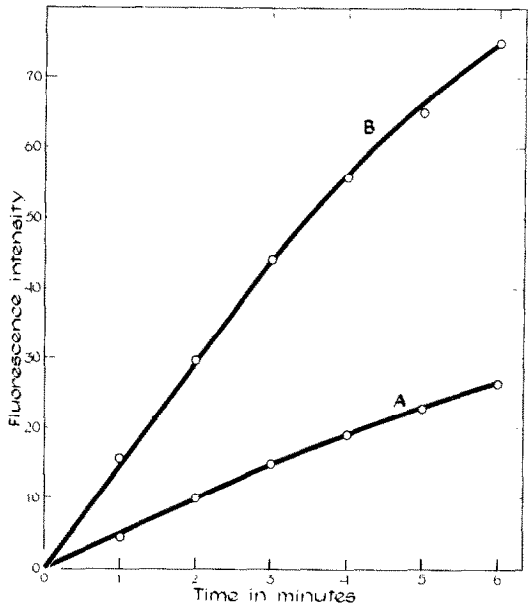

Fig. 1. Proportionality between fluorescence intensity and incubation time for alkaline phosphatase. Reaction mixture for curve A: 0.1 M Tris buffer, pH 10.4, 0.01 M $Mg(Ac)_2$, 2.03×10^{-4} M sodium α-naphthyl acid phosphate and alkaline phosphatase (Pentex) 7.1×10^{-10} moles* (based on 60% purity and a mol. wt. of 60,000, Schramm, G. and Armbruster, D., 1954); final vol. 1 ml, temp. 23°; activation λ 335 mμ, fluorescence λ 455; curve B: same as for curve A, except 2.03×10^{-4} M sodium β naphthyl acid phosphate; activation λ 350 mμ, fluorescence λ 410 mμ. All values were corrected for no enzyme controls.

*All values given for moles of enzymes are approximations.

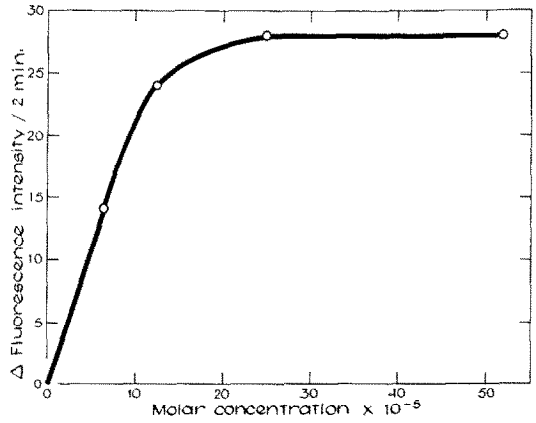

Fig. 2. Relationship between fluorescence intensity and sodium β-naphthyl acid phosphate concentration. Reaction conditions: same as Fig. 1, curve B.

kinetics with Km = 6.25×10^{-5}, which is in agreement with the value given by Moss and King (1962). By reduction of the final volume to 50 µl, it is easily possible, using a small-tube adapter for the Aminco Bowman spectrophotofluorometer, to assay 2.6×10^{-12} moles of enzyme (Table I),

TABLE I

Alkaline Phosphatase Activity Measured by Change of Fluorescence with Time at 23° and 37°.

Time (min)	Fluorescence Intensity*
23°	
2	5
4	12
6	17.5
8	23
10	27.5
37°	
10	5
20	10
30	15
40	20
60	30

*Values are means of triplicate analysis, corrected for no enzyme blanks.

Reaction conditions for 23° same as for Fig. 1, curve B, except 2.6×10^{-6} M sodium β naphthyl acid phosphate, 2.6×10^{-12} moles of enzyme and 50 µl final vol.; 37°: same as for 23°, except 2.6×10^{-7} M sodium β naphthyl acid phosphate and 5.5×10^{-14} moles enzyme.

linearity being maintained for 10 min. Calculations made from a concentration curve for pure β-naphthol (Sigma) indicate that the 10-min value seen in Table I (23°) is equivalent to the hydrolysis of 1 mµ mole of sodium β-naphthyl acid phosphate, an amount of product still within the limits of microscopic colorimetric analysis for phosphate (Norberg, 1942). A further reduction in the amount of enzyme to 10^{-14} moles still remains within range of the present fluorometric assay. By increasing incubation time and temperature to 1 hr and 37°, respectively, during which period linearity was maintained, this amount of enzyme was conveniently assayed (Table I).

Preliminary results of a fluorometric aminopeptidase assay can be seen in Fig. 3 in which the fluorescence intensity at 410 mμ was followed

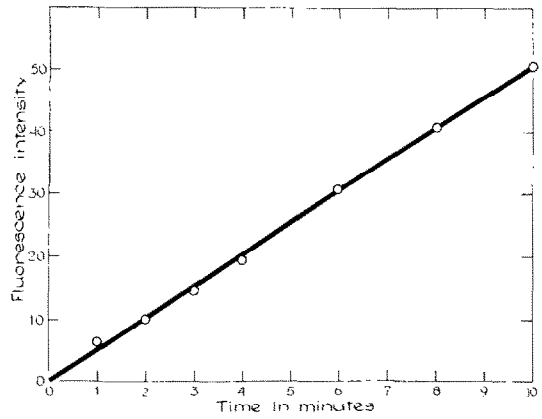

Fig. 3. Proportionality between fluorescence intensity and incubation time for aminopeptidase. Reaction conditions: 50 μl 0.1 M Tris buffer, pH 8.0, containing 2.56×10^{-3} M phenylalanyl β-naphthylamide; 50 μl of a 1:20 dilution of stock enzyme solution (1 mg leucine aminopeptidase/* ml 0.1 M Tris buffer, pH 8.0, containing 0.04 M $Mg(Ac)_2$) in 0.1 M Tris buffer, pH 8.0, preincubated for 10 min at 23°, equivalent to 8.0×10^{-12} moles of enzyme (based on mol. wt. of 300,000, Spackman et al., 1955). All values were corrected for no enzyme controls.

*Sigma Chem. Co., $C_1 = 14$

as a function of time at 23°, linearity being maintained during a 10-min period. Activation and fluorescence maxima for β-naphthylamine HCl (Sigma) were determined previously and found to be 335 and 410 mμ, respectively. Although the present assay conditions allow measurement of the activity of 10^{-12} moles of aminopeptidase, further increases in sensitivity have been attained, as in the case of alkaline phosphatase, and will be included in subsequent publication. The use of naphthylamine-linked substrates for demonstration of aminopeptidase activity by histochemical staining was evaluated by Smith and Hill (1960). Their main criticisms centered about low substrate solubility and low hydrolysis rates which prevent determination of proteolytic coefficients and

comparison with values obtained by employing native substrates. In the present assay, substrate solubility does not seem to be a problem because of the low substrate concentrations used in the fluorometric analysis.

Work is now in progress, not only toward the synthesis of naphthol- and naphthylamine-linked substrates for the analysis of other peptidases, β-glucuronidase, β-glucosidases, esterases and sulfatases, but also toward the further refinement of the present assay system, by employing the microdroplet technique described by Rotman (1961).

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