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SUBSTRATES FOR CYTOCHEMICAL DEMONSTRATION OF ENZYME ACTIVITY

V. KINETICS OF THE HYDROLYSIS OF THYMIDINE 3'-(5-BROMO-4-CHLOROINDOL-3-YL) PHOSPHATE BY PHOSPHODIESTERASE II

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SUMMARY

1. A comparison of the action of purified spleen phosphodiesterase II (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) on two synthetic substrates thymidine 3'-(*p*-nitrophenyl) phosphate (III) and thymidine 3'-(5-bromo-4-chloro-3-indol-3-yl) phosphate (V) is described. The utility of the latter (V) derives from a chromogenic reaction sequence that leads to the highly colored 5,5'-dibromo-4,4'-dichloroindigo (VII) *via* aerobic oxidation of the enzymically released intermediate 5-bromo-4-chloroindoxyl (VI). It is demonstrated that the rate of oxidation of VI to VII is significantly greater at pH 7.0 than enzymic release of VI. Therefore the oxidation step is not rate determining.

2. The rates of enzymic hydrolysis of III and V, effected at a substrate concentration of 150 μg/ml in 4.1 M acetate (pH 5.5) in accord with established procedures, are of the same order of magnitude (370 and 235 μmoles/h per mg protein, respectively). The K_m with III is about $0.25 \cdot 10^{-3}$ and differs by a factor of approx. 10 from that ($3 \cdot 10^{-3}$) recorded by Razzell and Khorana. The difference is ascribed to the improved method of purification which affords a stable enzyme free of contaminants.

3. A comparison of the kinetic parameters K_m and V for the two substrates indicates that V is an acceptable substrate for assay of phosphodiesterase II.

INTRODUCTION

Attempts to correlate cytochemical results with those of cell fractionation are difficult and fraught with danger. Nevertheless, efforts to remedy the deficiencies

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that frequently beset the cytochemical approach prompt a continuing search for substrates of greater specificity together with an improvement of existing methods. These considerations have sustained our continuing interest in the characterization of the phosphodiesterases I and II, the so-called exonucleases *via* cytochemical and biochemical techniques.

Cytochemical studies of (kidney orthophosphoric diester phosphohydrolase, EC 3.1.4.1) with the aid of the substrate thymidine 5′-(5-bromo-4-chloroindol-3-yl) phosphate (I)[1] afforded a localization of the corresponding indigo *(vide infra)* in rat tissue consistent with the subcellular distribution pattern(s) of the enzyme as deduced with thymidine 5′-(*p*-nitrophenyl) phosphate (II)[2,3]. Moreover, a comparison of the kinetics of hydrolysis of the two substrates by *(Crotalus adamanteus)* venom established[4] I as an acceptable substrate for intracellular localization of phosphodiesterase I.

The intracellular distribution of phosphodiesterase II (spleen exonuclease) in rat liver with the aid of thymidine 3′-(*p*-nitrophenyl) phosphate (III) shows that the enzyme is localized largely in the soluble fraction of the lysosomes and exhibits the latency typical of lysosomal hydrolases[3]. Attempts to localize this exonuclease with thymidine 3′-(α-naphthyl) phosphate (IV) were abandoned when the substrate proved to be totally resistant to this enzyme[5]. By contrast, thymidine 3′-(5-bromo-4-chloroindol-3-yl) phosphate (V) produces a striking histochemical reaction at pH 4.8–5.2 (acetate buffer) in tissues of rat and mouse that was localized chiefly in cell nuclei and was particularly prominent in spleen[1]. The chromogenic reaction sequence is initiated by the enzymic release of 5-bromo-4-chloroindoxyl (VI) from V (or I) which is rapidly and irreversibly oxidized to the highly colored and essentially insoluble 5,5′-dibromo-4,4′-dichloroindigo (VII, *cf.* Fig. 1) at the sites of activity. At pH 5.9, nuclear staining with V was reduced markedly; instead the reaction occurred mainly in the cytoplasmic granules of reticulum cells in liver, spleen, gastrointestinal tract and lung.

The sharp disparity in the histochemical response elicited by the synthetic sub-

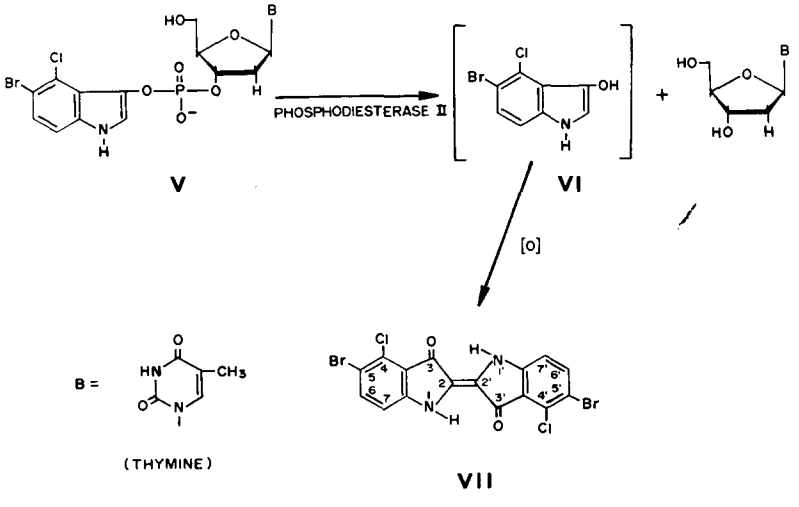

Fig. 1. Reaction sequence underlying the indiogenic principle.

strates IV and V prompted a comparison of the action of purified spleen exonuclease on III and V in an effort to ascertain the significance of our cytochemical findings[1].

MATERIALS AND METHODS

Substrates

Thymidine 3′-(*p*-nitrophenyl) phosphate was prepared according to the procedures of Turner and Khorana[6]. The synthesis of V has been described previously[7]. 3′-Phosphodeoxyribooligonucleotides were obtained from Miles Laboratory (Skokie, Ill., U.S.A.).

Enzyme preparation

Spleen exonuclease was purchased from Worthington (Freehold, N.J., U.S.A.). Stable enzyme preparations, free from phosphomonoesterase, adenosine deaminase, deoxyribonuclease and ribonuclease activities, were obtained according to the procedure of Bernardi and Bernardi[8]. The purified exonuclease retained 60% of its activity for as long as 3 months when stored at −15 °C. However, enzyme frozen for varying periods differs in specific activity so that rates in different experiments are not always comparable. A similar finding was recorded by Hilmoe[9].

A thermal inactivation curve[8] for the enzyme in 0.15 M acetate buffer (pH 5.0) containing 0.01 M EDTA showed, in accord with previous observations[8], that 50% inactivation occurred after heating at 58 °C for 20 min.

Enzyme assay

Spectrophotometric measurements were made with a Cary Model-11 recording spectrophotometer equipped with cell jackets thermostated by a Haake type F constant temperature bath. The jackets and the bath were joined in series to a Thermo–Cool heat exchanger. This arrangement provided a temperature regulation of ± 0.02 °C over the desired range (15–65 °C).

Exonuclease activity was assayed by measuring the liberation of mononucleotides from 3′-phosphodeoxyribooligonucleotides[8]. One activity unit is defined in this method as the amount of enzyme that liberates in 1 min mononucleotides having an absorbance at 260 nm equal to 1, under the conditions of the assay.

The specific activity was calculated by dividing the activity by the absorbance at 260 nm of the enzyme solution. Assays were performed using enzyme concentrations to obtain $A_{260\ nm}$ readings, corrected for blank, not higher than 2. Under these conditions, a linear relationship was obtained between enzyme concentration and mononucleotide liberation.

The chromatographic purification of (Worthington) spleen exonuclease derived from the procedures developed by Bernardi and Bernardi[8] led to protein of specific activity of 515. This value compares favorably with the optimum recorded value (545) (ref. 8). The latter in turn is comparable to the range of specific activities (995–2040) of the preparation by Razzell and Khorana[2]. In the comparison of activities as measured by the two procedures, one activity unit, as defined above, corresponds to the liberation of 1.8 μmoles of *p*-nitrophenol per h from III[8].

The assay of spleen exonuclease with III which follows the procedure of Razzell and Khorana[2], assumes an ε_{max} of 12 000 for *p*-nitrophenol. On this basis,

an increase in 0.20 absorbance unit corresponds to the hydrolysis of 0.1 μmole of substrate in the original mixture.

The rate of hydrolysis of V was followed by observing the formation of the indigo, VII, at 660 nm in a manner virtually identical with a previously described method[10]. The following procedure is considered typical. To 1.5 ml of a stock solution of acetate buffer (pH 5) which had been swept with N_2 (> 99.999% purity)[11] was added 1 ml of a deoxygenated spleen exonuclease solution of known concentration and 1 ml of a deoxygenated stock solution of V in 0.5% aqueous polyvinyl alcohol. The reaction mixture consisting of 3 μmoles of acetate buffer and enzyme was maintained at pH 5 under anaerobic conditions at 37 ± 0.02 °C.

The hydrolysis of V was terminated at the end of 30 min by submerging the reaction vessel in a water bath maintained at 100 °C to inactivate the enzyme[12]. Only in this manner was it possible to prepare solutions of the intermediate VI *(vide infra)*, of known strengths, *i.e.* free of oxidation product VII[10].

Shortly before spectrophotometric measurements were made (as described below) the indoxyl (VI) solution was withdrawn using a nitrogen-filled pipet which was emptied rapidly into 1 ml of oxygenated 0.5% polyvinyl alcohol. The reaction mixture was then quickly transferred to the cuvette.

The mixing and transfer required less than 15 s. The cuvette, after shaking, was inserted in the thermostated block of the spectrophotometer, and a reading of the absorbance was taken at 660 nm at various time intervals. This operation was repeated until sufficient data had been accumulated for an accurate determination of the oxidation rate. Although a short time elapsed in all such measurements, Cotson and Holt[10] have shown that there is sufficient oxygen in the saturated solution to maintain an adequate excess during the course of an experiment. In all measurements, the reference cell of the instrument contained a solution of the same concentration of polyvinyl alcohol and of salt and has the same pH as the reaction mixture. The pH of the latter solution was found to remain constant throughout the course of oxidation.

If an extinction coefficient (max.) for 5,5'-dibromo-4,4'-dichloroindigo (V) of 13 000 is assumed, an increase in absorbance of 0.3 corresponds to the liberation of 0.02 μmole of indigo or hydrolysis of 0.05 μmole of substrate in the original mixture[4]. A complete study of the non-enzymic oxidation step required a repetition of the previous procedure[4] over the pH range of 4–8.

Hydrolysis procedure

In essence, the procedure is identical with that described above for the oxidation step with the exception that the hydrolysis was effected under aerobic conditions. The reactants were elevated individually to the desired temperature by incubation in a constant temperature bath. In a typical run, as described above, the rate of appearance of VII was followed at 660 nm. The amount of substrate hydrolyzed could be calculated from the final concentration of indigo (VII).

The kinetic parameters, K_m and V, and the respective standard errors were evaluated with an IBM-360 computer using a program (Hyperb) described by Hanson *et al.*[13] which is based on a determination of maximum likelihood estimates for constants of the Michaelis–Menten equation by an iterative procedure.

RESULTS

The aerial oxidation of a number of mono- and dihaloindoxyls to the corresponding indigo has been studied kinetically over a pH range of 6–8.5 by Cotson and Holt[10]. The velocity constant for the oxidation of VI, which was generated from the hydrolysis of 5-bromo-4-chloroindol-3-yl acetate, was found to be $4 \cdot 10^{-3}\ s^{-1}$ at pH 8.0 and at 37 °C. However, the rate of oxidation was observed to decrease sharply below pH 7.4. Accordingly, it was first necessary to demonstrate that in the conversion of V to VII, the oxidation step (VI to VII) was not rate determining.

It was observed that the rates of oxidation were too rapid for accurate determinations above pH 8.2 and 37 °C. Measurements of $t_{1/2}$ for the oxidation of pH 7.6 and over the range of initial concentrations of 100 to 600 μM yielded a value of 82 $\pm$ 8 s (extrapolated) at 37 °C. By contrast, a $t_{1/2}$ of 68 $\pm$ 5 min was obtained for the enzymic conversion of V to VI under the same conditions. The rate data were extrapolated to 37 °C since the oxidation at this temperature is simply too rapid for accurate measurement by the technique employed in the present study. Since the

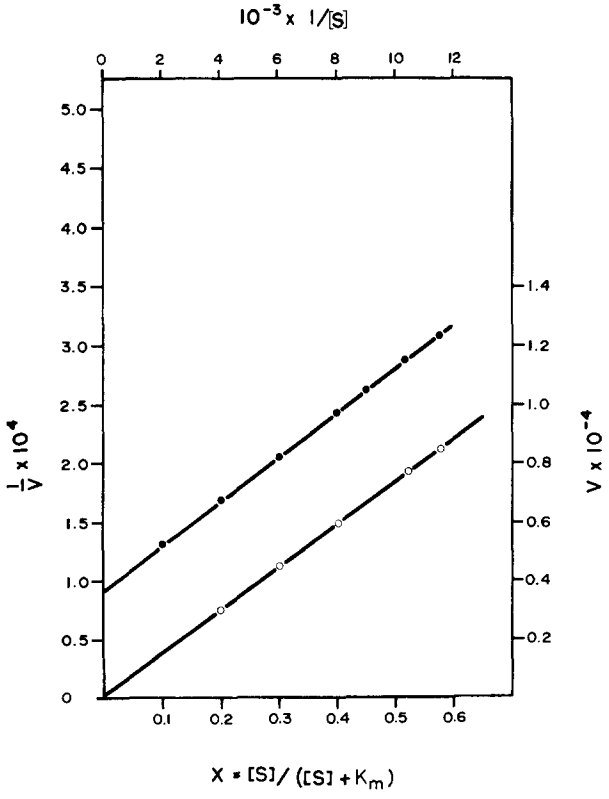

Fig. 2. Lineweaver–Burk plot[14] ($1/v$ *vs* $1/[S]$) for spleen exonuclease catalyzed hydrolysis of thymidine 3′-(5-bromo-4-chloroindol-3-yl) phosphate (●). Velocity, v, is expressed in μmoles/h per mg of protein; substrate concentration, $[S]$, in moles/l. Acetate buffer of pH 6.1. V = 10 100 $\pm$ 300 μmoles/h per mg protein, K_m = 278 μM. The lower curve (○) is derived from maximum likelihood estimates of v *vs* x, obtained by an iterative procedure[13]. The points are experimental and the line represents a weighted, leastsquare fit to data, obtained from program Hyperb.

activation energy of the oxidation is unlikely to vary over the relatively small temperature range (22–30 °C) the extrapolation appears justifiable.

It is apparent from this phase of study that the rate of oxidation (VI to VII) is significantly greater than the rate of hydrolysis of V by purified spleen phosphodiesterase at the corresponding pH. This result precludes the possibility that the (nonspecific) air oxidation is the slow step in the overall reaction. Therefore, the conversion of VI to VII has been ignored in the kinetics of the hydrolysis of V.

Determination of K_m and V

The rates of hydrolysis of the two diesters, III and V, by purified spleen exonuclease were studied at a fixed enzyme concentration and varying concentration of substrate. The data were plotted either as $1/v$ *vs* $1/[S]$ or as $[S]/v$ *vs* $[S]$ in order to search for regular deviations such as substrate or product activation or inhibition which might complicate the kinetics. No such phenomena were observed, and the data for both compounds gave good fits to straight lines when plotted according to either convention. A typical Lineweaver–Burk[14] plot is shown in Fig. 2. Values of K_m and V and the associated standard errors, as determined from computer analyses, are summarized in Table I.

TABLE I

THE KINETICS OF SPLEEN EXONUCLEASE-CATALYZED HYDROLYSIS OF THYMIDINE-3′-*p*-NITROPHENYL PHOSPHATE AND THYMIDINE 3′-(5-BROMO-4-CHLOROINDOL-3-YL) PHOSPHATE

The kinetic parameters K_m and V and the associated standard errors were determined from Hyperb (see Materials and Methods).

pH of acetate buffer	*Thymidine 3′-p-nitrophenyl phosphate**		*Thymidine 3′-(5-bromo-4-chloroindol-3-yl) phosphate**	
	$K_m \times 10^4$ (*M*)	*V (μmoles/h per mg protein)*	$K_m \times 10^4$ (*M*)	*V (μmoles/h per mg protein)*
4.8	2.52 ± 0.15	2380 ± 150	2.71 ± 0.10	6000 ± 300
5.2	2.58 ± 0.13	2500 ± 200	2.74 ± 0.12	8050 ± 250
5.7	2.54 ± 0.13	2740 ± 150	2.72 ± 0.15	9750 ± 300
6.1	2.62 ± 0.12	2800 ± 150	2.78 ± 0.15	11100 ± 300
6.7	2.59 ± 0.14	2600 ± 125	2.73 ± 0.13	10050 ± 400
7.2	2.52 ± 0.12	2330 ± 150	2.75 ± 0.15	7800 ± 200

* Spectrophotometric assay (see Materials and Methods).

Effect of pH

The rate of hydrolysis of III and V by spleen exonuclease (150 μg/ml in 1.4 mM acetate, pH 5.5) were 370 and 235 μmoles per h per mg of enzyme, respectively. However, the activity of spleen exonuclease at pH 4 toward these same substrates was about 25% of the cited values. This observation corroborates a previous report[8,15] that pH has a marked effect on the stability of dilute solutions of spleen exonuclease. The curves for the activity of purified preparations *vs* pH (Fig. 3) for the substrates III and V are similar, both exhibiting in succinate and phosphate buffers a pH optimum of 5.7; a higher value of 5.9 was found in acetate buffer. The presence of 0.02 M Mg^{2+} does not affect ostensibly the pH activity curves. A similar pH optimum

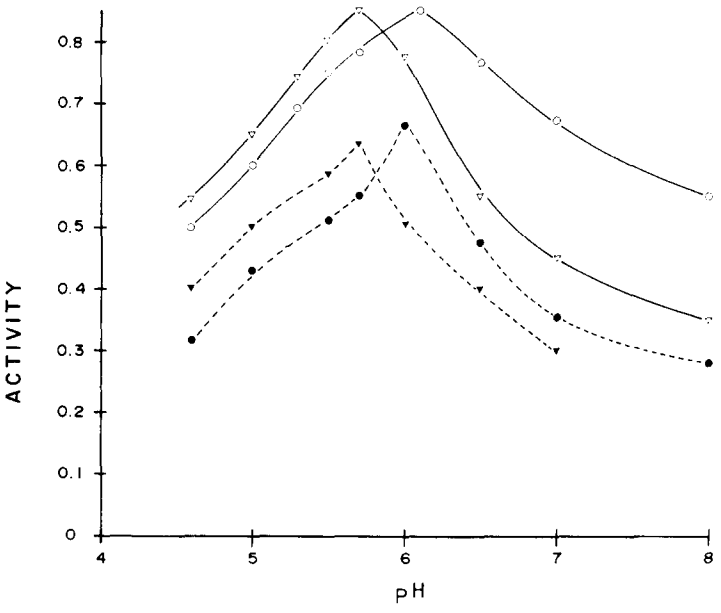

Fig. 3. Exonuclease activity on thymidine 3′-(*p*-nitrophenyl) phosphate; pH–activity ($A_{400\ nm}$) curves at 37 °C. Solvents were 0.15 M acetate (○—○); 0.15 M succinate (△—△). Exonuclease activity on thymidine 3′-(5-bromo-4-chloroindol-3-yl) phosphate: pH–activity ($A_{660\ nm}$) curves at 37 °C. Solvents were 0.15 M acetate (●---●), 0.15 M succinate (▲---▲).

has been reported previously[8,16]. EDTA enhances the enzymic activity without shifting the pH optimum[16].

DISCUSSION

The K_m ($0.25 \cdot 10^{-3}$ M, *cf.* Table I) with III for spleen exonuclease, purified according to the procedure of Bernardi and Bernardi[8], differs by a factor of about 10 from that ($3 \cdot 10^{-3}$ M) recorded by Razzell and Khorana[2]. This difference apparently stems from an improved method of purification which affords a stable enzyme free from several contaminating enzymes *(vide supra)*.

The rates of hydrolysis of III and V by spleen exonuclease at pH 5.5 are of the same order of magnitude (370 and 235 μmoles/h per mg protein, respectively). However, values of V for the two substrates show marked differences, particularly in the region of pH 6 (*cf.* Table I). The differences may in part be ascribed to the use of maximal initial reaction velocities, obtained *via* linear interpolation techniques, in best-fit Lineweaver–Burk plots. This consideration coupled with the fact that the kinetic parameters were determined from computer analysis would amplify differences that are not apparent in rate measurements. However, it is important to note that the scope of the present investigation precludes a firm conclusion relative to the sensitivities of the V for III and V over the pH range 4.8 to 7.2.

The present study provides the requisite corroborative evidence for cytochemical demonstration of spleen exonuclease of pH 5.9 with V. However, the

accuracy of localization* may be subject to question because of the tendency of the enzyme to leak from osmotically ruptured particles coupled with its true latency.

There remains the task of identifying the enzyme responsible for nuclear staining with V at pH 4.8. In this connection, it should be noted that thymidine 3'-(*p*-nitrophenyl) phosphate is hydrolyzed[17], albeit slowly, by acid deoxyribonuclease (EC 3.1.4.5) at pH 4.8. A study of the relative rates of hydrolysis of III and V purified by acid deoxyribonuclease is currently in progress.

In the course of preparation of this communication, Sierakowska and Shugar[18] reported that replacement of the *p*-nitrophenyl moiety by α-naphthyl in thymidine 3'-phosphate leads to a 150-fold decrease in the rate of hydrolysis by spleen phosphodiesterase II** and rat spleen supernatant. The observed histochemical resistance of IV *(vide supra)* could then be accounted for in terms of the significant disparity in the rates of hydrolysis of the two substrates.

The investigation of Sierakowska and Shugar also included a qualitative estimation of the "susceptibility" of thymidine 3'-(5-bromo-4-chloroindol-3-yl) phosphate (V) to purified phosphodiesterase II and spleen supernatant from which it was concluded that the reactivity of V to phosphodiesterase II approximates that of IV.

The results of the present study do not support this conclusion which, incidentally is drawn from kinetic information of questionable significance. The rates of hydrolysis of III and V (370 and 235 μmoles/h per mg, respectively), unlike III and IV, are first of all of the same order of magnitude. Moreover, the kinetic parameters (K_m and V, *cf.* Table I) derived in the comparison of III and V indicate that the indoxyl derivative (V) is an acceptable, if not in fact a superior substrate, for assay of phosphodiesterase II. Finally, the results of this investigation provide further amplification of the utility of the indigogenic principle in kinetic as well as cytochemical studies of hydrolytic enzymes.

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* Preliminary histochemical data (see ref. 1) were obtained with fresh frozen sections.
** It is not apparent whether the actual measurements utilized commercial or purified enzyme.

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