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SUBSTRATES FOR CYTOCHEMICAL DEMONSTRATION OF ENZYME ACTIVITY

IV. KINETICS OF THE HYDROLYSIS OF THYMIDINE-5'(5-BROMO-4-CHLOROINDOL-3-YL) PHOSPHATE BY SNAKE VENOM PHOSPHODIESTERASE*

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SUMMARY

1. A comparison of the action of venom phosphodiesterase I (orthophosphoric diester phosphohydrolase, EC 3.1.4.1) on the two synthetic substrates, thymidine 5'-*p*-nitrophenyl phosphate (II) and thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate (III), is described. The utility of the latter (III) is derived from a chromogenic reaction sequence that leads to the highly colored 5,5'-dibromo-4,4'-dichloroindigo (V) *via* aerobic oxidation of the intermediate 5-bromo-4-chloroindol-3-ol (IV), which is the product of enzymic hydrolysis. It is demonstrated that the rate of oxidation of IV to V is significantly greater than the enzymic hydrolysis of III by purified venom phosphodiesterase I. Therefore, the oxidation step is not rate determining.

2. The curves for activity of purified enzyme *vs.* pH for the two substrates similarly exhibit a broad optimum of 8.9 to 9.4. The kinetic parameters, K_m and v_{max}, as determined from computer analyses, are of the same order of magnitude for both substrates.

3. The effect of a relatively wide spectrum of inhibitors of enzyme activity and chelating agents is equally well reflected in both substrates. A similar situation prevails in heat inactivation studies and in the effect of the competitive inhibitor, $(pT)_3$.

4. It is concluded that thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate is an acceptable substrate for the intracellular localization of phosphodiesterase I.

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INTRODUCTION

It has been clearly demonstrated[1] that phosphodiesterase I (kidney orthophosphoric diester phosphohydrolase, EC 3.1.4.1) is analogous in general properties and in catalytic activity to the enzyme derived from snake venom and hog liver. The enzyme shows a specificity[2] for a nucleoside 5'-phosphoryl residue but is nonspecific with respect to both the nucleotide base and the substituent (R) attached to the phosphate moiety of the substrate. While the nature of R is not critical, it does have a great influence on the rate of hydrolysis[2].

Of the many synthetic derivatives of nucleoside 5'-phosphates prepared to date, none has received wider acceptance than thymidine 5'-*p*-nitrophenyl phosphate (II) for studies on the distribution of phosphodiesterase I in diverse materials as well as for detailed kinetic studies of the enzyme itself[3]. A recent report[4] from this laboratory described a procedure for the histochemical localization of phosphodiesterase I that utilized the synthetic substrate, thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate (III). The method is based on the enzymic release of 5-bromo-4-chloroindol-3-ol (5-bromo-4-chloroindoxyl, IV) which at the sites of activity is rapidly and irreversibly oxidized to the highly colored and relatively insoluble 5,5'-dibromo-4,4'-dichloroindigo (V, *cf.* Fig. 1). The histochemical findings were in accord with the intracellular localization of phosphodiesterase I as determined by cell fractionation techniques with II. The present communication describes the results of a comparison of the action of venom phosphodiesterase I on the same two substrates, and the findings lend credence to the conclusions reached in the histochemical investigation.

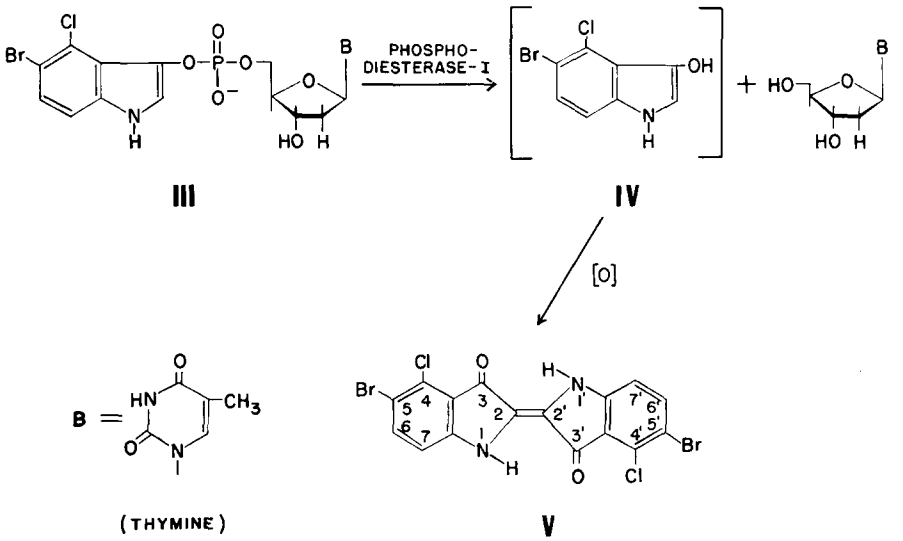


Fig. 1. Reaction sequence underlying the indigogenic principle.

MATERIALS AND METHODS

Substrates

The oligonucleotide $(pT)_3$* was provided by Dr. J. G. Moffatt, Syntex Institute for Molecular Biology, Palo Alto, Calif., whereas II was obtained from Calbiochem, Los Angeles (Lot No. 840173). The synthesis of III was modeled after that described by RAZZELL AND KHORANA[2] for II and will be described elsewhere together with related derivatives.

Enzyme preparation

Lyophilized *Crotalus adamanteus* venom was obtained commercially. Preparation of phosphodiesterase I, free from 5'-ribonucleotide phosphohydrolase (EC 3.1.3.5), was carried out by the procedure of KOERNER AND SINSHEIMER[5] which afforded a 60-fold purification over the crude venom. Enzyme preparations obtained on various occasions or frozen for varying periods differ in specific activity so that rates in different experiments are not always comparable. A similar finding was recorded by RAZZELL AND KHORANA[2].

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 $\pm$ 0.02° over the desired range (15–65°).

Hydrolyses of both II and III were performed in 3.5-ml cuvettes (10-mm path) containing 0.1 M Tris buffer, adjusted to a pH 8.9 with HCl, enzyme, and 0.5 mM substrate. The increase in optical absorbance due to liberation of *p*-nitrophenylate was measured at 400 nm. An increase in absorbance of 1.2 units is equivalent to the hydrolysis of 100 nmoles of substrate per ml (ref. 2). The assay of II is linear with time up to an absorbance of 1.1 and is nearly linear over a range of enzyme concentrations[6]. Thus 100, 250, 350 and 420 ng of phosphodiesterase I gave rates of 1.1, 2.6, 3.75, and 4.7 μmoles/h, respectively.

The rate of hydrolysis of III was followed by observing the formation of V at 660 nm. Beer–Lambert plots were utilized to ascertain the quantity of the indigo formed in the oxidation step. These plots, in turn, afforded a measure of the intermediate, IV, generated in the hydrolysis step. The procedure of COTSON AND HOLT[7] was adopted for the preparation of the plots which is based on the spectrophotometric measurement of the rate of appearance of V. When the oxidation is effected in aqueous media, the resultant indigo (dye) is deposited as a colloidal suspension, the stability of which is not suitable for optical measurement. The dye-sol can be stabilized by inclusion of 0.5% polyvinyl alcohol so that the optical property does not vary over several hours and certainly not for the duration of the kinetic measurements. It was found that the Beer–Lambert laws were obeyed by the polyvinyl alcohol-stabilized dye sol over the concentration range encountered. Accordingly, it was possible to utilize the optical absorbance directly in the calculation of the reaction rate of the

* The abbreviation is in accord with that adopted by *Biochim. Biophys. Acta*, 108 (1966) 1, for oligonucleotide nomenclature.

oxidation step. The following procedure is considered typical. To 2 ml of stock solution of Tris buffer (0.2 M, adjusted to pH 8.9 with HCl) which had previously been swept with (> 99.999% purity[8]) nitrogen, was added 1 ml of deoxygenated phosphodiesterase I solution of known concentration and 1 ml of a deoxygenated stock solution of III at 37 ± 0.02°. The stock solution was in turn prepared from deoxygenated 0.5% aqueous polyvinyl alcohol. The resulting reaction mixture consists of 0.1 M Tris, adjusted to pH 8.9 with HCl, enzyme, and 0.5 mM substrate under anaerobic conditions. The hydrolysis of III was terminated at the end of 1 h by submerging the reaction vessel in a water bath maintained at 100° to inactivate the enzyme[9]. Only in this manner was it possible to prepare solutions of the intermediate, IV (*vide infra*), of known strength, *i.e.*, free of oxidation product V (ref. 8).

Shortly before spectrophotometric measurements were made (as described below), the indoxyl (IV) solution was withdrawn using a nitrogen-filled pipet and 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 sec. 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[7] have shown that there was sufficient oxygen in the saturated solution to maintain an adequate excess during the course of the experiment. In all measurements, the reference cell of the instrument contained a solution of the same concentration of polyvinyl alcohol and of salt and had the same pH as the reaction mixture. The pH of the latter solution was found to remain constant throughout the course of the oxidation.

A complete study of the nonenzymic oxidation step required a repetition of the previous procedure over the pH range of 7.6 to 8.2 at temperatures of 15°, 18°, 23° and 26° in the presence and in absence of a number of various inhibitors.

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, 0.1 M Tris, adjusted to pH 8.9 with HCl, enzyme, and 0.5 mM substrate (III), were mixed, and the reaction mixture was transferred immediately to the spectrophotometer. The rate of appearance of V was followed at 660 nm. The amount of substrate hydrolyzed could be calculated from the final concentration of the indigo (V). Thus 100, 200, 350, and 420 ng of enzyme gave rates of 0.6, 1.8, 2.6, and 2.9 μmoles of substrate hydrolyzed per h, respectively.

The kinetic parameters, K_m and v_{max}, and the respective standard errors were evaluated with an IBM-360 computer using a program (Hyperb) described by HANSON *et al.*[10] which is based on a determination of maximum-likelihood estimates for the constants of the Michaelis–Menten equation by an iterative procedure.

RESULTS AND DISCUSSION

The aerial oxidation of a number of mono- and dihaloindoxyls to the corre-

sponding indigo has been studied kinetically over a pH range of 6 to 8.5 by COTSON AND HOLT[7]. The velocity constant for the oxidation of IV which was generated from the hydrolysis of 5-bromo-4-chloroindol-3-yl acetate was found to be $4 \cdot 10^{-3}$ sec^{-1} at pH 8.0 and at 37°. 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 III to V, the oxidation step (IV to V) was not rate determining.

It was observed that the rates of oxidation were too rapid for accurate determinations above pH 8.2 and at 37°. Measurements of $t_{1/2}$ for the oxidation at pH 7.6 and over the range of initial concentrations of 100 to 600 μM yielded a value of 68 $\pm$ 6 sec (extrapolated) at 37°. By contrast, a $t_{1/2}$ of 51 $\pm$ 4 min was obtained for the enzymic conversion of III to IV under the same conditions. The rate data were extrapolated to 37° since the oxidation at this temperature is simply too rapid for accurate measurement by the technique employed in the present study. Since the activation energy of the oxidation is unlikely to vary over the relatively small temperature range (15–26°), the extrapolation appears justifiable.

It is apparent from this phase of the study that the rate of oxidation (IV to V) is significantly greater than the rate of hydrolysis of III by purified venom phosphodiesterase I 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 IV to V has been ignored in the kinetics of the hydrolysis of III.

Determination of K_m and v_{max}

The rates of hydrolysis of the two diesters by a chromatographed preparation of *Crotalus adamenteus* venom were studied at fixed enzyme concentrations and varying concentrations 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[11] plot is shown in Fig. 2. Values of K_m and v_{max} and the associated standard errors, as determined from computer analyses, are summarized in Table I.

TABLE I

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

pH of Tris buffer	*Thymidine 5′-p-nitrophenyl phosphate**		*Thymidine-5′ (5-bromo-4-chloro-indol-3-yl) phosphate**	
	$K_m \times 10^4$** *(M)*	v_{max}** *(μmoles/h per mg protein)*	$K_m \times 10^4$** *(M)*	v_{max}** *(μmoles/h per mg protein)*
7.6	5.34 ± 0.21	9 800 ± 200	3.60 ± 0.14	7 300 ± 100
8.2	5.20 ± 0.19	22 700 ± 500	5.66 ± 0.28	18 600 ± 500
8.6	5.12 ± 0.18	30 900 ± 600	5.81 ± 0.18	20 600 ± 400
8.9	5.19 ± 0.17	34 400 ± 700	5.49 ± 0.15	22 900 ± 300
9.4	5.07 ± 0.21	32 900 ± 700	5.34 ± 0.09	21 800 ± 200
9.8	5.15 ± 0.14	32 000 ± 500	5.18 ± 0.14	20 900 ± 300

* Spectrophotometric assay (MATERIALS AND METHODS).

** The kinetic parameters K_m and v_{max} and the associated standard errors were determined from Hyperb (see MATERIALS AND METHODS).

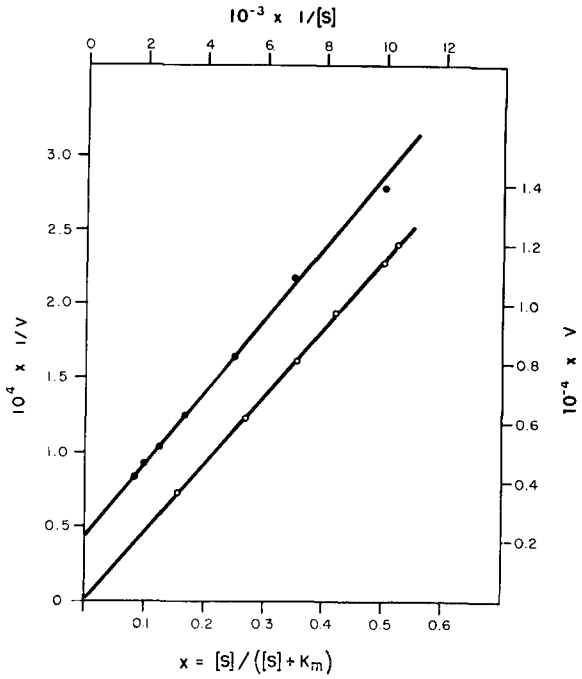


Fig. 2. Lineweaver–Burk plot[11] ($1/v$ *vs.* $1/[S]$) for phosphodiesterase I catalyzed hydrolysis of thymidine 5′-(5-bromo-4-chloroindol-3-yl) phosphate (●—●). Velocity, v, in μmoles/h per mg of protein; substrate concentration, $[S]$, in moles/l, and Tris buffer pH 8.9. $v_{max} = 22\,900 \pm 300$ μmoles/h per mg protein, $K_m = 549$ μM. The lower curve (○—○) is derived from maximum-likelihood estimates of v *vs.* x, obtained by an iterative procedure[10]. The points are experimental and the line represents a weighted, least-square fit to data, obtained from program Hyperb.

Effect of pH

The rates of hydrolysis of II and III by crude venom (200 μg/ml in 2 mM Tris, pH 8.9) were 580 and 385 μmoles/h per mg protein, respectively. However, the activity of venom at pH 6 toward these same substrates was only about 20% of the cited values. This observation corroborates a previous report[2] that pH has a marked effect on the stability of dilute solutions of the venom.

The curves for the activity of purified preparation *vs.* pH for the two substrates are similar, both exhibiting a relatively broad optimum of 8.9 to 9.4. A similar pH optimum has been reported previously[2].

Inhibition studies

RAZZELL AND KHORANA[2] have examined the effect of a relatively wide spectrum of inhibitors of the action of venom diesterase on uridine 5′-*p*-nitrophenyl phosphate It was of interest to extend these studies to II and III. However, it was first necessary to ascertain the effect of each inhibitor on the oxidation of the intermediate indoxyl IV to V in order to assess the true influence of the agent on enzymic activity. Using the procedure of COTSON AND HOLT[7] (*vide supra*), it was found that none of the inhibitors employed in this study exerted an effect on the oxidation rate over the pH range of 7.6 to 9.8.

TABLE II

A COMPARISON OF THE ACTION OF PHOSPHODIESTERASE I ON SOME NUCLEOSIDE 5'-ARYL PHOSPHATES IN THE PRESENCE OF REDUCING AGENTS

The system contained 0.5 mM substrate and 3 μg of phosphodiesterase I in 0.1 M Tris, pH 8.9, at 37°.

Additions	*Concn. (mM)*	*Thymidine 5'-p-nitrophenyl phosphate*		*Thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate*		*Uridine 5'-p-nitrophenyl phosphate**	
		Activity (μmoles/h)	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*
None		24.8	1.00	19.8	1.00	14.0	1.00
Cysteine	1	10.6	0.43	5.1	0.26	7.5	0.54
	3	7.8	0.32	3.4	0.17	3.9	0.28
	5	6.8	0.28	2.6	0.13	2.3	0.17
Glutathione	2	9.4	0.38	6.0	0.30	4.5	0.32
	5	5.4	0.22	3.4	0.17	2.2	0.16
Ascorbic acid	2	22.0	0.83	13.5	0.68	9.3	0.66
	5	14.4	0.58	7.7	0.39	6.4	0.46

* The data are that of RAZZELL AND KHORANA[2] which were obtained under comparable conditions.

It is apparent from Tables II and III that the magnitude of inhibition of phosphodiesterase I by both reducing agents and chelating agents, as determined with II and III, is comparable to that observed with uridine 5'-*p*-nitrophenyl phosphate. Moreover, the fact that the enzyme is only slightly stimulated by high concentrations of Mg^{2+} and Ba^{2+} is reflected in the activity of phosphodiesterase I toward the three mononucleotide esters (Table IV). The present study also confirms the previous finding[2]

TABLE III

A COMPARISON OF THE ACTION OF PHOSPHODIESTERASE I ON SOME NUCLEOSIDE 5'-ARYL PHOSPHATES IN THE PRESENCE OF CHELATING AGENTS

System as in Table II.

Additions	*Concn. (μM)*	*Thymidine 5'-p-nitrophenyl phosphate*		*Thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate*		*Uridine 5'-p-nitrophenyl phosphate**	
		Activity (μmoles/h)	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*
None		38.0	1.00	24.6	1.00	35.2	1.00
Sodium citrate	5000	36.8	0.95	23.8	0.97	33.1	0.94
EDTA	110	21.0	0.54	16.2	0.66	27.1	0.46
	260	12.4	0.32	9.6	0.39	6.7	0.19
	410	8.2	0.21	5.9	0.24	3.1	0.09
EDTA *plus* 2000 μM Mg^{2+}	410	20.2	0.52	13.5	0.55	14.6	0.41

* The data are that of RAZZELL AND KHORANA[2] which were obtained under comparable conditions.

TABLE IV

A COMPARISON OF THE ACTION OF PHOSPHODIESTERASE I ON SOME NUCLEOSIDE 5'-ARYL PHOSPHATES IN THE PRESENCE OF CATIONS

System as in Table II.

Additions	*Concn. (mM)*	*Thymidine 5'-p-nitrophenyl phosphate*		*Thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate*		*Uridine 5'-p-nitrophenyl phosphate**	
		Activity (μmoles/h)	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*
None		46.6	1.00	29.7	1.00	6.4	1.00
Magnesium acetate	0.4	49.8	1.07	32.6	1.10	8.4	1.31
	1.4	78.8	1.69	46.0	1.55	10.4	1.56
	10	96.0	2.06	59.4	2.00	13.3	2.08
	—**	46.6	1.00	29.7	1.00	6.4	1.00
Barium acetate	15	46.6	1.00	29.7	1.00	6.4	1.00

* The data are that of RAZZELL AND KHORANA[2] which were obtained under comparable conditions.

** Enzyme incubated with 0.04 M magnesium acetate, final Mg^{2+} concn., $8 \cdot 10^{-5}$ M, at pH 8.9 for 5–30 min, 37°.

that, while traces of salts and urea have little effect on the activity of phosphodiesterase I, large amounts of NaCl stimulate, but K_2HPO_4 and urea inhibit as shown in Table V.

Heat inactivation and competitive inhibition studies

It has been shown[1] that the activity of phosphodiesterase I from animal tissue toward both II and NAD^+ is a property of the same enzyme. This was established by

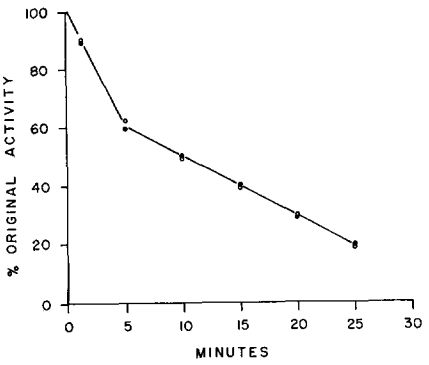
TABLE V

A COMPARISON OF THE ACTION OF PHOSPHODIESTERASE I ON SOME NUCLEOSIDE 5'-ARYL PHOSPHATES IN THE PRESENCE OF SALTS AND UREA

System as in Table II.

Additions	*Concn. (M)*	*Thymidine 5'-p-nitrophenyl phosphate*		*Thymidine 5'-(5-bromo-4-chloroindol-3-yl) phosphate*		*Uridine 5'-p-nitrophenyl phosphate**	
		Activity (μmoles/h)	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*	*Activity (μmoles/h)*	*Relative activity*
None		42.6	1.00	27.3	1.00	32.0	1.00
NaCl	0.10	48.0	1.13	28.6	1.05	38.4	1.20
	1.00	51.0	1.20	31.4	1.15	41.5	1.30
	2.00	52.8	1.24	32.2	1.18	41.5	1.30
LiCl	0.50	51.0	1.20	29.2	1.07	35.2	1.10
	2.00	33.2	0.78	21.8	0.80	16.2	0.51
K_2HPO_4	0.01	26.0	0.61	13.6	0.50	18.2	0.57
	0.025	11.8	0.28	6.0	0.22	7.4	0.23
Urea	2.40	39.2	0.92	26.0	0.95	27.2	0.85
	4.00	34.4	0.81	22.9	0.84	17.6	0.55

* The data are that of RAZZELL AND KHORANA[2] which were obtained under comparable conditions.



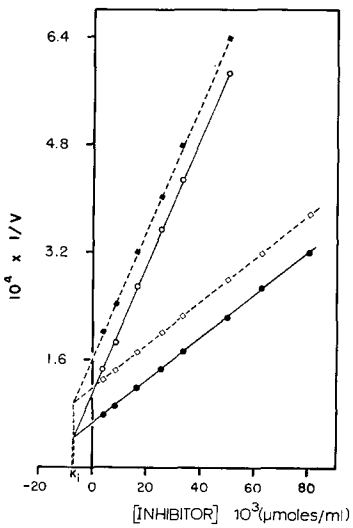


Fig. 3. Heat inactivation of venom phosphodiesterase I as determined with 5-bromo-4-chloro-3-indolyl-pT (○) and *p*-nitrophenyl-pT (●). Samples were heated in a water bath at 63°, then withdrawn and transferred to an ice bath at the indicated times. Each sample was assayed exactly 35 min after being removed from the heating bath.

Fig. 4. Competitive inhibition of thymidine 5′-*p*-nitrophenyl phosphate and thymidine 5′-(5-bromo-4-chloroindol-3-yl) phosphate by $(pT)_3$. Standard assay conditions were employed[2]; thymidine 5′-*p*-nitrophenyl phosphate at 200 μM (○—○), and 500 μM (●—●); thymidine 5′-(5-bromo-4-chloroindol-3-yl) phosphate at 200 μM (□— — —□) and 500 μM (■— — —■). Enzyme source, purified venom phosphodiesterase I.

TABLE VI

COMPETITIVE INHIBITION OF THE HYDROLYSIS OF THYMIDINE 5′-*p*-NITROPHENYL PHOSPHATE AND THYMIDINE 5′-(5-BROMO-4-CHLOROINDOL-3-YL) PHOSPHATE BY THE OLIGONUCLEOTIDE $(pT)_3$

System contained 0.5 mM substrate and required phosphodiesterase in 0.1 M Tris, pH 8.9 at 37°. Optical absorbance for thymidine oligonucleotide read at 267 nm.

Additions	*Concentration*		*Rate (p-nitrophenol, 400 nm)**		*Rate (5-bromo-4-chloro-indoxyl, 660 nm)***	
	Absorbance units/ml	*nmoles/ml*	*μmoles/h*	*%*	*μmoles/h*	*%*
None			3.00	100.0	3.00	100.0
$(pT)_3$	0.25	8.3	1.75	58.5	1.60	53.5
$(pT)_3$	0.50	16.0	1.17	39.3	1.27	42.3
Thymidine 3′-(5-bromo-4-chloroindol-3-yl) phosphate***		2500			0.42	14.0

* Concentration of phosphodiesterase I, 5 μg/ml.

** Concentration of phosphodiesterase I, 6 μg/ml.

*** The rate of hydrolysis of this agent was zero, in absence of thymidine 5′-(5-bromo-4-chloroindol-3-yl) phosphate.

measurement of the loss of kidney phosphodiesterase I activity during heat treatment as well as by competitive inhibition studies. Snake venom phosphodiesterase is similarly inactivated during heat treatment, as indicated by assays for activity (Fig. 3) on II and III.

Synthetic deoxyoligonucleotides with 3'-phosphate end groups, which are themselves slowly hydrolyzed, are potent inhibitors of venom phosphodiesterase[2]. It is apparent from Table VI that thymidine 3'-(5-bromo-4-chloroindol-3-yl) phosphate[4] is strongly inhibitory but is virtually resistant to hydrolysis. Fig. 4 indicates that the oligonucleotide, $(pT)_3$ is an equally effective competive inhibitor of the action of venom phosphodiesterase I on II and III. The K_i for $(pT)_3$ was determined according to the method of DIXON[12] in conjunction with the assay conditions of RAZZELL AND KHORANA[2].

The present data on competitive inhibition of hydrolysis of one substrate by another together with the heat inactivation studies lend additional support to the conclusion that there appears to be no difference in catalytic or general properties between venom and tissue phosphodiesterase I. Moreover, the results reported here corroborate the observation that phosphodiesterase I is but little affected by the substituent attached to the 5'-phosphate residue of the substrate. Finally, it is apparent that III is an acceptable substrate for biochemical as well as histochemical studies of the intracellular localization of the enzyme.

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