Fluorescence Study of Substrate Binding to Carboxypeptidase A*

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ABSTRACT: The kinetics of the enzymatic hydrolysis of β-carbonaphthoxy-L-phenylalanine (CNPhe) by carboxypeptidase A (CPA) was followed by observing the changes in the substrate and the product (β-naphtholate ion) fluorescence intensities.

The Michaelis constant and the catalytic constant were found to have the values $K_m = 5.5 \times 10^{-5}$ M and $k_3 = 6.0 \times 10^{-2}$ min^{-1}, respectively, at −2°. The amount of enzyme–substrate complex formed under conditions of enzymatic activity could also be directly measured from the decrease of substrate fluorescence in the presence of enzyme. Fair agreement between the data from kinetic studies and from direct fluorescence observations was achieved. Model studies indicate that of the various functional groups found near the active site of CPA only tyrosine is able to quench our substrate (CNPhe) fluorescence. This fact can be used to limit the number of possible substrate configurations with respect to the active site.

The structure and active site of carboxypeptidase A have recently been determined to a high degree of accuracy by sequence studies (Neurath *et al.*, 1970) and X-ray crystallography (Lipscomb *et al.*, 1970). On the other hand, the kinetics of its catalytic process has been investigated in great detail and mechanisms for its action have been proposed (Vallee *et al.*, 1970). It would now be important to find out exactly what interactions at the active site–substrate complex are involved in the catalytic step. This question could be studied profitably by using a substrate whose spectral properties are influenced by its being bound to the catalytic site of the enzyme (Stryer, 1968). In the case of carboxypeptidase A, a suitable substrate is β-carbonaphthoxy-L-phenylalanine (Ravin and Seligman, 1951; Wolf and Seligman, 1951) whose absorption and fluorescence spectra lie in a range unobstructed by the presence of the protein. In the present study we have tried to correlate the binding of this substrate as obtained from kinetic data to the change in its fluorescence intensity while the enzymatic process goes on. In the case of carboxypeptidase, where the overall topology of the active site is known, fluorescence studies can help us to pinpoint the actual structural relationship between the active site and substrate. With the aid of molecular models we were thus able to propose some of the specific interactions at the active site which, to our mind, are responsible for the decrease of substrate fluorescence.

Methods and Experimental Section

Carboxypeptidase A (CPA)[1] was obtained from Worthington Biochemical Corp. (enzymatic activity 55 units/mg). β-Carbonaphthoxy-L-phenylalanine (CNPhe) was a Fluka (Switzerland) purissimum grade product. All other chemicals were of analytical reagent grade. Triply distilled water was used throughout.

Solutions. The enzyme solution was prepared by centrifuging the original suspension, washing the crystals three times with water at 0°, and dissolving them in 2 M sodium acetate. The solution was very slowly filtered through a sintered-glass filter at 0° so as to avoid froth. The enzymatic activity of the solution was checked by measuring the rate of hippuryl-L-phenylalanine hydrolysis under standard conditions by the ninhydrin reaction (Cooking and Yemn, 1954). The enzyme concentration was determined from the optical density of the solution by assuming a value for the absorption coefficient of $\epsilon^{CPA}_{278\,nm}$ of 6.4×10^4 l./mole cm.

The substrate was dissolved in 0.05 M Tris buffer (pH 7.6). Fresh solutions were prepared before each experiment so as to minimize the spontaneous hydrolysis of the substrate. The concentration was again determined spectrophotometrically. A value of $\epsilon^{Phe}_{317\,nm}$ of 335 l./mole cm was used. Equal volumes of enzyme and substrate solutions were mixed at the beginning of the reaction.

Fluorescence was measured at right angles to the incident light in an apparatus described previously (Feitelson, 1964). The solutions had usually an optical density <0.1. If higher optical densities had to be used the results were corrected for inner filter effects.

Absorption spectra were measured with a Cary 14 spectrophotometer in 1-cm optical path quartz cells.

Kinetic measurements of the enzymatic reaction were carried out at −2°.

The substrate concentration was 1.5×10^{-5}–3.0×10^{-4} M and the enzyme concentration was in the range of 8.7×10^{-7}–2.2×10^{-6} M. At these concentrations the hydrolysis of substrate was found to be linear in enzyme concentration. The initial velocities of the enzymatic reaction were measured by following the increase in product (naphtholate) fluorescence intensity at λ 410 nm. The exciting wavelength was λ 317 nm.

The quenching of substrate fluorescence by the enzyme was also determined at −2°. In these experiments the substrate concentration was 1.5×10^{-5}–1.0×10^{-4} M and the enzyme concentration 3.0×10^{-5}–1.0×10^{-4} M. The change in fluorescence intensity as a function of time was followed both for the product (at λ 410 nm) and for the substrate (at λ 335 nm).

Results

The absorption and uncorrected fluorescence spectra of the substrate and product of the enzymatic reaction are presented in Figure 1. It is seen that in aqueous solution both the fluo-

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[1] Abbreviations used are: CPA, carboxypeptidase A; CNPhe, β-carbonaphthoxy-L-phenylalanine.

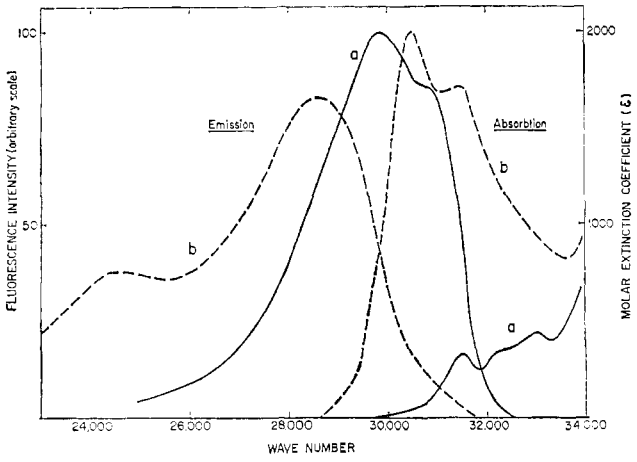

FIGURE 1: Absorption and fluorescence (uncorrected for instrument response) spectra of β-carbonaphthoxy-L-phenylalanine (curves a) and of β-naphthol (curves b).

rescence of naphthol (ROH*) and of the dissociated naphtholate ion (RO^{-}*) is observed. This is due to the fact that the naphthol molecule in the excited state is a much stronger acid (pK^* = 2.8) than in its ground state (pK = 9.23). At our experimental pH of 7.6, the dissociation (Weller, 1961)

$$ROH^* \cdot H_2O \underset{II}{\overset{I}{\rightleftharpoons}} RO^{-*} + H_3O^+ \qquad (1)$$

should almost entirely proceed to the right but, because of the comparatively slow forward reaction I, equilibrium is not fully established during the lifetime of the excited state of ROH* and both the fluorescence of ROH* and of RO^{-}* are observed. Weak acid anions which act as general bases in the Brønsted sense catalyze the dissociation reaction in eq 1 and thus help in establishing the equilibrium. We therefore used sodium acetate instead of the usual NaCl to provide the necessary ionic strength for the enzymatic reaction and thereby also transformed most of the free naphthol produced into naphtholate ions whose fluorescence is conveniently measured at 410 nm and gives a measure of the product concentration.

Enzymatic kinetics in which no back-reactions or inhibitory effects are observed are very often described by a Michaelis–Menten mechanism

$$E + S \underset{2}{\overset{1}{\rightleftharpoons}} ES \xrightarrow{3} Pr$$

in which a steady state with respect to the enzyme–substrate complex ES is assumed to be established. By plotting the kinetic data according to the well-known reciprocal Lineweaver–Burk (1934) plots (Figure 2), both the Michaelis constant

$$K_m = \frac{k_2 + k_3}{k_1} = \frac{[E][S]}{[ES]}$$

and the catalytic constant k_3 can be obtained. Since the two plots emphasize different regions of substrate concentration, the straight line was drawn in a way so that both yielded the same value of K_m; the least-square deviation was calculated. For a determination of k_3 the enzyme concentration is needed in addition to the slopes and intercepts of the above plots.

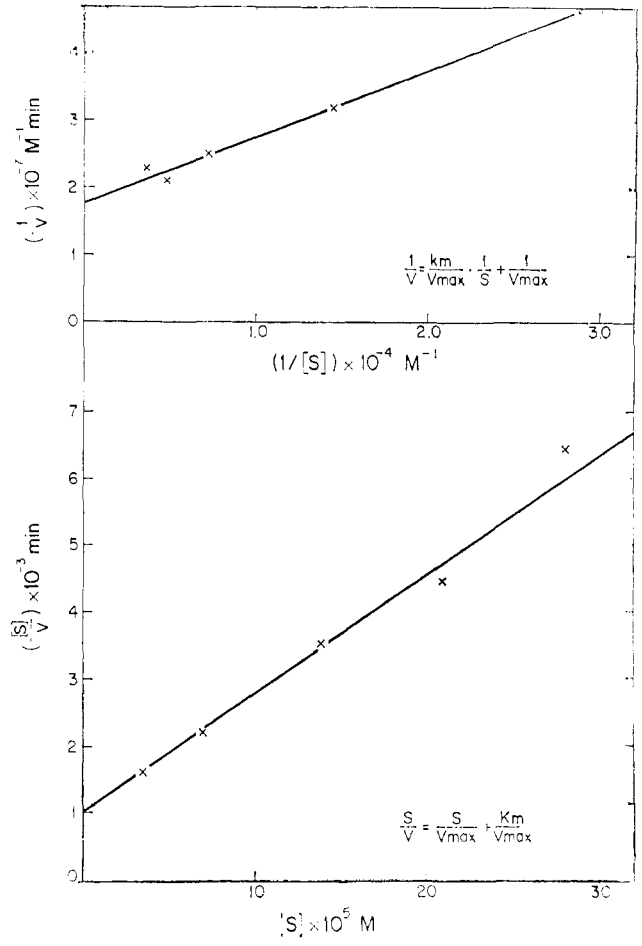

FIGURE 2: Lineweaver–Burk plots of β-carbonaphthoxy-L-phenylalanine hydrolysis by carboxypepetidase A at −2°.

Since this concentration is not accurately known to us the error in k_3 is somewhat greater than in K_m. We found K_m = (5.5 ± 0.8) × 10^{-5} mole/l. at −2°. At room temperature it was found to be K_m = (9.3 ± 0.7) × 10^{-4} mole/l. The catalytic constant k_3 = (6.0 ± 1.8) × 10^{-2} min^{-1} at −2°.

In the presence of comparatively large enzyme concentrations the fluorescence intensity of the substrate was found to decrease. In order to estimate the degree of this quenching effect the substrate fluorescence at 335 nm (see Figure 1) was measured. At this wavelength β-naphthol, which is produced in our experiments during the enzymatic process, has a comparatively low fluorescence. The reading at 335 nm can therefore be corrected for the naphthol emission by subtracting its value at λ 335 nm

$$F_{335}^{s} = F_{335} - F_{410}^{p}(f_{335}/f_{410})^{p} \qquad (2)$$

where F_λ is the fluorescence intensity at a specified wavelength and the superscripts s and p indicate the substrate and product, respectively. F_{335} is the fluorescence intensity measured. The quantity f_{335}/f_{410} is the ratio of naphthol emission at 335 and 410 nm. The fluorescence intensity at 410 nm is a measure of the product concentration, [P], in solution. The total concentration of the substrate present at time t in our system, $[S]_t$, is therefore obtained from the initial substrate concentration, $[S]_0$, and from $[P]_t$

$$[S]_t = [S]_0 - [P]_t \qquad (3)$$

TABLE I: Quenching of Substrate (CNPhe) Fluorescence by Enzyme (CPA) and Enzyme–Substrate Binding.

Enzyme Concn (M)	Substrate Concn (M)	Deg (%) of Fluorescence Quenching	% E–S Binding Calcd from Eq 7	% E–S Binding Calcd from Eq 6
3.18×10^{-5}	1.65×10^{-5}	29.5 ± 3	32.5 ± 1.7	32.5 ± 1.7
5.95×10^{-5}	5.0×10^{-5}	44.5 ± 2.5	41.4 ± 1.6	43.9 ± 1.6
1.02×10^{-4}	9.5×10^{-5}	56.8 ± 2.9	49.9 ± 1.6	52 ± 1.5

From our knowledge of the concentration dependence of the pure substrate fluorescence we calculate its value which should be expected when not quenched by the enzyme. The degree of substrate fluorescence quenching by the enzyme is then given by $(F_0^s - F_e^s)/F_0^s$, where F_0^s indicates the substrate fluorescence in the absence and F_e^s in the presence of enzyme. Columns 1–3 of Table I show the degree of quenching observed at various enzyme and substrate concentrations. These data are obtained from fluorescence measurements which were extrapolated to time zero, *i.e.*, the time of mixing enzyme and substrate at −2°.

As described in the Discussion we attribute the quenching of substrate fluorescence by the enzyme to the formation of an enzyme–substrate complex. In order to find which functional groups at the active site of the enzyme might be responsible for this quenching the influence of a variety of model substances on the substrate fluorescence in aqueous solution was tested. The reciprocal relative fluorescence of the substrate was plotted against concentration of quenching substance yielding the well-known Stern–Volmer constant (Stern and Volmer, 1919)[2] as a measure of quenching power of the particular substance. Sodium acetate (COO^-), dimethylacetamide ($CONH_2$), arginine, glycylglycine, phenylalanine, imidazole, histidine, and Zn^{2+} ions in the presence and absence of histidine all did not influence the fluorescence of CNPhe. Their Stern–Volmer constants k_{sv} had a value of less than 0.5 M^{-1}. *p*-Cresol, a model for the sparingly soluble tyrosine, and also L-tryptophan did quench the fluorescence of the substrate effectively. Their quenching constants at 15° were k_{sv}(cresol) = 89 ± M^{-1} and k_{sv}(Trp) = 67 ± 3 M^{-1} (Figure 3).

Discussion

We assume that at low enzyme and substrate concentrations the hydrolysis of β-carbonaphthoxy-L-phenylalanine by carboxypeptidase A proceeds by a Michaelis–Menten-like mechanism. The Michaelis constant expresses the ratio of the free enzyme and substrate concentrations to that of the bound ES complex in the system. If we know the initial enzyme and substrate concentrations $[E]_0$ and $[S]_0$ and the concentration of the product at time t, $[P]_t$, we can, with the aid of the value of K_m, calculate the amount of enzyme–substrate complex present in the steady state. On the other hand, if the fluorescence of the substrate is influenced by the fact of its being adsorbed at the active site in the form of an enzyme–substrate complex then the fluorescence measurements should yield an experimental means of determining the degree of substrate binding. We assume that the decrease in our substrate fluorescence in the presence of the enzyme can be wholly attributed to the formation of a nonfluorescent complex between them The total concentration of the substrate at time t, $[S]_t$, is obtained from the initial substrate concentration and the amount of product formed at time t (see eq 3). The fraction of $[S]_t$ which is bound in the form of the enzyme–substrate complex is given by $[S]_t$ multiplied by the ratio which expressed the degree of substrate quenching by the enzyme

$$[ES]_t = [S]_t(F_0^s - F_e^s)/F_0^s \quad (4)$$

The concentration of ES, the complex, as found by use of eq 4, is shown in curve c of Figure 4. The experiments were conducted at −2° since at this temperature we could still measure binding under conditions where the enzymatic process takes

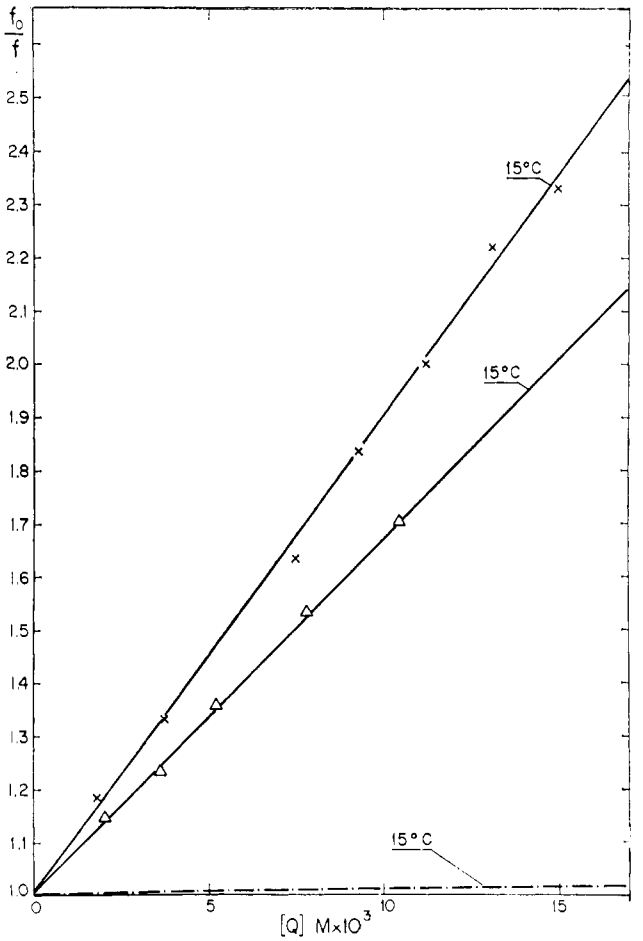

FIGURE 3: Stern–Volmer plots of β-carbonaphthoxy-L-phenylalanine quenching by *p*-cresol (×), L-tryptophan (Δ), and imidazole (broken line).

[2] $F^0/F = 1 + k_{sv}[Q]$; F^0/F is ratio of fluorescence intensity in absence and in presence of quenching agent Q.

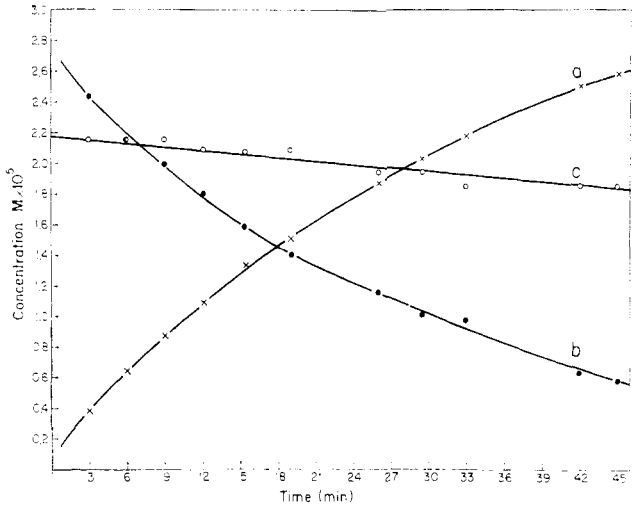

FIGURE 4: Progress of enzymatic hydrolysis of β-carbonaphthoxy-L-phenylalanine: (a) increase in naphthol concentration, (b) decrease of CNPhe concentration, and (c) concentration of enzyme–substrate complex as measured from fluorescence data.

calculated values is satisfactory and we therefore feel that it is permissible to use the fluorescence data in estimating substrate binding to the enzyme.

Figure 4 shows the changes in substrate and product concentrations, as well as in the concentration of the enzyme–substrate complex ES, during the hydrolytic process. It must be pointed out, however, that as the catalytic reaction progresses the agreement between observed and calculated concentrations of ES becomes worse. The degree of binding as measured by fluorescence quenching is larger than anticipated from K_m (eq 6) for comparable substrate and product concentrations. It seems therefore that the accumulation of products has an influence on the reaction and so we feel justified in using our enzyme–substrate binding data only for the very beginning of the catalytic process.

The results of our experiments with model substances indicate that of the various reactive groups present at the active site of the enzyme only Tyr-198 or the flexible Tyr-248 seem capable of quenching effectively the substrate fluorescence. This means that at least one of these residues must be located

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_2 + k_3)[ES] \tag{5}$$

$$[ES] = \frac{[E]_0 + [S]_0 + K_m - \sqrt{([E]_0 + [S]_0 + K_m)^2 - 4\left([E]_0[S]_0 - \frac{1}{k_1}\frac{d[ES]}{dt}\right)}}{2} \tag{6}$$

$$[ES]_{st,st} = \frac{[E]_0 + [S]_0 + K_m - \sqrt{([E]_0 + [S]_0 + K_m)^2 - 4[E]_0[S]_0}}{2} \tag{7}$$

place. On the other hand, the reaction was slowed down sufficiently so that we were able to follow conveniently the changes in reactant concentration. It also happens that because of the temperature dependence of K_m a more favorable complex to substrate ratio [ES]/[S] can be attained at a lower temperature.

In order to obtain a system where a significant fraction of the substrate is bound to the enzyme, much higher enzyme concentrations than usual in kinetic investigations have to be employed. However, the above-mentioned steady-state condition, which is assumed to exist in enzyme kinetics, requires a low enzyme–substrate complex concentration [ES] relative to that of the free enzyme and substrate. It follows that in our binding experiments, where an appreciable fraction of the substrate fluorescence is quenched, the reaction cannot proceed under steady-state conditions.

The differential equation describing the change in ES concentration with time (eq 5) cannot be equated to zero. However, if we solve it for [ES] we obtain eq 6, which differs from the corresponding value for steady-state conditions (eq 7) only by the correction term $4[(1/k_1)(d[ES]/dt)]$ under the square-root sign. Since $K_m = (k_2 + k_3)/k_1$, we see that this term will attain its maximal value if $k_2 \gg k_3$, when it becomes $4[(K_m/k_3)\ (d[ES]/dt)]$. The initial slope of [ES] *vs.* t is rather small, as seen from Figure 4, and we therefore felt that its value might be used in the correction term when calculating the substrate binding, *i.e.*, the concentration of the enzyme–substrate complex, by eq 6. Columns 3–5 of Table I show the values for substrate binding as obtained from the quenching of substrate fluorescence by the enzyme as well as the calculated results and the influence of the correction term.

We see that the agreement between the observed and the

in the vicinity of the naphthol ring of our substrate when the latter is bound to the enzyme. Furthermore for efficient quenching to occur it can be assumed that the tyrosine phenol ring would lie parallel to the aromatic system of our substrate. The X-ray studies determine roughly the position of the substrate at the active site and indicate which of various functional groups might be of importance in the hydrolytic process. We think that the fluorescence measurements described here add an additional limiting condition on the possible situation of the bound substrate. They might thus help in removing some of the ambiguity which still remains in the positioning of functional groups during the catalysis of carboxypeptidase A.

References

Cooking, E. C., and Yemn, E. W. (1954), *Biochem. J. 58*, xii.

Feitelson, J. (1964), *J. Phys. Chem. 68*, 391.

Lineweaver, H., and Burk, D. J. (1934), *J. Amer. Chem. Soc. 56*, 658.

Lipscomb, W. N., Reek, G. N., Hartsuck, J. A., Quiocho, F. A., and Bethge, P. H. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 177.

Neurath, H., Bradshaw, R. A., Petra, P. H., and Walsh, K. A. (1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 159.

Ravin, H. A., and Seligman, A. M. (1951), *J. Biol. Chem. 190*, 391.

Stern, O., and Volmer, M. (1919), *Phys. Z. 20*, 183.

Stryer, L. (1968), *Science 162*, 526.

Vallee, B. L., Riordan, J. F., Auld, D. S., and Latt, S. A.

(1970), *Phil. Trans. Roy. Soc. London, Ser. B 257*, 215.
Weller, A. (1961), *Progr. Reaction Kinetics 1*, 189.
Wolf, G., and Seligman, A. M. (1951), *J. Amer. Chem. Soc. 73*, 2080.

Multiple Cyclic Nucleotide Phosphodiesterase Activities from Rat Brain*

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ABSTRACT: A method has been developed to obtain good yields of cyclic nucleotide phosphodiesterase from rat brain cortex using sonication and high-speed centrifugation. The enzymatic hydrolysis of cyclic 3′,5′-adenosine monophosphate in a 20,000*g* supernatant fraction displayed anomalous kinetics indicative of two apparent dissociation constants. A theoretical basis for this kinetic disparity is discussed. Agarose gel filtration of the 20,000*g* supernatant indicated the presence of three active fractions: (1) an exclusion peak, (2) a high molecular weight fraction with a low affinity for cyclic 3′,5′-adenosine monophosphate (apparent Michaelis constant 1×10^{-4} M), and (3) a lower molecular weight fraction with a high affinity for cyclic 3′,5′-adenosine monophosphate (apparent Michaelis constant 5×10^{-6} M). Only in the high molecular weight fraction was there hydrolysis of cyclic 3′,5′-guanosine monophosphate. Competition studies support the hypothesis that the high molecular weight form of cyclic 3′,5′-adenosine monophosphate phosphodiesterase is actually a cyclic 3′,5′-guanosine monophosphate phosphodiesterase.

Cyclic 3′,5′-adenosine monophosphate phosphodiesterase was first studied in preparations from heart (Butcher and Sutherland, 1962). Further investigations of phosphodiesterase activity in heart (Nair, 1966), brain (Cheung, 1967, 1969; Williams, 1970), liver (Menahan *et al.*, 1969), fat cell (Hepp *et al.*, 1969), and frog erythrocytes (Rosen, 1970) have been reported, but extensive purification has not yet been achieved for any of these tissues. Published preparations of the enzyme which have included ammonium sulfate precipitation, ion exchange chromatography, or other methods of purification result in extremely low yields of the original activity from the tissue under investigation.

Interest in phosphodiesterase stems from the important position that this enzyme could play in controlling the levels of cyclic 3′,5′-adenosine monophosphate (cyclic AMP[1]) in various tissues and therefore its potential role in hormonal mechanisms. Clarification of this possibility requires an analysis of the properties of the enzyme. This analysis was unobtainable until recently because of low yields on preparation and the use of assay procedures insensitive to the low levels of cyclic AMP that are physiologically operative. Our initial investigation of the properties of cyclic AMP phosphodiesterase confirmed the observation of the anomalous kinetic behavior of the enzyme in homogenates of muscle (Kemp and Appleman, 1966), kidney (Senft *et al.*, 1968), brain (Brooker *et al.*, 1968), and adipose tissue (Blecher *et al.*, 1968). This investigation represents a detailed examination of this problem using a simple and sensitive assay procedure developed by a modification of the method of Kemp and Krebs (Delange *et al.*, 1968) and Brooker (Brooker *et al.*, 1968). The use of rat brain cortex for this investigation was prompted by the relatively high specific activity of phosphodiesterase in this tissue and the extensive information pertaining to subcellular localization of the enzyme (Breckenridge and Johnston, 1969; DeRobertis *et al.*, 1967). A preliminary report of this work has been presented (Thompson and Appleman, 1970).

Methods

Materials. ^{3}H-Labeled cyclic 3′,5′-adenosine monophosphate (specific activity 4.86 Ci/mmole) was obtained from New England Nuclear and purified by thin-layer chromatography on cellulose developed with 2-propanol–NH_4OH–H_2O (7:1:2, v/v). ^{3}H-Labeled cyclic guanosine monophosphate (specific activity 4.47 Ci/mmole) was obtained from New England Nuclear. Cyclic AMP and cyclic GMP were obtained from Schwarz and Calbiochem, respectively, and not further purified; 6% agarose (Bio-Rad Agarose A-5m) was equilibrated in 50 mM Tris-acetate and 3.75 mM 2-mercaptoethanol at pH 6.0 prior to use. Anion-exchange resin (Bio-Rad AG 1-X2, 200–400 mesh) was washed extensively in 0.5 N NaOH, 0.5 N HCl, and deionized water to a final pH of 5.0. Snake venom (*Ophiophagus hannah*, king cobra) was purchased from Sigma Chemical Co. All other chemicals and drugs were reagent grade or commercially available.

Preparation of Phosphodiesterase. Brains were removed from 200-g Sprague–Dawley rats killed by decapitation. Frozen brain tissue was not used since it yielded some inconsistent results. The cortex was homogenized for 1 min in eight volumes of 10.9% sucrose using a Sorval Omni-Mixer at maximum speed. All operations are performed at 4°.

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[1] Abbreviations used are: cyclic AMP, cyclic 3′,5′-adenosine monophosphate; cyclic GMP, cyclic 3′,5′-guanosine monophosphate; K_m, Michaelis–Menten constant; K_i, inhibition constant (Dixon plot); 5′-AMP, adenosine 5′-monophosphate.