(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*

W. Joseph Thompson and M. Michael Appleman†

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°.

* From the Departments of Biological Sciences and Biochemistry, University of Southern California, Los Angeles, California 90007. *Received August 31, 1970.* Supported by Public Health Service Grant AM-12684, National Institutes of Health Biomedical support grants, and the Diabetes Association of Southern California.

† To whom to address correspondence.

[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.

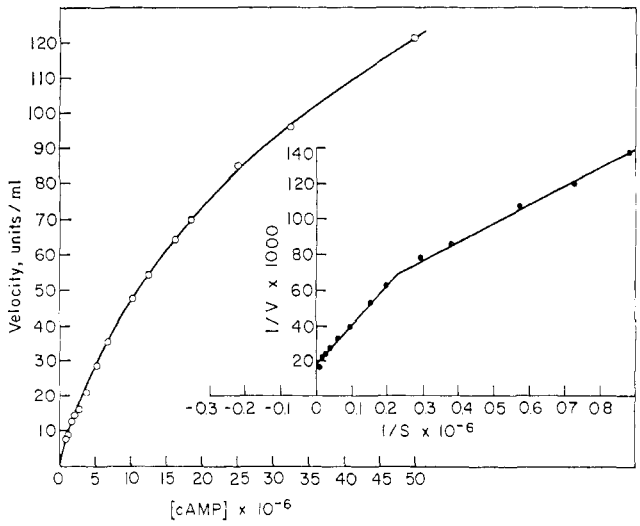

FIGURE 1: Kinetic plots of cyclic AMP hydrolysis by a rat brain sonicated supernatant.

TABLE I: Assay Procedure for Cyclic Adenosine Monophosphate Phosphodiesterase.

[³H]Cyclic 3′,5′-AMP $\xrightarrow[1]{\text{phosphodiesterase}}$ [³H]5′-AMP $\xrightarrow[2]{\text{snake venom}}$ [³H]Adenosine

Step 1: Phosphodiesterase Reaction
[³H]Cyclic adenosine monophosphate (200,000 cpm)
Cyclic adenosine monophosphate (1.25×10^{-7}–1.0×10^{-4} M)
$MgCl_2$ (0.005 M)
Tris-Cl (pH 8.0, 0.04 M)
2-Mercaptoethanol (0.00375 M) + enzyme in 0.4-ml final volume
10-min incubation at 30°, stopped by boiling 2.5 min

Step 2: Snake Venom Nucleotidase
Contents step 1 + 0.1 ml of snake venom (*Ophiophagus hannah*) (1 mg/ml)
10-min incubation at 30°, stopped by 1.0 ml of a 1:3 slurry Bio-Rad resin, AG1-X2, 200–400 mesh
Contents spun in clinical centrifuge and 0.5-ml alinuot counted by liquid scintillation

The homogenate could be centrifuged at 1000*g* and filtered through glass wool with no loss in activity, but this was not routinely necessary for brain tissues. The homogenate was sonicated in an ice bath with a Biosonic III at a setting of 40 for 15 min/30 ml in solution. This preparation was then adjusted to pH 6.0 with 1 M acetic acid while stirring over ice and centrifuged at 20,000*g* for 20 min. There was only a minor loss in activity at this point as compared to 50–60% losses if the preparation was not sonicated. This supernatant will be referred to as sonicated supernatant. This sonicated supernatant, the standard preparation of phosphodiesterase for this investigation, provides little purification over the homogenate, but it does permit studies of nearly 100% of the original activity.

Phosphodiesterase Assay. The assay for phosphodiesterase activity consists of a two step isotopic procedure as shown in Table I. Tritium-labeled cyclic AMP is hydrolyzed to 5′-AMP by phosphodiesterase under conditions shown in step 1. The 5′-AMP is then further hydrolyzed to adenosine by the nucleotidase in snake venom as shown in step 2. The anion-exchange resin binds all charged nucleotides and leaves [³H]adenosine as the only labeled compound to be counted. For kinetic analysis the concentration range is from 1.25×10^{-7} to 1×10^{-4} M. Activities termed low and high refer to assays at these limits of cyclic AMP concentrations. Assays for cyclic GMP hydrolysis are carried out by an identical procedure with the substitution of ³H-labeled cyclic GMP at concentrations of 2.5×10^{-7} to 4.54×10^{-5} M and total of 220,000 cpm/assay. Enzymes are diluted to provide linearity in an assay. Units of phosphodiesterase activity are in terms of picomoles of cyclic AMP or cyclic GMP hydrolyzed per minute per assay volume (0.4 ml).

Chromatography. Gel filtration was routinely carried out on Agarose A-5m columns of 2.2 × 170 cm dimensions. The flow rates for best separation did not exceed 12 ml/hr; fraction volumes were normally about 4 ml. The buffer conditions were 50 mM Tris-acetate (pH 6.0)–3.75 mM 2-mercaptoethanol. Column calibrations were run under the same conditions using Dextran 2000, human γ-globulin, and bovine hemoglobin; 8 ml of 12 mg/ml sonicated supernatant was routinely processed.

The activity in dilute fractions obtained from gel filtration was not stable, but if concentrated immediately using Amicon UM-2 ultrafiltration, the enzyme retained activity for several weeks at 4°. The individual concentrated fractions were then purified by gel filtration on Sephadex G-200 (1.5 × 90 cm columns) under the same buffer conditions as the Agarose gel filtration.

Results

Figure 1 shows the kinetic analysis of the sonicated supernatant fraction. Although the velocity *vs.* substrate plot shown in Figure 1 appeared superficially to follow classical Michaelis–Menten kinetics, the more sensitive Lineweaver–Burk double-reciprocal plot did not produce the expected linear results. Extrapolation of the linear portions of these plots yielded two apparent Michaelis constants for cyclic AMP, one of about 5×10^{-6} M and the other twenty times higher. This result was consistent with the reports of Brooker (Brooker *et al.*, 1968), Senft (Senft *et al.*, 1968), and Kemp (Kemp and Appleman, 1966).

The kinetic analysis of this system has proven to be difficult. This investigation has proceeded under the assumption that these anomalous double-reciprocal plots represent two enzyme systems with separate affinities for cyclic AMP rather than displaying the kinetics of one enzyme with multiple subunit sites of different apparent affinities.

Assuming that two independent enzyme systems of known Michaelis constant and maximum velocity are both operative and that each follows Michaelis–Menten kinetics, a theoretical curve can be mathematically synthesized. Figure 2 shows the types of Lineweaver–Burk plots that develop if the calculated individual velocities for the two enzymes with Michaelis constants similar to those of the cyclic AMP phosphodiesterase system are added at each substrate concentration. The four theoretical curves shown indicated the results obtained at four different ratios of maximum velocities of the two experimentally derived high and low plots. As can be seen from this figure and from the numerical values

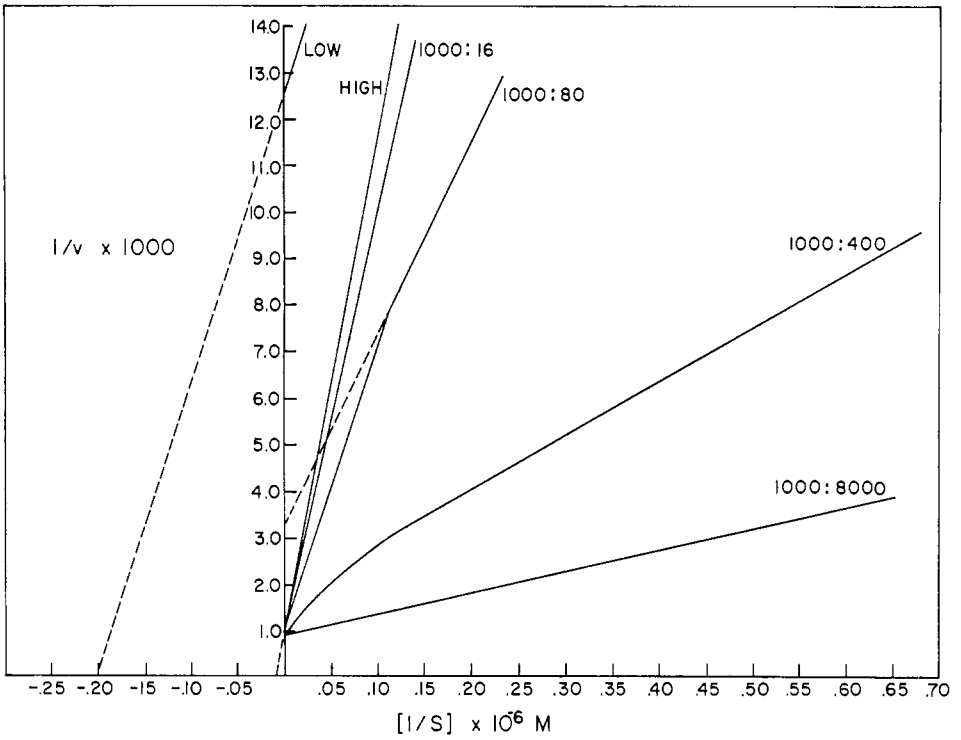

FIGURE 2: Calculated Lineweaver–Burk plots obtained by summation of the velocities for the individual enzymes acting on a common substrate both obeying Michaelis–Menten kinetics. Curves of each individual enzyme are included in addition to the four theoretical summation curves representing the plots obtained at the ratio (high K_m enzyme:low K_m enzyme) of maximum velocities of the individual enzymes. The individual enzyme characteristics are derived from experimental parameters of the rat brain sonicated supernatant. Slope and intercept calculations are by linear least-squares analysis.

TABLE II: Mathematical Analysis of a System of Two Michaelis–Menten Enzymes.[a]

	Constants[b]		Extrapolation from Summation Curves			
Curve in Fig. 2	V_{max_H}	V_{max_L}	V_{max_H}	V_{max_L}	$K_{m_H} \times 10^{-6}$ M	$K_{m_L} \times 10^{-6}$ M
High	1000		1000		104	
Low		80		80		5.10
1	1000	80	732	312	43.06	12.83
2	1000	16	856	360	77.5	28.6
3	1000	400	*c*	560	*c*	6.48
4[d]	1000	1000	*c*	1166	*c*	5.73
5	1000	1000		8196		5.17

[a] See text for explanation of procedures. [b] Michaelis constants used for calculation are: high $K_m = 1.04 \times 10^{-4}$ M and low $K_m = 5 \times 10^{-6}$ M. [c] No linear correlations are possible above concentration of 1×10^{-5} M. [d] Curve not shown in Figure 2.

obtained (Table II), there is very real danger that the observed kinetic constants obtained by extrapolation will differ greatly from those of the individual enzyme components of this system. The results of this type of kinetic analysis must therefore be interpreted as permitting the consideration of a particular multienzyme system as a possibility rather than confirming its existence. It must be emphasized that Figure 2 is only a mathematical analysis indicating that two enzymes operating independently can give a kinetic result similar to those observed in homogenates of brain. Physical evidence is necessary to confirm this hypothesis.

The results of the Agarose gel filtration are shown in Figure 3. There are three fractions with cyclic AMP phosphodiesterase activity: an exclusion peak, a high molecular weight fraction (approximately 400,000 molecular weight), and a low molecular fraction (approximately 200,000 molecular weight). Of these latter two, the higher molecular weight fraction displays greater activity at high concentrations of cyclic AMP while the lower molecular weight fraction has relatively greater activity at lower concentrations of cyclic AMP.

Note that only in the higher molecular weight region is there detectable hydrolysis of cyclic GMP at a concentration of 2.5×10^{-7} M. Resolution of these two molecular weight peaks is possible on Sephadex G-200 only by the use of the Agarose fractionated brain preparations and does not occur

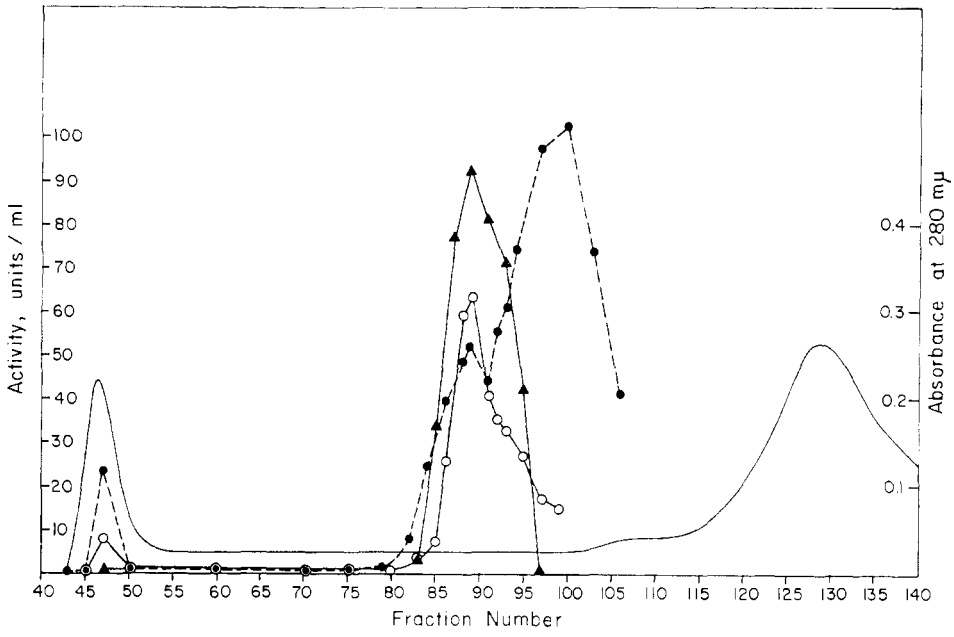

FIGURE 3: Six per cent Agarose gel filtration of a rat brain sonicated supernatant. (——) Absorbance at 280 mμ. Other expressed results are phosphodiesterase activities of various fractions assayed at differing cyclic AMP substrate concentrations of 0.125 μM (●----●; units × 10) and 100 μM (○——○; units × 0.5). These fractions were also assayed with cyclic GMP as substrate at a concentration of 0.25 μM (▲——▲; units × 1).

with the original sonicated supernatant probably caused by interaction with contaminating components of these brain preparations.

Figures 4 and 5 display the kinetics of the hydrolysis of cyclic AMP by the separated high and low molecular weight fractions. Figure 4 indicates that the higher molecular weight cyclic AMP phosphodiesterase activity obeys Michaelis–Menten kinetics, has a Michaelis constant of 1×10^{-4} M for cyclic AMP, and a Hill coefficient of 0.96 (Atkinson, 1966). This activity is completely excluded by Sephadex G-200 when concentrated and rechromatographed.

The kinetics of the pooled lower molecular weight fraction are shown in Figure 5. This fraction has an apparent Michaelis constant of 5×10^{-6} M for cyclic AMP and a Hill coefficient of 0.47 (Atkinson, 1966). Notice that in the higher concentration regions of the Lineweaver–Burk plot the points do not follow a straight line. This may be interpreted as indicative of negative cooperativity although it is not possible to completely rule out that this result may be due to contamination by the higher molecular weight form of phosphodiesterase. The theoretical portion of this discussion demonstrates that extreme caution should be exercised due to sensitivity of low molecular weight enzyme kinetics to high molecular weight enzyme contamination in the higher substrate regions. Additional purification and kinetic analysis of this lower molecular weight phosphodiesterase by the use of Sephadex G-200 have allowed almost complete separation of the low enzyme from the high molecular weight enzyme. This low molecular weight phosphodiesterase still appears to be a negatively cooperative enzyme.

It is interesting to note that when the Agarose gel filtration

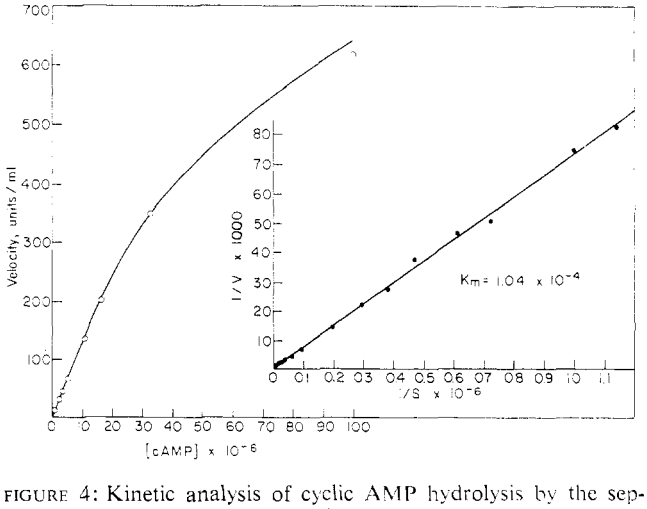

FIGURE 4: Kinetic analysis of cyclic AMP hydrolysis by the separated high molecular weight fraction.

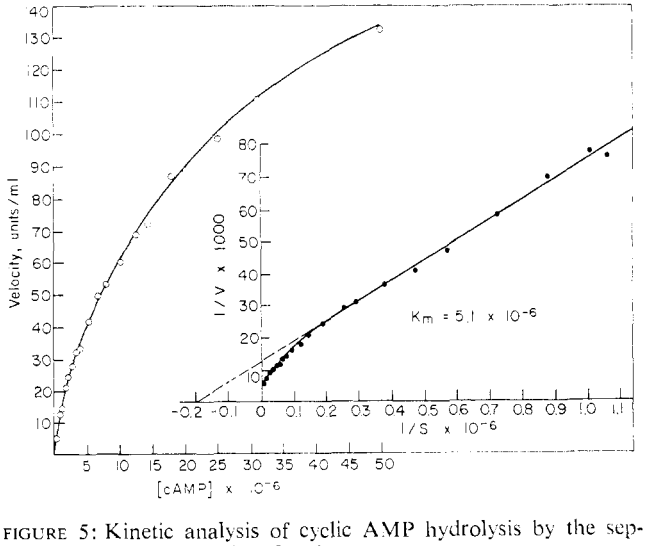

FIGURE 5: Kinetic analysis of cyclic AMP hydrolysis by the separated low molecular weight fraction.

of brain sonicated supernatant is carried out at a pH of 8.4 rather than 6.0, most of the low Michaelis constant enzyme activity migrates in the exclusion fraction of the column effluent without a change in its kinetic behavior. Discontinuous gradient centrifugation reveals this activity to be associated with particles of synaptosomal dimensions (Snyder *et al.*, 1965). This same method of analysis indictes that the sonication step in the preparation dissociates the low Michaelis constant enzyme from particles in the brain homogenates that have the same synaptosomal dimensions. The high molecular weight phosphodiesterase does not display this tendency to associate with membranous structure.

Insulin (236 μg/ml), imidazole (1×10^{-3} M), nicotinic acid (1×10^{-3} M), epinephrine (5.6×10^{-5} M), glucagon (2×10^{-7} M), ACTH (1×10^{-6} M), and serotonin (10^{-3} M) have no effect on either the high or low molecular weight phosphodiesterases. The inhibition constant, K_i, for theophylline is 1×10^{-4} M on the low molecular weight cyclic AMP phosphodiesterase. The high molecular weight enzyme has a K_i for ATP of 3.8×10^{-3} M.

An analysis of cyclic GMP hydrolysis by the high molecular weight fraction from Agarose columns indicates that the Michaelis constant for this nucleotide is 1.29×10^{-5} M. This is similar to the original Michaelis constant reported for cyclic GMP in rat brain (Brooker *et al.*, 1968). It is of interest to determine if the hydrolysis of cyclic GMP and cyclic AMP by this fraction are activities of a single enzyme.

Table III indicates the degree of competition between those two nucleotides for phosphodiestease activity. The fact that the K_i for each nucleotide as an inhibitor of the hydrolysis of the other agrees quite well with its Michaelis constant as a substrate indicates that only one active species of enzyme is present in the high molecular weight fraction. Additional evidence is provided by the fact that the half-life of the destruction of activity during incubation at 45° in the assay buffer is 9.5 min for cyclic AMP hydrolysis and 10.5 min for cyclic GMP hydrolysis.

Hydrolysis of cyclic GMP by the lower molecular weight phosphodiesterase is so slight as to be detectable only under altered assay conditions. The Michaelis constant for cyclic GMP phosphodiesterase activity is the same as the high molecular weight fraction, indicating that this may be due to slight contamination of the higher molecular weight phosphodiesterase overlap in the low molecular weight fraction even after G-200 chromatography. The K_i also agrees quite well with the Michaelis constant but displays noncompetitive inhibition with the low molecular weight cyclic AMP phosphodiesterase.

TABLE III: Kinetic Constants for Cyclic Nucleotide Hydrolysis by Phosphodiesterase Fractions Eluted from 6% Agarose Gels.[a]

Fraction	Cyclic Nucleotide	Michaelis Constant (M)	Inhibition Constant (M)
High molecular weight	Cyclic AMP	1.04×10^{-4}	0.95×10^{-4}
	Cyclic GMP	1.29×10^{-5}	1.23×10^{-5}
Low molecular weight	Cyclic AMP	5.0×10^{-6}[b]	
	Cyclic GMP	1.05×10^{-5}[c]	1.63×10^{-5}

[a] Pooled fractions were concentrated and rechromatographed on Sephadex G-200 before use. All values were determined by linear least-squares analysis and double-reciprocal Lineweaver–Burk plots (Michaelis constant) or Dixon plots (Inhibition constant) (Dixon, 1953). [b] Determined by extrapolation of the linear portion of the double-reciprocal plots. K_m values as low as 2.0×10^{-6} have been observed for this fraction. [c] Assay required doubling of standard radioactive label because of very low enzymatic activity.

Discussion

The results presented indicate that multiple cyclic nucleotide phosphodiesterase activities are present in rat brain. Preliminary investigation from this laboratory as well as other laboratories (Rosen, 1970) indicates that this phenomenon is not unique to brain tissues. The relationship between the multiple forms of cyclic nucleotide phosphodiesterase and their respective physiological functions is not completely clear. Although the integral ratio of the molecular weights indicates a possible subunit aggregation relationship, the fact that cyclic GMP hydrolysis is performed to any extent only by the high molecular weight fraction can be interpreted as evidence for the existence of two distinct enzyme systems.

It is important to point out that the role of phosphodiesterase in the regulation of the levels of cyclic AMP and cyclic GMP is not certain. That it may be critical is shown by the effects of methylxanthines on the level of cyclic AMP in various tissue presumably due to inhibition of cyclic AMP phosphodiesterase (Robison *et al.*, 1968; Sutherland and Robison, 1966). The methylxanthines potentiate or are a prerequisite for the action of most of the activators of the adenyl cyclase systems (Kakiuchi and Rall, 1968; Goldberg and Singer, 1969; Gilman and Rall, 1968; Fleischer *et al.*, 1969). The multiple nucleotide phosphodiesterases do show variable substrate affinity and inhibition indicating differential control. Another important aspect of phosphodiesterase involvement in control mechanisms concerns the discrete subcellular localization of the enzymes. It is interesting to speculate that the high-affinity cyclic AMP phosphodiesterase may be localized at a membrane site as indicated by its association with the particulate cell fractions under some conditions. If the low K_m phosphodiesterase is membrane bound, it could operate in conjunction with adenyl cyclase to control local steady-state levels of cyclic AMP as well as contributing to available and unavailable pools of cyclic AMP for various metabolic activities in line with the second messenger hypothesis.

The kinetics of the separated low molecular weight form do suggest a negative cooperative control system (Levitzki and Koshland, 1969; Conway and Koshland, 1968). If this is a reality, then the low K_m cyclic AMP phosphodiesterase would be very responsive to increasing cyclic AMP concentrations. It is also possible that cyclic GMP could control the velocity of cyclic AMP hydrolysis and *vice versa* (Rosen, 1970). Speculation on this aspect is difficult because of the lack of information concerning the response of cyclic GMP concentrations to hormonal stimulation (Goldberg *et al.*, 1969; Ishikawa *et al.*, 1969). Recently, however, it has been shown that cyclic GMP and cyclic AMP may vary independently in rat hearts after perfusion with acetycholine (George *et al.*, 1970).

It appears from our results that the high molecular weight enzyme which is probably the enzyme frequently studied as a high K_m cyclic AMP phosphodiesterase is very possibly a cyclic GMP phosphodiesterase. This enzyme is probably of a cytoplasmic origin and may serve to hydrolyze large excesses of cyclic GMP or cyclic AMP providing a level of control not necessitating a higher affinity for these nucleotides. It is interesting to note that the guanyl cyclase in most tissues (Hardman and Sutherland, 1969) and the cyclic GMP activated protein kinases of muscle (Kuo and Greengard, 1970) are also apparently cytoplasmic.

The variation in substrate affinity of the various enzymes which utilize or degrade cyclic nucleotides while superficially illogical will hopefully be eventually resolved when better information on subcellular localization and function is available.

Acknowledgments

The assistance and advice of Patricia Lissner, Dr. Cynthia Sevilla, Dr. Gary Brooker, Wesley Terasaki, Tom Russell, Christopher Lissner, Dr. William Walker, and Harrison Frank are gratefully acknowledged.

References

Atkinson, D. E. (1966), *Annu. Rev. Biochem. 35*, 85.

Blecher, M., Merlino, N. S., and Ro'Ane, J. T. (1968), *J. Biol. Chem. 243*, 3973.

Breckenridge, B. M., and Johnston, R. E. (1969), *J. Histochem. Cytochem. 17*, 505.

Brooker, G., Thomas, L. J., and Appleman, M. M. (1968), *Biochemistry 7*, 4177.

Butcher, R. W., and Sutherland, E. W. (1962), *J. Biol. Chem. 237*, 1244.

Cheung, W. Y. (1967), *Biochemistry 6*, 1079.

Cheung, W. Y. (1969), *Biochim. Biophys. Acta 191*, 303.

Conway, A., and Koshland, D. E., Jr. (1968), *Biochemistry 7*, 4011.

Delange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968), *J. Biol. Chem. 243*, 2200.

DeRobertis, E. D., Armaiz, G. R. D. L., Alberici, M., Butcher, R. W., and Sutherland, E. W. (1967), *J. Biol. Chem. 242*, 3487.

Dixon, M. (1953), *Biochem. J. 55*, 170.

Fleischer, N., Donald, R., and Butcher, R. W. (1969), *Amer. J. Physiol. 217*, 1287.

George, W. J., Polson, J. B., O'Toole, A. G., and Goldberg, N. D. (1970), *Proc. Nat. Acad. Sci. U. S. 66*, 398.

Gilman, A. G., and Rall, T. W. (1968), *J. Biol. Chem. 243*, 5867.

Goldberg, A. L., and Singer, J. J. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 135.

Goldberg, N. D., Dietz, S. B., and O'Toole, A. G. (1969), *J. Biol. Chem. 244*, 4458.

Hardman, J. G., and Sutherland, E. W. (1969), *J. Biol. Chem. 244*, 6363.

Hepp, K. D., Menahan, L. A., Wieland, O., and Williams, R. H., (1969), *Biochim. Biophys. Acta 184*, 554.

Ishikawa, E., Ishikawa, S., David, J. W., and Sutherland, E. W. (1969), *J. Biol. Chem. 244*, 6371.

Kakiuchi, S., and Rall, T. W. (1968), *Mol. Pharmacol. 4*, 367.

Kemp, R. G., and Appleman, M. M. (1966), *Biochem. Biophys. Res. Commun. 24*, 564.

Kuo, J. F., and Greengard, P. (1970), *J. Biol. Chem 245*, 2493.

Levitzki, A., and Koshland, D. E., Jr. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 1121.

Menahan, L. A., Hepp, K. D., and Wieland, O. (1969), *Eur. J. Biochem. 8*, 435.

Nair, K. G. (1966), *Biochemistry 5*, 150.

Robison, G. A., Butcher, R. W., and Sutherland, E. W. (1968), *Annu. Rev. Biochem. 37*, 149.

Rosen, O. (1970), *Arch. Biochem. Biophys. 137*, 435.

Senft, G., Munske, K., Schultz, G., and Hoffman, M. (1968), *Naunyn Schmiedebergs Arch. Pharmakol. Exp. Pathol. 259*, 344.

Snyder, S. H., Glowinski, J., and Axelrod, J. (1965), *Life Sci. 4*, 797.

Sutherland, E. W., and Robison, G. A. (1966), *Pharm. Rev. 18*, 145.

Thompson, W. J., and Appleman, M. M. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 1996.

Williams, R. H. (1970), *Amer. J. Med. Sci. 258*, 190.