THE OCCURRENCE AND POTENTIAL ECOLOGICAL SIGNIFICANCE OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN EPILIMNETIC LAKE WATER

By

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INTRODUCTION

This investigation is composed of two manuscripts written in the style acceptable to the journal Limnology and Oceanography. Part I is titled "Occurrence of particulate-associated and extracellular cyclic nucleotide phosphodiesterase in epilimnetic lake water". Part II is titled "Seasonal and diel dynamics of cyclic nucleotide phosphodiesterase in epilimnetic lake water: Relationship to cAMP dynamics".

Approval for presenting the thesis in this manner is based upon the Graduate College's policy of accepting a thesis in manuscript form at the request of the candidate and with the approval of the major professor.

OCCURRENCE OF PARTICULATE-ASSOCIATED AND EXTRACELLULAR CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY IN EPILIMNETIC LAKE WATER.

Running Head: cPDE in lake water

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ABSTRACT

Particulate-associated (15-60 nmol l^{-1} h^{-1}) and extracellular (15-25 nmol l^{-1} min^{-1}) cyclic nucleotide phosphodiesterase (cPDE) activity was identified in epilimnetic waters from a eutrophic reservoir. The pH optima (7.0-8.0), temperature optima (30-35° C), substrate specificity, and methylxanthine sensitivity of lakewater cPDE isolates paralleled characteristics of known animal cell and algal cPDE. Particulate cPDE appeared to be a surface-associated exoenzyme, rather than an intracellular enzyme. Simultaneous measurements of particulate and filtrate cAMP concentrations coupled with cPDE assays suggested that cPDE hydrolysis represented the major pathway of dissolved cAMP turnover in this system.

INTRODUCTION

Numerous correlative and pertubational studies conducted in situ and in the laboratory have demonstrated a putative mechanistic link between seasonal and diel variations in cyclic AMP (cAMP) concentrations and photosynthetic and heterotrophic carbon assimilation rates in algae and aquatic macrophytes (Francko 1984a,b,c; 1986; 1987a; Francko and Wetzel 1980, 1981b, 1982, 1984a,b). Recent work on cultured Selenastrum capricornutum, a unicellular chlorophyte alga, suggests that fluxes in both extracellular (dissolved) and intracellular cAMP induce changes in the algal transmembrane electrogenic potential, with concomitant perturbation of photosynthetic carbon assimilation (PCA) rates (Francko 1989 a,b). Alterations in both membrane potential and PCA rates could be induced in the laboratory using manipulations of dissolved and intracellular cAMP within the range of seasonal and diel variation occurring in freshwater and marine systems (a few pmol to over 0.1 μmol l^{-1} and ca. 10 pmol to over 1 μmol g^{-1} fresh wt., respectively; Ammerman and Azam 1981, 1982; Francko 1983, 1987a).

The operation of a putative cAMP mediated photosynthetic regulatory system in epilimnetic planktonic assemblages would be conditionally dependent on both cAMP production and release rates by biota (signal generation) and processes that remove the cAMP "signal" from cells and lake water. The above references contain data on cAMP production and release rates and demonstrate that biotic production by planktonic algae and bacteria, aquatic vascular plants, and attached epiphytes can account for most of the dissolved cAMP in lake water. To date however, almost nothing is known about the mechanisms responsible for cAMP degradation in

natural aquatic systems. This information is crucial for further elucidation of the ecophysiology of lacustrine cAMP metabolic pathways.

In bacterial and animal cells, cAMP degradation is enzymatically catalyzed by a class of cyclic nucleotide phosphodiesterases (cPDE). We are aware of only two definitive studies on cPDE in cultured algae, although numerous attempts to measure cPDE in terrestrial vascular plants have been attempted (reviewed by Amrhein 1977; Brown and Newton 1981; Francko, 1983). Fischer and Amrhein (1974) and Ownby and Kuenzi (1982) reported the presence of substantial cPDE activity in cultured Chlamydomonas reinhardtii and Anabaena variabilis, respectively. Like animal cell cPDE, enzymatic activity was strongly inhibited by the methylxanthine derivative theophylline and was highly specific for cAMP.

Circumstantial evidence for naturally occurring enzymatic hydrolysis of cAMP in freshwater systems was presented by Francko and Wetzel (1984a). Samples of littoral zone surface waters were filtered (0.6-μm filters) at time zero and incubated for 1 - 4 h in situ. Losses of cAMP in filtrates, presumably due to hydrolysis, ranged from 0.5 to 1.1 nmol l^{-1} h^{-1} in water samples containing 1 - 6 nmol of cAMP l^{-1}. Putative cAMP hydrolysis exhibited an optimum pH of 7.0 and a temperature optimum of 23° C. The capacity of lakewater filtrates to hydrolyze cAMP was irreversibly destroyed by heating water samples to 100° C for 5 minutes, but hydrolysis was not inhibited by 5 mM methyl-3-isobutylxanthine (MIX) a potent methylxanthine inhibitor of cPDE in animal cells (Amrhein 1977).

In the above study, the presence of cAMP hydrolytic activity was inferred through measurements of cAMP loss rates in filtered water; direct measurements of cPDE activity were not made. In this study we employed a specific cPDE assay system to examine the existence and

physicochemical characteristics of particulate-associated and extracellular cPDE within epilimnetic waters from a eutrophic reservoir. We also examined the relationship between cPDE activity and changes in cAMP concentrations in lake water, permitting limited speculation on the ecological importance of cPDE.

METHODS

Water samples were collected from the 0.1-m stratum of Sangre Isle Lake, (also known as Cedar Isle Lake), Payne Co., Oklahoma, a small (3.2 ha), shallow (mean depth 2.0 m), eutrophic reservoir with limnological characteristics resembling those of a natural eutrophic lake (Francko 1987b). Samples were collected at mid-day during May -October 1988, and during May of 1989 at an epilimnetic site located over the deepest portion of the reservoir. This system has been the site of previous studies on cAMP dynamics (Francko 1983, 1984a,b,c). Water temperature measurements (Thermistor) were made *in situ*, whereas pH measurements were made in the laboratory.

Water samples were returned to the laboratory within 20 minutes of collection and 150 ml portions were passed through 0.45-µm Millipore filters using a vacuum differential of 0.5 atm. Particulate material was rinsed off the filters and resuspended in 20 mL ice-cold 40 mM Tris-HCl, pH 7.6 containing 10 mM cysteine and 2 mM $MgSO_4$ in order to preserve the stability of putative cPDE activity (Ownby and Kuenzi 1982).

Filtrate and particle-associated cPDE activity were measured by preparative and assay techniques previously developed for cultured algae (Ownby and Kuenzi 1982). Resuspended particulate fractions were sonicated (Branson Model 185) on ice using four 10-s bursts on the highest power setting at 30-s intervals. The sonicated preparation was

centrifuged (3000 X g) for 5 minutes and resulting supernatants were assayed immediately. Control experiments indicated that additional cPDE activity was not released from particulate fractions by lengthier sonication.

The filtrate fraction was collected directly from the filtration apparatus and kept on ice until the assay was performed. Experiments demonstrated that sonication did not enhance putative filtrate cPDE activity.

Reaction tubes (1.5-ml polyethylene microfuge tubes) containing 100 µl of extract supernatant or filtrate (N = 3 for each fraction) and 200 µl of sufficient Tris-HCl to yield a final concentration of 40 mM, pH 7.6, 2 mM MgSO4, and 10 mM cysteine, were incubated at room temperature for 10 minutes. Additional replicate tubes were prepared as above and boiled for 15 minutes at time zero to serve as denatured controls. After incubation, 100 µl of a cAMP stock solution containing tracer quantities of 3H-cAMP (40 nmol cold cAMP and 1.3 X 10^3 Bq ml^{-1} of ^{3}H-cAMP) was added to each tube. After 90 minutes of incubation at 32° C, the tubes were boiled for 1 min, chilled on ice and centrifuged (3000 X g). Following centrifugation, replicate aliquots (200-µl) from the supernatant in each tube were removed and added to fresh microfuge tubes containing 200 µl of an aqueous solution of Ophiophagus hannah venom (1 mg mL^{-1}) to hydrolyze to adenosine any 5'-AMP formed through putative cPDE activity. Venom-containing aliquots were incubated for 20 min at 32°C, boiled, chilled, and centrifuged as before. Aliquots (200-µl) were taken from the supernatant of each tube and mixed with 1.2 ml of an ethanolic slurry of anion-exchange resin (Dowex AG1-X8) (Londesbourough 1976), shaken for 15 min, and centrifuged. ^{3}H-adenosine in sample supernatants was analyzed

by removing 200 µl from the supernatant of each tube for liquid scintillation spectroscopy. These data were used to compute cPDE activity (nmol cAMP hydrolyzed l^{-1} lakewater h^{-1}). Radioactivity in boiled controls was used to correct for non-enzymatic ^{3}H- cAMP breakdown and any unhydrolyzed 3H-cAMP not removed by the anion - exchange resin prior to calculations of enzymatic activity.

Although cPDE assays were typically conducted in assay mixtures buffered at pH 7.6, we also measured the pH-dependency of putative lakewater cPDE. Tris-maleate (pH 6.0 to 7.0) and Tris-HCl (pH 7.0 to 9.0) were used in this experiment. An additional series of experiments was conducted in water baths at non-standard temperatures to evaluate the temperature dependence of cPDE activity. The substrate specificity of lakewater cPDE was evaluated by using cyclic GMP (cGMP), glucose-6-phosphate and 5'-AMP as alternate substrates in the reaction mixture in addition of cAMP. In these experiments, 40 nmol of cAMP solution plus 40 nmol of alternative substrate were placed in reaction tubes. For the cAMP alone control, equivalent amount of assay buffer was added to correct for dilution effects. The methylxanthine sensitivity of lakewater cPDE activity was determined by incubating replicate particulate and filtrate samples amended by the addition of 10 mM theophylline (Francko and Wetzel 1981a; Ownby and Kuenzi 1982).

Chlorophyll a concentrations in each lakewater sample analyzed were performed in replicate on particulate fractions retained by 0.45-µm Millipore filters. Pigments were extracted in basic methanol (Holm-Hansen 1978) and assayed fluorometrically (Wetzel and Likens 1979). Portions of whole lakewater were preserved with Lugol's iodine solution for later analysis of planktonic community composition.

Concentrations of naturally-occurring cAMP in particulate matter and lakewater filtrates were simultaneously evaluated in replicate particulate and filtrate samples by preparative techniques and a competitive displacement assay previously published (Francko and Wetzel 1980; 1982).

RESULTS AND DISCUSSION

Our data indicated that enzymatic activity consistent with known characteristics of cPDE from cultured algae occurred in both particulate and filtrate fractions from epilimnetic Sangre Isle Lake waters. We conducted pH optimum characterization experiments in May, June and July 1988 and again in May 1989. Data for two sampling dates are shown in Figure 1, and indicate a broad pH optimum of about 7.0 and 7.5 in 1988 and 1989, respectively. Corresponding values for 4 June 1988 (pH 7.0) and 14 July 1988 (pH 8.0) were similar, although the range of one pH unit in the optimal values suggests that seasonally different isozymes of cPDE could have occurred. In all experiments, a precipitous drop in activity was noted when the pH was raised above 8.5. Since the pH of lakewater samples returned to the laboratory varied between 8.2 and 9.5, the above data suggest that cPDE activity values derived under standard assay pH conditions (7.6) may have overestimated *in situ* activity.

Temperature optimum experiments were conducted on 1 June and 6 June 1988. In each case, the temperature optimum was about 30-35° C (Figure 2) even though the *in situ* temperature of surface waters was about 25° C in early June. In each experiment incubation of samples at 50° C for 15 min or more resulted in total loss of enzyme activity.

Competition experiments indicated that lakewater cPDE preferentially

utilized cAMP as a substrate (Table 1). Of the three alternate substrates we evaluated, glucose-6-phosphate and 5' -AMP exhibited no evidence of competition. A slight depression in the calculated activity of the sample amended with cyclic GMP occurred, indicative of competition between the cyclic nucleotide substrates, but this compound reduced the activity toward cyclic AMP by only 10 - 15%. Particulate cPDE activity was reduced to undetectably low levels (<3 nmol l^{-1} h^{-1}) in samples incubated with 10 mM theophylline.

Taken together, the pH and temperature optima, substrate specificity, and methylxanthine sensitivity of cPDE activity we measured in Sangre Isle Lake samples closely paralleled that of cultured algae described in the literature (Fischer and Amrhein 1973; Ownby and Kuenzi 1982).

Temporal patterns of particulate-associated and filtrate cPDE activity for the period of study are shown in Figure 3 (upper panel). Particulate activity ranged from about 15 to 60 nmol l^{-1} h^{-1} while filtrate activity (15 to 25 nmol l^{-1} min^{-1}) varied less during sampling period. The ratio between particulate-associated cPDE and filtrate cPDE per liter lake water varied between 25 and 30, indicating that much more cPDE occurred in an exoenzymatic form on a volumetric basis.

When particulate cPDE levels per unit chlorophyll a were computed (Fig. 3 upper panel), a slightly different pattern emerged. Microscopic examination of Lugol's-preserved water samples indicated that two massive, nearly unialgal blooms of cyanophytes comprised the bulk of particulate biomass during the summer period (Anabaena spp. between late July and early September and Microcystis aeruginosa between mid-June and late July). Chlorophyll a-specific cPDE concentrations were highest

during the early and later stages of development of both surface blooms and lowest during the mid-point of the bloom periods. Biomass-specific cPDE remained elevated after the crash of the Anabaena bloom in mid-September.

Filtrate and particulate-associated cAMP concentrations (Fig. 3 lower panel) were similarly dynamic. The highest concentrations of particulate cAMP occurred in late July and mid-September corresponding to the late stages of the Microcystis and Anabaena blooms, respectively.

Dissolved cAMP levels were high early in the Microcystis bloom period, dropped during the middle stage of the bloom, and then increased again during the development and senescence of the Anabaena bloom. Similar relationships between seasonal phytoplankton assemblages have been reported in Sangre Isle Lake water (Francko 1983, 1987a), when cAMP can comprise up to 8 percent of the dissolved phosphorus pool.

From early June until mid-July an inverse relationship between particulate cPDE activity (which behaved as an exoenzyme during this investigation) and dissolved and particulate cAMP suggesting that this enzyme may have been important in regulating dissolved cAMP levels in situ during the study. This inverse correlation was not noted later in the study period.

Based on filtrate cAMP and cPDE levels from mid-July to mid-October the turnover time for dissolved cAMP in epilimnetic waters is much higher than previously cited studies on cAMP "loss" rates in epilimnetic filtrates from Lawrence Lake (Francko and Wetzel 1984a). As noted earlier, however, cPDE values reported here are likely overestimates of in situ hydrolytic rates since the ambient pH was always well above the measured pH optimum for naturally occurring cPDE in Sangre Isle Lake

waters. Taken together, our evidence suggests that cPDE hydrolysis may represent an important degradative pathway in epilimnetic waters.

Our evidence suggested that particulate-associated cPDE occurred largely as a surface-associated rather than intracellular enzyme. Total cell protein measurements were made by a reagent dye binding assay (Bradford 1976) on particulate-fraction subsamples prior to and following sonication. Sonicated aliquots contained several-fold more protein than non-sonicated samples, and a limit product was reached by the sonication procedure outlined in the Methods section. In contrast, particulate cPDE values were statistically similar ($P < 0.05$; t-test, $N = 2$) in both non-sonicated and sonicated aliquots, supporting the view that the enzyme was accessible to cPDE assay reagents even without cell disruption.

This finding has important implications for the formation of extracellular cPDE as well as for the potential role of cPDE in cAMP dynamics. If particulate cPDE is surface-associated, the actual filtrate cAMP turnover time might have been considerably shorter than that described earlier. We did not attempt to determine the source of epilimnetic extracellular cPDE in this study. However, if particulate cPDE is loosely associated with the outer surface of cells it might be readily released from planktonic biota into a dissolved form. Alternatively, cPDE activity present in epilimnetic filtrates may in part have been imported from the extensive littoral zone that comprises the majority of the surface area of Sangre Isle Lake. Macerated tissue samples from Ceratophyllum demersum, the dominant macrophyte species in this lake, contain measureable cPDE activity (Neighbors and Francko, unpublished data). On 12 July 1988, we made simultaneous measurements of filtrate cPDE levels at the epilimnetic sampling site and within an

adjacent Ceratophyllum bed. The littoral filtrate contained about five-fold more cPDE than the epilimnetic filtrate (69 versus 15 nmol l^{-1} min^{-1}), further supporting the plausibility of littoral exoenzymatic cPDE export.

The characteristics determined here for lakewater cPDE were similar to those of known cPDE isozymes from animal and algal systems, where this class of enzymes plays a central role in regulating cAMP-dependent physiological responses. Further characterization of lakewater cPDE and research on the ecological significance of cPDE activity appear warranted.

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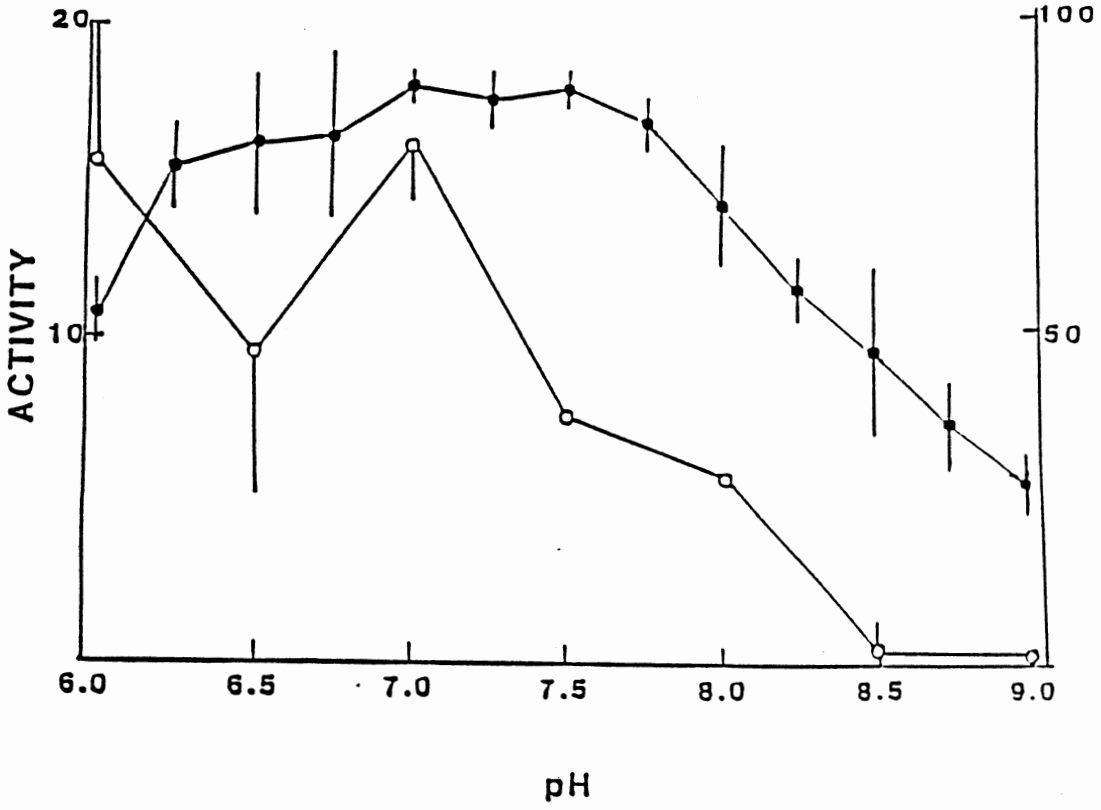
FIGURE LEGENDS

Figure 1. Effect of pH on the hydrolytic activity of particulate cPDE. Data shown in nmol l^{-1} h^{-1} ± SE (N = 3) for 8 June 1988 (○—○) and 21 May 1989 (●—●). Values on left side of ordinate refer to 1989 data whereas units on the right side correspond to 1988. Where error bars are not shown, SE was smaller than diameter of points.

Figure 2. Effect of temperature on hydrolytic activity of particulate cPDE, 1 June 1988. Data shown in nmol l^{-1} h^{-1} ± SE (N = 3). All SE values were smaller than diameter of points.

Figure 3. Seasonal dynamics of cPDE, and cAMP in particulate and filtrate fractions collected from the 0.1-m stratum of Sangre Isle Lake, 1988. Upper Panel. cPDE in particulate fractions shown in two scales, nmol l^{-1} h^{-1} (○—○, left ordinate) and nmol μg^{-1} Chl a (○--○, right ordinate). Filtrate cPDE shown in nmol l^{-1} min^{-1} (●—●, left ordinate). Error bars denote ± SE (N = 3).

Lower Panel. Particulate (○—○) and dissolved (●—●) cAMP values in nmol μg^{-1} Chl a and nmol l^{-1}, respectively. Error bars denote ± SE (N = 2) except where error was smaller than diameter of points.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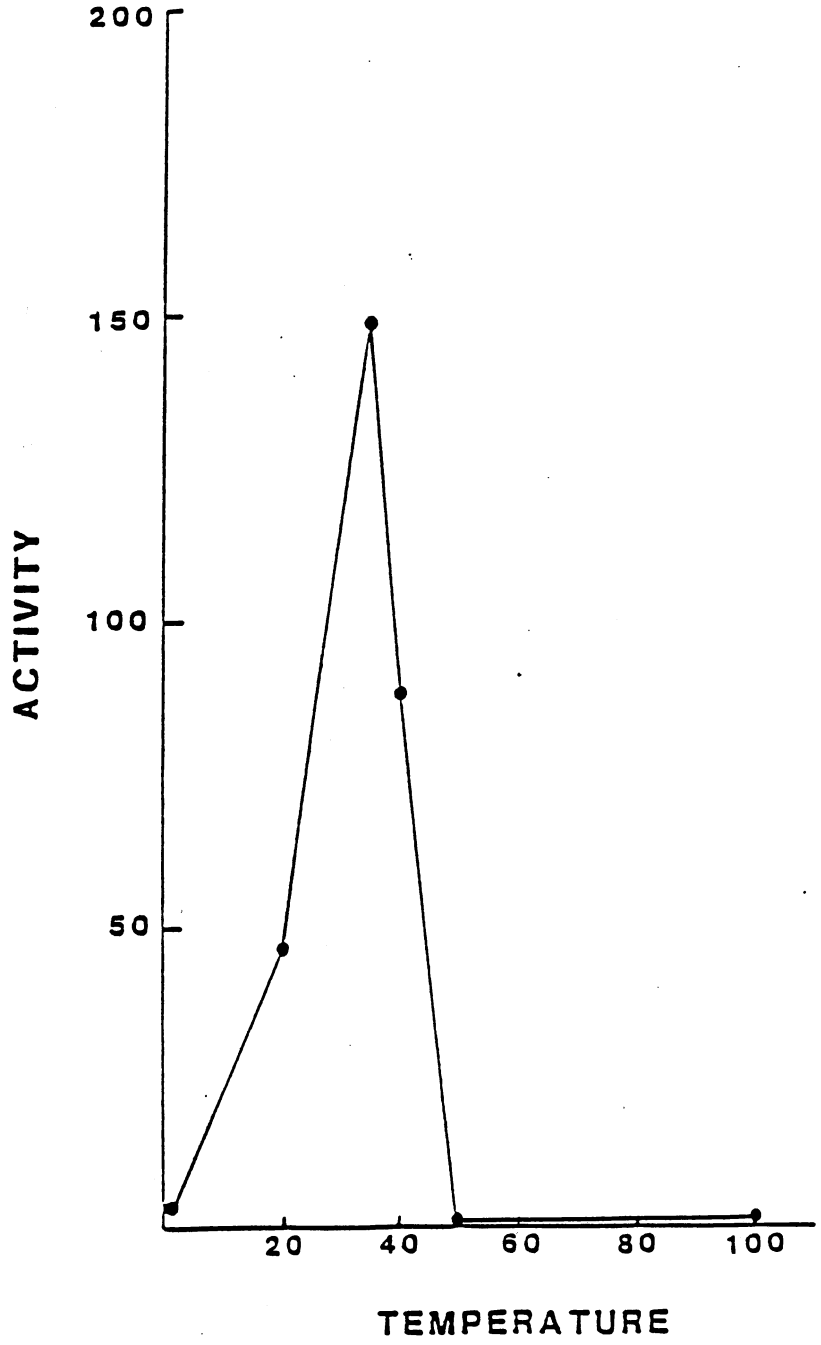
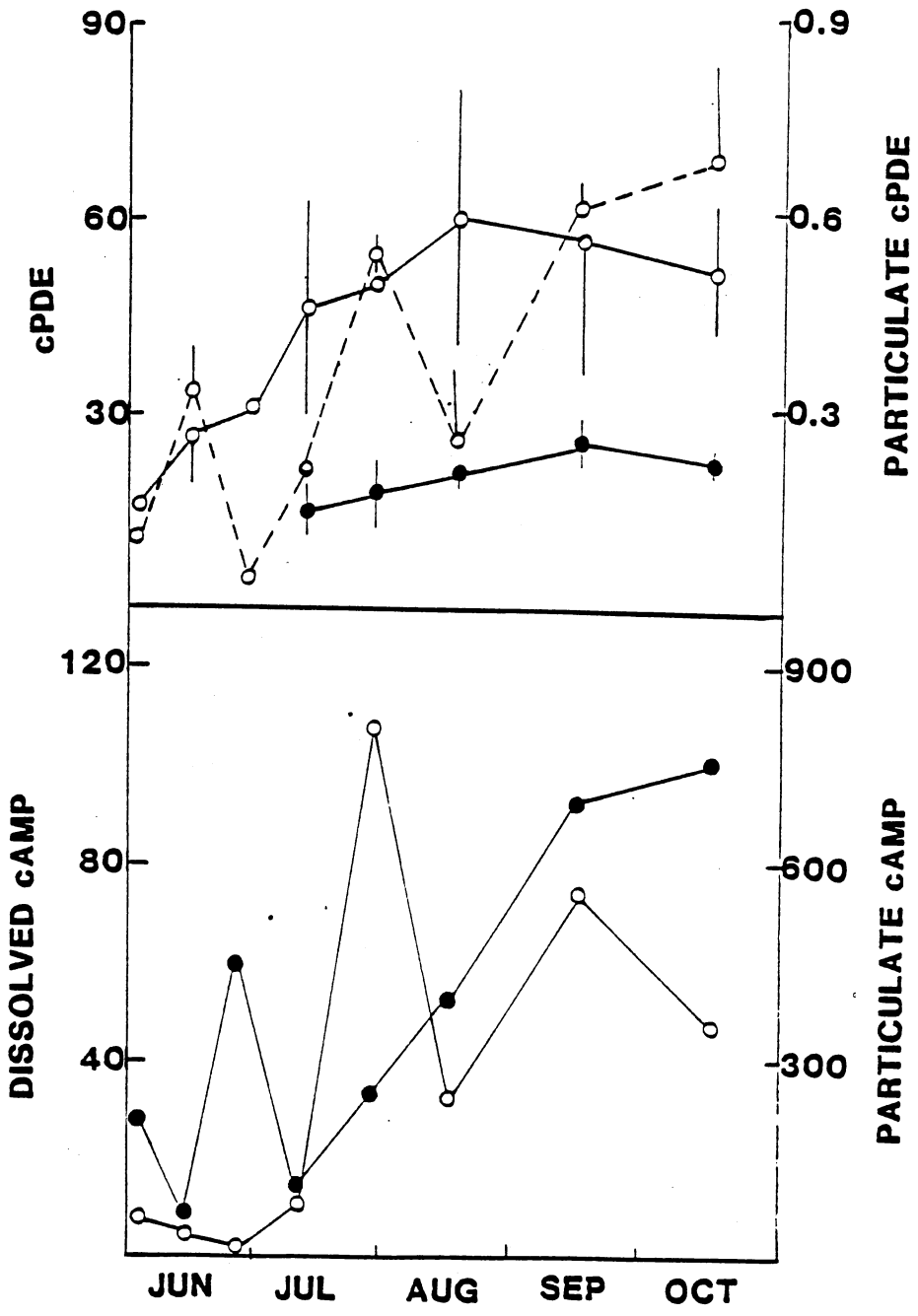


Figure 2.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30
cPDE
0.9
0.6
0.3
PARTICULATE cPDE
120
80
40
DISSOLVED cAMP
900
600
300
PARTICULATE cAMP
JUN
JUL
AUG
SEP
OCT

Figure 3

Table 1. Substrate specificity of cPDE activity in particulate fractions from epilimnetic water collected on 20 May 1988. Values shown as nmol l-1 of hydrolytic activity ± SD (N = 3) for each substrate.

SUBSTRATE	ACTIVITY
cAMP	96.4 ± 20.8
cGMP	82.2 ± 26.4
5'-AMP	96.5 ± 3.3
Glucose-6-P	89.9 ± 0.8

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SEASONAL AND DIEL DYNAMICS OF CYCLIC NUCLEOTIDE PHOSPHODIESTERASE IN EPILIMNETIC LAKE WATER: RELATIONSHIP TO cAMP DYNAMICS.

Running Head: cPDE dynamics in lake water.

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ABSTRACT

Cyclic nucleotide phosphodiesterase (cPDE) was assayed in epilimnetic lakewater filtrates and particulate fractions collected from an eutrophic reservoir. Thin-layer chromatography and kinetic experiments were conducted to further characterize this naturally-occurring enzyme system. Significant temporally-dynamic activity was observed on both seasonal and diel bases. We also found that 1) cPDE activity in both fractions is specific for cyclic 3':5' adenosine monophosphate, 2) Activity *in situ* for the 1988 season may be substantially lower than 1989 values even though laboratory values were 3-4 times higher, 3) virtually all measurable cPDE activity occurred during the late spring and summer months when the bulk of the phytoplankton was composed of cyanophytes, and 4) when *in situ* pH values were within the optimum range for cPDE activity, an inverse relationship between cPDE and dissolved cAMP was noted.

INTRODUCTION

Recent evidence suggests that cyclic adenosine 3':5'-monophosphate (cAMP) may regulate phytoplanktonic carbon assimilation rates via an electrogenic mechanism (Francko 1989 a,b). In cultures of the chlorophyte Selenastrum capricornutum, artificially - induced changes of extracellular (dissolved) and intracellular cAMP concentrations were paralleled by rapid, transitory perturbations in the plasmalemma electrogenic potential and proton flux, with corresponding perturbations in cellular photosynthetic capacity. These effects could be induced by manipulations of cAMP levels within the range normally found in epilimnetic lake and ocean systems (a few pmol to over 0.1 μmol $liter^{-1}$ and ca. 10 pmol to over 1 μmol g^{-1} fresh wt. particulate biomass) (Ammerman and Azam 1981, 1982; Francko 1983, 1987a). The cAMP-electrogenic model provides a potential mechanistic framework for understanding a large body of in situ and laboratory data correlating seasonal and diel cAMP dynamics in epilimnetic planktonic assembleges, aquatic macrophytes, and cultured algae to shifts in photosynthetic and heterotrophic carbon assimilation rates (cf. Francko 1984 a,b,c; 1986, 1987a; Francko and Wetzel 1982, 1984 a,b). The above studies also contain data on cAMP production and release rates in algae, aquatic macrophytes, and bacteria and demonstrated that most of the dissolved cAMP in surface waters is produced autochthonously.

If, however, dissolved or biota-associated cAMP represents a "signal" molecule capable of inducing metabolic changes in aquatic photoautotrophs, the ecological significance of this regulatory system requires knowledge of not only signal generation (production and release

by biota) but also signal removal.

Cyclic nucleotide phosphodiesterases (cPDE) represent a class of enzymes that hydrolyze cAMP in bacterial and animal cells, organisms in which cAMP plays a well-characterized regulatory role. Fischer and Amrhein (1974) reported the presence of substantial cPDE activity in a cultured chlorophyte alga, Chlamydomonas reinhardtii. Ownby and Kuenzi (1982) demonstrated that changes occur in the cPDE activity of cultures of Anabaena variabilis during different stages of growth and degrees of nitrogen starvation. The reported cPDE activity in both studies was highly sensitive to theophylline and exhibited an alkaline pH optimum, similar to many prokaryotic systems.

Barfield and Francko (1990) employed a specific enzyme assay to measure cPDE in particulate fractions and lakewater filtrates from the epilimnion of an eutrophic reservoir. The pH (7.0-8.0) and temperature (30-35°C) optima, substrate specificity, and methylxanthine sensitivity of our samples paralleled characteristics of animal cell and algal culture cPDE. Limited seasonal data suggested that cPDE could represent a major pathway for cAMP degradation in epilimnetic waters, and that the majority of cPDE occurred in lakewater filtrates and as a particle-surface exoenzyme.

Here we present a comprehensive examination of seasonal and diel cPDE dynamics in epilimnetic waters and its relationship to cAMP dynamics. We also characterized the primary hydrolytic products of lakewater cPDE and the reaction kinetics of dissolved and particulate cPDE fractions.

METHODS AND MATERIALS

Water samples were collected from the 0.1-m stratum of Sangre Isle Lake (also known as Cedar Isle Lake), Payne Co., Oklahoma, a small (3.2 ha), shallow (mean depth 2.0 m) eutrophic reservoir with limnological characteristics resembling those of a natural eutrophic lake (Francko 1987b). Samples were collected at mid-day during May - December 1988 and February - August 1989. Water temperature and pH were measured in the field and laboratory, respectively. Water samples were returned to the laboratory within 30 minutes of collection and portions (100 -250 ml) were passed through Millipore HA 0.45-μm filters using a vacuum differential of 0.5 atm. Filters were rinsed with 20 ml of ice-cold 40 mM Tris-HC1, pH 7.6, containing 10 mM cysteine and 2 mM $MgSO_4$ to resuspend particulate fractions.

Filtrate and particulate-associated cPDE activity was measured using preparative and cPDE assay techniques for cultured algae (Ownby and Kuenzi 1982) and modified slightly for analyses of natural samples (Barfield and Francko 1990). Resuspended particulate fractions were sonicated (Branson Model 185) using four 10-S bursts with 30-S intervals at the highest power setting. The sonicated preparations were centrifuged at 3000 x g for 5 minutes and the resulting supernatants were used immediately for assays of cPDE activity. Additional cPDE activity was not released from particulate fractions by lengthier sonication (Barfield and Francko 1990). Filtrates were kept on ice until assayed. We found that sonication did not enhance putative cPDE activity in the filtrate fraction.

Reaction tubes (1.5-ml polyethylene microfuge tubes) containing 100

µl of extract supernatant or filtrate (N=3) and 200 µl of Tris-HCl buffer to yield a final concentration of 40 mM, pH 7.6, 2 mM Mg SO_4, and 10 mM cysteine, were incubated at room temperature for 10 min. Additional replicate tubes were prepared as above and boiled for 15 min at time zero to serve as denatured controls. After incubation, 100 µl of a cAMP stock solution containing tracer quantities of ^{3}H-cAMP (40 nmol cold cAMP and 1.3 X 10^3 Bq ml^{-1} of ^{3}H-cAMP) was added to each tube. The reaction tubes were then incubated at 32°C for 90 minutes, boiled for 1 min, chilled on ice and centrifuged (3000 x g) to remove precipitated proteins. After centrifugation, replicate aliquots (200 µl) from the supernatant in each tube were removed and added to new microfuge tubes containing 200 µl of an aqueous solution of Ophiophagus hannah venom (1 mg ml^{-1}) to hydrolyze to adenosine any 5'-AMP formed through putative cPDE activity. These tubes were incubated for 20 min at 32° C, boiled, chilled, and centrifuged as above. Aliquots (200 µl) were taken from the supernatant of each tube and mixed with 1.2 ml of an ethanolic slurry of anion-exchange resin (Dowex AG1-X8; (Londesbourough 1976), shaken for 15 min, and centrifuged. Supernatants (200 µl) from each tube were removed to assay ^{3}H-adenosine by liquid scintillation spectroscopy. cPDE activity was then computed in units of nmol cAMP hydrolyzed $liter^{-1}$ lake water min^{-1} for dissolved activity and nmol $liter^{-1}$ h^{-1} for particulate activity. Boiled controls were used to correct for non-enzymatic ^{3}H-cAMP breakdown and any unhydrolyzed ^{3}H-cAMP not removed by the anion-exchange resin.

Chlorophyll a concentrations in lakewater samples were analyzed in replicate on the particulate fractions retained by 0.45-µm Millipore filters. Following extraction in basic methanol (Holm-Hansen 1978),

pigments were assayed fluorometrically (Wetzel and Likens 1979). Portions (20 ml) of whole lakewater were preserved with Lugol's iodine solution for later analysis of planktonic community composition. Particulate and dissolved cAMP levels were assayed by a previously published method (Francko and Wetzel 1980, 1982). Reaction kinetics (Km and Vmax) for cPDE in particulate and dissolved fractions were determined by varying the amount of "cold" cAMP in reaction mixtures from 0.5 to 80 nmol without changing the amount of radioactive label. The cPDE assay was then performed as above. Primary products of ^{3}H-cAMP hydrolysis in particulate extracts and lakewater filtrates were examined utilizing thin-layer chromatography (Ownby and Kuenzi 1982). Aliquots from reaction mixtures of both fractions along with 3'-AMP, 5'-AMP, 2':3' cAMP, 3':5'-cAMP, and adenosine standards were chromatographed on silica gel plates using isopropanol:NH_3:0.1 M H_3BO_3 (7:1:2 by volume) as the solvent system. The chromatogram was developed for 4h and sections (1.0 X 5.0 cm) were placed in vials for liquid scintillation counting.

RESULTS AND DISCUSSION

Considerable variation was noted in seasonal levels of CPDE activity (Figure 1). The highest levels of cPDE activity were measured in 1988 with a maximum of 28 nmol liter^{-1} min^{-1} in the dissolved fraction and 59 nmol liter^{-1} h^{-1} in the particulate fraction. Both activity peaks occurred in mid-September during a massive bloom of Anabaena sp. During the winter months virtually no activity was recorded in either fraction with exception of a major peak in early February when phytoplankton was dominated by green algae. Following this peak, activity levels fell until early June-late July of 1989 when algal communities were dominated

by Microcystis.

We also noted considerable temporal variation in the amount of particulate and dissolved cAMP (Figure 2). Dissolved cAMP levels were low early in 1988 reaching seasonal highs in mid-August and early October. Winter values fell to almost undetectable levels with the notable exception of a peak during February. In 1989, dissolved cAMP levels were consistently low (approximately 5 nmol cAMP $liter^{-1}$ lake water).

Biomass-specific particulate cAMP levels were similarly dynamic. In 1988, cAMP concentrations peaked in mid-June during a bloom of Microcystis, dropped following the crash of the bloom, then rose again to highs in September and October during the end of, and just after an Anabaena bloom (Figure 2). A dramatic decrease in cAMP levels was not observed over the winter months. In 1989, biomass-specific particulate cAMP levels were relatively consistent at approximately 100 pmol cAMP μg^{-1} chl a with a peak value of 250 pmol cAMP μg^{-1} chl a in mid-June.

During the 1988 spring-summer Microcystis bloom dissolved cPDE levels were inversely correlated with dissolved cAMP concentrations. However, this correlation broke down later in the summer during the Anabaena population maximum when cPDE and cAMP levels were both elevated. In an earlier study (Barfield and Francko 1990) we found that the activity of naturally-occurring cPDE from both particulate and filtrate sources was strongly inhibited by pH values above about 8.5. During much of the summer and fall of 1988, lakewater pH ranged from 9.3-9.6, far above the optimal range for this enzyme. Thus, the disparity between high laboratory cPDE levels conducted on samples buffered to a standard pH (7.6) and the high in situ cAMP levels we noted in 1988 could be due

to in situ pH inactivation.

Conversely, 1989 was a meteorologically anomalous year in northcentral Oklahoma, both much cooler and wetter than normal - epilimnetic water temperatures were generally about 5°C cooler during the late spring - late summer and massive algal blooms did not develop. The lakewater pH values ranged from 7.22 - 8.78 during 1989, but nearly all values were below pH 8.0. We hypothesize that although cPDE concentrations were lower in 1989 than in 1988, in situ hydrolytic rates of a community dominated by blue-green algae-June/July-(Microcystis) August/September (Anabaena -Oscillatoria) were probably much higher due to the more favorable extant pH, perhaps accounting for the decreased cAMP levels noted during 1989.

Diel analyses of changes in epilimnetic cPDE and cAMP were conducted on 15-16 July 1988 and 18-19 August 1988. Particulate and filtrate fractions were assayed for cPDE activity and cAMP levels seven times over a thirty-hour period.

Activity in all fractions and treatments exhibited a definite, reproducible pattern (Figs. 3 & 4). Enzymatic activity ranged from undetectable levels in mid-morning (detection limit = 3 nmol liter^{-1} h^{-1}) to almost 100 nmol liter^{-1} h^{-1} at midnight in the particulate fraction of both dates the experiment was performed. Enzymatic activity was not statistically different in sonicated versus non-sonicated fractions. In a previous paper, Barfield and Francko (1990) demonstrated that sonication does not significantly enhance cPDE activity though total protein levels in sonicated cell fractions increased several-fold, supporting the view that particulate cPDE may be a surface exoenzyme rather than an intracellular enzyme.

In the second diel experiment, the dissolved activity of lakewater filtrates was also measured. Dissolved activity ranged from a low of 3.5 to almost 40 nmol liter^{-1} min^{-1}. Dissolved activity rates were much faster than cell-associated activity rates (Figure 4).

Marked diel periodicity of dissolved and particulate cAMP levels was also observed (Figures 5 & 6). In general, dissolved cAMP levels were much higher than biomass-specific cAMP levels. During the first diel experiment, particulate cAMP levels (Figure 5, lower panel) ranged from 5 to 45 pmol cAMP μg^{-1} chl a (corresponding to 1-15 nmol liter^{-1} lakewater). Dissolved cAMP concentrations varied from 10 to 100 nmol cAMP liter^{-1} lake water (Figure 5, upper panel). In the second diel experiment, particulate cAMP levels were approximately the same as the first sampling date with the exception of the 12:00 AM sample which approached almost 200 pmol cAMP μg^{-1} chl a (Figure 6, lower panel). However, the concentrations of particulate cAMP uncorrected for biomass were much lower (Figure 6, upper panel). Dissolved cAMP levels in the second diel experiment were also much lower ranging from 2 to 12 nmol cAMP liter^{-1} lake water. It should be noted that the same pattern emerged in both replicates of this experiment. Low early morning levels were followed by a peak in mid-afternoon, then a dramatic increase up to a daily peak at midnight, followed by a return to early morning lows.

In both diel experiments a strong inverse relationship between enzymatic activity and dissolved substrate levels during the day was noted. However, during both diel experiments we found that lakewater pH rose above 9.0 during the evening hours, remaining elevated until the following morning. Like the aforementioned seasonal data, such pH data

support the view that diel shifts in the pH to levels far above the optimum range for the enzyme may temporarily elevate levels of cAMP during the night. This finding does contradict work by Francko and Wetzel (1984a) in which dissolved cAMP levels in Lawrence Lake, MI were lowest during the night period. However, in the Lawrence Lake study, pH values above 8.5 were generally found in the afternoon and early evening, and dropped considerably druing the nights hours.

Naturally-occurring particulate and dissolved cPDE fractions isolated from lake water in August 1989 exhibited Km values of 5.5×10^{-5} M and 2.25×10^{-5} M, respectively (Figure 7). The cell fraction was dominated by Anabaena and Oscillatoria. The dissolved cAMP concentration of 1.78 nmol $liter^{-1}$ at the time of these samples were analyzed and the observation that dissolved cAMP levels rarely exceeded 50 nmol $liter^{-1}$ during 1988 or 1989 suggests that the in situ enzyme may be operating at natural substrate levels below Km. Although this determination was only conducted one time, such data suggested that dissoved cPDE may arise from nonplanktonic sources, a thesis supported by the high littoral dissolved cPDE reported in our previous papaer (Barfield and Francko 1990).

Analyses of cPDE hydrolytic products in August 1989 samples (Figures 8 & 9) demonstrated that the primary hydrolytic product of both dissolved and particulate cPDE was 5'-AMP. In the particulate fraction, we found that the only product of ^{3}H-cAMP hydrolysis co-chromatographed with 5'-AMP (Figure 8). The majority of the radioactivity recovered co-chromatographed with 3':5' cAMP, perhaps indicative of relatively low levels of enzyme activity the date we performed the assay. The dissolved fraction was similar with the notable exception of a large peak corresponding to ^{3}H-adenosine (Figure 9). One explanation for the

recovery of such a large amount of radioactivity as ^{3}H-adenosine is that in the same water sample were high levels of alkaline phosphatase activity (Francko, unpublished data) and 5'-AMP produced by cPDE-catalyzed cAMP hydrolysis would be a substrate for alkaline phosphatase.

Taken with previously reported data on lakewater cPDE, our current work suggests that cPDE dynamics may in turn provide a major in situ regulatory pathway for cAMP degradation in epilimnetic waters. Further experimentation on the effect of cPDE activity on cAMP-modulated physiological responses in algae appears warranted as well as more in-depth characterization of in situ pH inactivation processes.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Seasonal distribution of cPDE in particulate and dissolved fractions collected from the 0.1 m stratum of Sangre Isle Lake June 1988 - July 1989. Particulate (●—●) values given as nmol l^{-1} h^{-1}. Dissolved (○—○) values given as nmol l^{-1} min^{-1}. Error bars denote ± SE (N=3) except where error was smaller than the diameter of points.

Figure 2. Seasonal distribution of cAMP in particulate and dissolved fractions collected from the 0.1 m stratum of Sangre Isle Lake June 1988 - July 1989. Particulate (●—● , right ordinate) values given as pmol cAMP μg^{-1} chl a. Dissolved (○—○ , left ordinate) given as nmol cAMP l^{-1} lakewater.

Figure 3. Diel dynamics of cPDE in sonicated (●—●) and non-sonicated (○—○) particulate fractions collected from the 0.1 m stratum of Sangre Isle Lake on 15-16 July 1988. Values shown as nmol l^{-1} h^{-1}. Errors bars denote ± SE (N=3), except where error was smaller than the diameter of the points.

Figure 4. Diel dynamics of cPDE in sonicated (●—●) and non-sonicated (○—○) particulate fractions and in the dissolved (□—□) fractions collected from the 0.1 m stratum of Sangre Isle Lake on 18-19 August 1988. Particulate values given in nmol l^{-1} h^{-1} (left ordinate). Dissolved values given in nmol l^{-1} min^{-1} (right ordinate). Errors bars denote ± SE (N=3), except where error was smaller that the diameter of the points.

Figure 5. Upper panel. Diel dynamics of particulate cAMP (●—● , left ordinate) and dissolved cAMP (○—○ , right ordinate) on 15-16

July 1988. Values on both ordinates are given as nmol cAMP l^{-1} lakewater. Lower panel Diel-dynamics of biomass specific-cAMP on 15-16 July 1988. Values shown as pmol μg^{-1} chl *a*.

Figure 6. Upper panel. Diel dynamics of particulate cAMP (○—○, left ordinate) and dissolved cAMP (●—●, right ordinate) on 18-19 August 1988. Values on both ordinates are given as nm cAMP l^{-1} lakewater. Lower Panel. Diel dynamics of biomass-specific cAMP on 18-19 August 1988. Values given as pm cAMP μg^{-1} chl *a*.

Figure 7. Hydrolytic kinetics of particulate cPDE (upper panel) and dissolved cPDE (lower panel). Data were fitted to an exponential curve.

Figure 8. Thin-layer chromatogram of dissolved cPDE activity following a 90 minute incubation with ^{3}H-cAMP and 4 h development. Data on ordinate are graphed as CPM x 10^{2}.

Figure 9. Thin-layer chromatogram of particulate cPDE activity following a 90 minute incubation with ^{3}H-cAMP and 4 h development. Data on ordinate are graphed as CPM x 10^{2}.

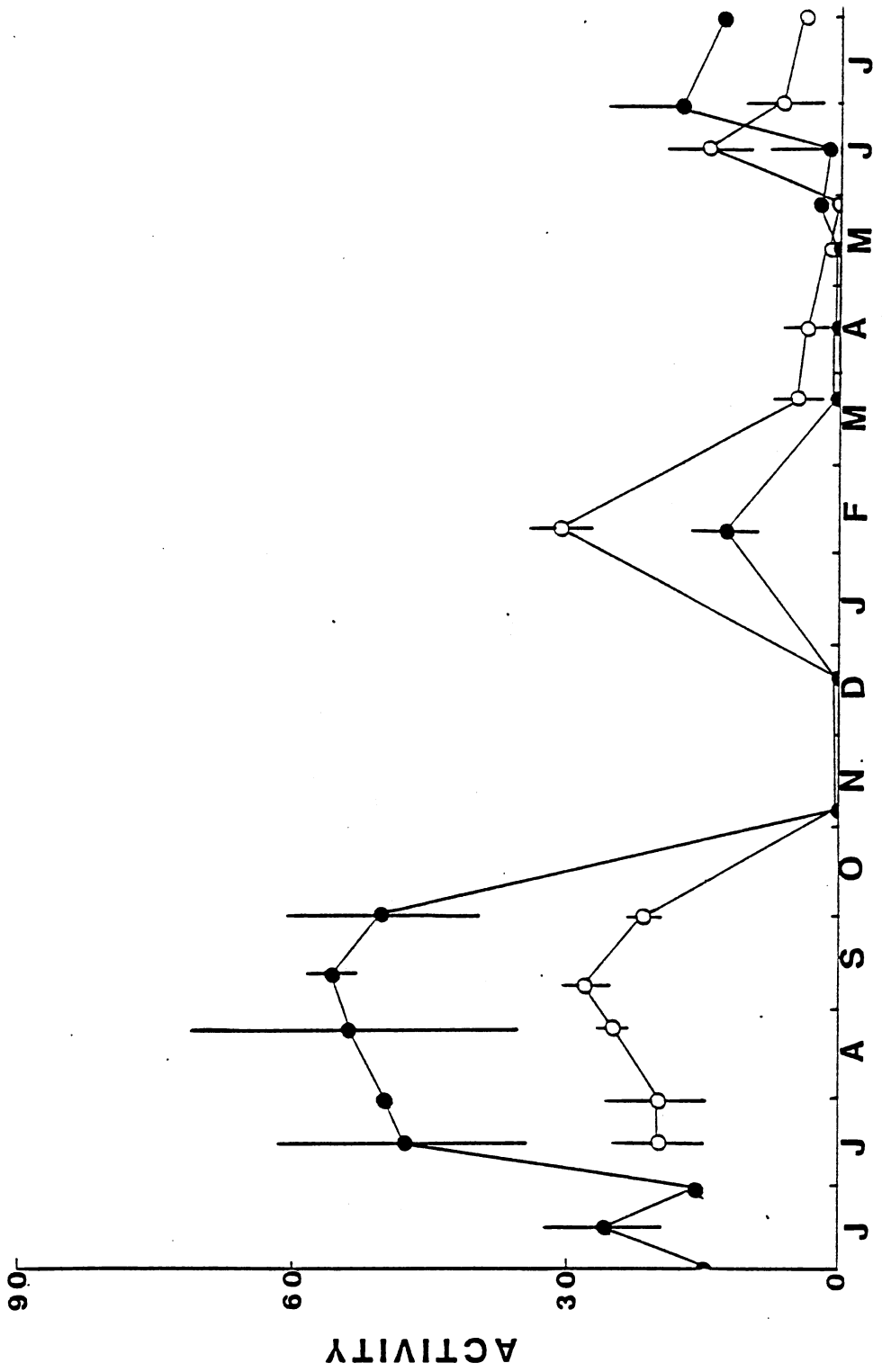

FIGURE 1.

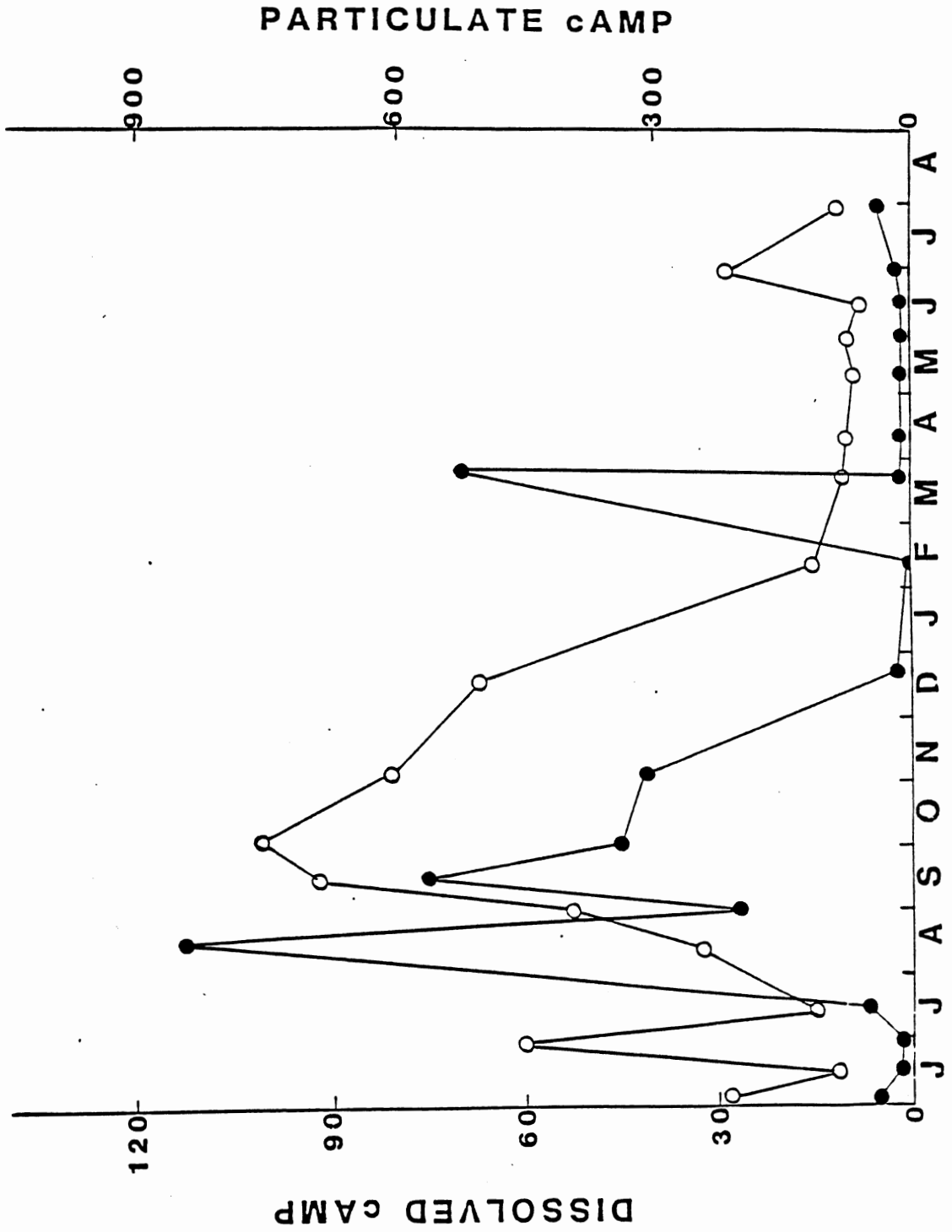
PARTICULATE cAMP
900
600
300
0
J J A S O N D J F M A M J J A
120
90
60
30
0
DISSOLVED cAMP

FIGURE 2.

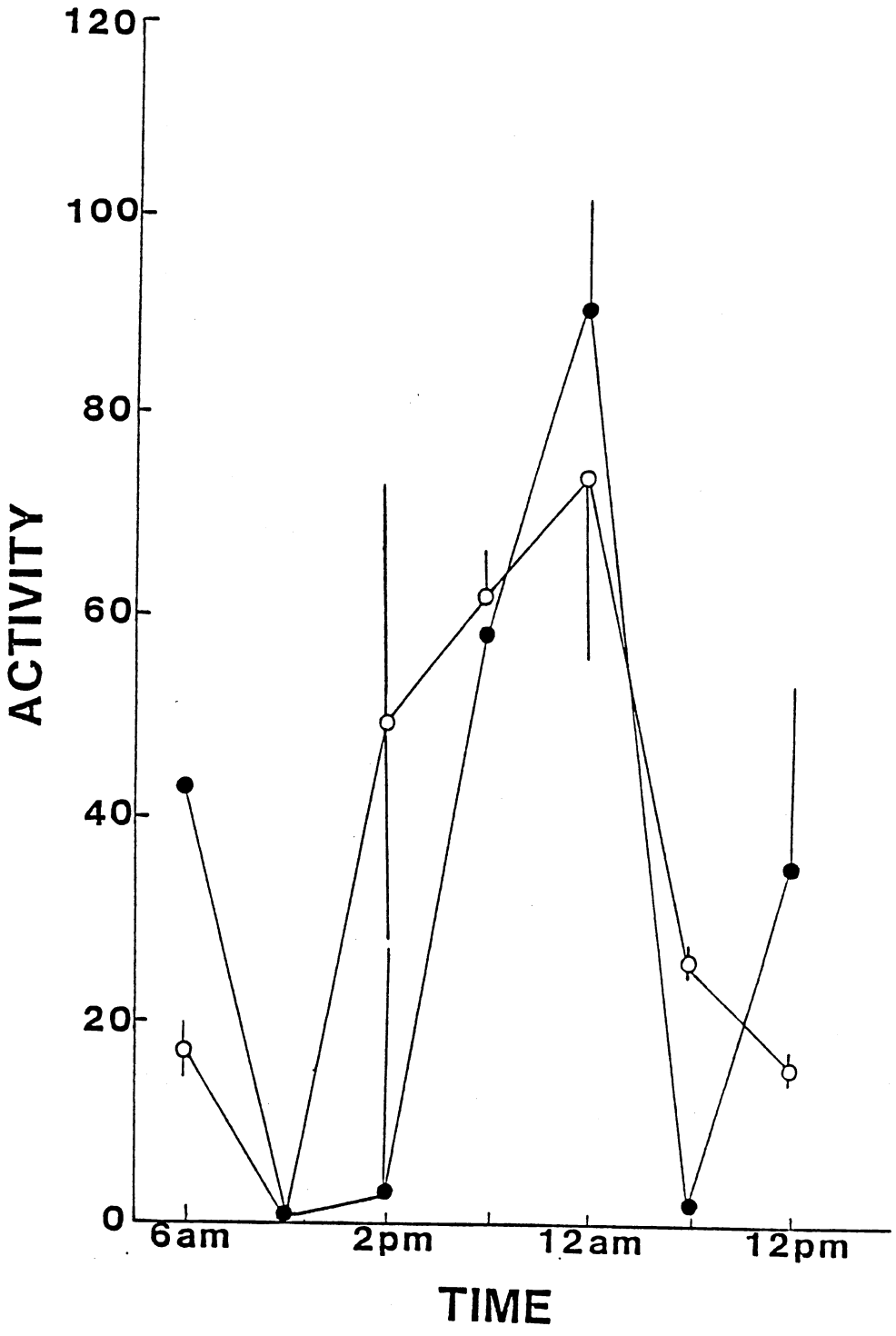

FIGURE 3.

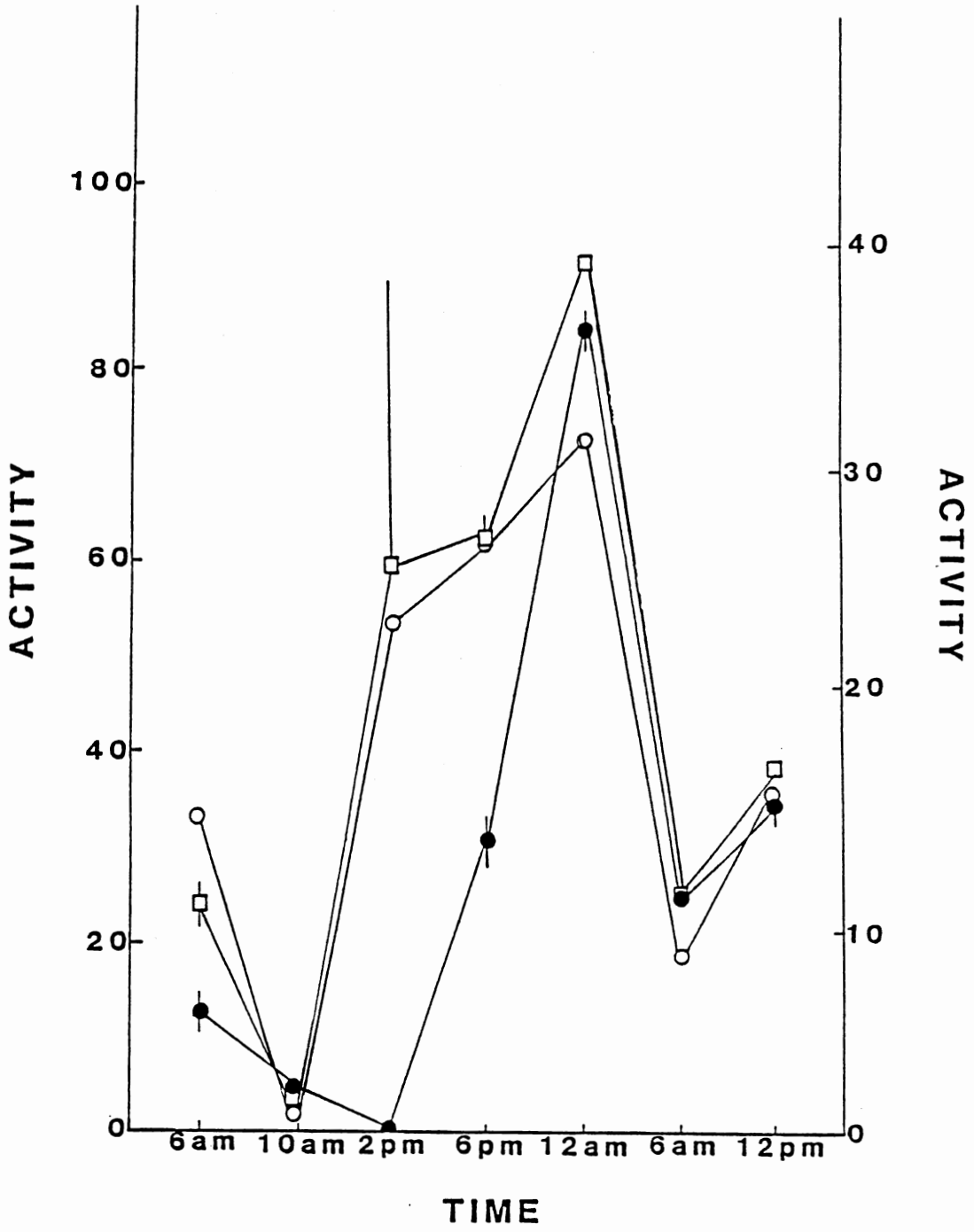
100
80
60
40
20
0
ACTIVITY
40
30
20
10
0
ACTIVITY
6am 10am 2pm 6pm 12am 6am 12pm
TIME

FIGURE 4.

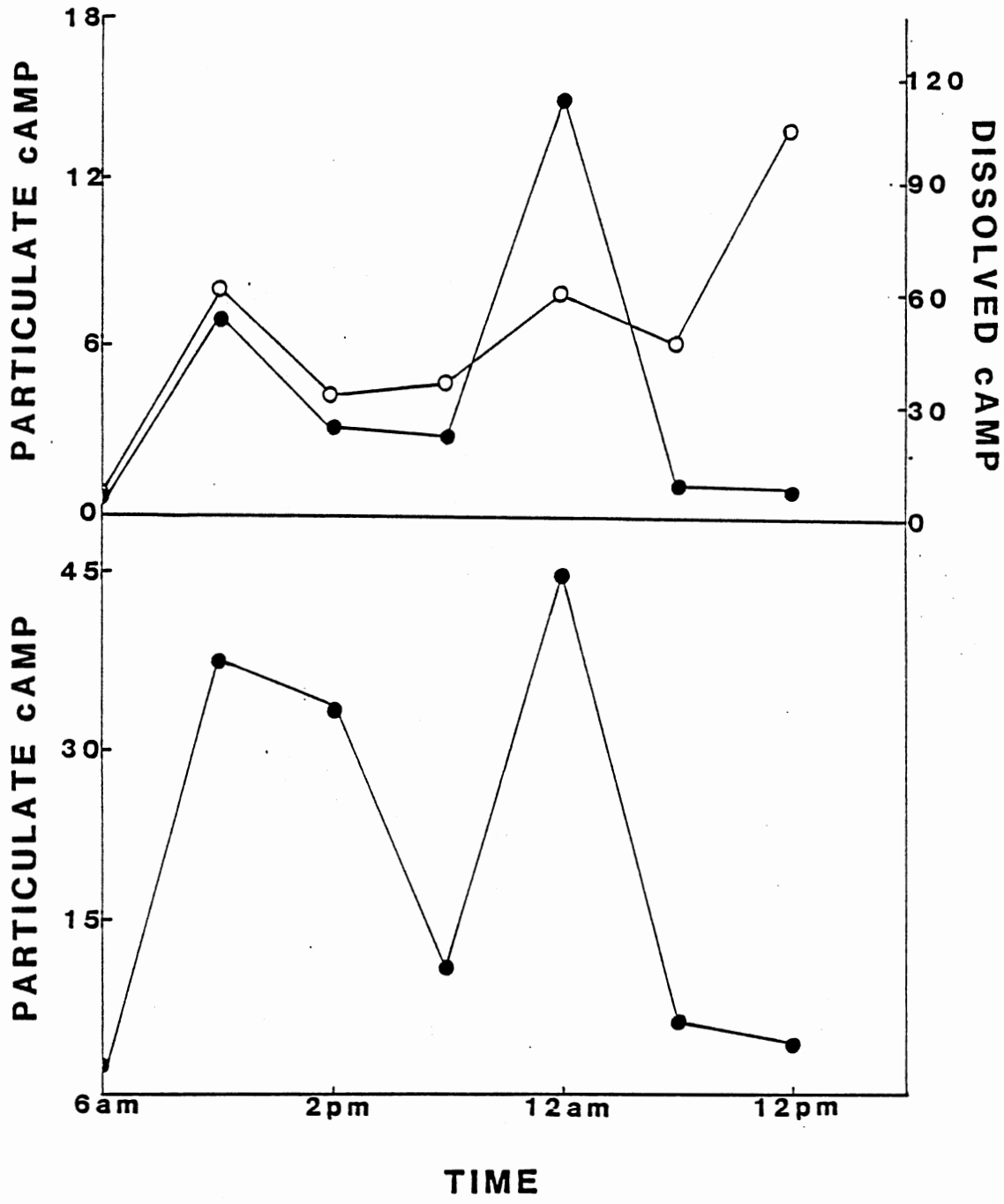
PARTICULATE cAMP
18
12
6
0
DISSOLVED cAMP
120
90
60
30
0
PARTICULATE cAMP
45
30
15
6am
2pm
12am
12pm
TIME

FIGURE 5.

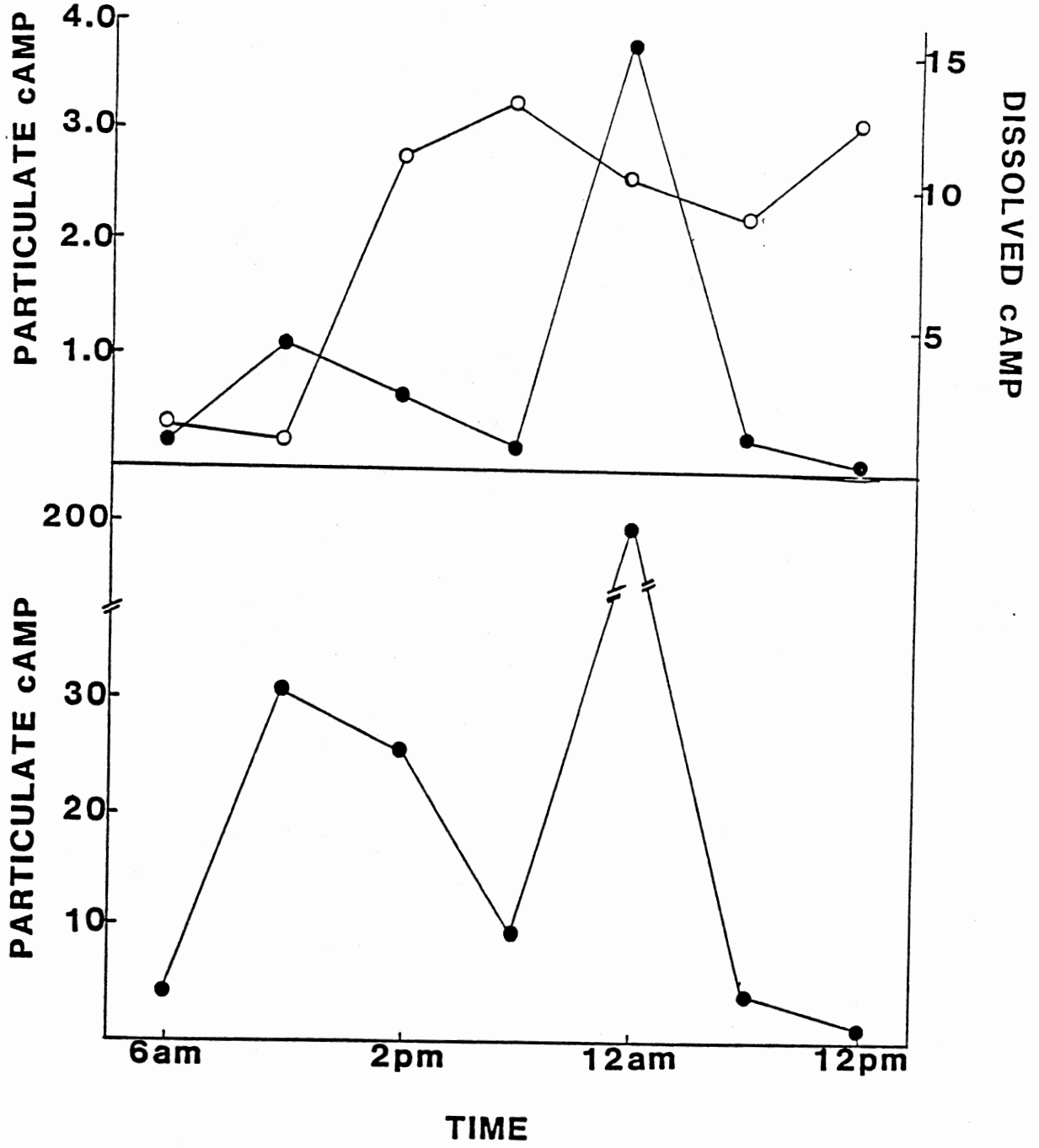
PARTICULATE cAMP
4.0
3.0
2.0
1.0
DISSOLVED cAMP
15
10
5
PARTICULATE cAMP
200
30
20
10
6am
2pm
12am
12pm
TIME

FIGURE 6.

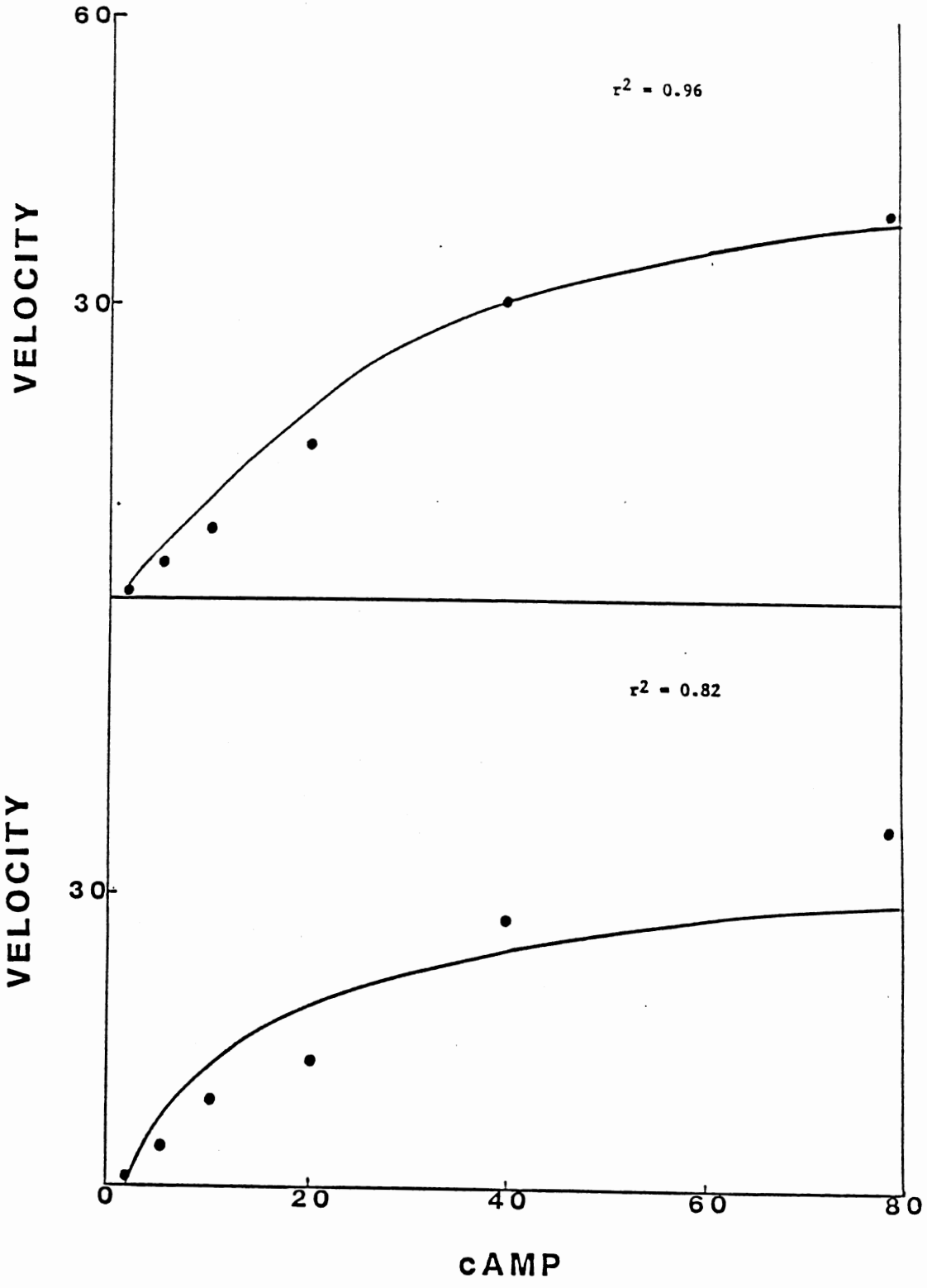

FIGURE 7.

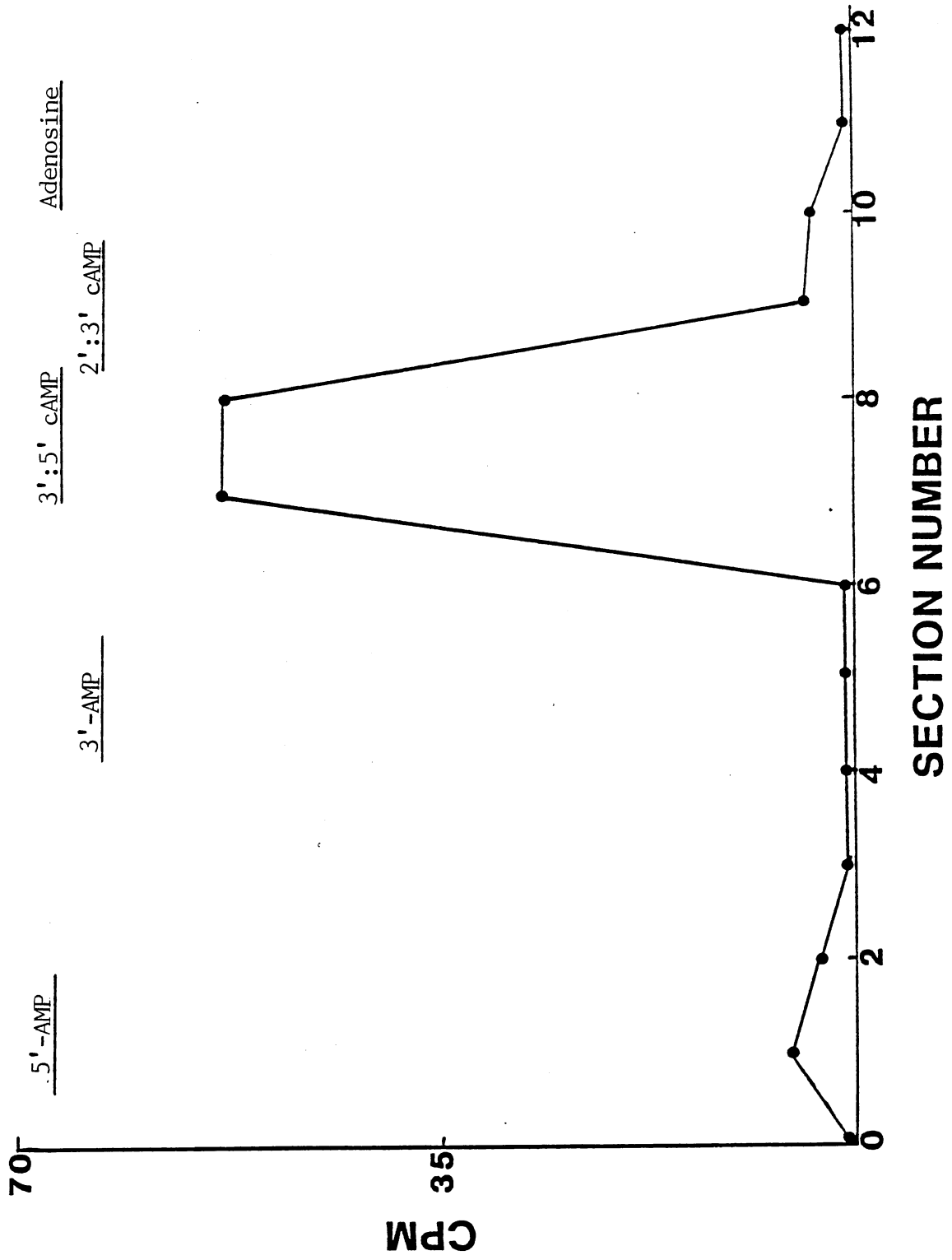
5'-AMP
3'-AMP
3':5' cAMP
2':3' cAMP
Adenosine
70
35
CPM
0
2
4
6
8
10
12
SECTION NUMBER

FIGURE 8

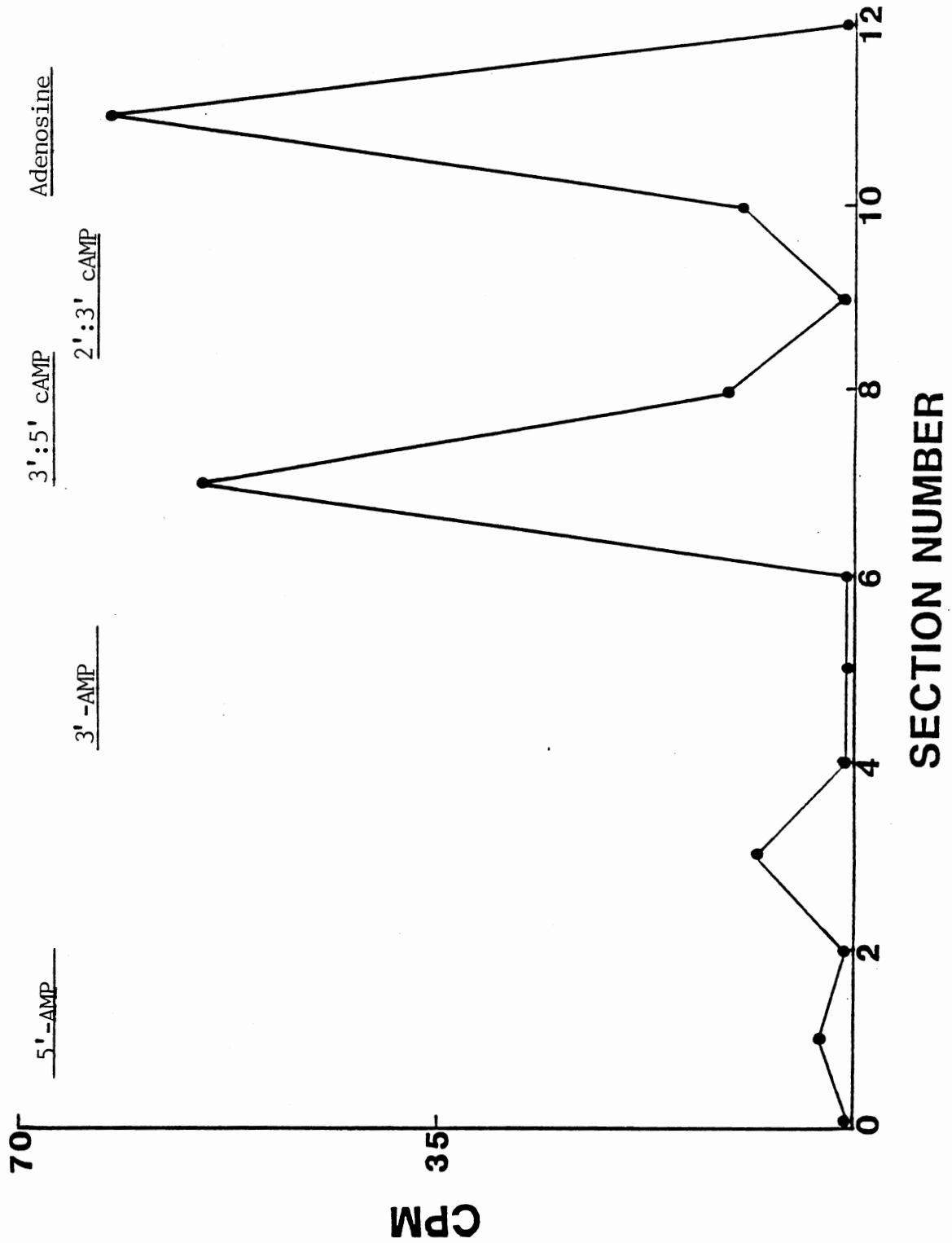
5'-AMP
3'-AMP
3':5' cAMP
2':3' cAMP
Adenosine
70
35
CPM
0
2
4
6
8
10
12
SECTION NUMBER

FIGURE 9

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