*Biochimica et Biophysica Acta*, 370 (1974) 498–509


BBA 67350

# STUDIES ON CYCLIC AMP PHOSPHODIESTERASE IN SEA URCHIN EGGS

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(Received June 12th, 1974)

## SUMMARY

1 Sea urchin eggs have multiple forms of cyclic AMP-phosphodiesterase (EC 3 1 4 17), 70% of which is localized in the post-microsomal supernatant However, in fertilized eggs at about first cleavage, there is a significant decrease in the amount (55%) of the enzyme detected in the post-microsomal supernatant This difference in the amount of phosphodiesterase present in the post-microsomal supernatant of cleaving eggs could be accounted for by a 2-fold increase in enzyme activity in heavy membrane fractions of cleaving eggs, as compared to that of unfertilized eggs

2 Kinetic studies show two apparent $K_m$ values for low and high cyclic AMP concentrations, i e 2 5 $10^{-6}$ and 1 4 $10^{-3}$ M, with $V_{max}$ values of 3 7 $10^{-8}$ and 3 2 $10^{-6}$ M, respectively

3 $Mg^{2+}$ is essential for the activity of the enzyme and the optimum pH is 7 60 Dibutyryl cyclic AMP, $N^6$-monobutyryl cyclic AMP and ADP were found to be inhibitory Methylxanthines also inhibited the sea urchin enzyme ATP was found to be the most potent physiological inhibitor of the sea urchin phosphodiesterase

4 A 4–5-fold increase in specific activity of phosphodiesterase was obtained in a 30–60% $(NH_4)_2SO_4$ fraction of the whole homogenate General properties of the partially purified enzyme were similar to those observed for the whole homogenate enzyme

5 Only a very weak cyclic GMP-phosphodiesterase activity was found in these egg-homogenates, which could be due to a non-specific activity of cyclic AMP-phosphodiesterase

## INTRODUCTION

We have previously reported on levels of cyclic AMP in fertilized and unfertilized sea urchin eggs [1] and on the changes in these levels in the presence of exogenously added cyclic AMP and $N^6$-monobutyryl cyclic AMP [2] Since cyclic AMP levels change after fertilization [1], an active regulatory system for metabolism of cyclic AMP must be present in these eggs Indeed, Castenada and Tyler [3] have shown that an adenylate cyclase is present in sea urchin eggs (some of this work has been confirmed in this laboratory (Amy, C and Rebhun, L I , unpublished observations) and we have examined some of the properties of cyclic AMP-phospho-

diesterase (EC 3 1 4 17) of these marine eggs Adenylate cyclase increases in activity after fertilization [3], but the degree to which this results in an increase in intracellular cyclic AMP concentration will depend on the activity and possibly, distribution of the phosphodiesterase as well We have therefore studied the cyclic AMP-phosphodiesterase before and after fertilization, examining its activity, intracellular distribution and response to a variety of potential pharmacological and physiological regulators of its activity

## MATERIALS AND METHODS

Eggs of the sea urchin *Strongylocentrotus purpuratus* were used in the present study Sea urchins were purchased from Pacific Bio-Marine Supply Co , Venice, Calif Cyclic [8-$^3$H]AMP (22 1 Ci/mmole) was obtained from The New England Nuclear Co Precoated mylar sheets of PEI-cellulose (POLYGRAM, CEL 300 PEI/UV$^{8}_{254}$) were purchased from Brinkman Instruments, New York The various nucleotides utilized in this study came from Sigma Chemical Co , St Louis Special enzyme-grade $(NH_4)_2SO_4$ was purchased from Schwarz-Mann All other chemicals were of reagent grade

Eggs of *S purpuratus* were collected and washed as described previously [1] In experiments with fertilized eggs, they were fertilized with an appropriately diluted suspension of fresh sperm and fertilization was routinely checked under the microscope Only those batches of eggs which showed 95–100% fertilization, were used for experimental purposes The eggs were gently stirred during and after fertilization and the temperature was maintained at about 18 °C Fertilized eggs were allowed to proceed to first cleavage (110–120 min at 18 °C), after which they were immediately centrifuged and the seawater was removed carefully The eggs were suspended and homogenized in an equal volume of ice-cold 0 10 M Tris buffer (pH 7 60), using a Dounce homogenizer In initial experiments, phosphodiesterase activity was assayed in whole egg-homogenates Subsequent kinetic studies utilized a 30–60% $(NH_4)_2SO_4$ fraction of whole homogenates, which was dissolved in 0 10 M Tris buffer (pH 7 60) and stored frozen in small aliquots at −20 °C In these preparations the specific activity was 4–5 times higher than in homogenates and the enzyme activity was stable for at least 2 weeks when stored frozen The 0–30% $(NH_4)_2SO_4$ fraction, which had a weak phosphodiesterase activity (15–20% of that in 30–60% $(NH_4)_2SO_4$ fraction) was discarded

Phosphodiesterase activity was also measured at different times during the course of early development of these eggs, i e at different time intervals after fertilization, until the first cleavage Enzyme assays in this case were done in whole homogenates or in 100 000 × *g* supernatants and their respective pellets

For studying the intracellular distribution of phosphodiesterase in sea urchin eggs, the eggs were homogenized in a medium containing 250 mM sucrose, 50 mM Tris buffer (pH 7 60) and 1 mM $MgCl_2$ and the homogenate was subjected to differential centrifugation The nuclear, mitochondrial and microsomal pellets were collected and suspended in the homogenizing medium mentioned above and the different fractions thus obtained were used as sources of the enzyme (Fig 1) The post-microsomal supernatant was also saved and phosphodiesterase assay was performed with the different subcellular fractions as described below The cell fractions

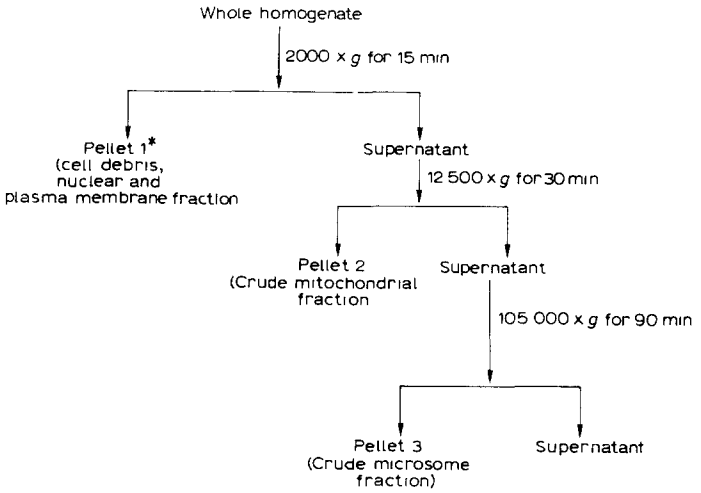


Fig 1 A schematic representation of the cell fractionation technique * Note that this pellet in the case of cleaving eggs does not contain the intact nuclei

were isolated in sequence in a Sorval RC-2B centrifuge and a Beckman L2-65B ultracentrifuge and were tested for their phosphodiesterase activity without further purification Homogenization of the eggs and all subsequent operations prior to the enzyme assay were carried out at 0–4 °C

Proteins were estimated by a microbiuret method [8], using bovine serum albumin as the standard

*Cyclic AMP-phosphodiesterase assay*

Phosphodiesterase was assayed according to a method modified from that of Cheung [4] Instead of measuring the inorganic phosphate released [4], cyclic [$^3$H]-AMP was used as the substrate and at the end of the reaction time, 5'-[$^3$H]AMP formed as the product was separated from the cyclic AMP by thin-layer chromatography on PEI-cellulose, using 0 85 M potassium phosphate (pH 3 40) as the developing solvent [5] Distinct separation of the various nucleotides (except between adenine and adenosine) was obtained when the chromatograms were developed for 3 h Nucleotides were carefully detected and marked out under an ultraviolet lamp and the spots were quantitatively scraped off, eluted with 0 5 ml of 0 3 M NaOH at room temperature and radioactivity counted in Bio-Solv toluene cocktail [6] Standard samples of pure cyclic AMP and 5'-AMP were routinely run in the chromatograms All phosphodiesterase assays were carried out at an excess of substrate concentration, so that the rate of reaction was linear throughout the length of the assay time The standard assay mixture contained in a final volume of 100 $\mu$l 50 mM Tris buffer (pH 7 60), 1 mM $MgCl_2$, 1 mM cyclic [$^3$H]AMP (115 000 cpm) and an appropriate amount of the enzyme (30–50 $\mu$g protein) Reactions were initiated by the addition of the enzyme, incubated for 30 min at 30 °C and finally stopped by the addition of 10 $\mu$l of 50% trichloroacetic acid Immediately after the addition of trichloroacetic acid, the tubes were placed in ice and all subsequent operations were carried out between 0–4 °C A 0-time sample, in which the trichloroacetic acid was

added before the addition of the enzyme, was routinely included in every assay Radioactive counts obtained at 5'-AMP spot of the 0-time sample were subtracted from the counts obtained at 5'-AMP spots of experimental samples All assays were run in duplicates

*Characterization of the cyclic AMP-phosphodiesterase*

In order to investigate the nature of phosphodiesterase(s) present in sea urchin eggs, i e whether multiple forms of the enzyme with different affinities for cyclic AMP exist in this system as in many others [9–14], kinetic measurements were performed over a wide range of concentration of cyclic AMP Initial $K_m$ determinations were made by plotting $1/v$ against $1/S$, according to the classical method of Lineweaver and Burke [7] and the results indicated the presence of two different forms of the enzyme with different affinities for cyclic AMP In order to further characterize the two different forms of the enzyme, phosphodiesterase assay was performed over a wide range of concentration of cyclic AMP ($10^{-7}$–2 $10^{-3}$ M) and the velocity-substrate concentration curves were graphed by plotting $v/S_0$ against $\bar{v}$, where $\bar{v}$ is the average velocity and $S^0$ is the initial substrate concentration Further analysis of the handling of data for phosphodiesterase kinetics is given in the Appendix of this paper For measuring the distribution of the high $K_m$ and the low $K_m$ phosphodiesterases, two widely different substrate concentrations, i e 2 $10^{-3}$ M and $10^{-6}$ M cyclic AMP concentrations were used Since in our case the low $K_m$ enzyme has a much lower $V_{max}$, it was assumed that at 2 $10^{-3}$ M cyclic AMP concentration, the rate of phosphodiesterase activity would be predominantly due to the high $K_m$ enzyme and interference from the activity of the low $K_m$ enzyme would be at a minimum Similarly, at the low substrate concentration, i e at $10^{-6}$ M cyclic AMP, the phosphodiesterase activity should be mainly due to the low-$K_m$ enzyme and the high-$K_m$ enzyme should contribute very little to the total phosphodiesterase activity at this low substrate concentration We must note here that the above assumption only follows if the low $K_m$ enzyme has also a $V_{max}$ considerably lower than that of the high $K_m$ enzyme, which is true in our case

## RESULTS

The phosphodiesterase activity in unfertilized or fertilized sea urchin eggs was found to be around 0 15–0 20 $\mu$mole per mg protein/min The activity was 4–5-fold higher in a 30–60% $(NH_4)_2SO_4$ fraction of the egg-homogenate, with which most of the kinetic studies have been performed Similar increases in specific activities in $(NH_4)_2SO_4$ fractions have been reported for cyclic AMP phosphodiesterases isolated from various mammalian systems [9–13] Under the experimental conditions described above, cyclic AMP hydrolysis was linear throughout the length of the incubation time (Fig 2) The optimum pH for the enzyme was found to be around 7 60 (Fig 3) and $Mg^{2+}$ were essential for its activity

It has been previously reported that unlike mammalian systems, e g skeletal muscle [9], puromycin and its purine analogue 6-dimethylaminopurine, do not inhibit the sea urchin egg-phosphodiesterase [1] However, in accord with other mammalian phosphodiesterases, the methyl xanthines caffeine, theophylline, aminophylline and methyl isobutyl xanthine, all inhibited the sea urchin enzyme (Table I) Methyl-isobutyl-

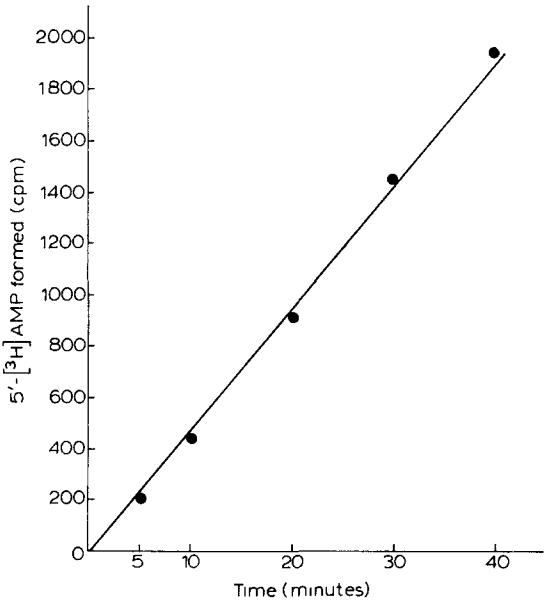


Fig 2 Linearity of the cyclic AMP-phosphodiesterase assay The assay mixture contained in a final volume of 100 $\mu$l 50 mM Tris buffer (pH 7 60), 1 mM $MgCl_2$, 1 mM cyclic [$^3$H]AMP (115 000 cpm) and an appropriate amount of the enzyme (30–50 $\mu$g protein) Other details are given in the text

xanthine was most potent in its inhibitory activity compared to the other methyl xanthines tested A number of other compounds were also tested for their effects on the enzyme activity Of all those tested, ATP was found to be the most potent inhibitor of the sea urchin enzyme Unlike the mammalian brain phosphodiesterases [14, 15], $Ca^{2+}$ did not stimulate (or inhibit) the sea urchin enzyme Dibutyryl

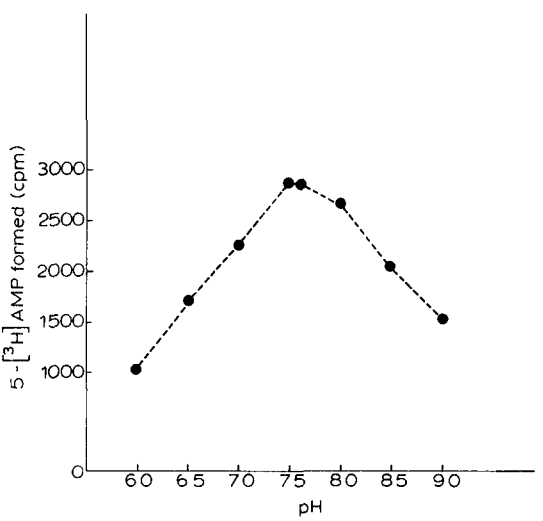


Fig 3 Effect of pH on sea urchin cyclic AMP-phosphodiesterase activity Assay conditions similar to Fig 2, except varying the pH as indicated in the graph

TABLE I

EFFECTS OF VARIOUS INHIBITORS OF PHOSPHODIESTERASE ACTIVITY IN SEA URCHIN EGGS

Assay conditions are the same as described in Fig 2

| Additions | Concn (mM) | % inhibition |
|---|---|---|
| Theophylline | 5 | 52 ± 3 4 |
| Aminophylline | 5 | 61 ± 5 2 |
| Caffeine | 5 | 51 ± 2 8 |
| 3-Iso-butylxanthine | 1 | 62 ± 3 4 |
| ATP | 1 | 78 ± 2 5 |
| ATP | 2 | 90 ± 1 7 |
| ADP | 1 | 50 ± 1 8 |
| Dibutyryl cyclic AMP | 1 | 47 ± 4 5 |
| Monobutyryl cyclic AMP | 1 | 48 ± 3 8 |

cyclic AMP, $N^6$-monobutyryl cyclic AMP and ADP were also found to be inhibitory at 1 mM concentration Effects of different inhibitors of the sea urchin egg-phosphodiesterase are listed in Table I

The total phosphodiesterase activity in sea urchin eggs remained essentially unchanged after fertilization, at least until the first cleavage (Fig 5) In our preliminary experiments, the cyclic AMP-phosphodiesterase seemed to undergo a decrease in its activity in fertilized eggs [1] This could have been due to incomplete elution of the nucleotide from the chromatograms Since then, the elution technique has been considerably improved in our present study by the addition of 0 3 M NaOH and incubation at room temperature, prior to the addition of scintillation cocktail

*Enzymatic parameters of cyclic AMP-phosphodiesterase*

Multiple forms of cyclic AMP-phosphodiesterase with different affinities for cyclic AMP have been widely reported in mammalian systems [9–14] In order to determine whether or not phosphodiesterases with different affinities for cyclic AMP exist in sea urchin eggs, kinetic measurements over a wide range of concentration of cyclic AMP ($2\ 10^{-3}$–$10^{-7}$ M) were performed When the values for the kinetic measurements at different cyclic AMP concentrations were plotted as described in Fig 4, the experimental points were distributed along straight lines of distinctly different slopes, indicating the presence of two phosphodiesterases with different affinities for cyclic AMP and different maximal velocities A phosphodiesterase with high affinity for cyclic AMP was found to have an apparent $K_m$ value of $2\ 5\ 10^{-6}$ M, with $V_{max}$ around $3\ 7\ 10^{-8}$ M The phosphodiesterase with a lower affinity for cyclic AMP had an unusually high $K_m$ value of $1\ 4\ 10^{-3}$ M, with a $V_{max}$ of $3\ 2\ 10^{-6}$ M Except for the extracellular slime-mold phosphodiesterase [16], the latter $K_m$ value seems to be at least one order of magnitude higher than those reported for the low-affinity enzyme in different systems [9–14]

*Intracellular distribution of phosphodiesterase(s)*

The different subcellular fractions were collected as described in Fig 1 and

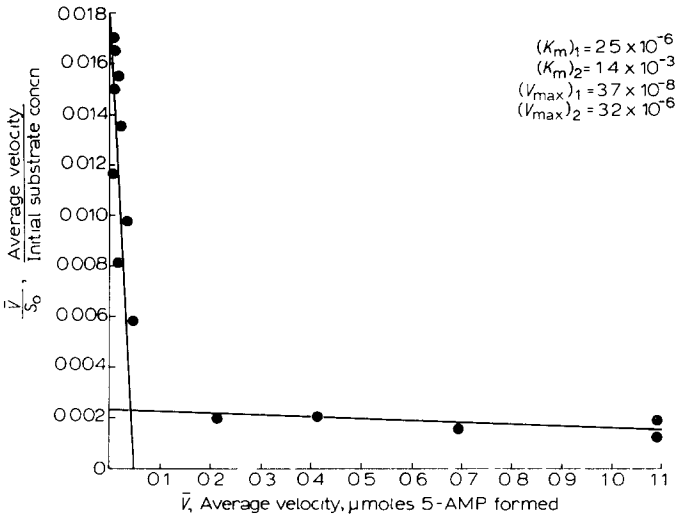


Fig 4 Kinetic properties of the cyclic AMP-phosphodiesterase Phosphodiesterase assay carried out as described in Fig 2 except varying the substrate concentration between 2 $10^{-3}$ and $10^{-7}$ M Kinetic analysis was done according to the details given in the Appendix of this paper $\bar{v}$ is the average velocity and $S_0$ is the initial substrate concentration

cyclic AMP-phosphodiesterase assay was carried out according to the procedure already described Most of the phosphodiesterase(s) in unfertilized sea urchin eggs was in the soluble fraction, 70% of the total activity having been recovered in the post-microsomal supernatant A much lower percentage of the total phosphodiesterse activity was recovered in the soluble fraction of the fertilized eggs (at first cleavage),

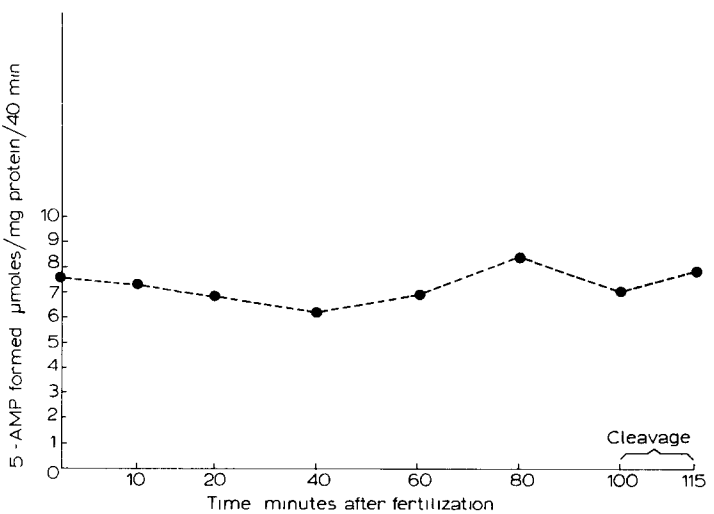


Fig 5 Cyclic AMP-phosphodiesterase activity in developing sea urchin eggs Phosphodiesterase assay was performed at different time intervals as indicated in the graph Assay conditions are same as described in Fig 2

TABLE II

INTRACELLULAR DISTRIBUTION OF HIGH $K_m$ AND LOW $K_m$ PHOSPHODIESTERASES IN UNFERTILIZED AND CLEAVING EGGS

Different cell-fractions were collected as described in Fig 1 and phosphodiesterase assay was performed as described in Fig 2 Results are expressed as percentages of the homogenates

| Phosphodiesterase type | Pellet 1* | | Pellet 2* | | Pellet 3 | | Supernatant | |
|---|---|---|---|---|---|---|---|---|
| | Unfertilized | Fertilized | Unfertilized | Fertilized | Unfertilized | Fertilized | Unfertilized | Fertilized |
| High $K_m$ | 10 ± 1 8 | 22 ± 3 2 | 5 ± 1 2 | 10 ± 2 8 | 15 ± 3 2 | 14 ± 2 8 | 70 ± 4 8 | 54 ± 4 5 |
| Low $K_m$ | 9 ± 2 2 | 17 ± 2 5 | 6 ± 1 5 | 12 ± 1 6 | 16 ± 3 6 | 16 ± 3 2 | 69 ± 5 4 | 55 ± 2 2 |

* Note changes between unfertilized and cleaving eggs

where only 55% of the total enzyme activity was detected in the post-microsomal supernatant and the rest of the activity was distributed in the various particulate fractions Study of the subcellular enzyme distribution in fertilized eggs at different time intervals (as in Fig 5) through first cleavage showed that the cyclic AMP-phosphodiesterase was redistributed, i e from a soluble form to a more particulated form, only around cleavage and not earlier after fertilization The subcellular distribution of the high $K_m$ and low $K_m$ phosphodiesterases is given in Table II Both the high $K_m$ and low $K_m$ enzyme activities are twice as high in heavy membrane fractions of cleaving eggs, as compared to that of unfertilized eggs Little change is seen in the enzyme activity of microsomal fractions of unfertilized compared to those of cleaving eggs

Assays for cyclic GMP-phosphodiesterase have also been performed, both in unfertilized and fertilized eggs Only a very weak activity could be detected in whole homogenates, which amounted to 5% of the total cyclic AMP-phosphodiesterase activity in these eggs This small activity may be due to a non-specific activity of the cyclic AMP-phosphodiesterase(s), which undoubtedly seems to be the major enzyme in these marine eggs Moreover, in our previous studies we could not demonstrate the presence of any quantity of cyclic GMP in these eggs [1] No detectable cyclic AMP (or cyclic GMP) phosphodiesterase activity is found in plasma membrane preparations isolated from these eggs Further characterization of the enzyme has not been undertaken

## DISCUSSION

An active cyclic AMP-phosphodiesterase is present in sea urchin eggs, the total activity of which does not change after fertilization, at least through first cleavage About 70% of the enzyme activity is found in the post-microsomal supernatant and the rest is distributed amongst the particulate fractions It should be noted however that as the different isolated cell fractions have not been washed further, (prior to the assay) a part of their phosphodiesterase activity could have originated from the adhering soluble supernatant

Unlike the cyclic AMP levels, or the adenylate cyclase activity in these eggs, the total phosphodiesterase activity does not increase on fertilization, a phenomenon, which could be significant in in vivo regulation of cyclic AMP levels after fertilization However, the intracellular distribution of phosphodiesterase(s) in fertilized eggs changes around the time of first cleavage The most noted difference as is evident from Table II, is a 2-fold increase in the activity of the phosphodiesterase(s) in both the nuclear and mitochondrial fractions isolated from fertilized eggs at first cleavage

The high $K_m$ enzyme (with an apparent $K_m$ of $1\,4\ 10^{-3}$ M) in sea urchin eggs seems to have a very low affinity for cyclic AMP Except for a slime mold phosphodiesterase [16], its $K_m$ is at least one order of magnitude higher than the $K_m$ reported for the low-affinity enzyme in different mammalian systems [8–14] An interesting feature in the kinetic parameter of the two phosphodiesterases of these eggs is the 100-fold difference in $V_{max}$ between the high-$K_m$ and the low-$K_m$ enzymes At the physiological levels of cyclic AMP in these eggs, i e around $10^{-7}$ M [1], the high-$K_m$ enzyme contributes very little to the total phosphodiesterase activity of these eggs However, by virtue of its higher $V_{max}$, it may still play some role in the regulation of

compartmentalized cyclic AMP inside the egg, particularly during fertilization and cleavage

It is worthwhile to draw attention to the highly inhibitory effect of ATP on phosphodiesterase from these eggs Physiological concentration of ATP inside an egg ranges between $10^{-3}$–$10^{-4}$ M [17], concentrations at which the phosphodiesterase is strongly inhibited (Table I) This may play an important role in regulating the balance between the in vivo adenylate cyclase and phosphodiesterase activities in these marine eggs It is possible, that phosphodiesterase remains inhibited to a significant extent under normal physiological conditions and it is the adenylate cyclase, which plays the major role in the regulation of in vivo cyclic AMP concentrations Reports have appeared in the literature [18] where phosphodiesterase activity remains essentially unchanged in the presence of various external stimuli, while adenylate cyclase activity increases, thereby causing a rise in cyclic AMP level

Higher concentration of phosphodiesterase in the heavy membrane fractions (but not in plasma membrane preparations) of cleaving eggs is difficult to explain at this time It may implicate some membrane-associated activities at cleavage, in which cyclic AMP may be involved [19, 20] The fact that this change in location of phosphodiesterase(s) (i e from a soluble to a more particulated form) occurs only around cleavage and not earlier on fertilization, is suggestive of such membrane-associated phenomenon

In conclusion, it may be said that these marine eggs have a complex enzymatic system for regulation for the intra-cellular cyclic AMP levels, but the exact role of this cyclic nucleotide in the various metabolic and developmental processes of these eggs is yet to be established

## APPENDIX*

Analysis of phosphodiesterase kinetics required solution of Eqn 1, the Michaelis–Menten equation for two enzymes acting simultaneously on the same substrate The analysis

$$-\frac{dS}{dt} = V = \frac{(V_{max})_2 S}{(K_m)_1 + S} + \frac{(V_{max})_2 S}{(K_m)_2 + S} \tag{1}$$

where $S$ is substrate concentration, $t$ is time, and $V_{max}$ and $K_m$ have their usual connotation, was most conveniently carried through in the integral form of the equation using the following transformation of variables

$$Y = \frac{1}{t} \ln \frac{S_0}{S}, \; X = \frac{S_0 - S}{t} \tag{2}$$

where ln represents the natural logarithm, $S_0$ initial concentration and $t$ is time The reader may verify that the integral form of the Michaelis–Menten equation for one enzyme is

$$Y = \frac{V_{max}}{K_m} - \frac{1}{K_m} X$$

in these variables

* Derivations by Dr N R Nath, Dept of Physics, University of Virginia, Charlottesville, Va, 22901, U S A

In order to represent the most general integral solution of Eqn 1 we introduce the following convenient abbreviations

$$\alpha_1 = \frac{1}{(K_m)_1},\ \alpha_2 = \frac{1}{(K_m)_2},\ \beta_1 = \frac{(V_{max})_1}{(K_m)_1},\ \beta_2 = \frac{(V_{max})_2}{(K_m)_2}$$

With these new constants and with the variables $X$ and $Y$, integration of Eqn 1 (after rearrangement of terms) between the limits of 0 and $t$ for the time variable and $S^0$ and $S$ for substrate concentration yields

$$Y = (\beta_1 + \beta_2) - \frac{\alpha_1\alpha_2(\beta_1 + \beta_2)}{\beta_1\alpha_2 + \beta_2\alpha_1} X - \frac{\beta_1\beta_2(\alpha_1 - \alpha_2)^2}{(\beta_1\alpha_2 + \beta_2\alpha_1)^2} \left\{ \frac{1}{t} \ln \left( \frac{S_0 + \dfrac{\beta_1 + \beta_2}{\beta_1\alpha_2 + \beta_2\alpha_1}}{S_t \dfrac{\beta_1 + \beta_2}{\beta_2\alpha_2 + \beta_2\alpha_1}} \right) \right\} \tag{3}$$

While data may be plotted directly with this equation, it is convenient to simplify it for use in certain limiting situations Preliminary plots indicated that the following conditions were approximated by our system

$$\alpha_1 \gg \alpha_2,\ \beta_1 \simeq \beta_2 \text{ (implying } (K_m)_1 \ll (K_m)_2) \tag{4}$$

If we simplify Eqn 3 under these conditions for low $X$ and for high $X$ (that is, for low substrate concentrations and for high substrate concentrations) with the additional restraint that $(S^0 - S) \gg S$) we obtain the following linear equations from which the $\alpha$'s and $\beta$'s may be obtained (and therefore, the $K_m$ and $V$ values)

$$\text{Low } X \quad Y \simeq (\beta_1 + \beta_2) - \frac{\alpha_1\beta_1}{\beta_1 + \beta_2} X \tag{5}$$

$$\text{High } X \quad Y \simeq \beta_2 - \beta_2 X \tag{6}$$

A more complex solution involving no assumption on substrate concentration can be obtained under the restrictions on $\alpha$'s and $\beta$'s given above This is

$$Y \simeq (\beta_1 + \beta_2) - \frac{\alpha_2}{\beta_2}(\beta_1 + \beta_2) X - \frac{\beta_1}{\beta_2} \left\{ \frac{Y}{\dfrac{X}{Y} + \dfrac{(\beta_1 + \beta_2)}{\alpha_1\beta_2}} \right\} \tag{7}$$

Determination of the constants was obtained by least squares fitting of the enzyme data with a NOVA 1200 computer $K_m$ and $V_{max}$ values obtained either from the simplified Eqns 5 and 6 or from the full Eqn 7 were very close For the best fit (in Eqn 7) the values of the constants were initially taken from the previous fits, and were systematically improved to obtain better chi-square values

ACKNOWLEDGMENT

This work was supported by funds from the National Institutes of Health and the National Science Foundation

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