# Cyclic 3′,5′-Adenosine Monophosphate Phosphodiesterase Produced by the Slime Mold Dictyostelium discoideum

Abstract. *An enzyme that hydrolyzes cyclic 3′,5′-adenosine monophosphate to 5′-adenosine monophosphate was found in the culture medium of the cellular slime mold* Dictyostelium discoideum. *The enzymatic activity shows a* p*H optimum of 7.5, and magnesium is required for maximum activity. The enzyme is not inhibited by caffeine. It has a Michaelis-Menten constant of* $2 \times 10^{-3}$M *and its molecular weight is around 300,000.*

One of the key events in the morphogenesis of the cellular slime molds is aggregation (*1*). Runyon (*2*) first demonstrated that aggregation is mediated by chemotaxis; this was firmly established by Bonner (*3*) who called the amoeba-attracting substance acrasin. Final proof was provided by Shaffer (*4*) who isolated the substance in vitro, reintroduced it, and obtained the orientation of sensitive amoebae. Furthermore, Shaffer (*5*) showed that the substance was degraded, presumably by an enzyme; this was confirmed by Sussman *et al.* (*6*).

Numerous attempts were then made to find the chemical identity of acrasin in *Dictyostelium discoideum* (*1*). There had been little progress until the recent discovery that the amoebae are attracted to cyclic 3′,5′-adenosine monophosphate (3′,5′-AMP) (*7*). It has been known for some time that bacteria give off an amoeba-attractant (*8*) and we know now that this attractant is cyclic 3′,5′-AMP (*7, 9*). Furthermore, we have demonstrated that the related species of cellular slime mold, *Polysphondylium pallidum,* secretes cyclic 3′,5′-AMP (*10*).

Cyclic 3′,5′-AMP was first discovered in animal tissues by Sutherland and Rall (*11*). This important substance is known to be implicated in numerous hormone reactions (*12*). In mammalian cells, it is formed from adenosine triphosphate by adenyl cyclase, an enzyme that is associated with the cell surface (*13*). For this study the pertinent step is the hydrolysis of cyclic 3′,5′-AMP to 5′-AMP by a specific phosphodiestrase (*14, 15*).

If cyclic 3′,5′-AMP is a naturally occurring acrasin (*10*), one would expect that the slime mold produces a phosphodiesterase; in fact, this should be the enzyme postulated by Shaffer (*5*) which destroys acrasin. In this study I have been able to show that *D. discoideum* does produce a phosphodiesterase, and it is interesting that the enzyme is released into the aqueous medium surrounding the amoebae. In the cellular slime molds it appears that both the attractant and the agent that destroys the attractant are almost entirely extracellular. This is reasonable, since these two substances must be partly responsible for the aggregation process that requires communication between cells that are initially separate from one another.

Amoebae of *D. discoideum* were grown on dead, autoclaved *Escherichia coli* in liquid culture (*16*). The *E. coli* (strain B/r) was first grown in a rich nutrient medium for approximately 40 hours at 22°C, washed once with 0.016*M* Sörensen buffer (*p*H 6.0), and suspended in the same buffer at a concentration of $10^{10}$ cells per milliliter. The bacterial suspension was autoclaved for 30 minutes at 121°C and was inoculated with amoebae that had been pregrown on autoclaved bacteria to give a concentration of $3 \times 10^4$ amoebae per milliliter. After about 4 days of growth at 22°C, when most of the bacteria had been eaten and the growth of amoebae had reached a stationary phase, the amoebae were harvested by centrifugation at 1500*g* for 5 minutes in a Sorvall refrigerated centrifuge. The supernatant obtained was centrifuged again at 10,000*g* for 10 minutes to remove all possible traces of *E. coli* debris.

This final supernatant was either dialyzed against 0.01*M* tris buffer (*p*H 7.5) and used directly as the source of the enzyme or fractionated with ammonium sulfate. In the latter case, the supernatant was brought to 90 percent saturation by adding crystalline ammonium sulfate at 0°C, with constant stirring. The precipitate was collected by centrifugation at 20,000*g* for 20 minutes and dissolved in one-tenth of its original volume of 0.01*M* tris buffer, *p*H 7.5. Some insoluble materials were removed by centrifugation before it was dialyzed in the same buffer solution. All these operations were carried out at 0° to 4°C. About 90 percent of the enzymatic activity of the medium was recovered in the fraction precipitated by ammonium sulfate, a fraction in which the increase in specific activity of the enzyme was approximately three- to fivefold.

The enzymatic activity of the amoebae was also examined. Amoebae were washed once with cold 0.01*M* tris buffer (*p*H 7.5), suspended in the same buffer, and broken by sonication. The entire broken-cell preparation was assayed without further fractionation.

The enzymatic activity was assayed according to the method of Butcher and Sutherland (*15*), with minor modifications. The reaction mixture contained 0.04*M* tris buffer (*p*H 7.5), 0.01*M* $MgCl_2$, 0.001 or 0.002*M* cyclic 3′,5′-AMP, snake (*Crotalus atrox*) venom (0.1 mg in 0.01*M* tris buffer, *p*H 7.5), and the enzyme in a final volume of 1.0 ml. The mixture was incubated for 30 minutes at 30°C, and the reaction was stopped by the addition of 0.4 ml of cold 20 percent trichloroacetic acid. After the removal of protein precipitates by centrifugation, the supernatant was analyzed for inorganic phosphate by the method of Fiske and SubbaRow (*17*). Methyl xanthines interfered with the phosphate analysis, but this was avoided by treating the supernatant solution containing trichloroacetic acid with norit. The *Crotalus atrox* venom contained a potent 5′-nucleotidase that hydrolyzed the 5′-AMP that formed in the reaction to adenosine and phosphate. Control experiments showed that the venom hydrolyzed neither cyclic 3′,5′-AMP nor 2′(3′)-AMP under the same conditions. The proper controls, in which either cyclic 3′,5′-AMP was omitted from the reaction mixture or trichloroacetic acid was added immediately after the enzyme, were included in each experiment to measure the background level of 5′-AMP and inorganic phosphate that might possibly be present in the enzyme preparations. Protein concentration was determined according to the method of Lowry *et al.* (*18*). One unit of enzyme activity is the amount of enzyme that catalyzes the hydrolysis of 1 μmole of cyclic 3′,5′-AMP to 5′-AMP in 30 minutes at 30°C.

The enzymatic activity appears in the supernatant of the culture medium. Usually, 1 liter of the supernatant of the medium contained 170 mg of protein and 800 units of the enzyme. Enzymatic activity of the preparation of broken amoebae was low—only 4 percent of that of the medium. Since the fraction precipitated by ammonium sulfate contained most of the enzymatic activity of the medium, this was the

one used for studying the properties of the enzyme.

The enzyme was tested for activity at various *p*H's with the use of tris-HCl as buffer and was found to have maximum activity at *p*H 7.5—a value close to that for phosphodiesterases of animal tissues (*14, 15*). It required $Mg^{2+}$ for maximum activity (Table 1); $Mn^{2+}$ could substitute for $Mg^{2+}$, but $Ca^{2+}$ was inactive. The enzymatic activity was completely inhibited in 1 m*M* ethylenediaminetetraacetate.

When the concentration of cyclic 3′, 5′-AMP was varied, a Lineweaver-Burk plot of concentration against activity gives a value of $2 \times 10^{-3}M$ for the Michaelis-Menten constant ($K_m$) of the enzyme and a maximum activity ($V_{max}$) of 24 unit/mg of protein (Fig. 1).

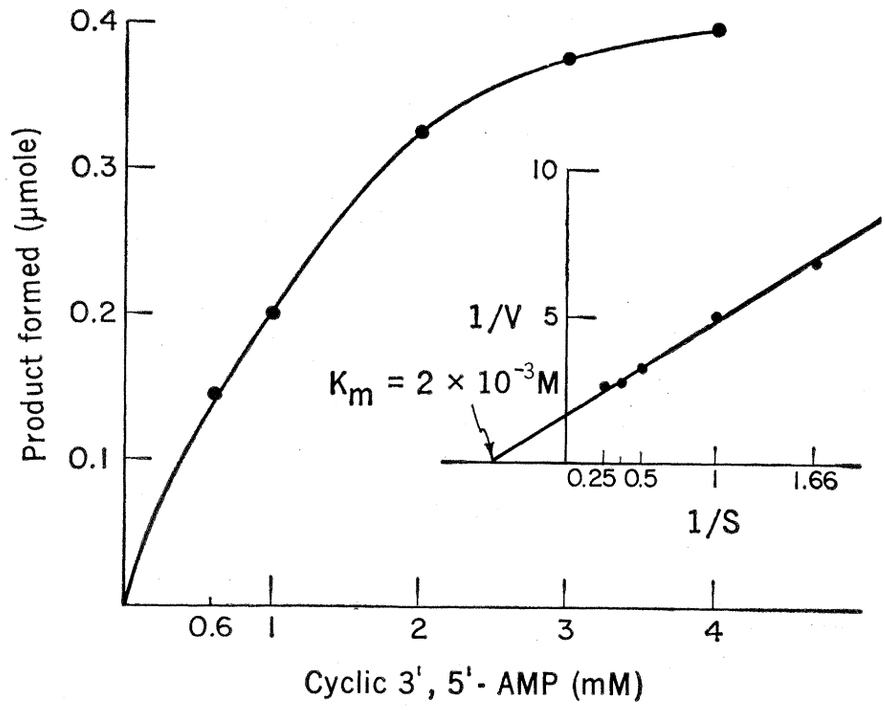


Fig. 1. Relation of the concentration of substrate to the activity curve and the Lineweaver-Burk plot of *Dictyostelium discoideum* phosphodiesterase. *V*, volume; *S*, substrate.

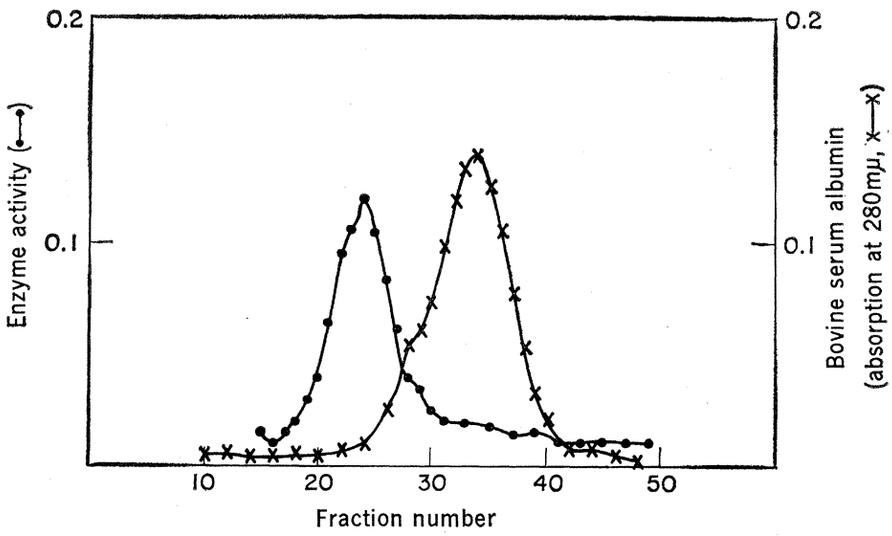


Fig. 2. Elution profile of phosphodiesterase activity and bovine serum albumin on a Sephadex G-200 column (2.4 by 38 cm). The volume of each fraction was 3.2 ml. The peak of blue dextran 2000 appeared at fraction 18 (void volume, 58 ml). See text for method of assay of phosphodiesterase activity. Bovine serum albumin was detected spectrophotometrically at 280 mμ. Elution was done at room temperature with 0.01*M* tris buffer, *p*H 7.5. Other experimental conditions were similar to those of Andrews (*20*).

Methyl xanthines are known as inhibitors for the phosphodiesterases of animal tissues (*15*). However, the amoeba enzyme was not inhibited by caffeine at a concentration of 10 m*M*. Theophylline (10 m*M*) in the assay mixture gave only 15-percent inhibition, whereas at this concentration it inhibits 90 percent of the beef heart phosphodiesterase activity (*15*). Diazoxide (7-chloro-3-methyl-1,2,4-benzothiadiazine 1,1-dioxide) (*19*) did not inhibit at 2 m*M*. Imidazole ($4 \times 10^{-2}M$) did not affect the enzyme, although it stimulates the activity of animal phosphodiesterase (*15*).

The product of the reaction catalyzed by the enzyme was identified in the following way. The enzyme was incubated with cyclic 3′,5′-AMP in the presence of $MgCl_2$ and tris buffer (*p*H 7.5) for various time intervals, and the reaction was terminated by addition of absolute ethanol to a final concentration of 80 percent ethanol. Samples of the reaction mixtures were analyzed by means of paper chromatography, the following four solvent systems being used: (i) 1*M* ammonium acetate and absolute ethanol (30 : 70, by volume); (ii) a mixture of isopropanol, ammonia, and water (7 : 1 : 2, by volume); (iii) a mixture of *n*-butanol, acetic acid, and water (5 : 2 : 3, by volume); and (iv) a mixture of saturated ammonium sulfate, 1*M* sodium acetate, and isopropanol (80 : 18 : 2, by volume). The nucleotides on the papers were detected under ultraviolet light. In all cases, 5′-AMP was found to be the only product of the reaction, and the concentration of 5′-AMP increased with the length of incubation time.

Molecular weight of the enzyme was estimated by Sephadex gel-filtration according to the method of Andrews (*20*). The enzyme was completely excluded from a Sephadex G-75 column; therefore, it was eluted on a Sephadex G-200 column, and bovine serum albumin was used as a standard protein of known molecular weight (Fig. 2). Molecular weight of the enzyme was estimated to be approximately 300,000, by using the curve for proteins of the elution volume ($V_e$) plotted against the log of the molecular weight provided by Andrews (*20*) for a Sephadex G-200 column. The estimation is a rough one, and more accurate determination of the molecular weight should be made

Table 1. Effect of divalent cations on the activity of phosphodiesterase.

| Cations added | Concentration (m$M$) | Activity (%) |
|---|---|---|
| | | 48 |
| $MgCl_2$ | 4 | 82 |
| | 10 | 100 |
| $MnCl_2$ | 4 | 92 |
| | 10 | 98 |
| $CaCl_2$ | 4 | 43 |
| | 10 | 43 |

by several other methods with a more purified form of the enzyme.

The enzyme was stable at room temperature for at least 5 hours and could be stored at −20°C for at least 6 weeks without loss of activity. Boiling the enzyme alone for 5 minutes completely destroyed the activity; however, the enzyme was partially protected from heat inactivation by the mixture of cyclic 3′,5′-AMP, $MgCl_2$, and tris buffer in the standard assay.

The fraction precipitated by ammonium sulfate does not contain nucleotidase activities, since it did not hydrolyze 5′-AMP or 2′(3′)-AMP.

In general, the properties of cyclic 3′,5′-AMP phosphodiesterase of slime molds resemble those of the phosphodiesterases of animal tissues. The one major difference is that phosphodiesterase of *D. discoideum* is relatively insensitive to the methyl xanthines. In this respect, it is similar to the phosphodiesterase of *E. coli* which is also not inhibited by caffeine (*21*). A minor difference is the fact that the $K_m$ of the *D. discoideum* phosphodiesterase is higher than the value obtained from other sources.

One important problem for the future is the role this enzyme plays in the normal development of the cellular slime mold. In addition, there is another aspect of this study which may have very general significance. This particular *D. discoideum* phosphodiesterase is liberated into the medium in large quantities and is remarkably stable. For these reasons this enzyme should be exceptionally useful in the study of the chemistry of cyclic 3′,5′-AMP.

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**References and Notes**

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## Rat Mammary Gland Differentiation in vitro in the Absence of Steroids

Abstract. *Mammary glands from several strains of immature female rats will form small lobules of alveoli when cultured in chemically defined media lacking steroid hormones but containing insulin and prolactin. The degree of lobulo-alveolar differentiation is increased if estradiol, progesterone, and aldosterone are also included in the culture media.*

Estradiol, progesterone, and prolactin previously have been considered to be the minimum hormone combination necessary for lobulo-alveolar differentiation in the mammary glands of rats (*1*) and mice (*2*). Organ culture experiments with both mouse (*3*) and rat (*4*) mammary glands have indicated a steroidal requirement for lobulo-alveolar development. However, lobulo-alveolar development has been reported in ovariectomized-adrenalectomized-hypophysectomized rats injected with large doses of prolactin and somatotropin (*5*). In recent experiments (*6*), steroids were found to be unnecessary for lobulo-alveolar differentiation in vitro of mammary glands from immature Long-Evans rats. The present report extends these results to other strains of rats.

The entire middle thoracic mammary glands from 3- to 4-week-old female Fischer, Long-Evans, Sprague-Dawley, and Wistar rats were removed and cultured in Waymouth's MB752/1 media containing penicillin G (35 μg/ml) and supplemented with the hormones insulin, or insulin + prolactin, or insulin + prolactin + estradiol-17β + progesterone + aldosterone. The culture method used was the same as that described by Ichinose and Nandi (*3*). Hormones were used at the following doses: crystalline bovine insulin, 0.124 unit/ml; prolactin (NIH P-S-6 and P-S-8), 0.124 unit/ml; estra-

Table 1. Effect of hormones on lobulo-alveolar development in vitro of mammary glands from immature rats of strains indicated.

| Hormones | Number of explants with indicated grade | | | | |
|---|---|---|---|---|---|
| | 0 | 0.5 to 1 | 1.5 to 2 | 2.5 to 3 | 3.5 to 4 |
| | | *Fischer* | | | |
| I | 4 | | | | |
| I-MH | 2 | 4 | 3 | | |
| I-MH-E-P-A | | 1 | 7 | 1 | |
| | | *Long-Evans* | | | |
| I | 6 | 4 | | | |
| I-MH | 1 | 2 | 8 | 3 | |
| I-MH-E-P-A | | | | 10 | 4 |
| | | *Sprague-Dawley* | | | |
| I | 4 | | | | |
| I-MH | 2 | 1 | 1 | 4 | 1 |
| I-MH-E-P-A | 1 | 3 | | 4 | 1 |
| | | *Wistar* | | | |
| I | 3 | | 1 | | |
| I-MH | 1 | 2 | 2 | 2 | 2 |
| I-MH-E-P-A | | 1 | 2 | 2 | 4 |