antibody used had specificity directed toward both the cyclopentane ring ($PGA_1 > PGE_1 > PGF_{1\alpha}$) and the side chains ($PGA_1 > PGA_2$ and $PGE_1 > PGE_2$).

This prostaglandin immunoassay system has not yet been fully characterized. However, it is already clear that by varying the antibody PGA_1, PGA_2, and PGE_1 can be measured in subpicomolar amounts. The present sensitivity of the immunoassay system and the differing degrees of prostaglandin cross reactivity justify the prediction that the radioimmunoassay system will be useful in evaluating the physiologic role of various members of the prostaglandin family.

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18 November 1970

Adenosine 3′,5′-Monophosphate Phosphodiesterase in the Growth Medium of Physarum polycephalum

Abstract. *The acellular slime mold* Physarum polycephalum *releases a soluble adenosine 3′,5′-monophosphate phosphodiesterase into the growth medium. Although this enzyme resembles the particulate diesterase of the same organism in kinetic properties and in inhibition by methyl purines, its greater stability, its insensitivity to stimulation by imidazole and to inhibition by adenosine triphosphate, and its selective release into the medium indicate a specific function, perhaps protection against exogenous cyclic nucleotide, for the soluble enzyme.*

Although adenosine 3′,5′-monophosphate (3′,5′-AMP) has been studied extensively in animal tissues as the probable mediator of the action of many hormones (*1*), recent work has also implicated 3′,5′-AMP in organisms not known to respond to hormones. Examples include the control of enzyme synthesis in bacteria (*2*) and the stimulation of aggregation (*3, 4*) and possibly differentiation (*5*) of *Dictyostelium discoideum* amoebae. Thus, in bacteria and in slime molds, 3′,5′-AMP appears to have highly specialized functions related to normal growth and development. The slime molds in particular may normally be expected to exist in environments providing exogenous sources of 3′,5′-AMP (for example, from dead bacteria). Because an exogenous supply of this nucleotide might interfere with normal regulatory mechanisms, such organisms may have evolved protective mechanisms. Thus *Dictyostelium* amoebae release into the growth medium a phosphodiesterase that hydrolyzes 3′,5′-AMP to 5′-AMP (*6*).

The present results show that *Physarum polycephalum* plasmodia contain a largely particulate 3′,5′-AMP phosphodiesterase and also release a potent phosphodiesterase into the growth medium; the properties of the two enzymes are significantly different.

Physarum polycephalum was grown in submerged culture (*7*); 30 ml of growth medium was inoculated with about 1 ml of a 3-day culture. When required, surface cultures were established on filter paper in contact with liquid medium (*8*). To obtain material for enzyme extraction, the mold was allowed to settle in the culture flask, collected with a Pasteur pipette, and washed by again settling through fresh medium. After removal of excess medium on filter paper, the mold was homogenized for 30 seconds in a Potter-Elvehjem homogenizer in 50 m*M* tris(hydroxymethyl)aminomethane (tris) (*p*H 7.4) and centrifuged at 20,000*g* for 10 minutes. The residue was washed once in 50 m*M* tris (*p*H 7.4) and finally suspended in the same buffer. To test for extracellular phosphodiesterase the growth medium was separated from the mold by decanting after 3 days of growth in shake culture and centrifuged at 20,000*g* for 15 minutes. The phosphodiesterase in the growth medium could be concentrated by precipitation between 40 and 60 percent saturation with ammonium sulfate followed by dialysis against 500 volumes of 10 m*M* tris (*p*H 7.4) for 16 hours; unless otherwise stated this concentrated enzyme was used in all studies with the growth medium enzyme.

Enzyme activity was routinely assayed by separation of the reaction products by paper chromatography. The reaction mixture contained, in a final volume of 0.03 ml, 0.05*M* tris buffer (*p*H 7.4), 0.01*M* $MgCl_2$, 0.35 μc of tritiated 3′,5′-AMP (specific activity, 1.4 c/mmole), approximately 10 μg of snake venom 5′-nucleotidase, 2.9 m*M* 3′,5′-AMP unless otherwise stated, and enzyme. Incubations were carried out at 30°C, and reactions were stopped with 0.01 ml of concentrated HCl. Samples (0.05 ml) were chromatographed on Whatman 3-MM paper in a solution of 1*M* ammonium acetate and ethanol (15 : 35, by volume); radioactivity associated with adenosine was measured by liquid scintillation counting. Control assays indicated that the 5′-nucleotidase preparation did not contain significant 3′,5′-AMP phosphodiesterase activity. The activity of 5′-nucleotidase was measured in similar assays without 3′,5′-AMP and 5′-nucleotidase and including 0.4 m*M* [8-^{14}C]AMP (specific activity, 1.2 μc/μmole). Protein was measured by the method of Lowry *et al.* (*9*).

Preliminary experiments indicated that 3′,5′-AMP phosphodiesterase activity was mainly located in a particulate fraction of *Physarum* and was also

extensively released into the growth medium. The relative capacities of the different fractions to hydrolyze 3′,5′-AMP and AMP are shown in Table 1; the results are expressed as total activities in the growth medium and in the entire mold harvested from the medium. A high proportion of the mold phosphodiesterase was localized in the particulate fraction. The relatively low activity of 5′-nucleotidase in the growth medium indicates some degree of specificity of protein release from the mold in shake culture, and the tenfold excess of diesterase activity in the medium suggests continuous excretion of enzyme during the growth period.

Specificity of release is also indicated by the high specific activity (up to 800 nmole/min per milligram of protein) of the concentrated growth medium enzyme. This specific activity is similar to that obtained with the extracellular *Dictyostelium* enzyme (*6*) and higher than that of purified phosphodiesterase preparations from several mammalian sources (*10, 11*).

Extracts made from surface cultures of *Physarum* indicated no change in activity of phosphodiesterase during the division cycle of the mold. Samples were taken to cover the period from 2 hours before the first synchronous mitosis until 3 hours after mitosis. During this period the concentration of 3′,5′-AMP in the plasmodium has been observed to undergo marked fluctuations (*5*).

The concentrated growth medium enzyme was stable for at least 4 weeks at −20°C with frequent thawing and freezing, but the particulate enzyme lost 70 percent of its activity after storage for 4 days at −20°C. In addition, the growth medium enzyme lost no activity on storage at 25°C in growth medium (*p*H 4.6) for 5 days. The high activity of 5′-nucleotidase in the particulate preparations prevented identification of 5′-AMP as the reaction product of the phosphodiesterase, although this is implicit in the assay used to measure the diesterase. However, the growth medium enzyme contained only low 5′-nucleotidase activity, which completely vanished after 7 day's storage at −20°C; thus the product of the reaction catalyzed by this enzyme could be isolated. Assays were carried out with concentrated growth medium enzyme (50 μg of protein) for 10 minutes under standard conditions, except that 5′-nucleotidase was omitted and the concentration of 3′,5′-AMP was 0.12 m*M*. The reaction was stopped by addition of 15 m*M* ethylenediaminetetraacetate (EDTA) and heating for 5 minutes in a boiling water bath. Muscle AMP deaminase (20 units) was added to one half of the reaction mixture, and incubation was continued for a further 30 minutes. Chromatography of portions in a mixture of ammonium sulfate, 0.1*M* sodium phosphate buffer (*p*H 6.8), and *n*-propanol (30 : 50 : 1, weight to volume to volume) and liquid scintillation counting indicated that 5′-AMP was the only product of phosphodiesterase action, and that this was quantitatively converted into 5′-IMP (inosine monophosphate) in the presence of 5′-AMP deaminase. The relative migrations of 5′-IMP, 5′-AMP, and 3′,5′-AMP in the above solvent were 1.0, 0.47, and 0.14, respectively.

Table 1. Activities of phosphodiesterase and 5′-nucleotidase in *Physarum* and in its growth medium. One unit of enzyme is defined as the amount catalyzing the production of 1 μmole of product per minute.

Sample	Phosphodiesterase (total units)	5′-Nucleotidase (total units)
Growth medium	7.2	0.72
Resuspended mold residue	0.78	16.4
Soluble mold fraction	0.09	32

The optimum *p*H for 3′,5′-AMP hydrolysis was tested with both the particulate and the growth medium enzymes in tris-HCl buffers; both enzymes had a broad *p*H optimum between 7.5 and 8.0. When assayed at *p*H 4.6 in sodium acetate buffer, the growth medium enzyme was 52 percent as active as at *p*H 7.4. Both enzymes had about 50 percent activity in the absence of Mg^{2+}; the addition of 1 m*M* EDTA to assays containing no added Mg^{2+} completely inhibited phosphodiesterase activity. Maximum activity was obtained with 1.6 m*M* $MgCl_2$, and no inhibition was observed at concentrations up to 10 m*M*.

Variation of the concentration of 3′,5′-AMP yielded values of the Michaelis-Menten constant (K_m) of 0.5 m*M* for both enzymes; maximum velocity of the concentrated growth medium enzyme was 610 to 800 nmole/min per milligram of protein and that of the particulate enzyme was 4 to 6 nmole/min per milligram of protein. The K_m value was similar to that reported for animal tissue 3′,5′-AMP phosphodiesterases (*10*), but lower than the value of 2 m*M* found with the extracellular phosphodiesterase produced by the cellular slime mold *Dictyostelium discoideum* (*6*).

Both enzymes were inhibited to the same extent by caffeine and theophylline; inhibition of phosphodiesterases from many sources by methylated purines is well known (*10–12*). With 0.14 m*M* 3′,5′-AMP, 50 percent inhibition was obtained with 3 m*M* caffeine and with 0.2 m*M* theophylline. In common with other phosphodiesterases (*10, 12*), the particulate enzyme from *Physarum* was stimulated by imidazole; 12.6 and 18 percent stimulation was obtained with 8 and 32 m*M* imidazole, respectively. The same concentrations of imidazole inhibited the enzyme from the growth medium by 6.5 and 16 percent.

Preliminary experiments also indicated that the particulate phosphodiesterase was inhibited by ATP. In assays containing 0.12 m*M* 3′,5′-AMP, 50 percent inhibition was obtained with 1.2 m*M* adenosine triphosphate (ATP); however, quantitation was difficult as particulate fractions rapidly hydrolysed ATP, and adenosine diphosphate was not an inhibitor. Therefore it is likely that the present results underestimate the inhibition by ATP. The growth medium enzyme was not inhibited by ATP concentrations as high as 2 m*M*. Inhibition of 3′,5′-AMP phosphodiesterase from rat brain (*11*) and bovine heart (*13*) by ATP has been reported.

Inhibition of the particulate phosphodiesterase by ATP provides some evidence for regulation of its activity in the mold and provides a clear-cut difference in the properties of the particulate and the growth medium enzymes. Both the differences in properties of the particulate and growth medium phosphodiesterases and the relatively high specific activity of the latter enzyme suggest that the enzyme found in the medium is a specific enzyme that is specifically secreted. Thus the organism must gain some advantage from the action of this enzyme in its environment. This conclusion is reinforced by the unusual stability of the excreted enzyme. Many of the previously studied extracellular enzymes catalyze first steps in the degradation of potential nutrients. However, the existence and properties of the secreted phosphodiesterase suggest a different type of function. In such slime mold habitats as rotting logs or other decaying organic matter, both the general level of metabolic activity and the diversity of organisms will be high. Under these conditions the release of various physiologically active com-

pounds, from either living or dead organisms, is inevitable, and the evolution of protective mechanisms against the more potent of these compounds may have been necessary. Destruction of a compound in the medium would be the most direct way to prevent it from disrupting the organism's normal regulatory mechanisms. It appears that the excreted phosphodiesterases of *Dictyostelium* and *Physarum* may be examples of a functional class of enzymes that protect an organism from the damage that would result from an exogenous source of a compound that participates in the regulation of the organism's metabolism or development.

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27 July 1970; revised 8 September 1970

Allergic Encephalomyelitis: New Form Featuring Polymorphonuclear Leukocytes

Abstract. *The passive transfer of allergic encephalomyelitis can be produced in a single day. In work described, the procedure was made to coincide with a transient drug-induced deficiency of lymphocytes. As a result, the lesions of this autoimmune disease contained a predominance of polymorphonuclear leukocytes instead of the usual mononuclear cells. Not only is this a new histologic form of the disease, but the ready recognition of polymorphonuclears as reactive cells provides a powerful new tool for investigating the roles of immunologically specific effector cells and nonspecific reactive cells in production of tissue damage.*

The perivascular inflammatory exudate of the autoimmune disease experimental allergic encephalomyelitis (EAE), like other forms of delayed hypersensitivity, contains far more mononuclear than polymorphonuclear leukocytes (*1*). Further dissection of the composition of the infiltrate can be accomplished with passive transfer of EAE by injection of lymph node cells from immunized donors into normal, unimmunized recipients. With the aid of this technique, it has been found that specifically sensitized mononuclear cells from the donor are far less common in the EAE lesions than nonspecific mononuclears from the donor or from the recipient (*2*). The importance of nonspecific host cells is indicated also by suppression of passive transfer of EAE by cytotoxic drugs or radiation applied to the *recipients* in advance of the cell transfer (*3*). We report a particular treatment of the recipient that has produced a morphologically new form of passive EAE in which the inflammatory leukocytes are predominantly polymorphonuclear. These polymorphonuclears can be of host origin only.

The method was based on three considerations. First, the passive transfer system permits manipulation of the recipient without affecting the encephalitogenic potency of the donor cells. Second, the cytotoxic drug cyclophosphamide reduces the level of circulating mononuclears faster than it reduces the level of polymorphonuclears (*4*). Therefore, it is easy to obtain a transiently selective lymphopenia in the recipients.

Third, the lesions of EAE can be detected in a scant 24 hours after passive transfer if recipients are prepared by a physical injury of their brains which lowers the threshold of disease (*5*). This rapid transfer of EAE can be accomplished during the period of selective lymphopenia.

Donor female Lewis rats (Microbiological Associates) were immunized in the right hind feet with guinea pig spinal cord, Freund's complete adjuvant, and pertussis vaccine (an ancillary adjuvant) (*5*). Seven days later, when many animals had early clinical signs of EAE, the draining lymph nodes were harvested and processed into a cell suspension. The living cells were injected intravenously into unimmunized, histocompatible male Lewis recipients. The recipients had been prepared beforehand with thermal injuries of the brain, and some of them had been given cyclophosphamide intraperitoneally. The recipients were killed 24 hours after passive transfer, and their brains were examined histologically.

In the control recipients that did not receive cyclophosphamide, veins and brain parenchyma adjacent to the zone of thermal coagulation necrosis were heavily infiltrated with mononuclear cells (*5*). These EAE lesions were identical with those observed in the usual protracted forms of EAE. In recipient rats that were given cyclophosphamide (125 mg/kg) 1 day before cell transfer, the location of the lesions was unchanged, but mononuclear cells were reduced in number and were largely restricted to the walls of a very few veins. In their place, polymorphonuclear leukocytes were present in large numbers (*6*). These cells were observed in vessel lumens, walls, and perivascular

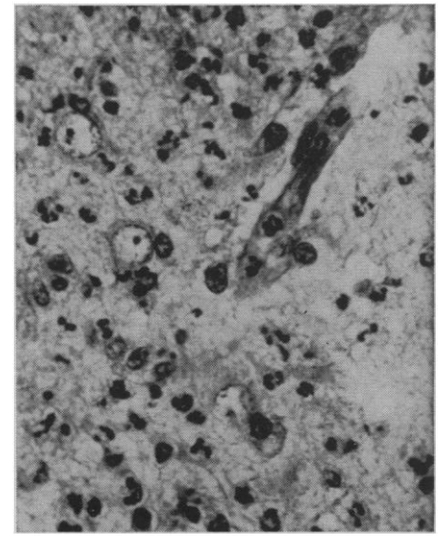

Fig. 1. The new form of allergic encephalomyelitis. There are four polymorphonuclear leukocytes in the lumen of a capillary (upper right), and at least 50 others are recognizable in the brain parenchyma of this one field. The large, pale nuclei with prominent nucleoli are neuronal. Medium-sized nuclei are probably mostly glial. Inflammatory mononuclear cells, which characterize conventional forms of EAE, are rare. Periodic acid–Schiff–hematoxylin stain (× 680).