To the Graduate Council:

I am submitting herewith a dissertation written by Leslie Robert Ballou entitled "Cyclic AMP and Calmodulin as Post-Translational Modulators of Fruiting in Coprinus cinereus." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

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We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Vice Chancellor
Graduate Studies and Research

CYCLIC AMP AND CALMODULIN AS POST-TRANSLATIONAL MODULATORS OF FRUITING IN *COPRINUS CINEREUS*

A Dissertation

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Doctor of Philosophy

Degree

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Leslie Robert Ballou

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ABSTRACT

Dikaryotic mycelia of Coprinus cinereus, when incubated in 12 hour alternating light-dark cycles on the appropriate defined media, will begin to differentiate fruit bodies in 6 days which will sporulate 5 days later. Mycelia incubated in continuous light or continuous darkness will not give rise to normal fruiting structures. In fruiting cultures a diurnal fluctuation of cAMP content associated with mycelial extracts was detected in which levels of the nucleotide increased during light exposures and decreased during dark exposures. The amplitude of cAMP fluctuations decreased with culture age on a dry weight basis. Continuous light incubated mycelia contained an initially higher level of cAMP than continuous dark incubated mycelia and in both cases levels of the nucleotide decreased with culture age. In general, the activities of glycogen phosphorylase, triacylglycerol lipase and protein kinase were negatively affected in the presence of high levels of cAMP. The activity associated with a Ca^{++}-dependent modulator protein, now called calmodulin, was very low or absent in non-initiating cultures but high in the mycelia of fruiting cultures, especially 24 hours prior to the first sign of differentiation in alternating light-dark incubated cultures. Associated with the pre-initiation increase in calmodulin activity was an increase in the phosphorylation of histone by kinases associated with crude mycelial extracts. A comparison of the activities of the post-translational modulators examined during the differentiation of Coprinus indicated that a decreasing, diurnally

fluctuating pattern of cAMP content, high calmodulin and histone phosphorylation activity were biochemical events that were critical to the initiation of the differentiation of fruit bodies.

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LIST OF ABBREVIATIONS

cAMP = adenosine 3',5'- cyclic monophosphate

EGTA = ethylene glycol bis(beta-aminoethyl ether)-N,N'-tetraacetic acid

EDTA = (ethylenedinitrilo)-tetraacetic acid

HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

Tris = tris(hydroxymethyl)aminomethane hydrochloride

TCA = trichloroacetic acid

DEAE = diethylaminoethyl

Cam = calmodulin

ATP = adenosine triphosphate

ScAMP-TME = succinyl tyrosine [^{125}I] methyl ester derivative of cAMP

RIA = radioimmunoassay

PDE = cyclic nucleotide phosphodiesterase

Bovine-PDE = activator-deficient cyclic nucleotide phosphodiesterase from bovine heart tissue

CHAPTER I

INTRODUCTION

Extensive studies on the formation of fruiting bodies in higher fungi have been undertaken to obtain basic genetic and biochemical information concerning the regulatory mechanisms associated with sexual morphogenesis (41, 42, 44, 62-67, 69-70). The salient features of a generalized life cycle of a Basidiomycete are an alternation of an indefinite haploid phase with a single celled diploid phase, usually with a dikaryotic phase interposed. Only under the appropriate environmental conditions will the dikaryon be induced to produce fruiting bodies containing basidial cells in which karyogamy, meiosis, and spore formation take place in rapid succession (41).

Some attempts have been made to identify the proteins regulated by the genes known to be responsible for particular stages of the sequence of morphogenetic events in higher fungi (41, 42). However, limited success has been achieved in the search for those particular proteins and to identify the specific activities of such proteins as being definitely associated with the initiation of fruiting.

More recently, several reports have indicated that the addition of culture media or mycelial extracts of the same or different species of fungi was effective in the initiation of fruiting in monokaryotic mycelia (18, 19, 62-67). The substance most effective in initiating the fruiting process has been isolated from mycelia or fruiting bodies of the same or entirely unrelated species of fungi, purified in some

cases (18, 19, 64), and identified as adenosine 3', 5'-cyclic monophosphate (cAMP) (18, 19, 49, 62-67).

Awareness of the importance of cAMP began in 1956 with studies on the molecular basis of hepatic glycogenolysis (40). Since then, a great amount of information has been amassed relative to the seemingly ubiquitous presence of cAMP in living organisms and its apparent universal function as an extremely versatile regulatory compound (2, 14, 45, 46). Cyclic AMP quickly became known as the intracellular mediator or "second messenger," of the action of many hormones in animals (45). Since then, however, cAMP has been implicated in a great number of non-hormonally regulated functions (14, 38, 45, 46) and furthermore, has become regarded as a part of the complex system by which cells are able to respond to environmental stimuli. In "primitive" organisms such as E. coli, cAMP would appear to be primarily involved as an intracellular signal which mediates the regulation of catabolite repression (43) and in Dictyostelium discoidium regulating the aggregation of amoebae which have been deprived of nutrients (25).

In all systems studied thus far, with the possible exception of E. coli (43), the activation of protein kinases is the only well established mechanism for the molecular action of cAMP (47). However, cAMP has been demonstrated to also inhibit and/or have no effect on protein kinases in many organisms (8, 9, 26-30, 47, 66) which makes generalizations about the mechanism of action of cAMP difficult at best. Protein phosphorylations regularly occur at serine residues (7, 47), and many such phosphorylated proteins serve as modulators or effectors of a great number of cellular responses (47).

Calcium ions have long been implicated as a potent, universal regulator of a number of cellular processes (73). However, little was known about the mechanism of action of calcium ions until the recent discovery of a multifunctional, calcium-dependent modulator protein, now called calmodulin (5-7). Calmodulin is now recognized as a primary receptor for calcium ions (5, 7, 10, 31, 71-73), much in the same way that cAMP-dependent protein kinases act as receptors for cyclic AMP (47). Often, the effects of cyclic nucleotides and calcium ions are interdependent since it has been discovered that calmodulin functions not only as a calcium ion receptor but also functions as a regulator of Ca^{++}-dependent adenylate cyclase and phosphodiesterase, the two enzymes responsible for the metabolism of cAMP (7). Thus, two very different kinds of regulators, calcium ions and cAMP, are intimately associated with one another through the action of calmodulin (7).

Since the initial discovery of calmodulin as an activator of phosphodiesterase (5), many more calmodulin regulated enzymes or cellular processes have been preliminarily identified and these include: adenylate cyclase, Ca^{++}-ATPase, guanylate cyclase, myosin light chain kinase, phosphorylase kinase, Ca^{++}-dependent protein kinase, NAD kinase, membrane phosphorylation, microtubule disassembly, phospholipase A_2, neurotransmitter release and others (7). Thus, it would appear that as organisms have become more complex, increasing the problems associated with intercellular communication, the number of cellular regulators has not increased proportionally, leaving cyclic nucleotides, calcium ions and hormones as three important regulators of cellular activities. Furthermore, all three interact in such manner such that the metabolism

of one modifies the metabolism of the other two (7).

Because it was possible to synchronize the fruiting of Coprinus cinereus by the appropriate light-dark incubations by an as yet poorly understood mechanism, this system was ideally suited to study the interrelationships of cAMP, which has already been shown to be involved in the induction of fruiting in higher fungi (5, 7, 10, 31, 71-73), protein kinase which have been shown to mediate the actions of cAMP (47) and calmodulin, which modulates the actions of cAMP (5-7), during the inductive period. Synchronous fruiting enables one to attempt to correlate metabolic events with specific changes in morphology. In the case of higher fungi, the production of fruit body primordia signals a change from vegetative to reproductive growth of the dikaryotic mycelia, a change that is no doubt triggered by a set of biochemical events that must take place prior to the onset of differentiation of primordia.

Previous attempts to correlate specific biochemical events to the onset of fruiting in higher fungi have met with little success because of the complex nature of the interactions of those compounds considered to be most likely involved in the fruiting process. Most significant is the fact that this is the first investigation on the subject of fruit body initiation to include a study and demonstration of calmodulin activity during development in higher fungi and because of this protein's effects on cAMP metabolism directly and cAMP modulated systems is an integral component in the sequence of biochemical events which ultimately determine whether initiation will or will not take place. The complex nature of the metabolic events which appear to be involved in the initiation of fruiting, with special reference

to the cyclic AMP-calmodulin-calcium ion interaction, are probably best illustrated by the general diagram of Cheung (7) (see Figure 1).

The presence of hormones or the possible role of such compounds in the fruiting of higher fungi has not been well established. In such organisms, cAMP may well act as a "first messenger" (23) instead of the classical, hormonally mediated "second messenger" (40) functions associated with the nucleotide in mammalian tissues. However, it is clear that other regulators of cAMP metabolism than hormones, such as Ca^{++}, calmodulin, and modulator binding proteins are all involved in ultimately eliciting a specific physiological response (7). It is the intention of this study to examine the interrelationships of cyclic nucleotide metabolism, protein kinases, and calcium-binding proteins in terms of fruit-body initiation in Coprinus.

Proposed Mechanism of Action of Cyclic AMP and Calmodulin

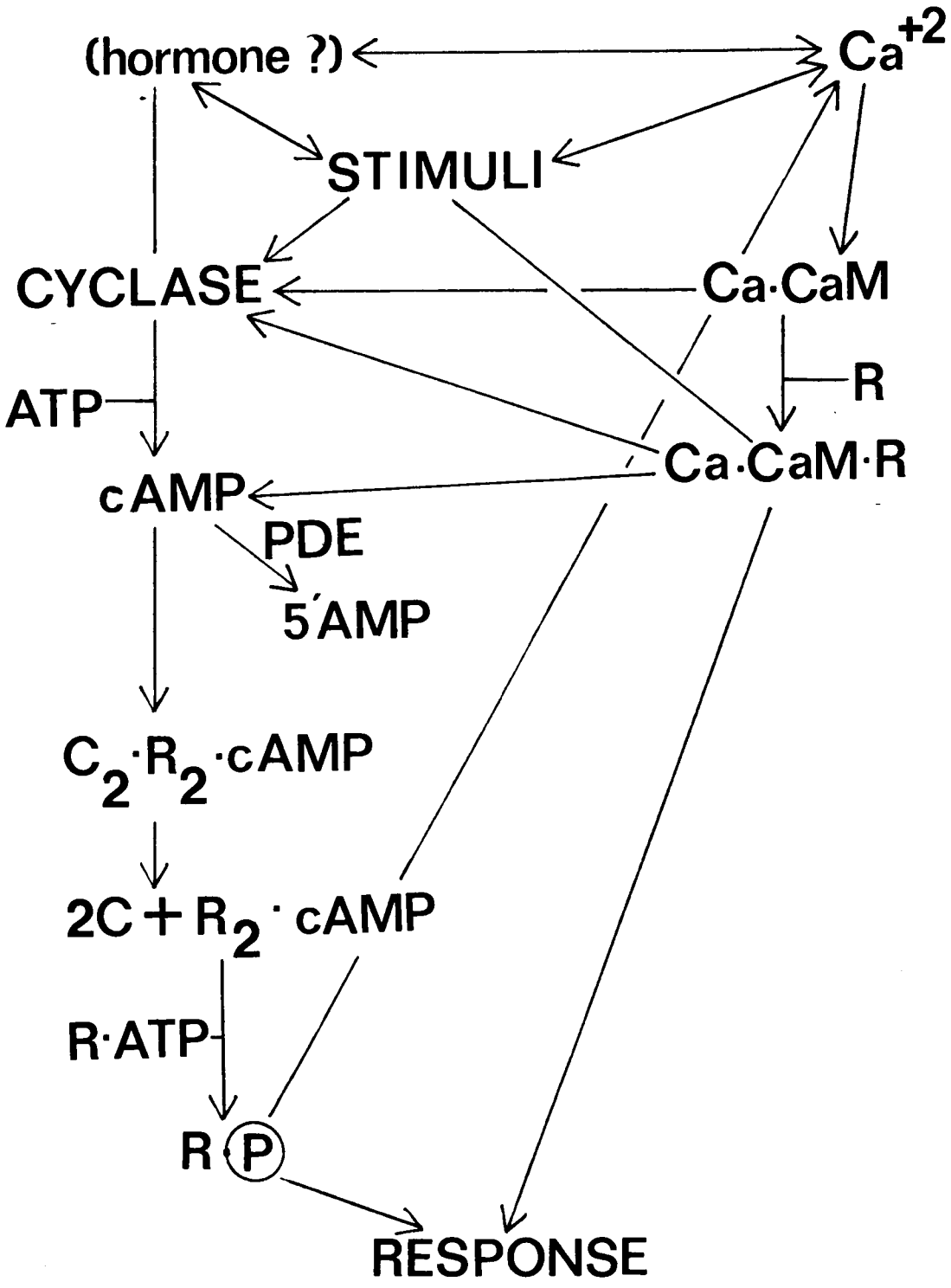


Figure 1. A generalized scheme of the mechanism of action of cyclic AMP and calmodulin. R, receptor; R_2, regulatory subunit; C, catalytic subunit; Ca, calcium ions; P, phosphorous. Modified from Cheung (7).

CHAPTER II

MATERIALS AND METHODS

Organism and Culture Procedures

Dikaryotic mycelia of the basidiomycete Coprinus cinereus (RK 246/1) were used throughout this study. The initial cultures of this organism were obtained from Dr. R. F. O. Kemp, University of Edinburgh, Edinburgh, Scotland.

Only after extensive preliminary culture studies was a defined media found that would support both vigorous vegetative growth as well as normal fruit body development. Initially, several different types of complex media were tested which were reported to support fruiting of closely related Coprinus species (33-35, 44, 63). However, since none of the media tested would support the fruiting of Coprinus cinereus in a predictable manner it became necessary to develop a defined media which would support such growth. The actual development of a complex defined media involved a process of trial and error beginning with, and eventually by modifying in various combinations the published culture techniques of Lu (33), Robert (44), Madelin (34), and Manachere (35). Ultimately, a combination of aspects of all four methods listed above were incorporated such that Coprinus cinereus was able to produce normal fruiting structures in a synchronous manner when vegetative mycelia were exposed to the appropriate light and dark incubations. The media is designated Coprinus Fruiting Media (CFM) and is composed of the following constituents in one liter of distilled water: 10 gm

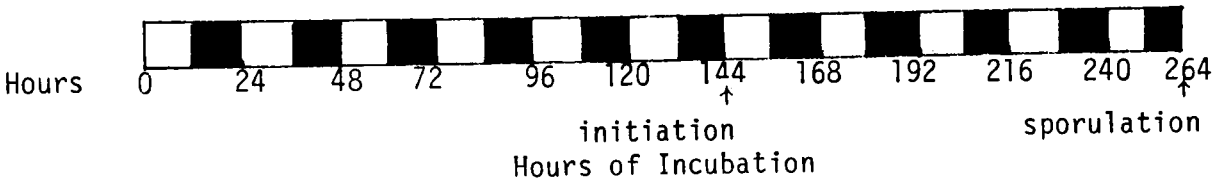
glucose; 0.65 asparagine; 1.0 gm alanine; 0.5 gm $Ca(NO_3)_2$; 0.5 gm $MgSO_4$; 0.25 gm K_2HOP_4; a stock solution of the following micronutrients was prepared so that when diluted to one liter it contained: 0.55 mg $ZnCl_2$; 3.5 mg $FeCl_3$; 1.2 mg H_3BO_4; 0.275 $CaCl_2$; 0.105 mg $(NH_4)_6MoO_4$; 0.10 mg $CuSO_4$ and 100 µg thiamine.

Thirty grams of agar was used per liter when solid media was desired. The 3% agar content of the media was firm enough to facilitate the easy harvesting of the mycelia from the surface of the agar while, at the same time, did not affect adversely the growth of the organism.

For most purposes mycelia were grown in 10 cm sterile plastic Petri dishes containing approximately 30 ml of media. Agar plugs removed from vigorously growing cultures with a sterile cork bore (7 mm diameter) were routinely used as inocula by simply inverting the agar plug on the surface, centrally on the plate to be inoculated. When larger quantities of mycelia were required mycelia were grown in low form culture flasks containing 500 ml of liquid media.

One of the most important requirements in the development of the system under which the organism was cultured was the necessity to be able to regulate the time of initiation of fruiting of individual cultures by the manipulation of a single environmental parameter. Following extensive experimentation it was found that the relative lengths of light and dark exposures during successive 24 hour periods exerted profound control over the growth of the organism and the eventual ability of the organism to produce normal fruiting structures. Exposure of the mycelia to periods of alternating dim light (100 fc/sq.) and total darkness of 12 hours each resulted in the synchronous

production of fruit body initials (hyphal knots) near the end of the 12 hour dark period terminating at 144 hours of total incubation time. Furthermore, if the alternating 12 hour light and dark exposure periods were continued, synchronous sporulation of mature fruit bodies would take place during the fifth 12 hour dark exposure following fruit body initiation (depicted in the following diagram):



Variation in the length of the respective light-dark exposures resulted in the disruption of the synchronous nature of the fruiting process, yet fruiting would still take place if the changes in the light/dark cycles were not drastic. Cultures grown in continuous light or continuous darkness never produced normal fruit bodies. Nonetheless, such cultures were grown and used routinely throughout this study as a basis for comparison of the metabolic events taking place in fruiting versus non-fruiting cultures prior to and just following the initiation of fruiting in alternating light-dark grown cultures at 144 hours of incubation.

In all experiments using solid media the mycelia were harvested by simply scraping the mycelia from the surface of the agar with a plastic spatula or if liquid culture, the mycelia were simply decanted from the culture flasks and rinsed with distilled water. Harvesting of alternating light-dark incubated cultures took place after 72, 84, 96, 108, 120, 132, 144, 156, 168, 180, 192 hours of incubation respectively. Mycelia grown in continuous light or continuous darkness were

harvested at 24 hour intervals beginning with 72 hour cultures and ending with 192 hour cultures. All cultures were incubated at 25°C.

Unless otherwise stated all data points represent average values and the vertical lines represent the range of experimental variation when appropriate.

Cyclic AMP Assay Procedure

The technique used for the assay of endogenous cyclic AMP levels in Coprinus was essentially that of Steiner et al. (56). All of the reagents (chemicals, antigens, and antibodies) were obtained from New England Nuclear Corporation. This technique employs a radioimmunoassay (RIA) based upon the principle of competition between radioactive and non-radioactive antigen (sample) and a fixed number of antibody binding sites. If increasing amounts of nonradioactive antigen are allowed to react with a constant amount of antibody, a decreasing amount of radioactive antigen is bound to the antibody and this result is expressed in terms of unlabeled antigen. The labeled antigen is a succinyl tyrosine [^{125}]I-methyl ester derivative of cyclic AMP (ScAMP-TME). Using this technique measurement of between 0.0025 and 25 picomoles of cyclic AMP can be assayed per assay tube.

The following procedure (56) was used for the extraction and measurement of cyclic AMP associated with dikaryotic mycelia of C. cinereus:

I. Mycelia were harvested at the intervals described earlier from cultures incubated in alternating light/dark, continuous light or continuous dark. Mycelia were homogenized by means of a Potter-Elvehjem homogenizer in several 1 ml aliquots of cold 6% TCA.

II. To determine the recovery of cyclic AMP during extraction approximately 4,000 CPM of [^{3}H]-cyclic AMP was added to the TCA extract of several of the samples. This amount of cyclic AMP was later subtracted when calculating the cyclic AMP content of those individual samples.

III. The samples were centrifuged at 2,500 x g for 15 minutes at 4^{o}C.

IV. The supernatant was collected and extracted 4 times with 5 ml of water-saturated diethyl ether. The ether phase was discarded.

V. The samples were placed in a water bath at 70-80^{o}C and evaporated to dryness under a stream of air.

VI. The residue was dissolved in 1 ml of 0.05 M sodium acetate buffer, pH 6.2 and 100 µl were used in each assay. The final assay mixture contained the following: 100 µl extract; 100 µl sodium acetate buffer; 100 µl of ScAMP-TME-[^{125}I] containing normal rabbit serum and 100 µl of the antiserum complex.

VII. The assay tubes were covered with aluminum foil and incubated for 18 hours at 2-8^{o}C.

IX. Following incubation 1 ml of cold buffer was added to each assay tube, mixed well with a vortex mixer and centrifuged: in the cold for 15 minutes at 2,000 x g. The supernatant was decanted by inversion of the assay tubes over absorbent paper for 1 minute making sure no liquid adhered to the lip of the tube. Care was taken to not disturb the antibody complex at the bottom of the assay tubes.

X. All tubes, including the appropriate blanks, were counted

in a gamma counter for one minute and compared with standards assayed at the same time.

Glycogen Phosphorylase (α-1,4-glucan-ortho-phosphate glucosyltransferase, EC 2.4.1.1) Assay Procedure

Appropriately incubated mycelia were harvested and homogenized as stated earlier in 5 mM potassium phosphate, 5 mM α-sodium glycerol phosphate buffer, pH 6.8, 0.2 mM dithiothreitol (DTT) and assayed within 30 minutes of homogenization. The reaction catalyzed by glycogen phosphorylase is reversible; however, evidence suggests that the enzyme functions *in vivo* in glycogen breakdown (2, 9, 14, 45, 46).

The assay of glycogen phosphorylase activity used in this study was a modification of the technique of Firtel and Bonner (12) utilizing a linked enzyme system containing phosphoglucomutase, glucose-6-phosphate dehydrogenase and 6-phosphogluconic acid. The production of NADPH was measured by the increase in absorbance at 340 nm per minute using a Beckman DU spectrophotometer coupled to a Gilford 242 chart recorder.

The final reaction mixture contained the following in a total volume of 1.0 ml: 20 μmoles sodium glycerol phosphate; 45 μmoles potassium phosphate, pH 6.8; 6 mg purified glycogen; 5 μmoles $MgCl_2$; 1.6 μmoles $NADP^+$; 1 μg glucose-6-phosphate dehydrogenase; 10 μg phosphoglucomutase; 100-250 μg protein from crude extract. The incubation was carried out at 25°C. A molar extinction coefficient of 6.22×10^3 was used to calculate units of enzyme activity which were defined as the amount of enzyme required to produce 1 nmole of NADPH per minute. Appropriate dilutions of the enzyme were made in order to

insure that all assays were performed at concentrations such that product formation was linear with respect to time.

Triacylclycerol Lipase (EC 3.1.1.3) Assay Procedure

The procedure used to assay lipase activity was essentially that of Tietz and Fiereck (61). This procedure is based on the hydrolysis of triacylglycerides in olive oil into fatty acids, diglycerides and to some small extent into monoglycerides and glycerol. The amount of fatty acid formed under the specific conditions of the assay was a measure of the lipase activity in the sample. The fatty acid formed was determined by titration with a standardized NaOH solution. Since each enzyme sample contained materials with titerable value it was necessary to subtract this value from values obtained from incubated samples.

The reaction mixture of each lipase assay contained the following in a total volume of 6.5 ml: 2.5 ml distilled water; 3 ml substrate (Sigma); 1 ml of 0.2 M Tris buffer (pH 8.0). Incubation was carried out at 37^{o}C for 6 hours. After incubation the contents of each assay tube were emptied into clean flasks and the assay tubes washed with 3 ml 95% ethanol. Each assay mixture was then titrated with NaOH (0.05 M). All lipase activity was expressed as International Units per liter by simply multiplying the number of mls of NaOH required to neutralize the fatty acids liberated during incubation by 280.

Protein Kinase (ATP: Protein Phosphotransferase, EC 2.7.1.37) Assay Procedure

Appropriately incubated mycelia were harvested and homogenized by means of a Potter-Elvehjem homogenizer in 0.05 M Tris-HCl buffer,

pH 7.4. The resulting homogenate was centrifuged at 1,000 x g for 20 minutes in the cold. The supernatant was used as the crude enzyme extract.

The protein kinase standard reaction mixture in a total volume of 0.2 ml contained the following: 10 moles of sodium acetate buffer (pH 6.5); 1.5 nmoles [γ - ^{32}P] ATP specific activity 9.5 Ci/mmol (New England Nuclear); 2 µmoles $MgSO_4$; 0.4 mg histone (RIA-grade), 1 µmolar cAMP and 0.2 ml crude extract. The above reaction mixture is a modification of the assay procedure of Uno and Ishikawa (66). Assays were performed in the presence and absence of exogenous [^{32}P] receptor (histone), cAMP and calmodulin (Sigma Chemical Co.). The reaction was initiated by the addition of labeled cAMP and terminated by the addition of 4 ml of cold 4% TCA. All samples were incubated at 25^{o}C for 30 minutes. As a carrier protein 0.2 ml of 0.63% bovine serum albumin was added after the addition of the TCA. This mixture was allowed to stand for 5 minutes at 4^{o}C and was then centrifuged. The precipitate was dissolved in 0.2 ml of 1 M NaOH and the solution was reprecipitated by the addition of 2.0 ml of cold 5% TCA. This procedure was repeated twice. Following the third wash the precipitate was dissolved in 200 µl of 1 M NaOH and an aliquot of this solution was taken to be counted by liquid scintillation. One unit of activity was defined as the amount of enzyme which would catalyze the incorporation of 1 picomole of [^{32}P] into protein in one minute. All assays were performed under conditions in which product was formed in a linear

fashion with respect to time. All liquid scintillation counting was carried out in low K^+ glass scintillation vials (20 ml) containing Scinti-Verse scintillation fluid (Fisher Scientific Company).

Bio-Gel Column Chromatography of Crude Protein Kinase Extracts

Liquid grown cultures were harvested when fruiting had begun and the mycelial mats were washed repeatedly with distilled water. All operations were carried out at 4^oC. The mycelial mats were homogenized in a Waring blender in 500 ml of 0.05 M Tris-HCl buffer, pH 7.4 and the homogenate was then centrifuged at 1,000 x g for 20 minutes. The supernatant was then divided into 100 ml aliquots and further centrifuged for 20 minutes at 15,000 x g. The supernatant was then treated with 72 gm of solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in 10 ml of 0.05 M Tris-HCl buffer, pH 7.4. After removal of the precipitate by centrifugation a 5 ml aliquot of the supernatant containing approximately 25 mg of protein was applied onto a Bio-Gel A 0.5 (100-200 mesh) column (2.5 cm x 40 c cm) which had been equilibrated with Tris buffer. Elution of the column was carried out with the same buffer and seventy 3 ml fractions were collected using an Isco fraction collector. The flow rate of the buffer was 12-15 ml per hour. The elevate was monitored at 280 nm using an Isco UV Analyzer coupled to a Beckman chart recorder.

A 0.2 ml aliquot of each column fraction was then assayed for protein kinase activity as described earlier. Histone (RIA purity

Sigma) was used as protein substrate in all assays and each fraction was assayed in the presence and absence of 1 μM cAMP.

Purification of Coprinus Cinereus Calmodulin by Fluphenazine-Sepharose 4B Affinity Chromatography

The following procedure for the isolation of calmodulin from crude protein extracts is a modification of the procedure of Charbonneau and Cormier (4). Liquid grown cultures were harvested and homogenized immediately in 500 ml of 50 mM Tris buffer, pH 8.0 which had been pre-heated to 100°C. This homogenate was filtered through two layers of cheese cloth while under vacuum on a large Buchner funnel. The residue remaining in the cheese cloth was squeezed by hand to remove any remaining liquid. The filtered material was then centrifuged at 7,500 x g for 20 minutes at 4°C.

The supernatant was treated with 1.1 volume of 95% ethanol while being stirred at 4°C for 10 minutes followed by centrifugation at 7,500 x g for 20 minutes. This supernatant was again treated with 1.45 volume of 95% ethanol and stirred for 10 minutes in the cold followed by centrifugation at 7,500 x g for 20 minutes. The pellet resulting from the centrifugation of the second ethanol treated supernatant was suspended in 10 mM HEPES, 0.5 mM $CaCl_2$, pH 7.0 and dialyzed extensively against the same buffer at 4°C. The dialyzed sample was then centrifuged at 13,000 x g for 30 minutes in the cold. The resultant pellet was used as the crude calmodulin preparation.

Fluphenazine (Squibb & Sons) was coupled to Sepharose 4B (Pharmacia) using the bisoxirane procedure (4). In this method, fluphenazine was attached to the agarose matrix via a 12 atom spacer provided by a diglycidyl ether coupling reagent. The following is a description of the procedure used for the activation and coupling of the fluphenazine to the agarose matrix: Sepharose 4B was washed with distilled water on a glass filter and suction dried under vacuum. Activation of the agarose was accomplished by mixing 50 gm of suction dried Sepharose 4B with 50 ml of diglycidyl ether, 50 ml of 0.6 N NaOH containing 3 mg/ml sodium borohydride. This mixture was incubated at room temperature with gentle shaking. After 8 hours, the gel was washed with 4 liters of distilled water. Following this wash, the coupling reaction was performed by mixing the activated gel with 80 ml of 0.1 M carbonate buffer, pH 11.0 containing 202 mg of fluphenazine-2HCl. This mixture was heated with gentle shaking for 48 hours at $70^{o}C$. After coupling, the Sepharose mixture was washed on a glass funnel with 1 liter of acetone to remove unreacted fluphenazine. The acetone wash was followed by a wash with 1 liter of absolute ethanol. During this wash, the agarose was exposed to the ethanol for at least 30 minutes to allow any excess oxirane groups to react with the ethanol. The coupling procedure and all subsequent uses of the coupled gel were carried out protecting the gel from light exposure with aluminum foil as fluphenazine is photosensitive.

Affinity Chromatography

The crude, dialyzed calmodulin extract was applied onto a column (2 cm x 19.8 cm) containing fluphenazine-Sepharose equilibrated

with 10 mM HEPES, 0.5 mM $CaCl_2$, pH 7.0. After application of the sample, the column was washed with approximately 100 ml of column buffer. Following this wash the column was eluted with 2 liters of 0.5 m NaCl in buffer. The salt wash was continued until absorbance at 280 nm was less than 0.005. Coprinus calmodulin was eluted from the column with 10 mM Tris, 5 mM ethylene glycol bis(beta-aminoethyl ether)-N,N'-tetraacetic acid (EGTA), pH 8.0. All chromatography was done at room temperature in the dark.

Cyclic Nucleotide Phosphodiesterase (EC 3.1.4.17) Assay Procedure

The procedure used to assay for cyclic nucleotide phosphodiesterase activity associated with crude mycelial extracts and with extracts from fresh bovine heart tissue is a modification of the procedure of Thompson and Appleman (60). In the first step of the assay tritium labeled cyclic AMP is hydrolyzed to 5'-AMP by the phosphodiesterase. The 5'-AMP is further hydrolyzed in the second step by snake venom nucleotidase to adenosine. Following snake venom hydrolysis, anion-exchange resin is added which binds all charged nucleotides leaving only uncharged [^{3}H]-adenosine to be counted. The reaction mixture of step one contained the following: [^{3}H]-cAMP sp. act. 36.4 Ci/mmol (200,000 CPM); 1 µM cAMP; 5 mM $MgCl_2$; 40 mM Tris-HCl, pH 8.0; 3.75 mM 2-mercaptoethanol; and an appropriate amount of enzyme in a final volume of 0.4 ml. Incubation was initiated by addition of labeled cAMP and was 10 minutes in duration at 30°C. The reaction was stopped by boiling for 2.5 minutes. Step 2 was initiated by the addition of 0.1 ml of snake venom (Ophiophagus hannah) to the contents of step 1. This mixture was then incubated at 30°C for 10 minutes. This incubation

was stopped by the addition of 1 ml of a 1:3 slurry or Bio-Rad resin, AG1-X2 (200-400 mesh). This mixture was then centrifuged in a clinical centrifuge and a 400 µl aliquot counted by liquid scintillation. The enzyme concentration used in each assay was such that product formation was linear with respect to time. In specific cases either calmodulin and/or EGTA was added to the initial reaction mixture as was 1 mM Ca^{++}.

Isolation of Calmodulin-Dependent Cyclic Nucleotide Phosphodiesterase from Bovine Heart Tissue

The procedure used for the purification of calmodulin (activator) deficient cyclic nucleotide phosphodiesterase (PDE) is a modification of the technique of Ho et al. (16).

Bovine heart tissue was obtained fresh (Lay Packing Company) and kept refrigerated until it was cut into small pieces (200 grams total weight) and homogenized in a Waring blender in 2.5 volumes of buffer containing 20 mM Tris and 1 mM EDTA, pH 7.5. The homogenate was centrifuged at 365 x g for 30 minutes, the supernatant collected, and its pH adjusted to 8.0. The supernatant was brought to 60% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 11,000 x g for 30 minutes in the cold. The resultant pellet was suspended in 20 mM Tris, 1 mM imidazole, 1 mM $MgAc_2$, 0.1 mM EGTA, 10 mM 2-mercaptoethanol buffer, pH 7.5 (buffer A) and dialyzed against the same buffer overnight. The dialyzed sample was then centrifuged at 40,000 x g for 1 hour and the supernatant applied onto a DEAE cellulose column (4 cm x 30 cm) which had been equilibrated with buffer A. The column was first eluted with 2 column volumes of Buffer A containing 0.05 M NaCl and then with a linear gradient of 0.05-0.40 M NaCl. Total volume of the gradient was

1 liter. All fractions collected were dialyzed against 40 mM Tris, pH 7.5 and then assayed for cyclic nucleotide phosphodiesterase activity in the presence and absence of calmodulin obtained from Sigma Chemical Company or from extracts of Coprinus in the presence or absence of EGTA.

Assay for Calmodulin Activity

Calmodulin isolated from C. cinereus was assayed according to its ability to stimulate the activity of calmodulin-deficient cyclic nucleotide phosphodiesterase from bovine heart (bovine-PDE). The reaction mixture of 0.4 ml contained 20 mM Tris, 1 mM imidazole, 1 mM $MgSO_4$, 1 μM cAMP, 10 μM $CaCl_2$, [^{3}H]-cAMP (300,000 CPM), pH 7.5, 80 g bovine-PDE and an appropriate amount of calmodulin. To this basic reaction mixture other components were added in order to investigate various aspects of the reaction including 10 mM EGTA, 1 mM EDTA and 50 mM caffeine. The amount of calmodulin added in routine assays was diluted such that the stimulated activity of the bovine-PDE (activator-deficient) resulted in linear product formation with respect to time. For preparations of bovine-PDE possessing activator-independent phosphodiesterase activity, this activity was measured separately and subtracted from the total calmodulin stimulated activity. One unit of calmodulin activity is defined as the amount of calmodulin required to stimulate the hydrolysis of 1 picomole of cAMP per minute above the basal activity level of activator-deficient bovine-PDE. Specific activity is expressed as units per mg of protein.

Time course experiments were conducted using crude extracts from C. cinereus in which no exogenous bovine-PDE was added to the

reaction mixture so that endogenous activator-deficient phosphodiesterase activity could be measured in the presence of 10 mM EGTA.

Protein Determination

Unless otherwise stated, total soluble protein was determined by the procedure of Bradford (3) or if interference of protein binding to Coomassie Blue was suspected the procedure of Lowry was employed (32). Bovine serum albumin served as the standard in either case.

Disc Polyacrylamide Gel Electrophoresis

The electrophoretic procedure used in this study was a modification of the procedure of Davis (11) using 7-1/2% acrylamide gels. The only modification of the procedure used in this study was that the sample was applied onto the spacer gel in a sucrose solution (1:1) instead of polymerizing the sample in the gel. Both the anode and cathode buffers were Tris-glycine, pH 8.9. All electrophoresis was carried out at $4^{o}C$ and the current used was $\simeq$ 2 mAmps per tube. Three hours were required for each run. Protein was stained with Coomassie Brilliant Blue R-250, destained and stored in a 10% acetic acid/10% isopropanol solution.

CHAPTER III

RESULTS

The Effect of Culture Condition on the Growth of *Coprinus*

Dikaryotic mycelia of *C. cinereus* grown as described in the methods section were harvested and dry weights and total soluble protein of aliquots were determined. Dry weight determinations are presented in Figure 2. Under all incubation conditions an initial lag phase was followed by a period of rapid growth. Cultures grown in alternating light/dark continued to grow rapidly throughout the experimental growth period while the growth rates of cultures grown in continuous light and continuous dark began to decrease (relative to light/dark cultures) at 144 hours of incubation.

Total soluble protein content of the mycelia (Figure 3) followed a linear course over the entire experimental incubation period for both continuous light and continuous dark-incubated cultures. Mycelia grown under alternating light/dark conditions had similar protein content compared to continuous light and dark-grown mycelia prior to 144 hours of incubation. A slight diurnal response was detected with respect to protein content in light/dark-incubated cultures with the greatest increases taking place during dark periods. After 144 hours of incubation, alternating light/dark cultures began to accumulate significantly higher amounts of protein than continuous light or dark-grown cultures so that by the end of the incubation period alternating

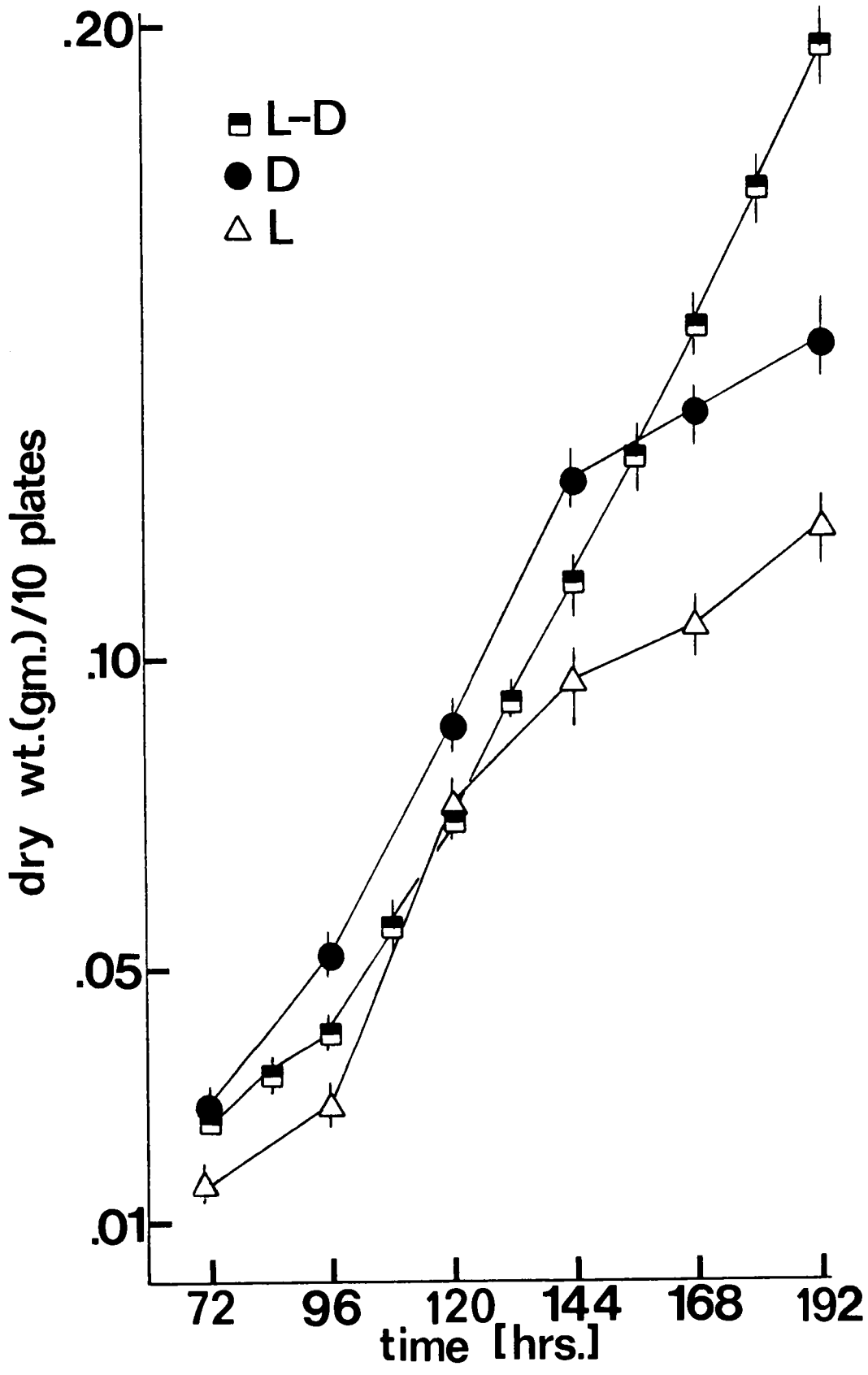


Figure 2. Dry weight curves for dikaryotic mycelia grown under different conditions. L-D, alternating light/dark; D, continuous darkness; L, continuous light.

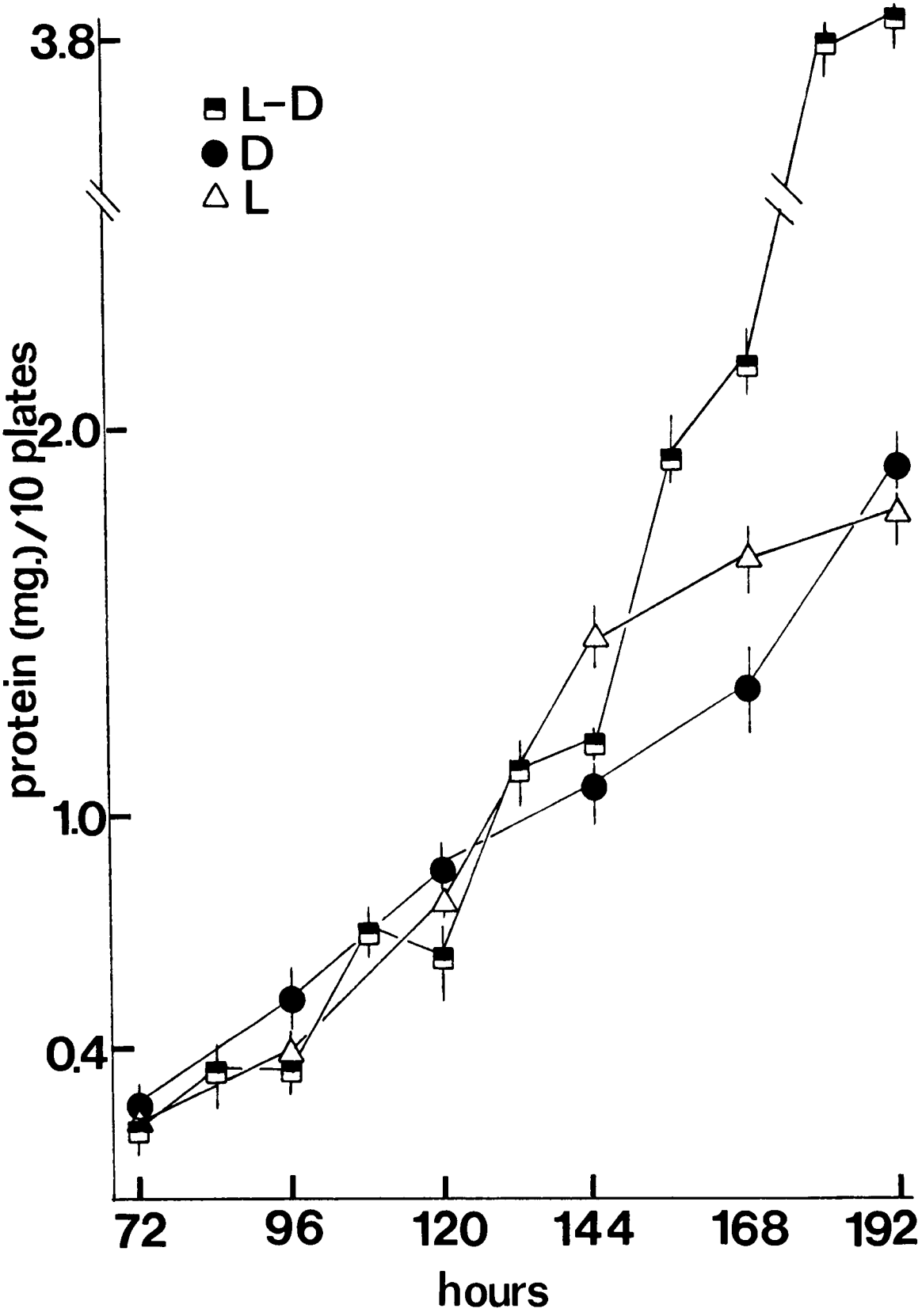


Figure 3. Total protein content of dikaryotic mycelia grown under various conditions. L-D, alternating light/dark; D, continuous darkness; L, continuous light.

light/dark cultures contained approximately twice as much protein as continuous light or continuous dark grown cultures. In young cultures the total protein content made up as much as 20% of the total dry weight while in older cultures only 1-2% of the total respective dry weights was protein.

In those cultures grown under alternating light/dark conditions initiation of fruiting (hyphal knotting) always took place in the center of the plate between 140-144 hours of incubation and these primordia would eventually give rise to normal fruit bodies (Figure 4). Cultures grown in continuous light would produce a few initials anytime between 132 and 168 hours of incubation but such primordia would never develop into normal fruit bodies. Dark-grown cultures would not usually initiate. Rarely, a dark incubated culture would give rise to an abnormal fruit body produced at the edge of the plate, which possessed an elongated stipe and greatly reduced pileus.

Endogenous cAMP Concentration in Extracts from Dikaryotic Mycelia of Coprinus

Cyclic AMP was extracted and assayed as described earlier. All assays were performed in duplicate from two separate crude extracts prepared from 2 groups of appropriately incubated mycelia. In those cases when harvesting was routinely done at 24 hour intervals intermediate assay points (12 hour) were determined to insure an accurate representation of endogenous levels of cAMP in those cultures. Figure 5 graphically displays the relationship between endogenous cAMP concentration, incubation condition and mycelial age. Most interesting was the diurnal rhythm in relative amounts of cAMP seen in mycelia grown



Figure 4. Young Coprinus cinereus fruit bodies after 216 hours of incubation in alternating light/dark exposures. The fruit bodies are 2-3 cm in height.

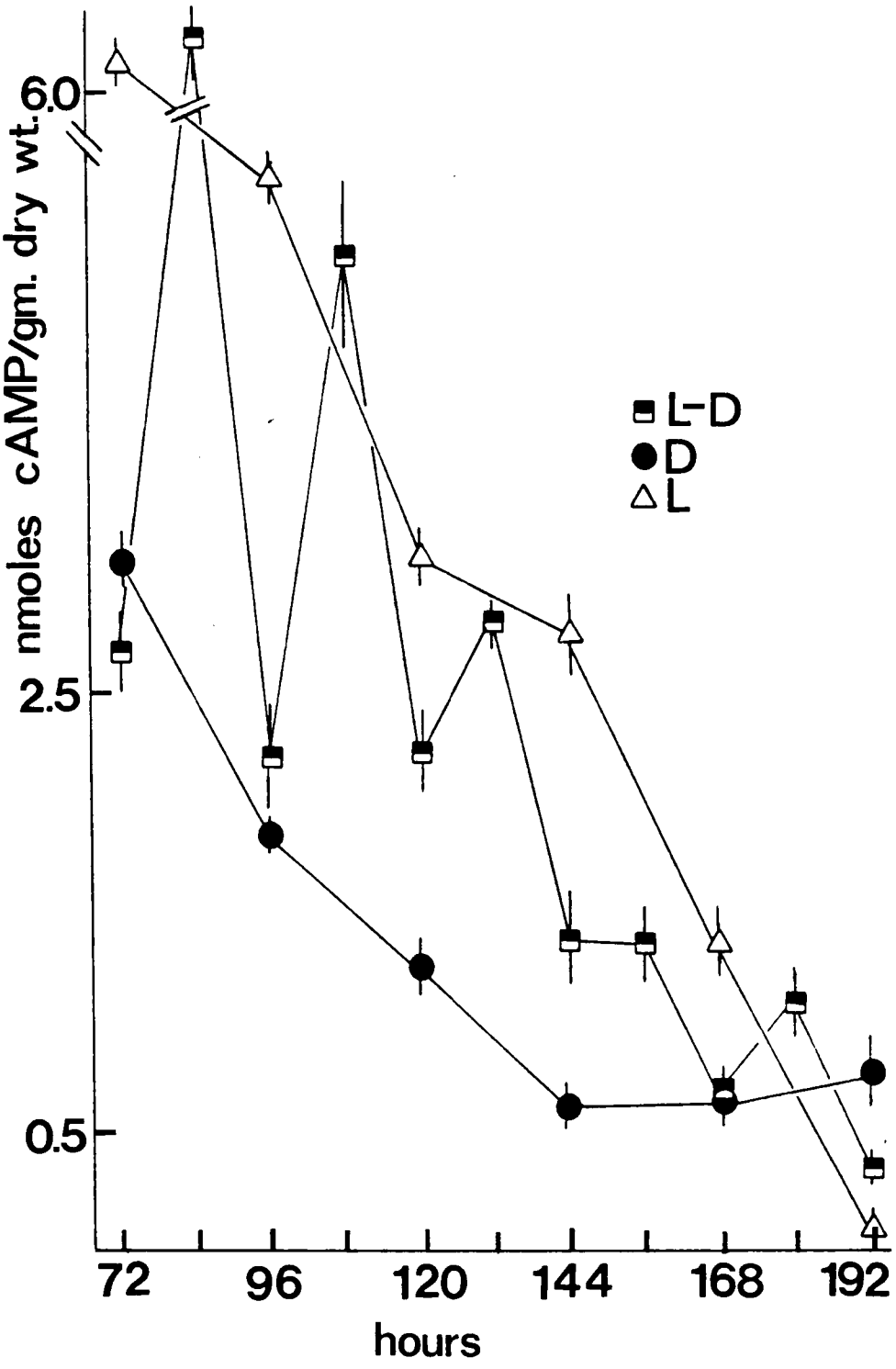


Figure 5. Cyclic AMP levels in crude extracts from dikaryotic mycelia grown under different conditions. L-D, alternating light/dark; D, continuous darkness; L, continuous light.

in alternating light and dark cycles. In each 24 hour period studied cAMP levels always increased during 12 hour light exposures and declined during 12 hour dark treatments. The greatest difference in amplitude of this rhythm occurred in the youngest cultures followed by a gradual decrease in the amplitude of the rhythm with increasing mycelial age. A general decline in cAMP concentrations was also observed with increase in culture age.

Cultures grown in continuous light showed an almost linear decline in cAMP concentration with respect to culture age. The amount of cAMP detected in light-grown mycelia was generally comparable to the amounts detected in alternating light/dark cultures when cAMP levels were determined at the end of each light treatment.

Cultures grown in continuous darkness had declining amounts of cAMP with respect to culture age. Initial concentrations of the nucleotide were approximately half the levels found in continuous light-grown cultures. Initially cAMP levels in dark-grown cultures were comparable to levels of cAMP in light/dark-grown cultures when assayed at the end of respective dark treatments (72 and 96 hours respectively). In 120 and 144 hours dark-grown cultures the cAMP level was found to be lower than either continuous light-grown cultures or alternating light/dark-grown cultures, a result of the general decline in cAMP levels associated with increasing culture age regardless of light/dark treatment. However, after 144 hours of incubation the cAMP concentration remains relatively unchanged in dark grown mycelia while during the same period light and light/dark-grown mycelia cAMP content continued to decline to levels that were ultimately lower than dark grown

mycelia levels at 192 hours of incubation.

The Effect of Culture Condition on Glycogen Phosphorylase Activity in Crude Extracts from Coprinus

Glycogen phosphorylase in crude extracts from variously grown dikaryotic mycelia was assayed as described earlier. In Figure 6a the results of the determination of total glycogen phosphorylase activity with respect to endogenous cAMP concentration in variously grown cultures are presented. In alternating light/dark incubated cultures there initially was a diurnal rhythm of phosphorylase activity such that activity would decrease during the light periods and increase during the dark periods until 132 hours of incubation at which point the activity increased dramatically with respect to culture age regardless of the light/dark treatment. In cultures incubated in continuous light phosphorylase activity remains relatively constant, gradually increasing until 120 hours of incubation, decreasing slightly at 144 hours and finally increasing between 144 and 192 hours of incubation but never attaining levels of activity detected in light/dark grown cultures. In cultures grown in continuous darkness phosphorylase activity remained relatively constant with respect to culture age and cAMP concentration.

Total activity of glycogen phosphorylase (Figure 6b) shows that in alternating light/dark cultures reached a peak at 132 hours of incubation following an initial decrease from 72 to 108 hours of incubation.

Phosphorylase activity in cultures grown in continuous light initially increased with respect to culture age until 120 hours of

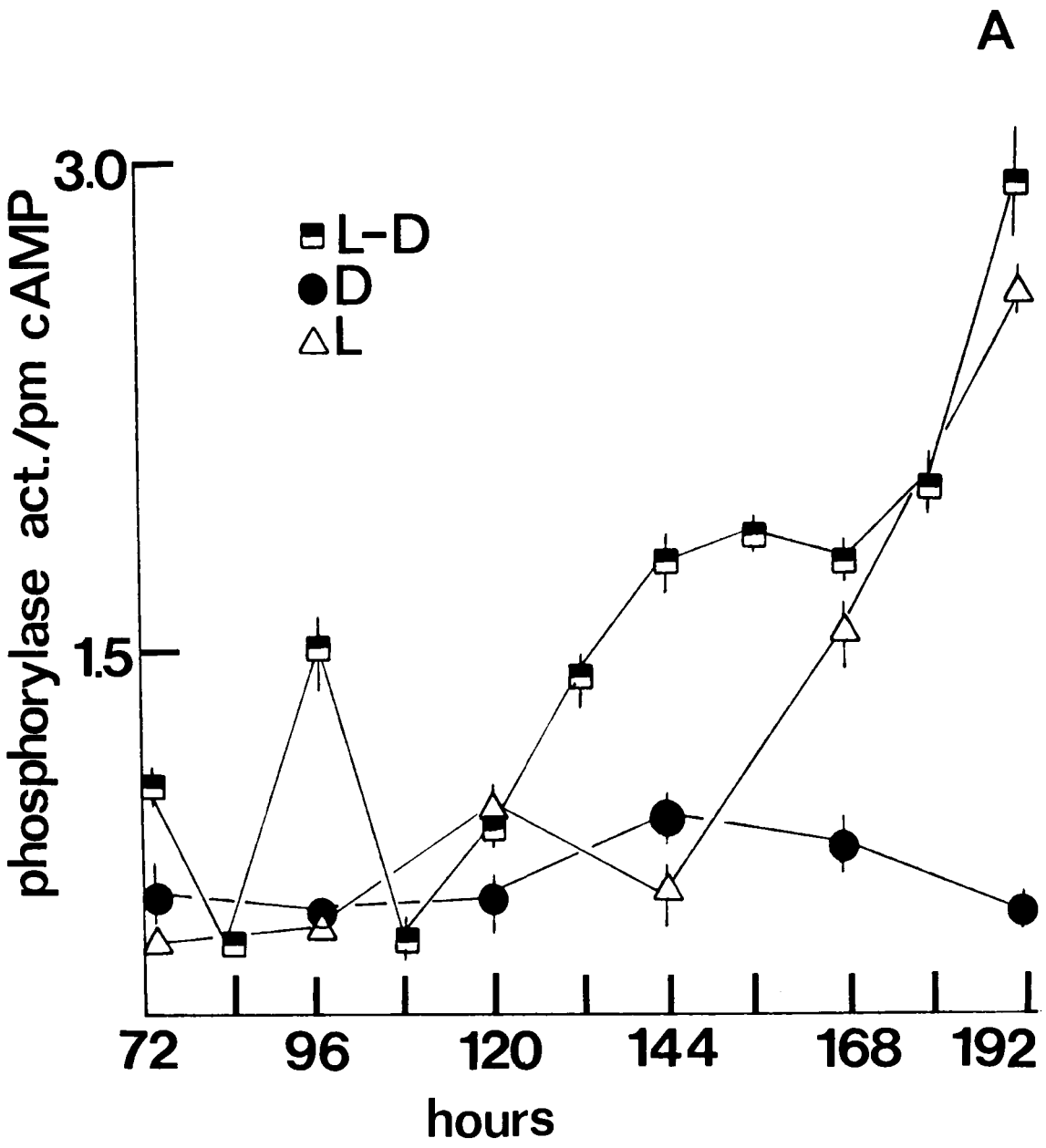


Figure 6. Total glycogen phosphorylase activity associated with crude extracts from dikaryotic mycelia. (A) Expressed in terms of endogenous cAMP levels during the experimental period; (B) grown under different conditions.

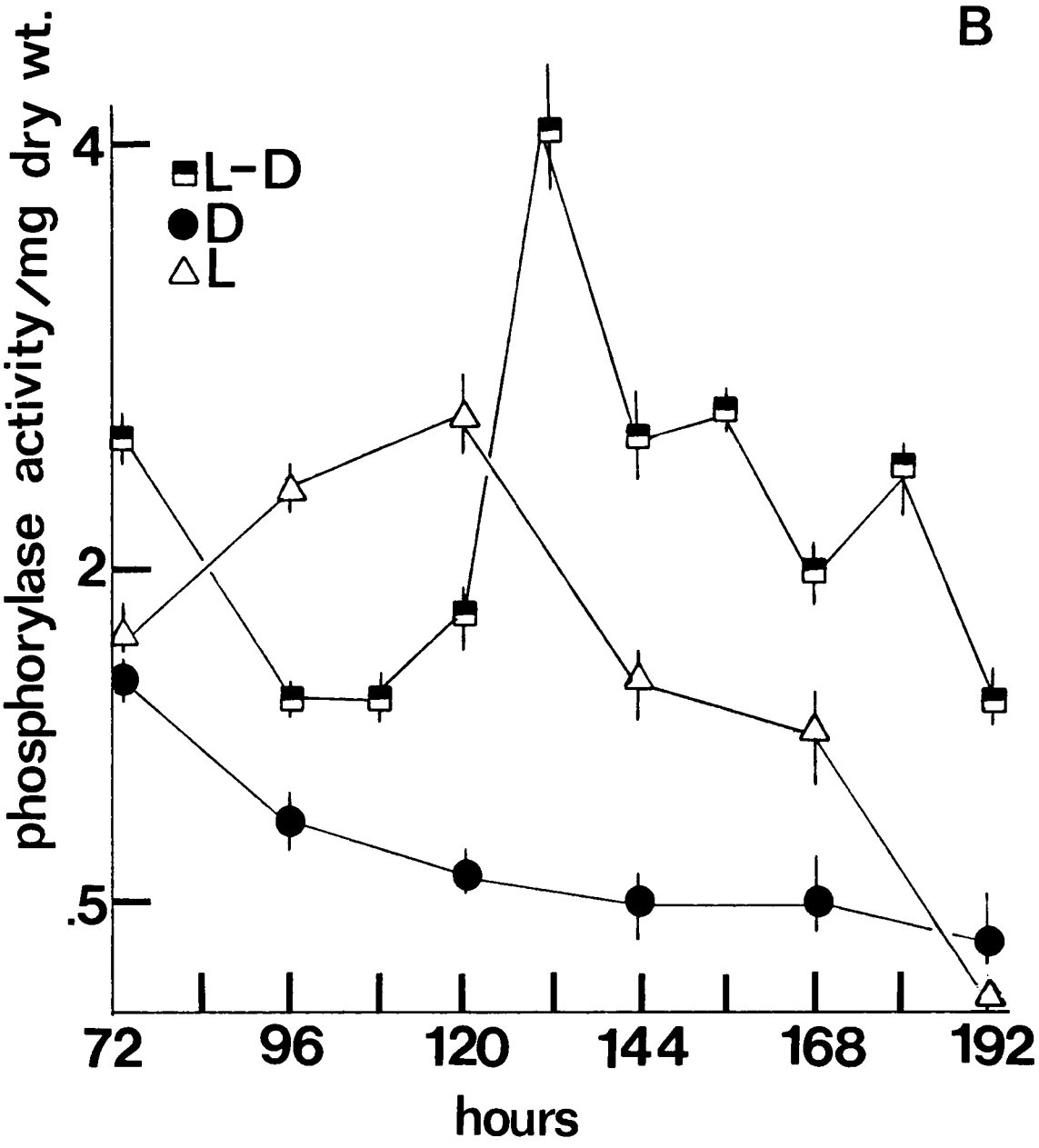


Figure 6 (continued)

incubation after which time there was a general decline in activity relative to 120 hour levels. Activities associated with dark grown mycelial extracts was initially lower than either continuous light or alternating light/dark activities and declined gradually over the entire incubation period.

The Effect of Cyclic AMP Concentration on Purified Glycogen Phosporylase Activity

Purified glycogen phosphorylase (Sigma Chemical Co.) activity was assayed with respect to varying concentrations of cAMP and the results are shown in Figure 7. The specific activity of the purified enzyme was not stimulated at any cAMP concentration tested. Cyclic AMP concentrations of 10^{-8} M had no effect on activity while lower concentrations of cAMP (10^{-9} M and 10^{-10} M) slightly inhibited activity and higher cAMP concentration (10^{-7} M and 10^{-6} M) inhibited activity to a small degree. At cAMP concentrations higher than 1 μm, phosphorylase activity was inhibited dramatically. Millimolar quantities of cAMP totally inhibited phosphorylase activity.

The Effect of Culture Condition on Endogenous Triacylglycerol Lipase Activity from Crude Extracts of Coprinus

Endogenous lipase was assayed in extracts from appropriately incubated mycelia as described earlier. The results are presented in Figure 8a and it can be easily seen that the general pattern of lipase activity was in general similar to glycogen phosphorylase activities shown in Figure 7a with respect to cAMP concentration. In alternating light/dark conditions, lipase activity decreased initially to 108

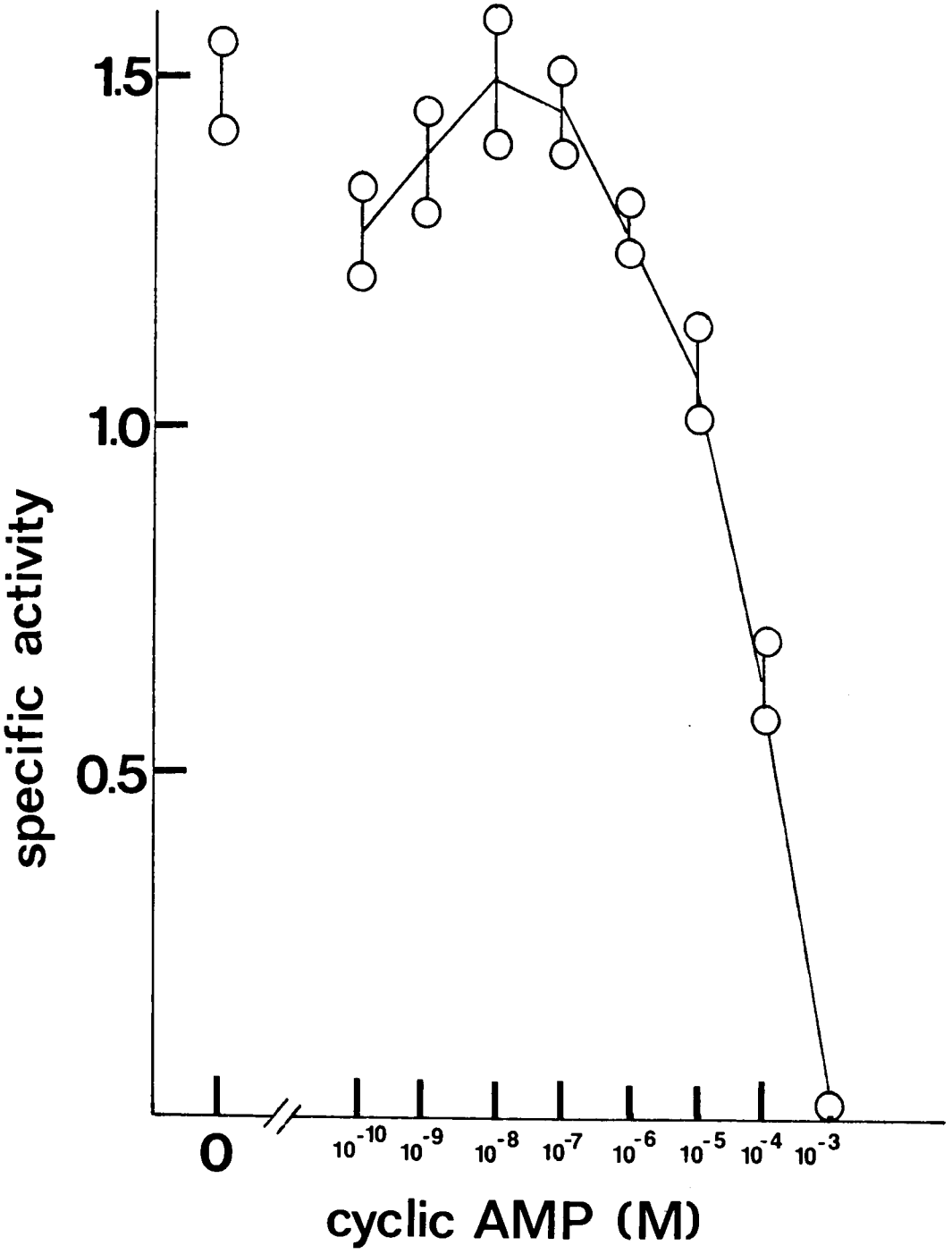


Figure 7. The effect of cAMP on the activity of purified samples of glycogen phosphorylase (Sigma Chemical Co.).

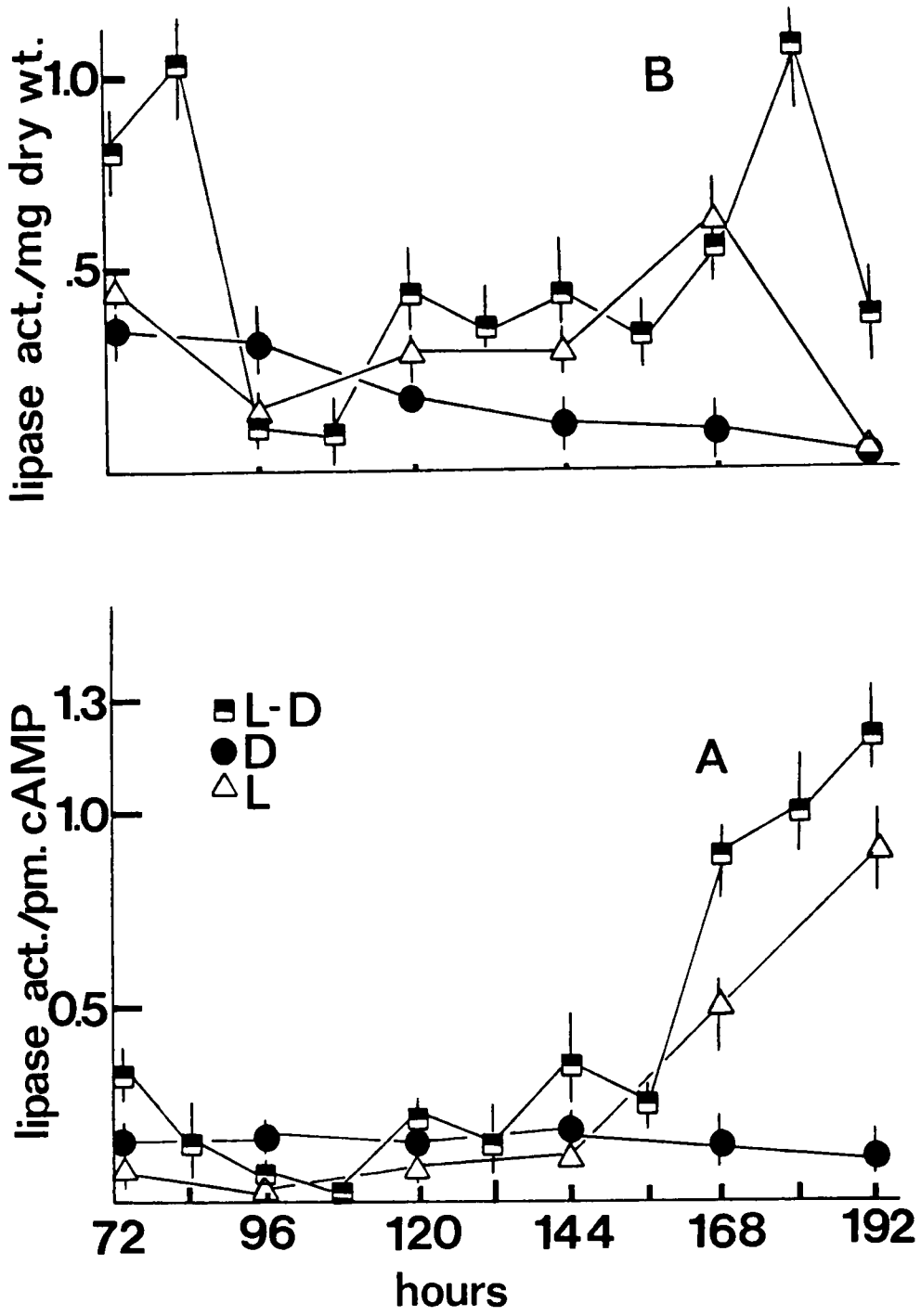


Figure 8. Total lipase activity per pmol cAMP (A) and total lipase activity (B) associated with crude extracts from dikaryotic mycelia grown under different conditions.

hours of incubation, after which time lipase activity increased gradually, fluctuating diurnally until 106 hours of incubation. Lipase activity increased after 156 hours of incubation.

Cultures grown in continuous light possess low initial ipase activity which increased gradually until 144 hours of incubation after which time the lipase activity increased very rapidly at a rate similar to the increase detected in alternating light/dark-incubated cultures. Cultures grown in continuous darkness showed little variation in lipase activity with respect to culture age. The activity remained relatively low throughout the entire incubation period.

Total lipase activity (Figure 8b) associated with alternating light/dark cultures was initially high but decreased after 96 hours of incubation and remained low until an increase of activity at 120 hours of incubation. From 120 througn 168 hours of incubation lipase activity remained relatively unchanged until 180 hours of incubation at which time activity increased to initial levels followed by a decrease in activity at 192 hours of incubation. Activity detected in continuous light-grown cultures decreased during the initial experimental period but then gradually increased until 168 hours of incubation. The final 24 hour incubation period resulted in a significant decreased in lipase activity. Activity in continuous dark grown mycelia was comparable to levels in light-grown cultures but decreased gradually throughout the incubation period.

The Effect of Incubation Condition on Protein Kinase Activity Associated with Crude Extracts of Coprinus

Protein kinase activity was assayed as described earlier under three different assay conditions: (1) endogenous activity (no histone added to the reaction mixture), (2) excess histone added, and (3) excess histone plus 1 μm cAMP. The data thus obtained is presented in Figures 9, 10, and 11 for alternating light/dark, continuous light and continuous dark-incubated cultures, respectively.

Under alternating light/dark incubation conditions initial endogenous levels of protein kinase activity underwent significant diurnal fluctuations between 72 and 108 hours of incubation. The kinase activity decreased approximately 70% during light exposures and increased to the same degree during dark periods. After the initial 36 hour period total kinase activity decreased to less than 0.05 units of activity and remained at relatively low levels for the rest of the incubation period. When histone was added as an exogenous [^{32}P] receptor the same initial diurnal fluctuation in kinase activity was detected but the amplitude of the fluctuations was reduced compared to endogenous activity between 72 and 108 hours of incubation. However, unlike endogenous kinase activity, the diurnal rhythm of histone kinase activity continued beyond the 108 hour point by an additional 48 hours after which time the activity decreased in general for the duration of the incubation period. When 1 μm cAMP was added to the reaction mixture a general inhibition of kinase activity was observed for the first 48 hours of incubation compared to endogenous and histone supplemented kinase activity. Beginning with 108 hours of

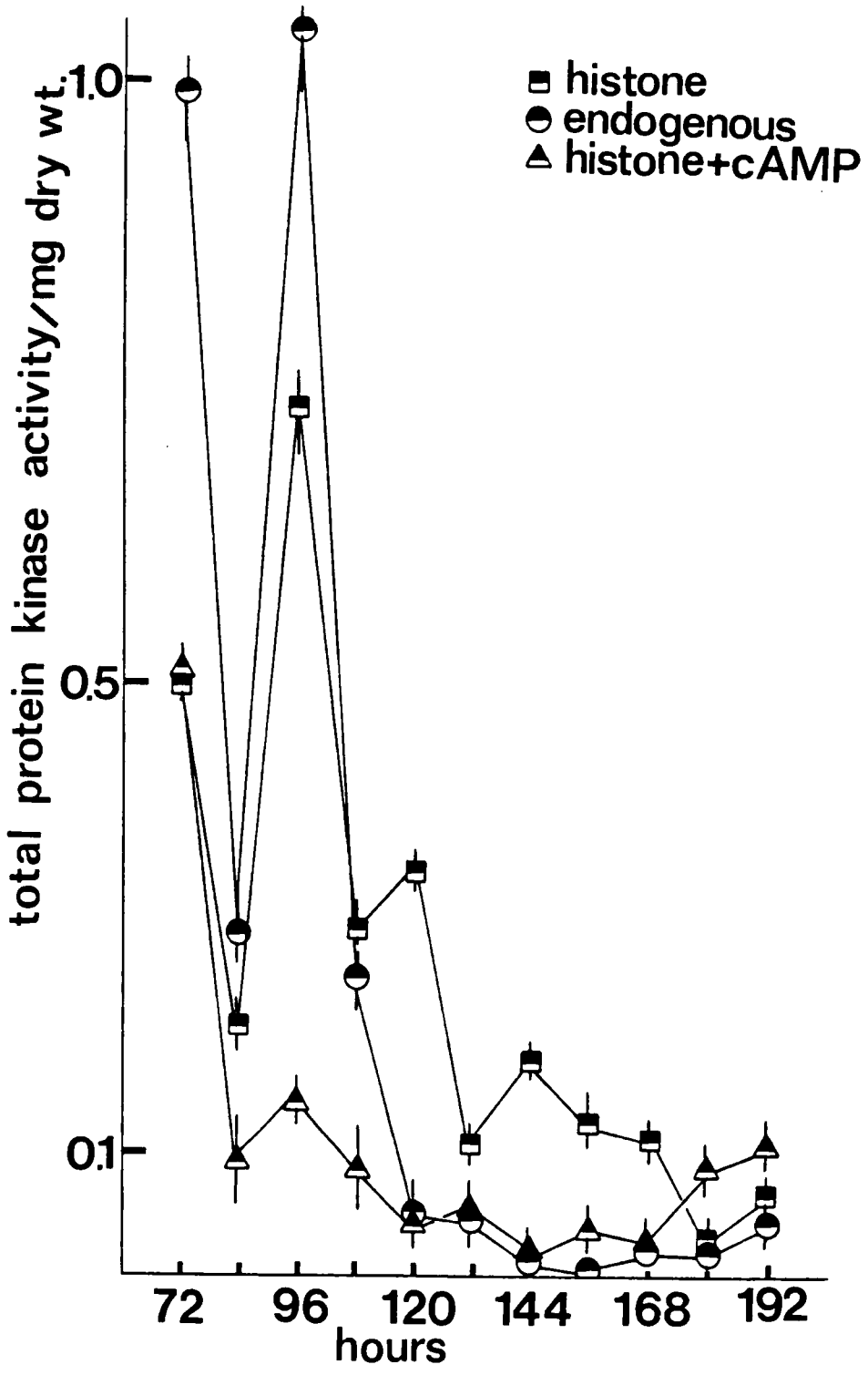


Figure 9. Total protein kinase activity associated with crude mycelial extracts from dikaryotic hyphae grown under alternating light/dark conditions. Activity is expressed in terms of pmols labeled P incorp. per minute per mg dry weight.

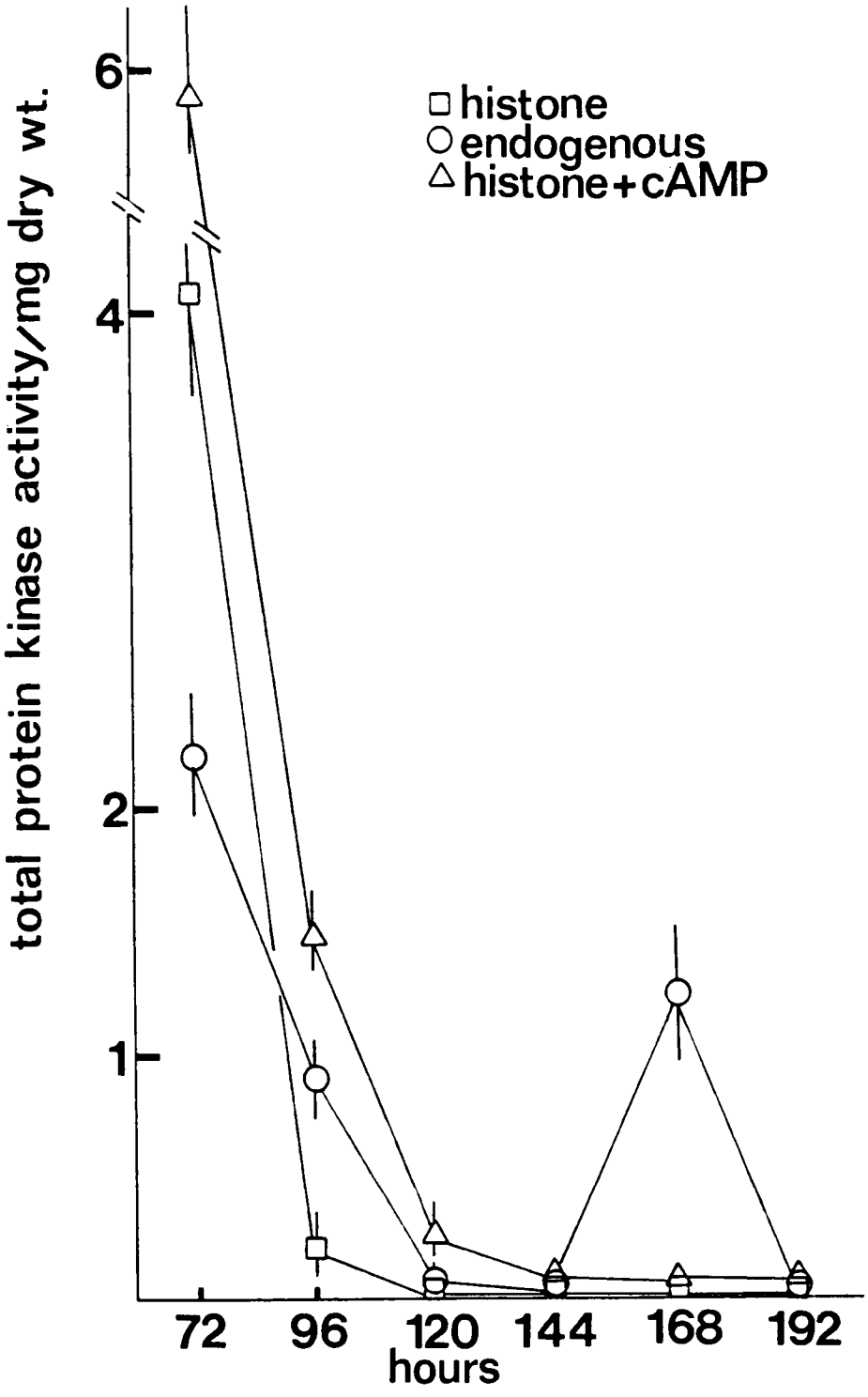


Figure 10. Total protein kinase activity associated with crude extracts from dikaryotic mycelia grown in continuous light.

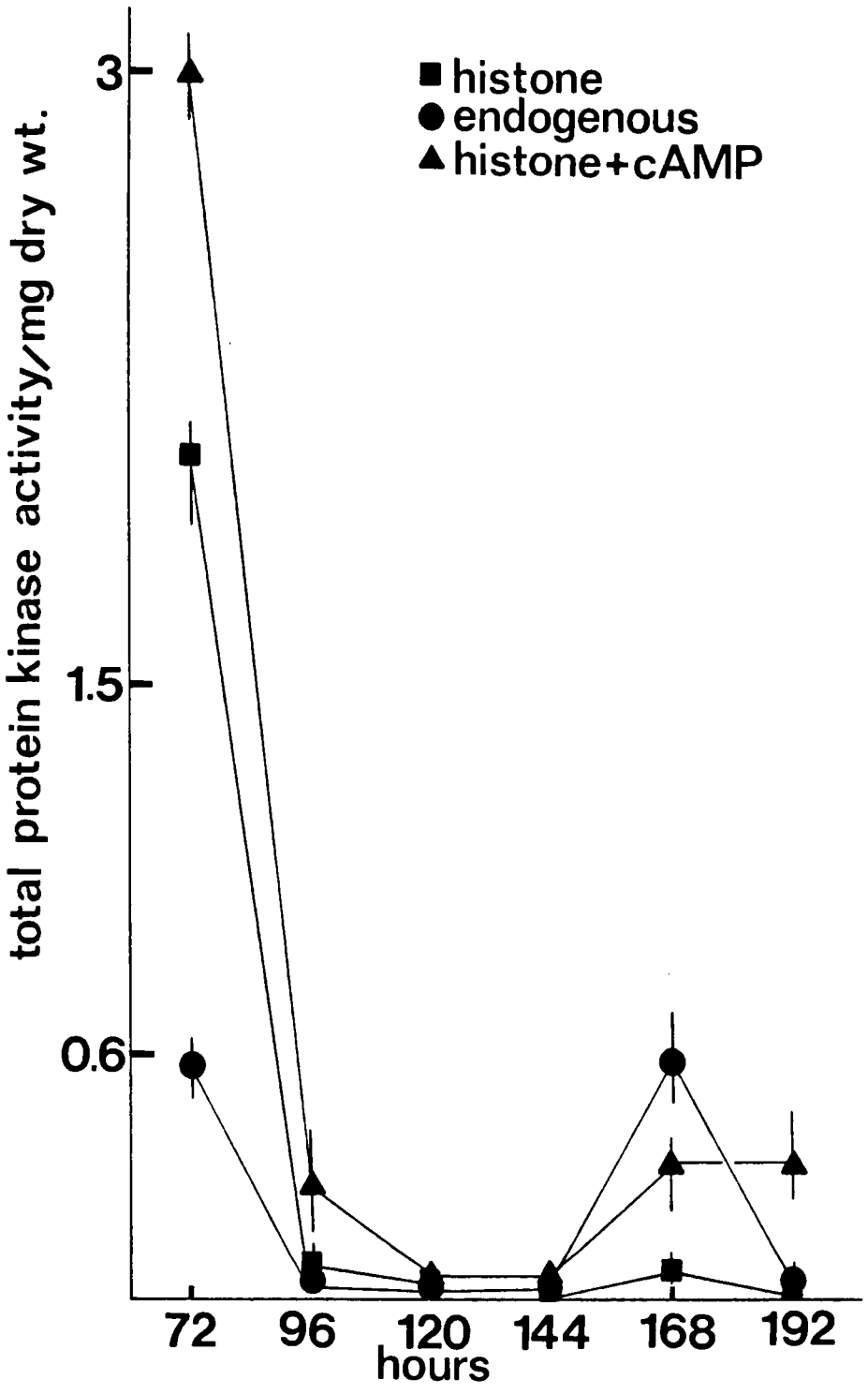


Figure 11. Total protein kinase activity associated with crude extracts from dikaryotic mycelia grown in continous dark.

incubation to the end of the incubation period cAMP added kinase activity was comparable to endogenous kinase activity both of which were well below histone supplemented levels of kinase activity for that time period. Cyclic AMP appeared to stimulate kinase activity slightly at 156, 180 and 192 hours of incubation respectively.

Cultures incubated in continuous light (Figure 10) possessed significantly higher protein kinase activity than alternating light/dark-grown cultures after 72 hours of incubation regardless of the reaction mixture of the assay. Most striking is the stimulation of kinase activity by cAMP over endogenous activity by approximately 3 times which is the reverse of the situation observed in alternating light/dark cultures. Under all assay conditions protein kinase activity decreased rapidly with culture age to remain relatively constant except for some increase in endogenous kinase activity at 168 hours of incubation. In general, kinase activities were on the order of 5 times greater in light grown cultures as compared to alternating light/dark-grown cultures and 2-4 times as great as dark-grown cultures depending upon the assay mixture.

Cultures grown in continuous darkness (Figure 11) had protein kinase activities that followed the same general pattern as did those activities detected in continuous light grown cultures with respect to culture age. In general, initial kinase activities were approximately 60% of the continuous light-grown cultures in the presence of cAMP and/or histone. The same cAMP stimulation of kinase activity as was seen in light grown cultures was observed in dark grown cultures over the incubation period except for the peak of endogenous

activity at 168 hours of incubation which also occurred in light-grown cultures. In dark-grown cultures cAMP stimulated kinase activity initially approximately 5 times over endogenous levels while in light grown culture the stimulation was approximately 2.5 times those of endogenous levels. As in light-grown cultures, kinase activities decreased with respect to culture age but for the exception at 168 hours noted above.

A Comparison of Histone Phosphorylation Activity and Endogenous Cyclic AMP Levels in Cultures Grown Under Alternating Light/Dark Conditions

Data obtained from the radioimmunoassay of cAMP levels and the protein kinase assay described earlier with histone added are shown together in Figure 12a. As can be clearly seen there is a very definite inverse relationship between cAMP concentration and protein kinase activity at least through 144 hours of incubation at which time initiation of fruiting takes place. Between 144 and 168 hours that relationship is not apparent but was restored between 168 and 192 hours of incubation. Histone phosphorylation with respect to cAMP concentration (Figure 12b) shows high initial activities followed by a decrease between 84 and 120 hours of incubation. Activity increased significantly at 132 hours of incubation and remained relatively high until 180 hours of incubation.

The Ability of Protein Kinase in Crude Extracts of C. cinereus to Phosphorylate Histone

Data obtained by assaying the crude enzyme extracts of the organism for the ability to phosphorylate histone minus the

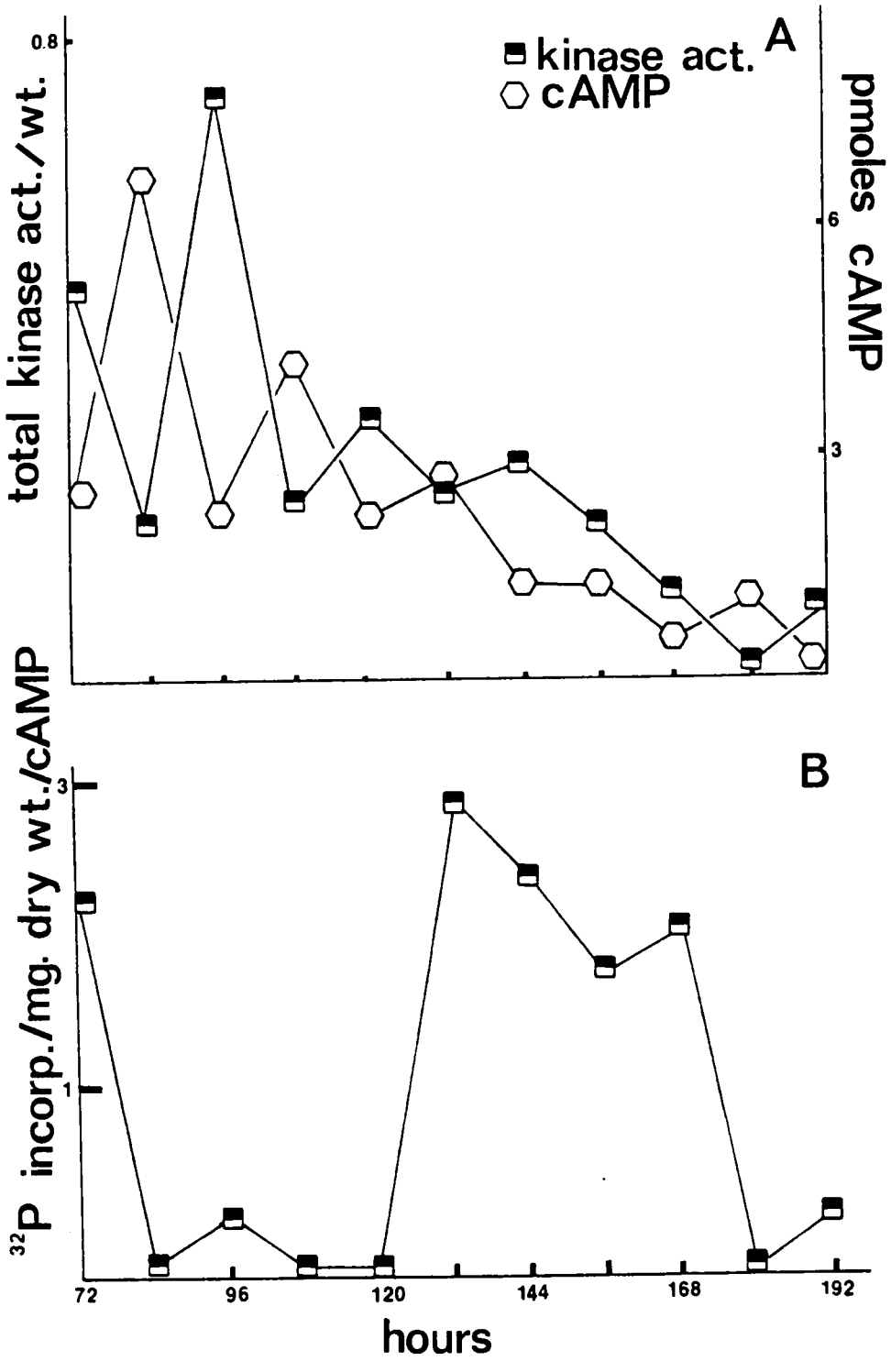


Figure 12. Total histone phosphorylation activity compared to endogenous cAMP levels . (A) Associated with alternating light/dark grown mycelia; and (B) histone phosphorylation/pmole cAMP.

phosphorylation of endogenous protein in the presence and absence of 1 μm cAMP is shown in Figure 13. The ability of continuous light or dark-grown cultures to phosphorylate histone follows a similar pattern with respect to culture age in the presence of cAMP. Histone phosphorylation was highest in very young mycelia and was further stimulated by the presence of cAMP. Light-grown cultures subsequently did not phosphorylate histone over endogenous levels from extracts from mycelia over 72 hours of age. Continuous dark grown cultures showed the same initial decrease in activity after 72 hours of incubation but remained able to phosphorylate histone except at 168 hours of incubation with an increase in activity at 192 hours of incubation.

A very different pattern of histone phosphorylation was observed in alternating light/dark-grown cultures. Cyclic AMP inhibited histone phosphorylation throughout the incubation period except at 180 and 192 hours of incubation. In the absence of exogenous cAMP histone phosphorylation activity increased rapidly to 120 hours of incubation decreased in the subsequent light period, increased slightly at 168 hours of incubation during a dark period, followed by a decrease in activity reaching zero at 180 hours of incubation.

Time Course of Histone Phosphorylation in the Presence and Absence of Cyclic AMP

A crude extract obtained from liquid grown cultures was assayed for its ability to phosphorylate histone in the presence and absence of cAMP. The results of the assays are shown in Figure 14. Most of the phosphorylation took place during the first minutes at similar rates following initiation of the reaction. It is clear that cAMP

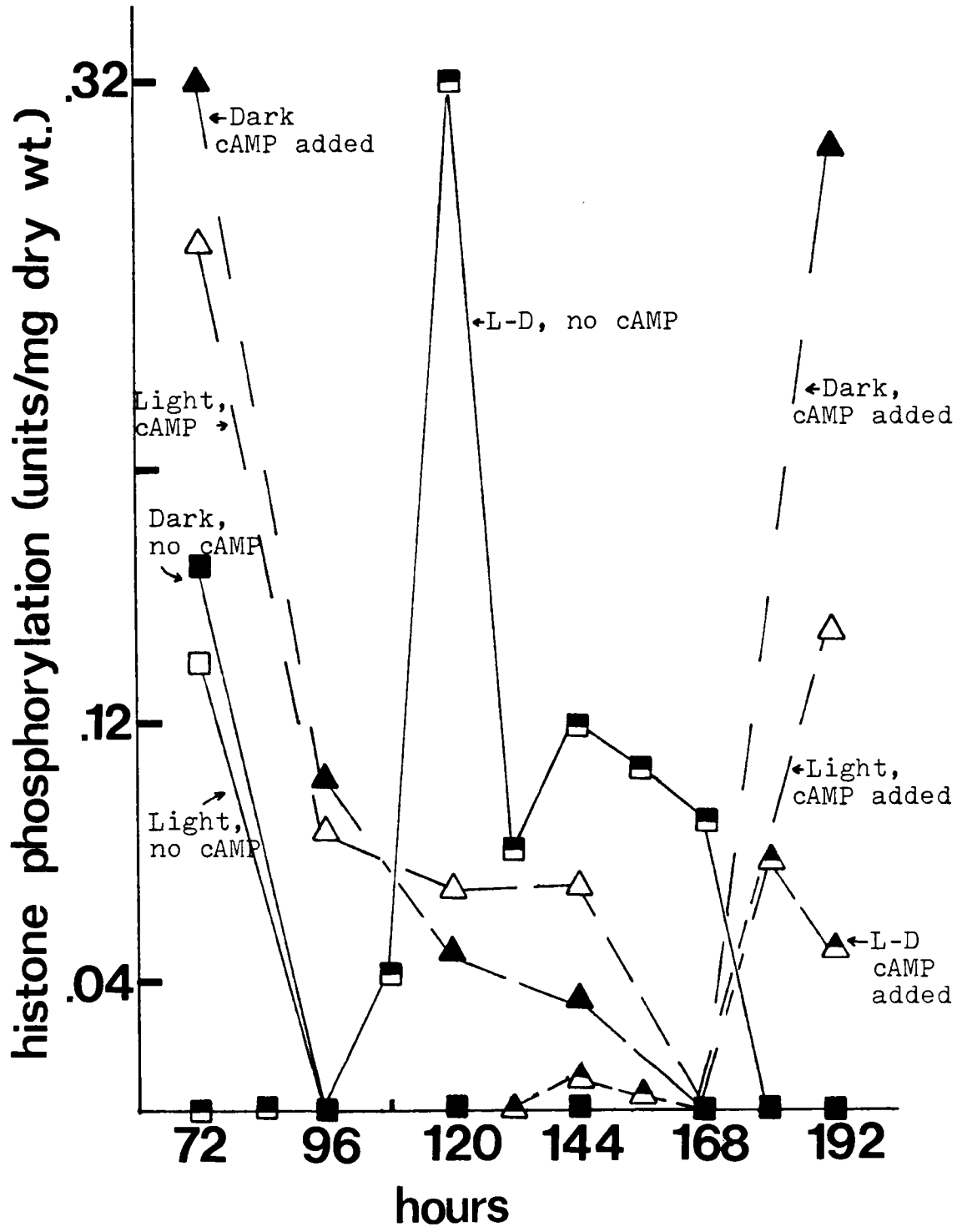


Figure 13. Histone phosphorylation corrected for endogenous phosphorylation of unknown substrates associated with crude extracts from dikaryotic mycelia grown under different conditions.

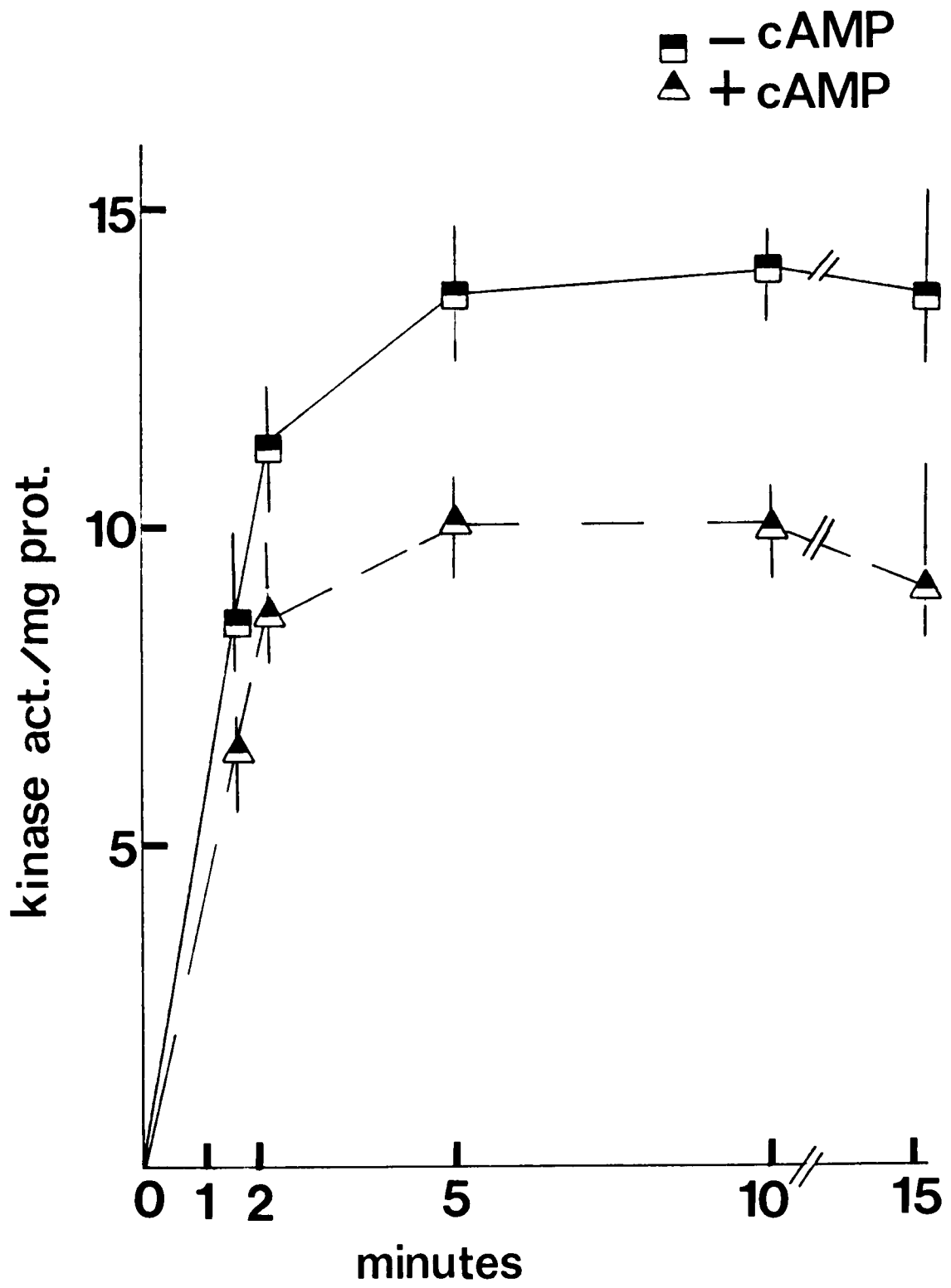


Figure 14. Time course of total endogenous protein kinase activity associated with crude extracts of mycelia grown under alternating light/dark conditions in the presence and absence of cAMP micromolar and histone.

inhibits the ability of the crude enzyme preparation to phosphorylate histone as incubation time increases. The inhibition was approximately 30% at maximum histone phosphorylating activity (10 minutes). Similar initial reaction rates followed by cAMP inhibition suggests that the formation of a molecular complex involving cAMP is involved in the inhibitory mechanism.

Gel-Filtration of Crude Coprinus Protein Kinase Extract

An aliquot of the same extract used in the time course experiment described above was fractionated by Bio-Gel (Bio-Rad) A 0.5 column chromatography (described earlier) and each 3 ml fraction was assayed for protein kinase activity in the presence and absence of 1 μm cAMP using histone as the substrate. The results are shown in Figure 15. Seven major peaks of histone phosphorylation were detected and were designated as peaks I through VII. Activities detected of less than 0.003 pmole [^{32}P] incorporated per minute were regarded as basal levels.

Histone phosphorylation activity could be divided into 3 classifications: cAMP--dependent, peaks I and VII, cAMP--inhibited, peaks II, III, IV and V; and cAMP--independent, peak VI. Activity of peak I and VII were stimulated approximately 6 times by 1 μm cAMP. Peaks II, III, IV and V activities were inhibited from 5 to 2.5 times depending upon the fraction. Peak VI activity was affected by only 18% in the presence or absence of cAMP.

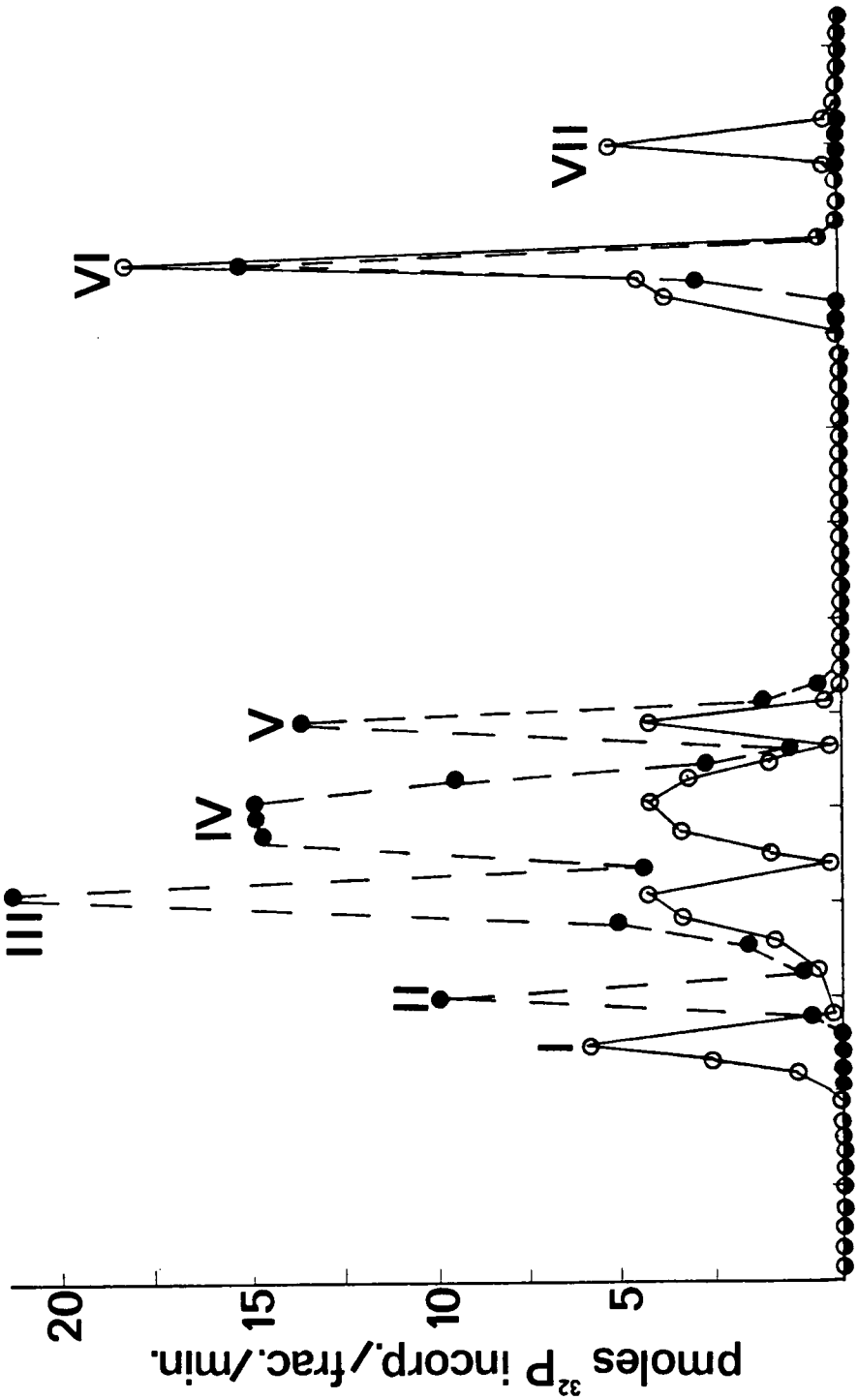


Figure 15. Fractionated crude protein kinase extract assayed for kinase activity in the presence (————) and absence (- - - - - -) of cAMP. Extracts were taken from dikaryotic mycelia grown in alternating light/dark conditions.

The Effect of cAMP Concentration on Protein Kinase Fractions I (cAMP-Stimulated), III (cAMP-Inhibited) and VI (cAMP-Independent)

Aliquots from peak I, III, and VI were assayed for protein kinase activity in the presence and absence of varying concentrations of cAMP (10^{-9} M - 10^{-6} M) (Table 1). Peak I kinase activity was stimulated by all concentrations of cAMP tested by approximately five times. Peak III kinase (cAMP-inhibited) was inhibited by 10^{-6} M and 10^{-7} M cAMP approximately 3.5 times. Lower concentrations of the nucleotide (10^{-8} M and 10^{-9} M) inhibited ^{32}P incorporation as well, 1.8 times and 1.2 times respectively. Peak VI kinase (cAMP-independent) activity was slightly stimulated at all concentrations of cAMP tested, averaging approximately a 10% increase in activity.

The Combined Effects of Cyclic cAMP and Calmodulin on cAMP-Inhibited and cAMP-Stimulated Protein Kinases

Aliquots from cAMP-stimulated (Peak I) and cAMP inhibited (Peak III) protein kinase were assayed for the ability to phosphorylate histone in the presence and absence of 1 μM cAMP, 1 μM calmodulin (Sigma Chemical Company), 10 mM EGTA, 1 mM Ca^{++} by the technique described earlier. The results of this experiment are presented in Table 2. When cAMP-inhibited protein kinase activity was assayed in the presence of 1 μm cAMP activity decreased by approximately 53%. Incubation of the enzyme with calmodulin resulted in a slight stimulation response of approximately 20%. However, kinase activity assayed in the presence of both cAMP and calmodulin resulted in an increase in enzyme activity of almost 2.5 times basal activity.

TABLE 1. ^{32}P incorporated into histone/30 minutes at varying concentrations of cAMP by protein kinase fractions I, III, and VI (Figure 15) isolated by Bio-Gel column chromatography as described in the Methods Section.

Protein Kinase Type	^{32}P Incorp. into Histone (CPM x 10^3) at Varying cAMP Concentrations (M)				
	0	10^{-6} M	10^{-7} M	10^{-8} M	10^{-9} M
Peak I					
cAMP-Stimulated	2.1	10.1	10.1	10.7	10.4
Peak VI					
cAMP-Independent	22.0	26.2	26.0	25.2	25.0
Peak III					
cAMP-Inhibited	37.7	8.0	8.0	13.6	19.9

TABLE 2. Bio-Gel protein kinase fractions assayed for histone phosphorylation activity under various conditions. Data is expressed in terms of ^{32}P incorporated into histone in 30 minutes (CPM x 10^3).

Protein Kinase Type	The Effect of Cyclic AMP, Calmodulin and EGTA on Protein Kinase Activity			
	Endogenous	cAMP Only	CaM Only	cAMP Plus CaM
Peak I				
cAMP-Stimulated	4.9	23.0	33.8	23.1
cAMP-Stimulated Plus EGTA	5.0	21.7	5.1	22.0
Peak III				
cAMP-Inhibited	21.0	9.9	25.1	62.5
cAMP-Inhibited Plus EGTA	2.0	9.6	21.2	10.3

Aliquots taken from the same kinase fraction were then assayed in the presence of EGTA, a calcium ion chelator. This chelator had little effect on kinase activity in the presence of absence of cAMP but tended to reverse the stimulatory effects of calmodulin alone or even more dramatically when cAMP and calmodulin were incubated together. Cyclic AMP-stimulated protein kinase incubated in the presence of 1 μM cAMP showed an increase in activity of 4.7 times over basal levels. Calmodulin alone stimulated kinase activity more than did cAMP alone by increasing the activity by 7 times over basal levels. Activity of the kinase in the presence of both cAMP and calmodulin stimulated activity over basal levels to the same degree as cAMP alone but less than calmodulin alone, a 32% decrease. The addition of EGTA to this protein kinase appeared not to affect enzyme activity except in the case of calmodulin only added assays in which case the EGTA totally reversed the stimulation of activity detected when calmodulin was incubated with enzyme extracts in the absence of EGTA.

The Effect of Culture Condition on Endogenous Levels of Cyclic Nucleotide Phosphodiesterase Activity

Appropriately incubated cultures were extracted and assayed for phosphodiesterase activity as described earlier. The results are shown in Figure 16. Activity associated with extracts from continuous light-grown hyphae had very high initial enzyme activities, 2 to 7 times greater than activities in alternating light/dark and dark-grown cultures during the period from 72 to 120 hours of incubation. With increasing culture age, after 96 hours of incubation, enzyme activity decreased rapidly in the cultures. Extracts from dark-grown hyphae

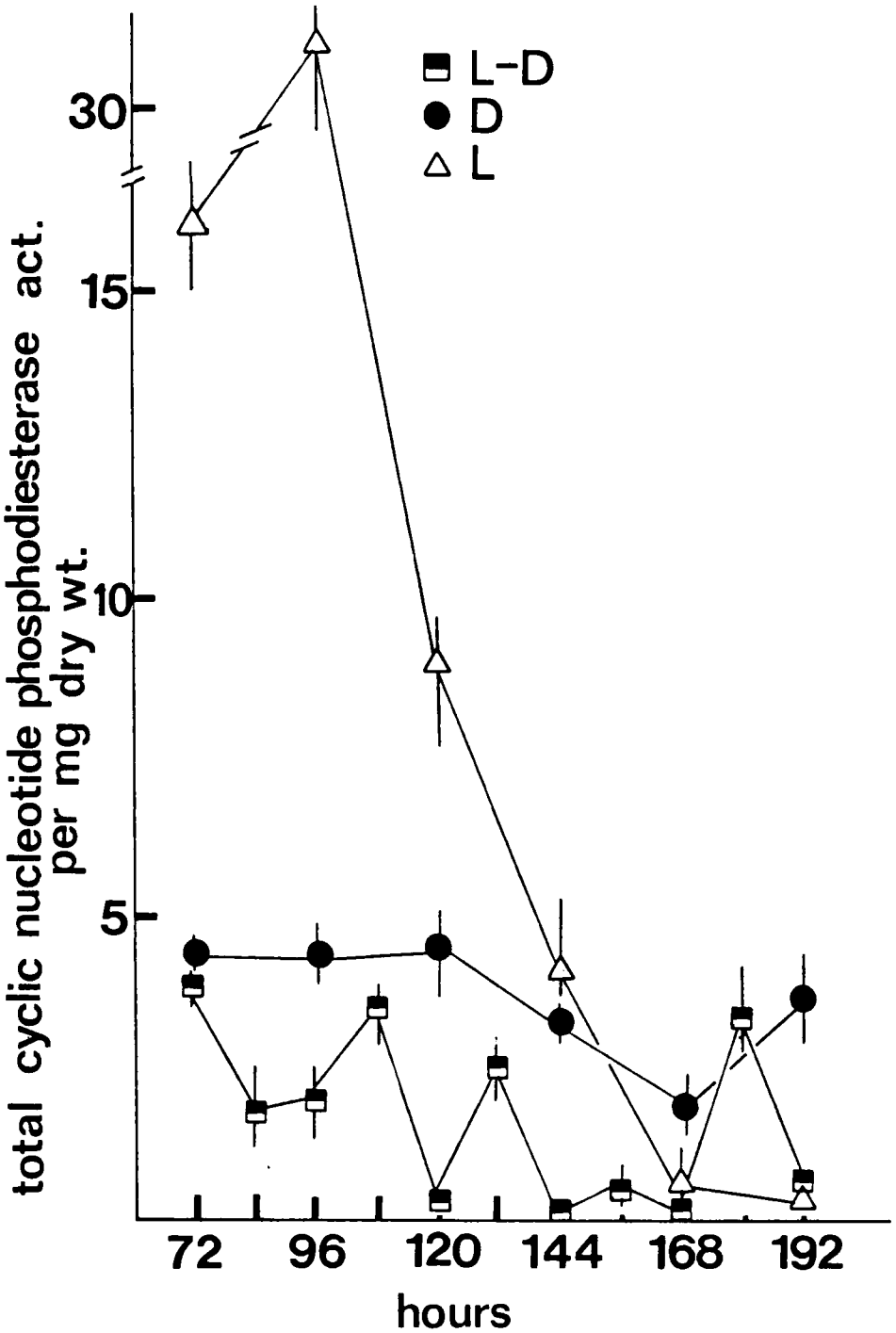


Figure 16. Total cyclic nucleotide phosphodiesterase activity associated with crude extracts from dikaryotic mycelia grown under different conditions. L-D, alternating light/dark; D, continuous darkness; L, continuous light.

had relatively stable enzyme activity throughout the incubation period at a level significantly lower than initial continuous light grown mycelia but in the same range as alternating light/dark cultures. Phosphodiesterase activities assayed in extract from alternating light/dark grown cultures were generally lower than continuous light or continuous dark-grown cultures. After an initial decrease from 72 to 84 hours of incubation a diurnal rhythm of activity began with 96 hours of incubation such that activity increased during light and decreased during dark exposures throughout the remainder of the incubation period.

Studies on a Calcium-Dependent Activator of Cyclic Nucleotide Phosphodiesterase: Calmodulin from Coprinus

Calmodulin activity is expressed in terms of its ability to stimulate, in the presence of Ca^{++}, the activity of cyclic nucleotide phosphodiesterase (PDE) which had been previously dissociated from endogenous activator. Activator-deficient phosphodiesterase was isolated from bovine heart tissue by DEAE-cellulose column chromatography as described in the Materials and Methods section. Calmodulin was isolated from Coprinus by fluphenazine-agarose affinity column chromatography as described earlier. The results of the affinity chromatography are shown in Figure 17. Fractions were assayed for calmodulin activity and it was found that all of the activity was eluted with the EGTA pulse and this fraction was designated as Coprinus calmodulin A and used in future experiments. A purification scheme of Coprinus calmodulin from the crude extract is shown in Table 3. Coprinus calmodulin specific activity was increased by 434

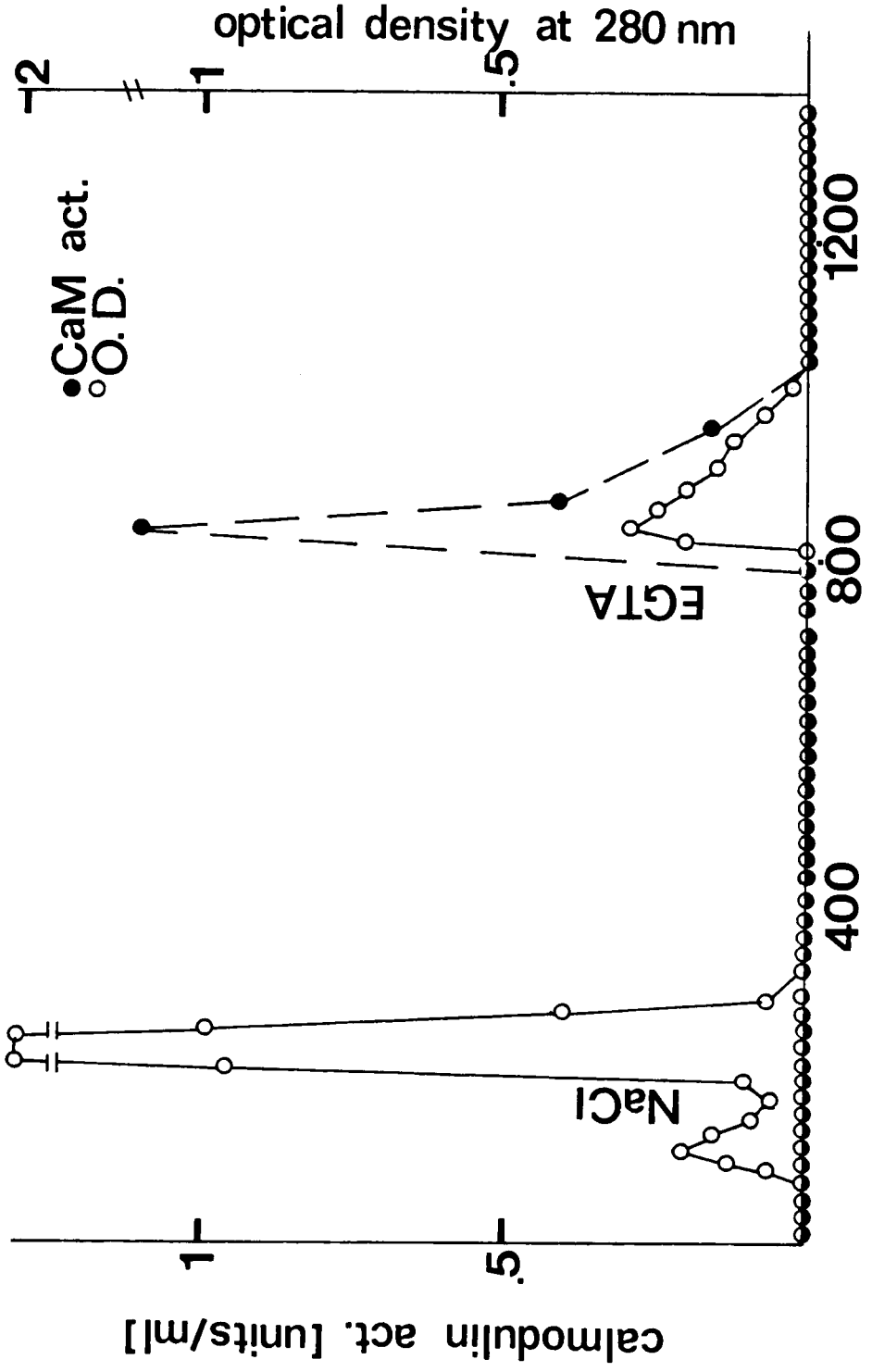


Figure 17. Elution profile of a crude Coprinus extract by fluphenazine-Sepharose affinity chromatography. Optical density (————), calmodulin activity (- - - - - -).

TABLE 3. *Coprinus* calmodulin purification scheme from light/dark grown crude mycelial extracts. Calmodulin activity is expressed in terms of its ability to stimulate the activity of cyclic nucleotide phosphodiesterase.

Fraction	Volume (ml)	Protein (mg)	Total Activity	Specific Activity	Purification
Heat-Treated Crude	474	686.0	6253	9.2	--
Second Ethanol ppt.	100	42.9	5821	135.2	14.7
Fluphenazine Column	40	1.1	4456	3979	433.4

times using this affinity column procedure.

Aliquots of Calmodulin A were subjected to electrophoresis to check for purity of the protein and the results are shown in Figure 18. Only one protein band could be visualized with either coomassie blue or silver stain.

Isolation of Activator-Deficient Cyclic Nucleotide Phosphodiesterase from Bovine Heart Tissue

In order to study Coprinus calmodulin in greater detail activator-deficient phosphodiesterase was isolated using the procedure of Ho et al. (16) as described earlier. Each DEAE-celluose fraction was assayed for phosphodiesterase activity in the presence and absence of EGTA and the presence and absence of calmodulin A. The results of this experiment are shown in Table 4. All fractions collected after elution with a linear salt gradient began showed phosphodiesterase activity in the absence of EGTA or calmodulin A. Addition of EGTA to the reaction mixture inhibited all Bovine-PDE fractions to varying degrees, totally deactivating fraction #1 enzyme. The addition of Coprinus calmodulin A (1 µg/assay) in the absence of EGTA stimulated bovine PDE from all fractions except for fraction #2. Bovine-PDE in fraction 4 was stimulated the greatest amount, 11.15 times over basal activity. When each bovine-PDE fraction was incubated with EDTA and Coprinus calmodulin A phosphodiesterase activity previously stimulated by calmodulin was greatly reduced except for fraction 2 activity which was stimulated. In some cases calmodulin plus EGTA activities were lower than initial endogenous activities. In the presence of 50 mm caffeine all PDE activity was lost. Since the bovine-PDE associated

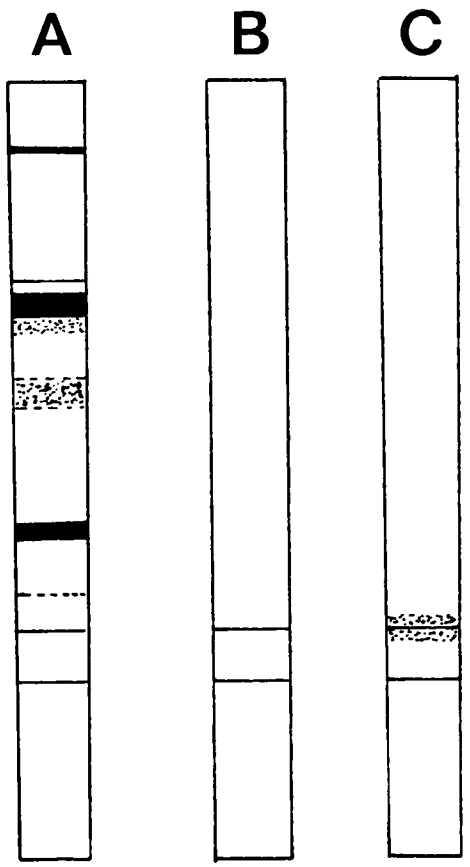


Figure 18. Gel electrophoresis of (A) second ethanol precipitation of crude Coprinus extract, (B) protein eluted from fluphenazine column with EGTA, stained with Coomassie Blue and (C) fraction B stained with silver stain.

TABLE 4. Bovine-PDE fractions from a crude extract of bovine heart tissue assayed for cyclic nucleotide phosphodiesterace activity under various conditions. Data is expressed in terms of specific activity (picomoles of cAMP hydrolyzed per minute/mg protein).

DEAE-Cellulose Fractionation of Cyclic Nucleotide Phosphodiesterase From A Crude Extract of Vocine Heart Tissue					
Fraction Number	Endog-enous	Plus EGTA	Plus Coprinus CaM	Plus EGTA and CaM	Stimulated Activity
1	24.7	0	138.9	88.9	5.6
2	36.0	6.2	27.3	102.1	0
3	326.8	40.4	676.7	231.8	2.1
4	68.7	20.0	761.9	18.9	11.2
5	543.4	309.4	618.3	94.2	1.1
6	334.0	270.0	863.0	290.0	2.6
7	41.1	15.6	103.1	23.6	2.5
8	64.0	31.6	183.6	8.7	2.9
9 - 10	68.0	20.4	129.9	25.4	1.9
11	200.5	19.1	272.7	156.4	1.4
12	53.6	26.7	148.4	78.7	2.8
13	20.9	181.4	558.5	304.1	2.8
14	96.6	39.9	265.5	179.8	2.8

with fraction #4 was most stimulated by Coprinus calmodulin A and the stimulation could be greatly reduced with EGTA it was used in studies to further investigate Coprinus calmodulin. In general those fractions eluted at low NaCl concentration (0.15 M) were most affected by calmodulin and/or EGTA while those fractions eluted at higher salt concentrations were least calmodulin stimulated and/or EGTA inhibited.

Time Course of the Interaction Between Bovine PDE and Coprinus Calmodulin A

Bovine PDE was incubated in the presence and absence of Coprinus calmodulin A, 10 mM EGTA, and 50 mM caffeine. Phosphodiesterase activity was determined at intervals from 0 to 10 minutes after initiation of the reaction as described earlier. The results of this experiment are shown in Figure 19. Assays of endogenous activity associated with bovine-PDE showed that most of the hydrolysis of cAMP took place during the first minutes of the assay (59.25 pmol) followed by only slight increases to a maximum level after 5 minutes (71.52 pmol cAMP hydrolyzed). Incubation of the bovine-PDE with 10 mM EGTA inhibited activity such that after one minute only 11.71 pm of cAMP had been hydrolyzed. In the presence of EGTA maximum activity was reached after 3 minutes (42.85 pmoles cAMP hydrolyzed) after which time no further increases in enzyme activity were detected. Bovine-PDE incubated in the presence of 50 Pm caffeine resulted in no cAMP hydrolyzed. The addition of excess amounts of Coprinus calmodulin A to the bovine-PDE ultimately led to an increase in bovine-PDE activity reaching a maximum after 3 minutes of 2.45 pmoles cAMP hydrolyzed, an increase of 297% over the maximum activity of activator-less enzyme.

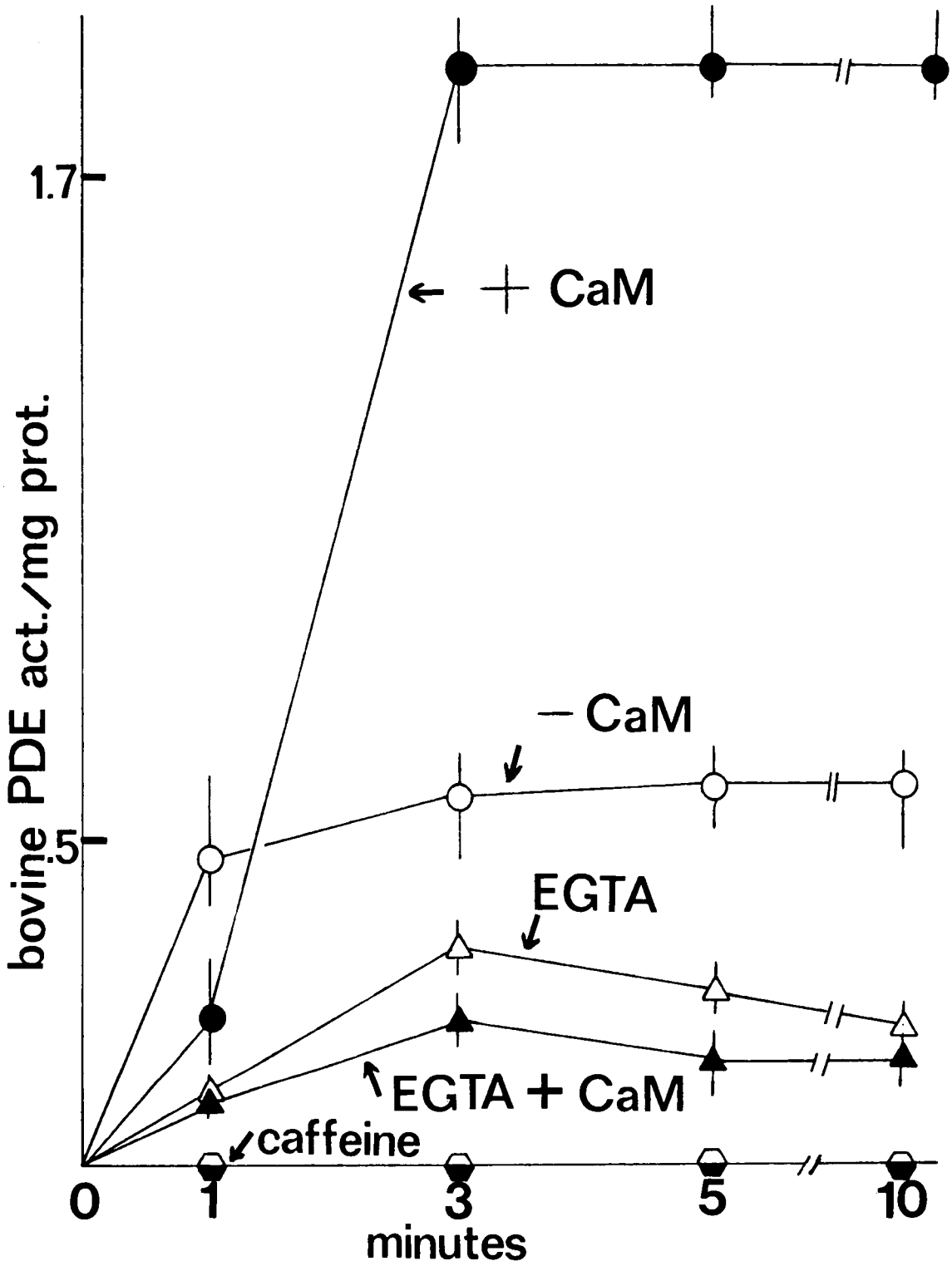


Figure 19. Time course of the effects of EGTA and caffeine on activated and activator-deficient bovine-PDE. Bovine-PDE isolated from bovine heart and calmodulin isolated from Coprinus cinereus.

However, after 1 minute calmodulin-bovine-PDE activity was 53% lower than bovine-PDE activity alone due to an unexplained interaction of CaM and bovine PDE. When EGTA was added to the bovine-PDE-calmodulin reaction mixture activity was inhibited to about the same extent as was bovine-PDE alone. Caffeine totally inhibited enzyme activity in the presence and absence of calmodulin.

Dose-Response Curve of the Stimulation of Bovine-PDE by Coprinus Calmodulin A

Different amounts of Coprinus calmodulin A were incubated with a fixed amount of bovine-PDE activity, were assayed as described earlier, were corrected for endogenous activity, and are shown in Figure 20. Bovine-PDE was stimulated by as little as 2.5 mg of Coprinus calmodulin A (the least amount tested) by a factor of 8.8% of maximal stimulation. Maximal stimulation was obtained by addition of 25 μg of activator and the addition of more calmodulin did not result in any further stimulation of bovine-PDE activity. Fifty percent maximal activity was obtained by the addition of approximately 15 nanograms of activator.

The Effect of Culture Condition on Endogenous Calmodulin Activity in Crude Extracts from Coprinus

Crude extracts from appropriately incubated cultures were assayed for cyclic nucleotide phosphodiesterase activity in the presence and absence of 20 mM EGTA as described in the Methods section. The difference in activity between non-EGTA and EGTA containing reaction mixtures was designated as stimulated activity due to endogenous calmodulin. One unit of calmodulin activity was equal to

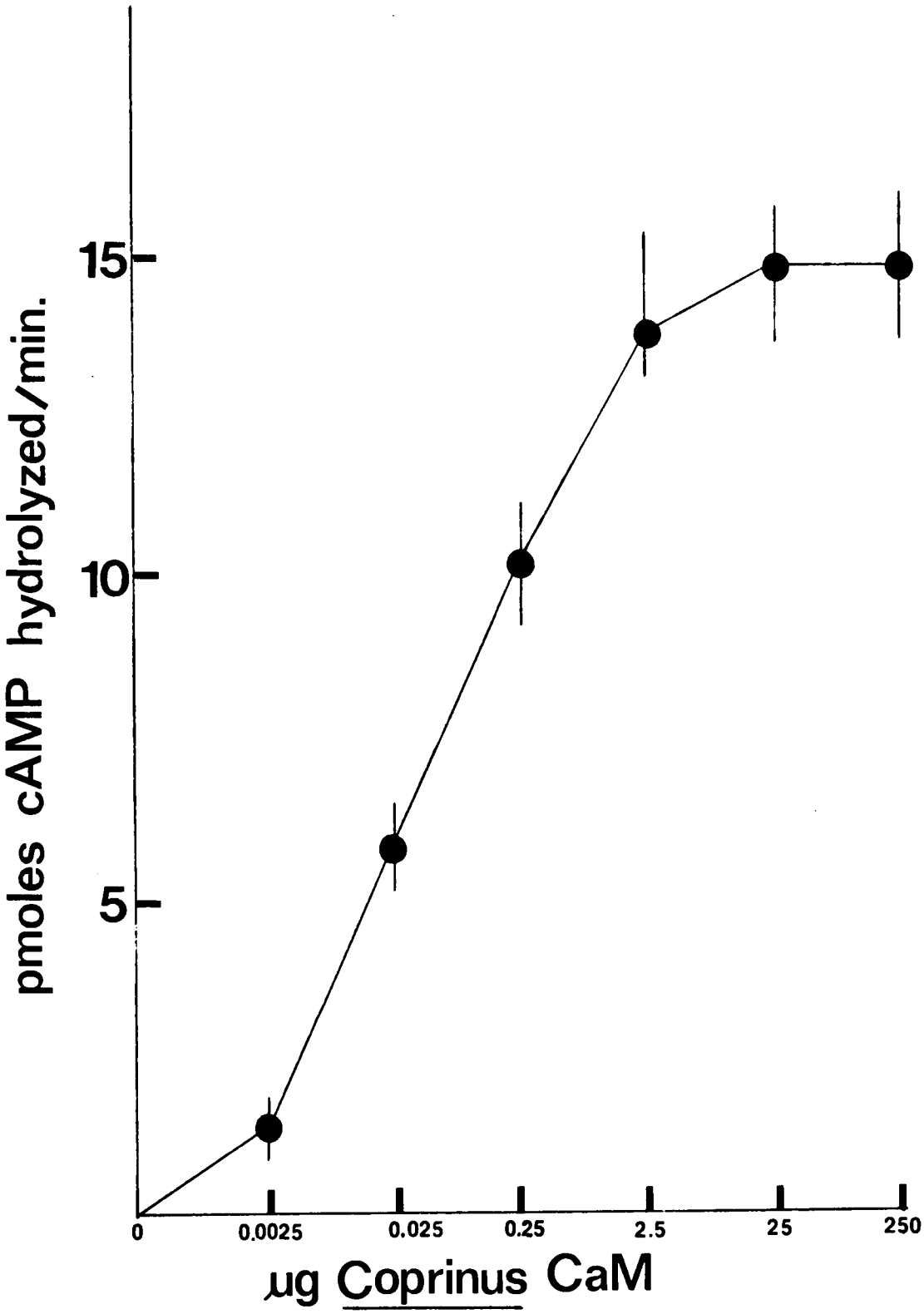


Figure 20. Dose-response of bovine-PDE to Coprinus calmodulin. 100 µg of bovine-PDE was used per assay.

50% stimulation of phosphodiesterase over non-activated levels. The results of these experiments are shown in Figure 21. Endogenous calmodulin activity associated with alternating light/dark grown cultures exhibited a fluctuating activity pattern without apparent condition to individual light or dark treatments. However, total activity peaked after 120 hours of incubation, one light/dark cycle prior to initiation of fruit body primordia. Following 120 hours of incubation calmodulin activity decreased but again peaked at 161 hours of incubation at about 50% of the 120 hour level of activity. Extracts from cultures grown in continuous light had little calmodulin activity compared to alternating light/dark extracts, which remained low at rather constant levels throughout the incubation period. Dark-grown mycelia exhibited no endogenous calmodulin activity at anytime during the growth cycle.

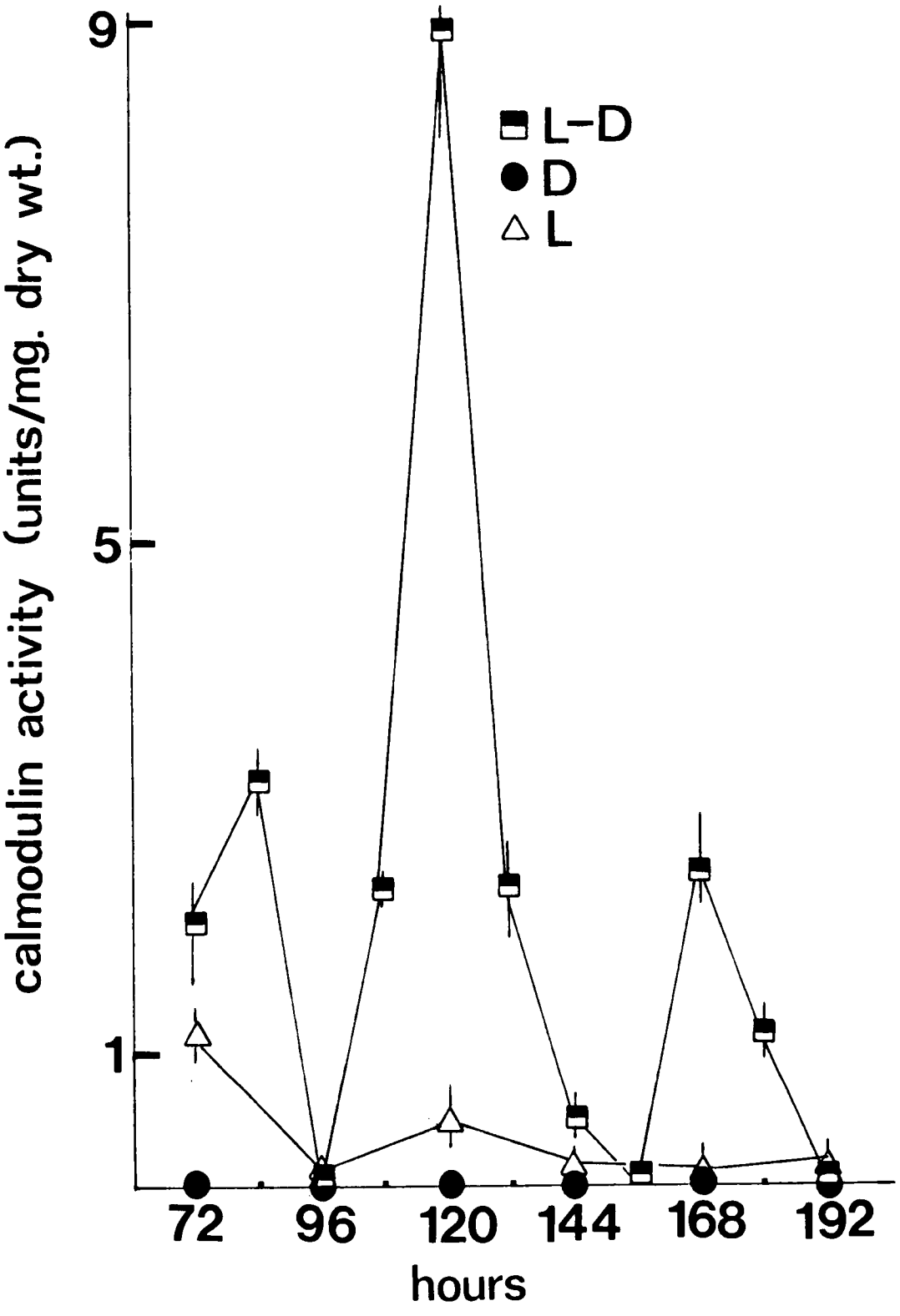


Figure 21. Endogenous calmodulin activity associated with crude extracts from dikaryotic mycelia grown under different conditions.

CHAPTER IV

DISCUSSION

The results shown in Figures 22, 23, and 24 illustrate the complex nature of many of the basic metabolic events which occurred during the development of dikaryotic mycelia of Coprinus cinereus grown under different environmental conditions. It is difficult at best to identify those specific biochemical events occurring in undifferentiated dikaryotic mycelia as those biochemical events which are unique to, and ultimately result in, a shift from vegetative to reproductive growth with associated changes in morphology, from those biochemical events that must simply be considered as part of the basic metabolic character of the organism. The problem is further complicated when one considers the inherent difficulties associated with studying post-translationally regulated, long-term processes, such as the induction of fruiting in higher fungi, since such processes usually involve cascades of biochemical events (2, 14, 45, 46) with individually very short response times (seconds) but regulating a developmental process requiring days or even weeks to complete. For example, the synthesis of a single compound such as cAMP has been shown to result in a sequential series of biochemical events ultimately resulting the activation or deactivation of one to many metabolic processes (2, 14, 38, 45, 46).

Much of the information available relative to the role of cAMP and fruiting in Coprinus has come from the laboratory of Uno and

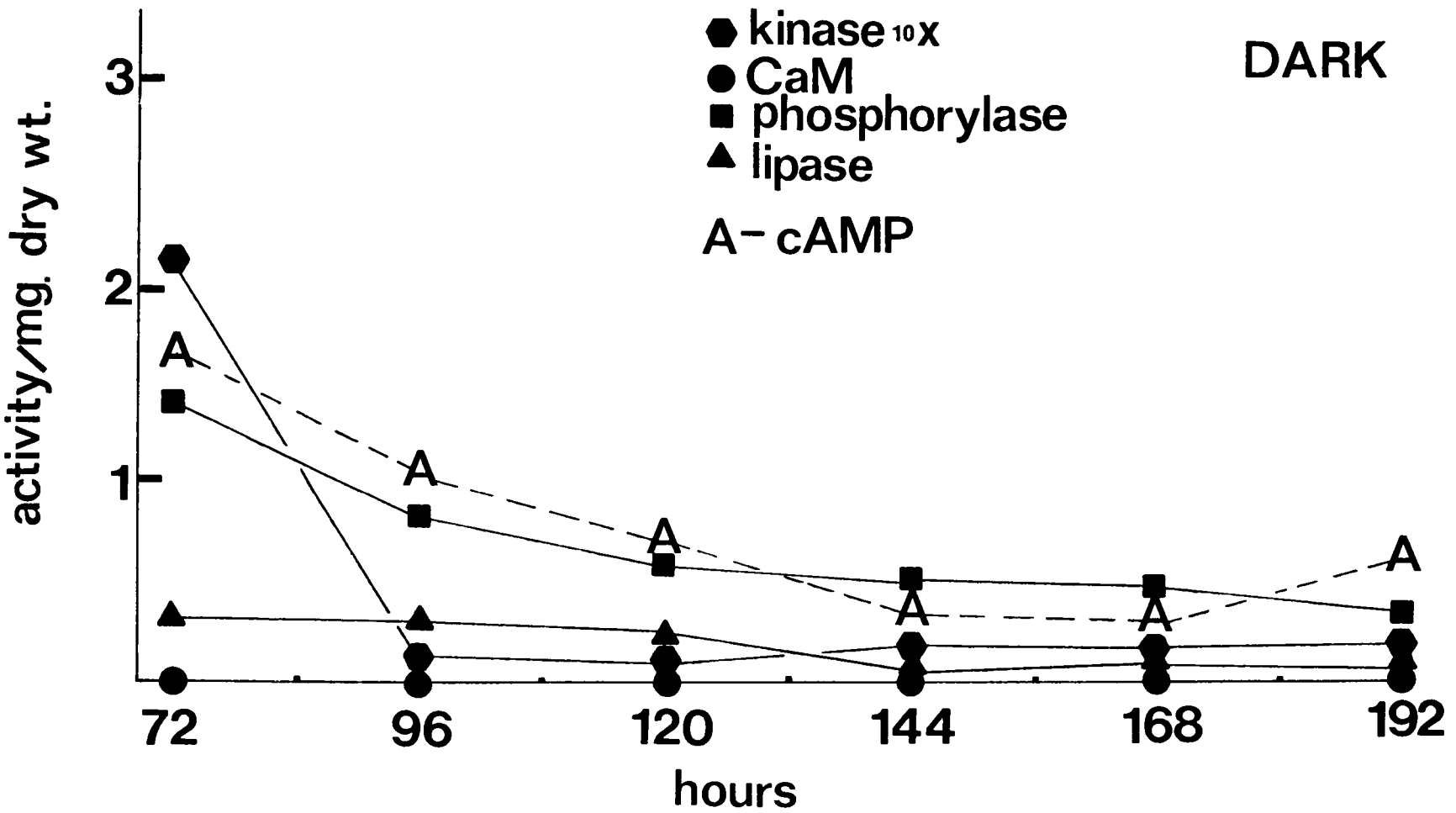


Figure 22. A comparison of the activities of several enzymes relative to cAMP concentration (pmoles) associated with dikaryotic mycelia incubated in continuous darkness. No initiation takes place.

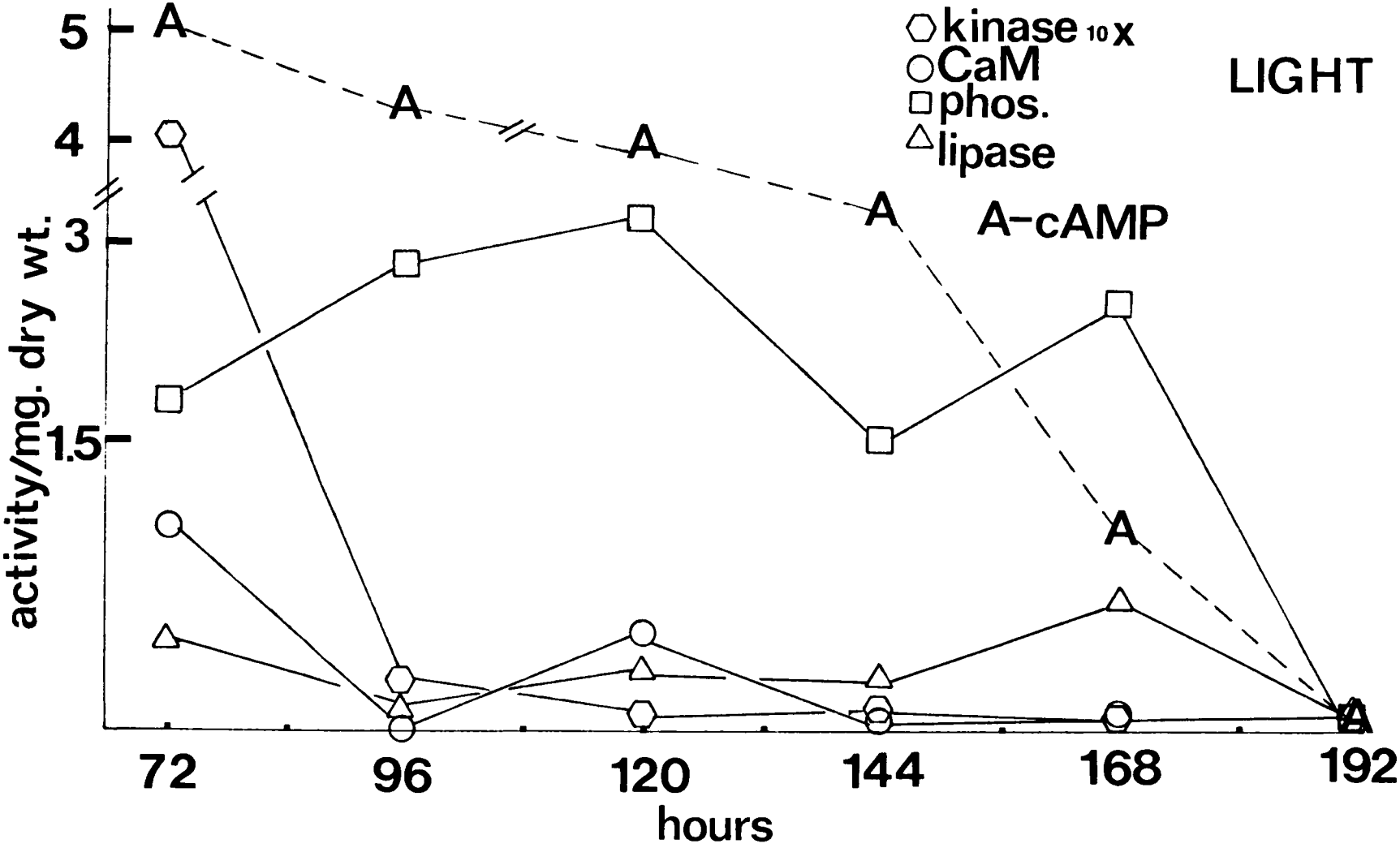


Figure 23. A comparison of the activities of several enzymes relative to cAMP concentration (pmole) associated with dikaryotic mycelia incubated in continuous light. Some initiation takes place.

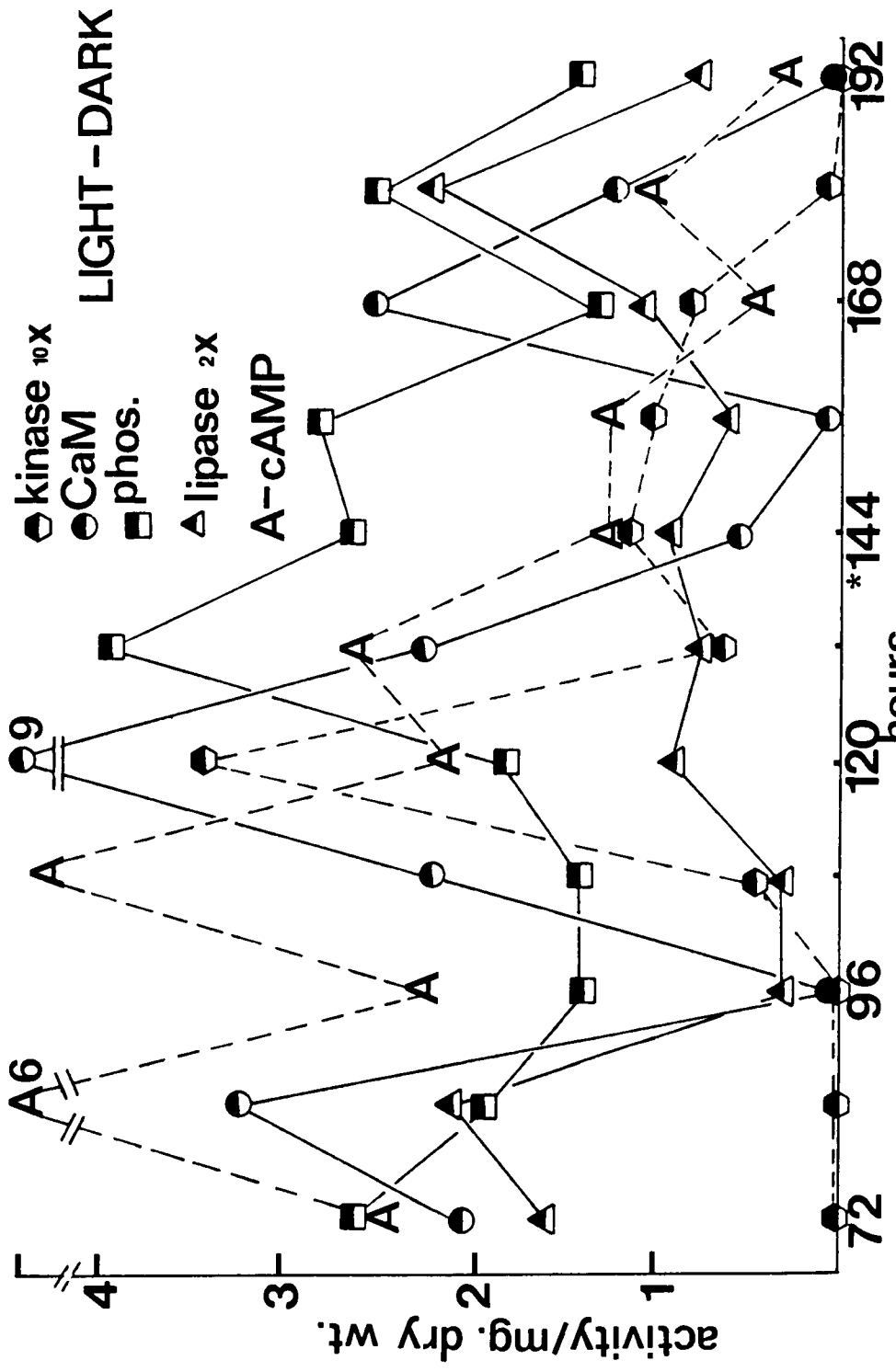


Figure 24. A comparison of the activities of several enzymes relative to cAMP concentration (pmoles) associated with dikaryotic mycelia grown under alternating light/dark conditions. Cultures initiate synchronously at 144 hours of incubation as indicated (*).

Ishikawa (62-67). Most of the data presented by those workers was obtained using mutant monokaryons grown in continuous light on undefined liquid media. Such mutant organisms would appear to differ significantly from normal dikaryons in terms of their respective basic metabolic activities (41).

According to Ishikawa and Uno (18, 19) mycelia of Coprinus sp. (N or N+N) which were able to produce fruit bodies accumulated cAMP in their hyphae while those mycelia unable to produce fruit bodies did not accumulate the nucleotide. This implied that the enzymes involved in the metabolism of cAMP, adenylate cyclase and cyclic nucleotide phosphodiesterase, were in essence responsible for the regulation of fruiting since those enzymes ultimately controlled the cAMP levels present in the mycelia at any given time (62-67). Cyclic AMP accumulation was inhibited by darkness or elevated glucose levels in the culture medium (64-67). Data obtained in the current study indicated that all dikaryotic mycelia, regardless of culture condition were able to metabolize and accumulate cAMP (Figure 5, page 27). Clearly, the levels of cAMP detected in continuous light and alternating light/dark-grown mycelia were at times significantly higher than those levels detected in dark-grown mycelia. These data indicated that more than the simple presence or absence of the nucleotide was necessary for the production of fruit bodies. Furthermore, it would appear that more was involved in the induction of fruiting than the production of a critical amount of cAMP since continuous light-grown mycelia had levels of the nucleotide comparable to the maximum levels of cAMP detected in alternating light/dark-grown mycelia yet, continuous

light/grown cultures did not fruit normally. Higher cAMP levels associated with cultures exposed to light is consistent with data indicating that adenylate cyclase is light activated (67). This is very clearly demonstrated in data from alternating light/dark-grown mycelia (see Figure 5, page 27).

Clearly, some factor associated with the very definite diurnal fluctuation of cAMP accumulation (light) and breakdown (dark) characteristic of alternating light/dark cultures was important in the inductive process rather than continuously high levels of the nucleotide. In actuality the amount of cAMP present at any given time is a direct function of the ratio of activities of both the cyclase and phosphodiesterase which are not only affected by light and dark treatments but by chemical regulators as well (2, 7, 14, 45, 46). It has been reported that the activities of cyclase and phosphodiesterase do not appear to parallel cAMP concentrations measured during ontogenetic development (54) which suggests the presence of a number of regulators of cAMP biosynthesis and degradation. Figure 16, page 52, clearly demonstrated the difficulty in trying to draw parallels between enzyme activity and nucleotide concentration present at any given time. Others have reported greater success in this regard using sub-cellular fractions for the comparison of cyclase and phosphodiesterase activity to cAMP levels during development (54).

The general pattern of decreasing levels of cAMP with respect to culture age was consistent with results reported by Uno and Ishikawa (63) as were the absolute amounts of the nucleotide found in mycelia during this study. Because the initiation of fruiting would appear to

either be set in motion or repressed rather early in the developmental cycle of this organism (prior to 144 hours of incubation) it could be argued that all of those biochemical events modulated by cAMP directly involved during the inductive period became decreasingly active as initiation approached resulting in a decreased ratio of cAMP to total protein or dry weight. It has been well established that cAMP binds directly to specific proteins (2, 8-10, 13, 17, 21, 22, 24, 26-29, 38, 39, 43, 45-57, 57, 58, 66, 67) and this may affect the ability to accurately measure the total cAMP content of a given crude extract and while this must be considered, the amount of cAMP bound to protein at any given time is small when compared to the total levels of "free" cAMP (data not presented).

It has been reported (65) that high glucose levels (2%-5%) in the growth media inhibits the synthesis of cAMP in mycelia otherwise capable of production of the nucleotide. Results obtained in the current study indicated that cAMP levels were highest in young mycelia when exogenous glucose levels were highest. Of course, Coprinus fruiting media contained 1% glucose which may be well below inhibitory levels of the sugar. Furthermore, the diurnal fluctuation of cAMP levels in alternating light/dark-grown mycelia indicated that glucose levels alone found in the culture media could not account for cAMP levels detected in mycelia at any given time.

In a wide variety of cell types, cAMP mediates its action through the activation or deactivation of protein kinases which, in turn, through the phosphorylation of cellular proteins, control various cellular functions (2, 7, 8-10, 13-15, 17, 21-23, 26, 30, 38, 47, 57,

67, 68). Phosphorylase kinase is one such protein and it catalyzes the formation of active glycogen phosphorylase a from the inactive glycogen phosphorylase b and thus stimulates glycogenolysis. This, assays of glycogen phosphorylase a activity are also indirect measurements of phosphorylase kinase activity. This kinase is composed of 4 subunits designated α, B, γ and δ (8). The α and B subunits have been shown to be the components phosphorylated by cAMP-dependent protein kinase (8). The δ subunit has been shown to be identical to calmodulin (10) but yet, the enzyme can be stimulated by further additions of calmodulin to the assays (10). Thus, glycogen phosphorylase a activity is modulated by both cAMP and calmodulin through the action of phosphorylase kinase.

Glycogen phosphorylase a activity associated with extracts from Coprinus (Figures 6a and 6b, pages 30 and 31) varied significantly with respect to mycelial age and culture condition. Significantly, there was an increase in total phosphorylase a activity after 132 hours of incubation in alternating light/dark-grown mycelia, just 12 hours prior to the formation of fruit body primordia in these cultures. Wright and Park (74) have reported that the activity of this enzyme controlled the speed of differentiation of Dictyosteluim discoidium. Because continuous light or dark-grown cultures displayed no such increase in activity this may be regarded as a significant metabolic event relative to the initiation of fruiting.

When total activity of phosphorylase a was normalized for cAMP concentration (Figure 6a, page 30), activity appeared to be generally inhibited by the nucleotide. This would appear to be the reverse of

the expected if cAMP were in fact a positive modulator of phosphorylase kinase activity (9, 10). This phenomenon can be explained that since cAMP effects are mediated by an intermediary compound, a kinase, the limiting factor in the stimulation of glycogen phosphorylase a may well be due to the availability of a kinase and not the total amount of free cAMP present.

Considering the apparent inhibition of glycogen phosphorylase a activity by cAMP, purified enzyme was incubated with varying amounts of the nucleotide (Figure 7, page 33). The results obtained indicated that cAMP could bind directly to phosphorylase a, inhibiting its activity in the absence of phosphorylase kinase, by an unknown mechanism, such as a conformational change. The direct binding of cAMP to protein has been extensively reported and has been shown to affect the activity of many molecules (8-10, 15, 17, 27-29, 43, 51, 54, 55).

A comparison of phosphorylase a activity with calmodulin activity (Figure 21, page 66) must be undertaken with care for two reasons. First, the subunit of phosphorylase kinase is calmodulin (10) and secondly, calmodulin activity is defined in terms of its ability to stimulate phosphodiesterase. The stimulation of PDE activity may or may not be proportional to calmodulin's ability to stimulate phosphorylase kinase, and in turn phosphorylase a activity. Nevertheless, high calmodulin activity associated with 120 hours alternating light/dark-grown mycelia was followed by an increase in total phosphorylase a activity at 132 hours and was repeated to a lesser degree at 168 and 180 hours of incubation respectively. This suggested that increased calmodulin activity affected subsequent

phosphorylase activity by activating the phosphorylase kinase which could explain the apparent delay in increased phosphorylase a activity.

The phosphorylation of nuclear proteins, such as histone, has been suggested to play a role in the control of various cellular functions including transcription (38, 39, 59). The occurrence of protein kinase which was activated by cAMP was initially described by Walsh et al. (68) and similar enzymes have been detected in many different prokaryotic and eukaryotic cells (2, 7-10, 13-15, 17-19, 20-23, 25-30, 38, 39, 45-47, 53, 58, 59, 66). It is currently held that cAMP-activated protein kinases are receptors for cAMP and are responsible for the effects of this nucleotide (47). Protein kinases that are independent of or inhibited by cAMP respectively have since been reported although the biological function of these kinases is virtually unknown (17, 27).

Results obtained in this study confirmed the presence of multiple protein kinase activities associated with crude mycelial extracts of C. cinereus. Total protein kinase activity varied with respect to mycelial age and culture condition (see Figures 9, 10, 11, and 15, pages 37-39, and 47). These results suggested that the relationship between cAMP and protein kinase associated with alternating light/dark-green mycelia was significantly different from the situation in continuous light or dark-grown mycelia (Figure 13, page 44). Addition of the nucleotide was generally inhibitory with respect to alternating light/dark extracts from young mycelia (Figure 15, page 47) while being stimulatory with respect to extracts from young continuous light or dark-grown mycelia. Extracts from alternating light/dark-grown

mycelia had levels of total protein kinase activity that varied inversely with respect to cAMP concentration (Figure 12a, page 42). Protein kinase activity normalized to cAMP (Figure 12b, page 42) showed a significant increase in histone phosphorylation between 120 and 180 hours of incubation, which was just prior to and during initiation of fruiting. No such increase in activity during this critical period was observed in continuous light or dark-grown mycelia. This suggested that glycogen phosphorylase a activity during induction and initiation was essential for normal fruiting to occur.

The fractionation of crude mycelia extracts indicated the existence of at least 3 types of protein kinase (Figure 15, page 47), all of which have identical catalytic function but are differentially regulated by cAMP. This occurrence of both cAMP-stimulated and cAMP-inhibited protein kinases have been reported in acellular slime molds (27), mammalian tissue (17) and Coprinus (66). There is reason to believe that the cAMP-independent protein kinase found in fractionated crude extracts is the catalytic subunit of the cAMP-dependent kinase from the same extracts (47). Both cAMP-stimulated and cAMP-inhibited protein kinase were affected by concentrations of cAMP in the known range of intracellular concentrations of the nucleotide (Table 1, page 49).

Calmodulin has been shown to activate several protein kinases including myosin light chain kinase (MLCK) (55) muscle phosphorylase kinase (8, 15, 53) and glycogen phosphorylase kinase (10, 26). In the present study it was found that both cAMP-stimulated and cAMP-inhibited protein kinase were calmodulin stimulated in the presence

or absence of cAMP (Table 2, page 50). There appeared to be a synergistic type of cooperativity between cAMP and calmodulin with respect to cAMP-inhibited kinase activity (Fraction I, II, III, IV, Figure 15, page 47). This phenomenon has not been previously reported, but cAMP has been shown to increase the affinity of calmodulin for various target enzymes (7). Cyclic AMP-stimulated kinase activity was diminished in the presence of both cAMP and calmodulin, below levels of activity associated with calmodulin only. These data strongly suggest the direct binding of both cAMP and calmodulin to protein kinase with each modulating the effect of the other. Clearly, more work needs to be done in this area but since direct binding of these modulators to an enzyme is taking place, changes in enzyme activity are probably due to conformational changes.

If one compares protein kinase activity associated with alternating light/dark-grown mycelial extracts with endogenous calmodulin activity associated with similar extracts (Figure 21, page64) there would appear to be a clear relationship between calmodulin activity and increased histone phosphorylation (Figure 13, page 44) at 120 hours of incubation and 132 hours of incubation respectively.

Much of the evidence presented thus far points to the possibility that cAMP and calmodulin effects are mediated through phosphorylation reactions. However, since the specific intracellular substrates of the multiple protein kinases present in crude mycelial extracts from C. cinereus have not been determined it is not possible to conclude beyond doubt that phosphorylation reactions either stimulate or inhibit the induction of fruiting. Yet, the character of

protein kinase activity in fruiting cultures differed from non-fruiting cultures so significantly that one must consider the importance of these reactions in the inductive process.

Lipase activity associated with mycelial extracts (Figure 8a and 8b, page 34) varied significantly with respect to mycelial age and culture condition. Cyclic AMP has been shown to be an inhibitor of lipase activity, while at the same time, the enzyme is calmodulin stimulated (7, 48). A comparison of lipase activity and calmodulin activity during the experimental period does not reveal the existence of direct relationship. However, lipase activity did tend to increase in older mycelia when cAMP levels were lowest and a period following increased calmodulin activity, at least in alternating light/dark-grown mycelia.

If one compares lipase activity no glycogen phosphorylase a activity with respect to cAMP concentration (Figures 8b and 6b, respectively, pages 34 and 31) it would appear that the respective activities of the enzymes follow the same general pattern. This similarity is most interesting when one considers that lipase is cAMP-inhibited and calmodulin stimulated (7, 48) while glycogen phosphorylase activity is both cAMP stimulated and calmodulin stimulated (7, 8, 26). This suggests the presence of very complex regulatory mechanism involving a number of different factors.

Calmodulin has been detected in all nucleated cells examined (7). Therefore, the isolation of calmodulin from Coprinus cinereus was not surprising although this is the first report of that protein from this genus. Coprimus calmodulin was purified over 400 times

(Table 3, page 55) by simply passing a heat-treated crude extract through a fluphenazine-Sepharose-4B column. Charbonneau and Cormier (4) reported the purification of peanut calmodulin by a factor of about 800 times using a similar technique.

The elution of Coprinus calmodulin from the column was essentially identical to the elution of calmodulin from Agaricus, porcine brain and Renilla reniformis (4). Subsequent electrophoresis of the eluate following the EGTA pulse indicated the presence of a single protein (Figure 19, page 60) which was shown to be authentic calmodulin by several criteria.

By definition calmodulin activity is measured in terms of the ability of that protein to stimulate the activity of cyclic nucleotide phosphodiesterase over its inactivated state (5, 6, 31). In addition to phosphodiesterase, calmodulin has been found to activate a number of other enzymes, often in response to Ca^{++} ion flux (7) such as lipase (7, 44), MLCK (55), phosphorylase kinase (8, 15, 49), NAD kinase (7) ATP-ase (7) adenylate cyclase (7) and guanylate cyclase (7). Furthermore, by regulating cAMP metabolism, calmodulin indirectly regulates the numerous reactions modulated by that nucleotide. Clearly, the interaction of calmodulin with the enzymes listed above is tightly regulated by the cell because it has been found that calmodulin is present in excess amounts in all tissues examined (20, 54). Furthermore, the activator is specific and lacks tissue or species specificity (20, 54).

The ability of Coprinus calmodulin to stimulate bovine heart

phosphodiesterase (bovine-PDE) which was activator deficient was significant (Table 4, page 58). During a preliminary purification of bovine-PDE (data not presented) addition of Coprimus calmodulin increased bovine-PDE activity but the activation was further increased by EGTA instead of the expected reversal of the calmodulin activation. Sharma et al. (50-52) and others (71-73) have reported the existence of proteins which bind to calmodulin and are referred to as modulator binding proteins which, in essence, modulate the activity of the modulator. Further purification however resulted in a bovine-PDE preparation essentially activator-deficient and free of interferring contaminants (Table 4, page 58). Those bovine-PDE fractions eluted at low salt concentrations (less than 0.15M NaCl) as previously reported by Ho et al. (16) and Sharma et al. (52) were most stimulated by calmodulin since under such conditions the bovine-PDE eluted was activator-deficient.

Activator-deficient bovine-PDE could be stimulated by a factor of about 11 times basal activity, within ranges of calmodulin-stimulated activities reported by Ho et al. (16) but higher than reported by Lin et al. (3). Stimulated activity of the bovine-PDE was reversed by addition of the Ca^{++} ion chelator EGTA which indicated that the stimulation was Ca^{++}-dependent as first reported by Cheung et al. (5). Caffeine (50 mM) inhibited bovine-PDE totally and calmodulin did not stimulate the enzyme in the presence of this inhibitor as previously reported (3). All of these data indicated that the fluphenazine column fraction designated Coprinus calmodulin A was in fact the heat stable, Ca^{++}-dependent stimulator of cyclic nucleotide phosphodiesterase.

Dose response activation by the activator (Figure 20, page 63) was similar to that reported by Lin et al. (31). In the present study it was found that 2.5 nanograms of calmodulin stimulated enzyme activity and maximum stimulation was obtained with the addition of 2.5 µg of activator per 100 µg of enzyme. Lin et al. (31) reported most stimulation occurred between 0.1 and 0.5 µg of activator per 100 µg of enzyme which would result in a much steeper dose response than detected in this study. Differences most probably resulted from use of a different assay procedure, different bovine-PDE source and purity of activator-deficient enzyme. Lin et al. (31) reported a 1700 fold purification of porcine brain calmodulin using a preparative electrophoretic technique.

Clearly, mycelial age and culture condition significantly affected endogenous calmodulin activity associated with crude mycelial extracts (Figure 21, page 64). Significantly, there was no detectable calmodulin activity associated with crude mycelial extracts (Figure 21, page 64) nor with dark-grown mycelia and there was little calmodulin activity associated with continuous light-grown cultures. Conversely, alternating light/dark-grown mycelia had specific periods of high calmodulin activity, especially 24 hours prior to initiation in such cultures. Few studies have been done examining the relation of calmodulin activity to developmental change since sub-cellular fraction, not crude extracts are preferred for such investigations (31). Yet, data obtained in this study suggested that calmodulin activity was crucial to the normal development of Coprinus. Those cultures which did not demonstrate such calmodulin activity did not develop normally.

Jarrett et al. (23) have postulated that in plants light stimulates an unknown membrane bound receptor which results in increased Ca^{++} uptake raising the intracellular concentration of that ion. The Ca^{++} flux in turn stimulates calmodulin which then further may stimulate a number of target enzymes (Figure 25). In the current study it has been shown that light is necessary for normal development and for calmodulin activity but light alone was less important than alternating light/dark exposures. Thus, the Jarrett model of calmodulin activation in plants is not directly applicable to Coprinus but there would appear to be some interesting similarities between the two systems.

An Overview

Figures 22, 23, and 24 (pages 66, 67, and 68) represent a comparison of the respective activities of triacylglycerol lipase, glycogen phosphorylase protein kinase(s) and calmodulin respectively, compared to endogenous cAMP concentrations during development of Coprinus cinereus. Alternating light/dark-grown mycelia (Figure 24, page 68) initiated synchronously after 144 hours of incubation. Continuous light-grown mycelia (Figure 23, page 67) never resulted in the production of normal fruit bodies. Dark-grown mycelia (Figure 22, page 66) did not typically initiate.

A comparison of the metabolic parameters studied during this investigation in cultures grown under differing light and dark conditions clearly shows that the activities of those compounds studied varied significantly depending upon culture condition. Much work has been done on the role of light in the development of fungi (57) but as

HYPOTHETICAL MODEL OF THE INDUCTIVE PROCESS

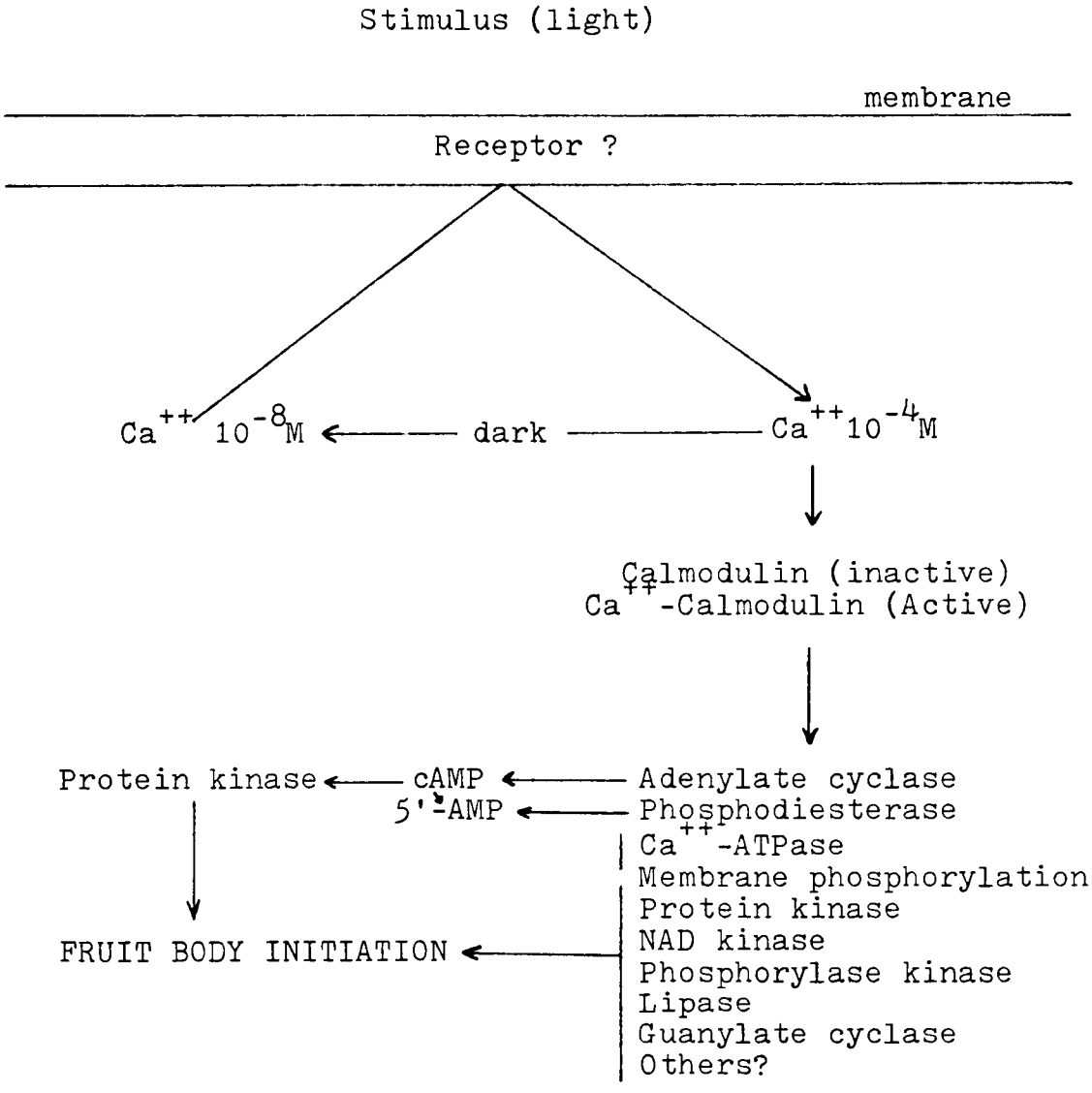


Figure 25. A hypothetical model for the induction of fruiting. Modified from Jarrett et al. 1980.

yet the mechanism of light reception and subsequent stimulation of target molecules is unknown. In fact, in this study it was shown that cultures grown in continuous light grew slightly more slowly than cultures grown in continuous light grew slightly more slowly than cultures grown in continuous dark, while alternating light/dark cultures grew more rapidly than either continuous light or dark cultures, especially after initiation. Thus, it would appear that both light and dark are important to normal development and the relative length of each exposure appeared to be very important to normal development especially with respect to young cultures. The very different metabolic character of mycelia grown under each respective culture condition, while not being reflected significantly in terms of initial growth rates, profoundly affected the ability of respective cultures to eventually produce normal basidiocarps. Preliminary experiments indicated that if young mycelia were transferred from non-fruiting conditions to fruiting conditions prior to a critical time, fruiting would occur. If the transfer was made after that critical time the organism was irreversibly committed to an asexual life cycle. Conversely, cultures initially incubated in fruiting conditions longer than a critical period would initiate even if placed in darkness, however light was required after initiation for normal fruit body development. The question then becomes what is it that commits the organism to a sexual or asexual pattern of reproduction during the early development of the mycelia. Clearly, it is something which occurred in alternating light/dark-grown mycelia and not in continuous light or dark-grown mycelia.

Many of the actions of cyclic AMP are mediated through protein

kinases (47), including the transcription of genetic material (38). In continuous light or dark-grown mycelia protein kinase activity was found to be significantly higher initially than activity associated with alternating light/dark-grown mycelia. Furthermore, in young mycelia cAMP positively modulated histone phosphorylation in continuous light or dark-grown mycelia but negatively modulated the same process in alternating light/dark-grown mycelia. Clearly indicated here is that a very different metabolic pattern was established during the inductive period between alternating light/dark-grown mycelia on the one hand, and continuous light or dark-grown mycelia on the other (see Figure 11, page 39).

Changes in lipase activity during growth and development of this organism remained relatively minor, especially during the inductive period. Thus, this enzyme's role in the inductive process is not clear.

Glycogen phosphorylase a has previously been shown to be positively modulated by cAMP (9). In this study, however, cAMP appeared to inhibit activity of this enzyme. Only after cAMP levels decreased over initial levels did enzyme activity increase but other factors than cAMP were probably involved in the increase of activity.

Significantly, calmodulin activity reached a very high level 24 hours prior to initiation along with very high levels of histone phosphorylation activity in alternating light/dark-incubated cultures. No such metabolic events occur in either continuous light or dark-grown cultures. Thus, the increases in calmodulin and protein kinase activity 24 hours prior to initiation would appear to be the most

likely candidates if one had to choose the metabolic events unique to normally fruiting cultures. Uno and Ishikawa have previously implicated protein kinase activity in the fruiting process (66), but no information regarding protein kinase, calmodulin and fruiting is available other than that presented in this report. Because calmodulin modulates a great number of cellular process its specific relationship to fruiting is not clear.

As tempting as it would appear to replace cAMP as the "Fruit Inducing Substance" (FIS), a phrase coined by Uno and Ishikawa (62) great care must be exercised. Determination of a single FIS is difficult considering the complexity of the fruiting process. How can cAMP be the FIS if calmodulin regulates cAMP metabolism? How can calmodulin be selected as the FIS if calmodulin activity is regulated by Ca^{++} ion flux? Calcium ion flux has in turn been shown to be a result of membrane depolaization (1, 37, 73) which is a cAMP modulated process in some cases. The argument can become rather circular. The basic problem in selecting a single "inducer" molecule is that as the complexity of intercellular communication increases there is not a parallel increase in the nuber of cellular regulators (7), few regulators modulating many processes.

Areas of continued investigation that would appear to be most promising would be relative to factors affecting Ca^{++} ion flux, namely rapid transient changes in membrane potential. Also, work regarding the mechanism of light reception in fungi and subsequent stimulation of target molecules would be very interesting.

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VITA

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