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Cyclic AMP and Enzyme Induction in Aging Cultures of Aspergillus ornatus

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CYCLIC AMP AND ENZYME INDUCTION IN AGING CULTURES OF *ASPERGILLUS ORNATUS*

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by

Dennis F. Unger

1977

APPROVAL SHEET

This thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Arts

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Approved, March 1977

Bradner W. Coursen

Robert E. L. Black

Joseph L. Scott

DEDICATION

... for my parents

TABLE OF CONTENTS

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LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABSTRACT

The presence of endogenous cyclic AMP was demonstrated in mycelial mats of Aspergillus ornatus Raper grown on cellulose-xanthate membranes overlying a defined agar medium.

Cyclic AMP activity was detected in 30 separate samples. Purification techniques increased the cyclic AMP concentration in excess of eight times the level observed in crude extracts. In the presence of exogenous phosphodiesterase, cyclic AMP was only slightly detectable in crude extracts. The concentration of endogenous cyclic AMP declined by approximately 60% in non-growing aging mycelial mats.

The inducible enzyme, 2, 3 dihydroxybenzoic acid carboxylyase (o-pyrocatechuic acid carboxylyase) was detected in crude extracts. The addition of 0.1% L-tryptophan to the defined agar medium increased the enzyme activity to five times the observed level in un-induced mats. With increasing age in non-growing mycelial mats, the level to which the enzyme could be induced decreased, while the basal enzyme activity remained only slightly changed.

A relationship between the age-related decline in cyclic AMP and o-pyrocatechuic acid carboxylyase (OPCA carboxylyase) may exist. However, addition of 10^{-3} M cyclic AMP to the culture medium neither increased OPCA carboxylyase activity nor increased endogenous cyclic AMP levels at early stationary phase. Likewise, the addition of 0.1% L-tryptophan to the defined agar medium did not alter endogenous cyclic AMP levels at early stationary phase. A discussion of the role of these effects on aging is included.

CYCLIC AMP AND ENZYME INDUCTION IN AGING CULTURES OF *ASPERGILLUS ORNATUS*

INTRODUCTION

The study of aging presents a unique problem. There is a distinct contrast between the vigorous physical appearance and abilities of a mature youth of 18 and the overall deterioration of the 85 year old adult. Despite the clear visual cues which lead to a public acceptance of some sort of biological mechanism which we call aging, many scientists are unwilling to accept the process as a separate entity, but prefer to think of it as part of the developmental process. This has caused investigators such as Strehler (1962) and Sorokin (1966) to establish criteria which measure aging free from the influence of concurrent processes. The gerontologist must try to isolate, or at least minimize the effects of developmental or morphological influences from his study. He must also account for the environmental changes such as intracellular interactions or alterations in the growth medium or conditions. Hayflick (1973) has reviewed several modern theories of aging, but perhaps the most widely accepted view is that held by Medawar (1951) and Medvedev (1964) who consider aging to be a decrease in the ability of an organism to adapt to its environment after attaining reproductive maturity.

The study of whole organisms often clouds the perspec-

tives on aging, therefore many studies have been devoted to analyzing cellular changes. Organelles such as mitochondria (Ozelkok and Romani, 1974; Sohal, 1975), chloroplasts (Harnischfeger, 1973) and other membrane systems (Grinna and Barber, 1972) have been shown to undergo alterations in structure as well as in biochemical contents. Accumulation of cellular materials such as lipids (Foster, 1949; Van Etten and Gottlieb, 1965), pigment granules (Hamperl, 1934; Strehler, 1964) and free radicles (Harman,1971), as well as a decrease in solubility of biological polymers due to cross-linkage and crystallization (Bjorksten, 1972) all show a definite relationship to the aging phenomenia.

Basic cellular mechanisms such as protein synthesis (Dickman and Gordon, 1975; Holliday, 1969), respiration (Calabrese and Stoffolano, 1973; Van Etten, et al., 1965) and photosynthesis (Harmischfeger, 1975) have been selected as points of study, since they measure a cell's ability to function in response to a particular environment. A uni-directional flow of biochemical events is not always evident, but in general, when comparing young and aged cells, a definite contrast in content, ratios, and efficiences of these processes can be found.

Mediating these cellular processes are the enzymes which also have been observed to undergo age related alterations. Because of an increased occurrence of errors in biochemical translation, transcription and DNA synthesis with age, biologists have examined the possible role of accumula-

tion of somatic mutations in the aging process (Strehler, 1962). These eventually lead to mistakes in protein synthesis that cause an irreversible breakdown of the cellular machinery for the production of protein. According to Orgel (1963), a final and lethal "error catastrophe" eventually occurs. In support of this hypothesis, Holliday and Tarrant (1972) have demonstrated an age-related accumulation of heat labile enzymes. A less drastic approach to the change in enzymatic machinery with age, is the possibility of the production of isozymes. Studies on isocitratelyase and cytochrome oxidase (Gershon and Gershon, 1970; Reiss and Rothstein, 1975; and Alberghia and Guarnieri, 1975) have demonstrated the accumulation of several different populations of the enzyme. These isozymes have a wide range of ability to function or an affinity for a particular substrate. As the organism ages, the less efficient or completely inactive forms tend to predominate, thus reducing a cell's ability to utilize the substrate.

If aging is defined as a loss of an organism's ability to adapt to its environment and inducible enzymes are elicited by the environment, such enzymes present an excellent tool for the investigation of aging. Compiled studies (Adelman, et al., 1972) have shown that as age progresses, an organism loses its ability to produce these enzymes in response to an inducer in the environment.

The mechanism for this decrease of inducibility has yet to be fully explored. A decrease in the ability of the

inducing substance to enter the cell through a reduction of membrane permeability or loss in the ability to actively transport it has been suggested by Langheinrich and Ring (1976). Alternatively, Gershon and Gershon (1970) have shown that if isozymes exist for a particular enzyme, and if the less efficient forms accumulate with increasing age, then although the inducer (substrate) may readily enter the cell, the induced enzyme's ability to break down the substrate has been altered causing a lowering of the specific activity of the enzyme. Still another possibility to explain the loss of enzyme inducibility exists at the operon level of gene control. The concentration of a chemical regulator (such as a repressor) influences the amount of production of the inducible enzyme. Extensive work (Pastan and Adhya, 1976) has been done on prokaryotes with this aspect in mind.

Cyclic adenosine 3', 5'-monophosphate (cyclic AMP) is a well known chemical regulator and has been implicated in many biochemical functions. It is known as a first messenger of hormonal action in animal tissues and organs (Turner and Bagnara, 1971). It acts as an important mediator in permeability, secretion, control of the nervous system, and in regulating body metabolism (Greengard and Robison, 1972). Cyclic AMP has been found in higher plants (Brewin and Northcote, 1973; Brown and Newton, 1973) as well as algae (Anrhein and Filner, 1973). Most importantly, a tremendous volume of experiments has examined its role as a regulator of gene transcription in prokaryotes (Pastan and Perlman, 1972; Pastan and Adhya, 1976).

Within the last few years information has begun to accumulate on the role of cyclic AMP in fungi. Several studies have linked cyclic AMP to morphological changes. In Mucor racemosus, dimorphism was found to be dependent on endogenous cyclic AMP levels (Larsen and Sypherd, 1974; Paznokas and Sypherd, 1975). The yeast bud form was present with high cyclic AMP levels, whereas lower concentrations led to hyphal development. In Coprinus macrorhizus, endogenous cyclic AMP as well as its binding protein were required to induce formation of sexual fruit bodies (Uno and Ishikawa, 1973a; 1973b). With exposure to light, previously dark grown C. macrorhizus accumulated in order, adenyl cyclase and cyclic AMP, followed by fruit body formation (Uno, et al., 1974a). On the other hand, Schwalb (1974) demonstrated that the addition of exogenous cyclic AMP to Schizophyllum commune caused cessation of normal development of fruit bodies. Wold and Suzuki (1973b) found that exogenous cyclic AMP in Aspergillus niger promoted adhesiveness of conidia, and pellet formation in liquid culture instead of a fluffy growth which was also characteristic of growth during unfavorable nutrient conditions.

A series of studies (Tsuboi, et al., 1972; Tsuboi and Yanagishima, 1973; 1975) showed that glucose inhibited sporulation in Saccharomyces cerevisiae, but the addition of cyclic AMP reversed this repression. This response is the same as would be expected from the "glucose effect" present in the prokaryote, Escherichia coli (Pastan and Perlman, 1972).

Sporulation requires energy supplied through respiration (Croes, 1967) and respiratory chain enzymes are sensitive to glucose repression. It would appear that many of the reported morphological changes in fungi such as sporulation control by cyclic AMP levels may be the result of that nucleotide's effect on respiration.

Studies have begun on cyclic AMP effects on metabolic processes in fungi. Fang and Butlow (1970) showed in S. cerevisiae that cyclic AMP addition derepressed the low rates of cyanide sensitive respiration and deficiences in cytochromes of the mitochondrial electron transport chain caused by high glucose levels. Glycogen metabolism was increased in C. macrorhizus by exogenous cyclic AMP's ability to cause a 3-fold rise in glycogen phosphorylase activity (Uno and Ishikawa, 1976). Citric acid accumulation was enhanced by endogenous increases of the cyclic AMP concentration in A. niger (Wold and Suzuki, 1973a). It was further demonstrated that zinc and cyclic AMP may be regulatory partners in A. niger. According to Wold and Suzuki (1976a; 1976b), A. niger has two distinct phases, growth and citric acid accumulation. The concentration of zinc determines the metabolic phase, and the concentration of cyclic AMP enhances growth or citric acid accumulation depending on the phase as determined by the zinc concentration. Cyclic AMP by itself has no effect on phase determination. Hence, as zinc determines the physiological state and cyclic AMP regulates the activity, a system is established in a fungus which is in accord with the

second messenger amplifier of hormone action present in animal systems.

The role of cyclic AMP in enzyme induction has been well documented in prokaryotes (Pastan and Adhya, 1976). Investigations have been initiated to demonstrate this relationship in fungi. Van Wijk and Konijn (1971) found higher levels of endogenous cyclic AMP corresponded with an increased ability to induce ∝-glucosidase and succinate- dehydrogenase in Saccharomyces carlsbegensis. Wiseman and Lim (1974) confirmed the increased inducibility of ∝-glucosidase with higher levels of cyclic AMP in S. cerevisiae. A similar phenomenon of the inducibility of D-serine deaminase and tryptophanase in C. macrorhizus was also observed (Uno and Ishikawa, 1974). Cyclic AMP thus appears to be involved as a morphological inducer and a metabolic regulator in fungi.

The functions of cyclic AMP seem as diverse as the hypothetical causes of aging. This coupled with its apparent universal presence should lead to consideration of cyclic AMP as an agent of aging, particularly in a basic mechanism such as a loss of enzyme inducibility. Its role in inducibility has been established (Pastan and Perlman, 1972; Pastan and Adhya, 1976). Some authors have also reviewed its role in transport of amino acids (Jost and Rickenberg, 1971). However, only a few investigators have tried to determine cyclic AMP levels at various ages (Kessler and Levinstein, 1974; Haslam and Goldstein, 1974). Although some investigators have noted age-related alterations in hormonally medi-

ated enzymes (Adelman, et al., 1972; Adelman and Freeman, 1972), little work has been aimed at establishing a possible role in age-related changes in the induction process correlated with cyclic AMP.

Aspergillus ornatus is an exceptional organism in which to study aging. Methods of controlling the fungus to procure an aging system have been previously reviewed (Spiegelman, 1974). The presence of an inducible enzyme, 2, 3 dihydroxybenzoic acid carboxylyase (o-pyrocatechuic acid carboxylyase) has been established and its ability to be induced has been shown to decline with age (Spiegelman and Coursen, 1975).

According to some authors, measurement of aging should not begin until reproductive maturity has been attained (Medawar, 1951) or until mitosis has ceased as determined in filamentous fungi through estimating relative cell numbers by the quantity of DNA (Gottlieb and Van Etten, 1966). It would appear that these conditions are met when the stationary phase of growth is reached as measured by DNA concentration.

The multiple spore spray inoculation technique can be used to obtain a homogeneous mycelial mat composed of cells of similar physiological ages (Yanagita and Kogané, 1963). However, a considerable length of time may still exist before the onset of the stationary phase. Further reductions in lag phase may possibly be obtained by preincubation of the spores in a germination promoting environment before in-

oculation by adding extra nutrients to the growth medium, and by utilizing a liquid form of the medium.

Addition of growth factors such as thiamine may stimulate early increases in growth. Thiamine is the most common vitamin deficiency in free living fungi (Burnett, 1968).

This paper does not directly deal with establishing aging as a separate entity, although several steps have been taken to avoid morphological changes. Gottlieb and Van Etten (1966) have correlated DNA quantities in fungi as a good representation of cell number. A continuing increase in DNA concentration would indicate mitosis is taking place, while a decrease in concentration with time would suggest autolysis. Therefore, an aging system is viewed here as one in which the total DNA concentration remains constant through time. The loss of A. ornatus'ability to adapt to adapt to its environment is shown through its decreased ability to produce o-pyrocatechuic acid carboxylyase with age. In these studies, it is proposed to establish a reliable aging system, to determine whether cyclic AMP is present in A. ornatus, to determine if there is any age-relationship between endogenous cyclic AMP levels and the induction of o-pyrocatechuic acid carboxylyase.

MATERIALS AND METHODS

Organism. Stock cultures of Aspergillus ornatus Raper, isolates of a culture originally obtained from D.J. Schwemmin, were stored in the dark at 4°C on 1.5% agar slants containing a complex organic medium (Schwemmin, 1960). Stocks were transferred to fresh slants every two months and subcultures were made every two weeks. Hyphae were taken periodically from subcultures to prepare slants for conidia production.

Experimental media. Fresh cultures used for age-related studies were grown either on a defined agar or liquid medium as described by Schwemmin (1960). The composition of the medium was as follows:

		Trace Elements	
KH_2PO_4	1.0 gm	$Na_2B_4O_7 \cdot 10H_2O$	88
$MgSO_4 \cdot 7H_2O$	0.5 gm	$CuSO_4 \cdot 5H_2O$	393
NaCl	0.1 gm	$FeSO_4 \cdot 6H_2O$	910
$CaCl_2$	0.1 gm	$MnCl_2 \cdot 4H_2O$	72
$(NH_4)_2$ tartrate	5.0 gm	$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	37
glucose	40.0 gm	$ZnSO_4 \cdot 7H_2O$	8800
trace elements	1.0 ml	double-distilled H_2O	1000
tyrosine	0.2 gm		
double-distilled H_2O	1.0 L		

The pH was adjusted to 4.8 with dilute HCl before autoclaving. When used, the concentration of agar was 1.5%. In some growth experiments, a ten-fold dilution series of thiamine, ranging from 1 to 10^{-6} g/L, was used. Cyclic AMP (Sigma Chemical Corp., St. Louis, Mo.) at a concentration

of 10^{-3} M was added to some preinduction media either before autoclaving or by adding filter sterilized cyclic AMP Millipore GSWP-02500) to the autoclaved medium. All induction media contained 0.1% L-tryptophan.

Preparation of growth chambers and Petri plates for inoculation. In all experiments, mycelial mats were grown at 24°C on Dupont 193 PUDO cellulose-xanthate membrnaes over liquid or agar medium. Before use, membranes were pre-soaked in double-distilled H_2O for 12 to 24 hrs to remove any potential water soluble inhibitors.

A. Liquid medium. Growth chambers were prepared by attaching the membrane to 100 mm diameter glass collars with pre-soaked size 84 rubber bands. The growth chambers were floated on distilled H_2O and autoclaved. After cooling, the growth chamber membranes were inoculated with spores and transferred to 2 liters of defined liquid medium in cut-off 4 L Erlenmeyer flasks. The chambers were floated on the surface of the medium surrounded by an ultrahigh-molecular-weight polyethlylene ring. The ring was used to eliminate contact between the chamber and the wall of the flask.

B. Agar medium. Each 9 cm Petri plate contained 50 ml of a 1.5% defined agar medium (Schwemmin, 1960). Circular membranes were autoclaved in double-distilled H_2O and aseptically placed over the solidified agar growth medium in such a way to allow a 1-3 mm lip to lie against the inside shoulder of the plate. Air bubbles between the membrane and the medium were removed.

Inoculation. Conidia were produced by growing subcultures of A. ornatus on complex agar medium in the dark for 72 hrs, exposing them to a 24 hr light period, and returning them to the dark for an additional 72 hrs. Conidia were collected by gently agitating the sporulating surface of the mycelial mat with a sterile loop under a 0.01% solution of sodium lauryl sulfate. The conidial suspension was sedimented at 12,000 rpm for 10 min at 20°C in a RC2-B Sorvall refrigerated centrifuge using a SS-34 fixed angle rotor (Ivan Sorvall Inc., Norwall, Conn.). The pellet was washed in sterile double-distilled H_2O and adjusted to an optical density of 0.8 at 450 nm as determined by a Spectronic 20 colorimeter (Bausch and Lomb, Rochester, N.Y.). The adjusted conidial suspension was sprayed onto membranes using a glass atomizer (#422550, Kontes Glass Co.) pressured by air pumped through a $HgCl_2$ trap at sufficient pressure to insure even coverage of the conidia on the membrane. Two 1-second bursts of spray were applied, with the growth chamber or Petri plate rotated 180° between bursts. The atomizer was held 12 inches from the target. Experimental cultures were incubated at 24°C in light or dark, depending on the nature of the experiment.

Dry weight. Complete mycelial mats were removed from the cellulose membranes at 24 hr intervals beginnig at hour 36 and until hour 252. The mats were dried in crucibles at 55°C for 48 hrs and weighed.

DNA extraction and assay. One hundred mg of dried mycelia were placed in a 75 ml glass disruption flask (VWR #34007-066, VWR Scientific, Baltimore, Md.) with 50 gms of 0.45-0.50 mm glass beads (VWR #34007-146) and 30 ml of cold 5% trichloroacetic acid (TCA). The cells were homogenized for 90 sec in a Braun MSK Mechanical Cell Disrupter, while being cooled with a gas-liquid mixture of CO_2. An 18-ml sample of the homogenate was sedimented at 10,000 rpm for 10 min at 4°C. The pellet was resuspended in 10 ml of 5% TCA and stored at 4°C for 12 hrs. This procedure was repeated three additional times for 8, 2, and 2 hr periods, followed by two consecutive 1 hr washings with a 3;1 (v;v) 75% ethanol-ether solution. At the end of the final incubation period the extract was sedimented. The pellet was resuspended in 2 ml of 10% TCA and boiled for 30 min. The heated solution was sedimented again and the supernatant was analyzed for DNA using the cysteine-HCl method of Stumpf (1947). The solution was sedimented at 15,000 rpm for 10 min. Color was allowed to develop for 1 hr, and the optical density of the reaction mixture was determined at 490 nm using a Zeiss PMQ II Spectrophotometer.

Glucose and pH determination. The glucose concentration in the medium was determined by the 3, 5 dinitrosalicylate reagent method (Clark, 1964). Optical density was read at 540 nm on the Spectronic 20 colorimeter. PH was measured by the immersion of a pH electrode (Corning #4136-M-10) into the medium and read on a pH meter (Corning M-10).

Cyclic AMP extraction and assay. The procedure followed was a modification of the methods utilized by Gilman (1970; 1974), Johnson (1972), Schultz, et al. (1974), and Weinryb (1972). A 200 mg sample of fresh mycelial mats was placed in a 75 ml glass disrupter flask with 50 gms of 0.45-0.50 mm glass beads and 30 ml of cold 5% TCA. The sample was homogenized for 60 sec in the Braun MSK Disrupter while cooled with a gas liquid CO_2 mixture. A 20 ml sample of the resulting slurry was sedimented at 2,000 rpm for 10 min at 4°C and the pellet discarded. Two ml of 1N HCl was added to 18 ml of the remaining supernatant. The acidified extract was then washed 6 separate times with 50 ml of fresh ethyl ether to remove the TCA. The resulting aqueous phase was heated at 85°C for 6 min. The partically purified extract was allowed to cool before further treatment.

A 2 ml sample of the extract was placed on a 30 x 0.62 cm Dowex column (AG-50W-X8, 100-200 mesh) H^+ form (Bio. Rad, Richmond, California) and eluted with 0.1N HCl. A flow rate of 35 ml/hr was held constant by maintaining a 25 ml eluate heat above the resin bed regulated by a Cole-Parmer peristalic pump (#WZ1R031, Chicago, Il.). The eluate was collected in 5 ml fractions utilizing an automatic fraction collector (ISCO #270). Fractions 9 through 21 were lyophilized to dryness in a Kontes glass lyophilization apparatus with four 500 ml flasks. The residue was reconstituted and combined in 9 ml of 0.05 M Tris/4 mM EDTA, pH 7.5

buffer.

This purified sample was again fractionated on a second Dowex column and the lyophilization and reconstitution steps repeated. All extracts at intermediate purification steps were quick-frozen in liquid N_2 and stored at -20°C. A 0.21 unit/mg protein phosphodiesterase (Sigma) solution was added to some of the samples.

The cyclic AMP assay was performed using the Amersham/Searle kit (#TRK-432) which utilizes a competitive binding protein, charcoal absorption principle. The reaction mixture consisted of a 50 µl ^3H cyclic AMP solution, 50 µl of the sample, and 100 µl binding protein which were combined while in a 0°C ice/water bath. The solution was vortexed and incubated at 4°C for 2 hrs. A 100 µl charcoal suspension was added to remove the unbound nucleotide. The charcoal was sedimented at 1200 rpm using the GLC-1 Sorvall centrifuge with an HL 4 rotor placed in a 4°C cold room. A 200 µl sample of the reaction mixture was added to 10 ml of PCS cocktail (A/S-#196096) in glass Spectravials (A/S-#00332-6) and the activity was determined on a Beckman Scintillation Counter (#LS-3133T, Irvine, California).

Induction. Mycelia mats were transferred at 3 different ages (156, 204, and 252 hours) to an appropriate induction medium. At each induction age, 4 different induction treatments were performed as follows: One-fourth of the total number of mycelial mats at each age were placed on a cyclic AMP defined agar medium; ¼ were placed on a fresh

defined agar medium, and the remaining ½ of the samples were left undisturbed. All transferred mats were incubated at 24°C in the dark for 15 hrs. At the end of this period, mats preinduced on cyclic AMP were transferred to a cyclic AMP/ tryptophan induction medium. These were labeled as A/T samples. Mats preinduced on the defined agar medium were transferred to an induction medium containing tryptophan only, and labeled as D/T. Mats which were previously undisturbed were transferred either to a tryptophan induction medium (T) or a fresh non-inducing defined agar medium (D). Theses cultures were incubated at 24°C in the dark for 6 hrs.

O-pyrocatechuic acid carboxylyase (OPCA carboxylyase) induction - extraction and assay. All mycelial mats were induced for 6 hrs on a tryptophan defined medium. A 3 gm sample of the fresh induced mycelia was placed in a 75 ml glass disruption flask containing 50 gm of 0.45-0.50 mm glass beads and 15 ml of a phosphate buffer at pH 7.0. The cells were disrupted for 60 sec in the braun MSK disrupter while being cooled with a gas-liquid mixture of CO_2. The slurry was sedimented at 8,000 rpm for 10 min at 4°C in a Sorvall RC2-B refrigerated centrifuge. An 0.8 ml sample of the supernatant liquid was added to a reaction tube containing a mixture which consisted of 1.0 ml citrate-phosphate buffer (pH 5.2), 0.2 ml 0.01 M OPCA (k&K Labs), and 0.2 ml double-distilled H_2O. An inactivated blank was prepared by substituting 0.2 ml of a 5% $HgCl_2$ solution for the H_2O. The blanks and the reaction mixtures were incubated at 30°C for

18 min. One ml of 1 N HCl was added to each tube to stop the reaction. The OPCA was extracted with 10 ml of ethyl acetate and separated at 3100 rpm for 10 min on a clinical centrifuge (International-Cl, Needham Heights, Mass.). The disappearance of OPCA was measured on the Zeiss PMQ II Spectrophotometer at 320 nm (Subba Rao, et al., 1970).

Protein determination. Total protein was determined using a modification of the biuret method of Gornall (1949). One ml of the remaining homogenate used for the OPCA carboxylyase assay was added to 4 ml of the biuret reagent. The samples were sedimented at 15,000 rpm for 10 min at 20°C in a Sorval RC2-B centrifuge. Color was allowed to develop for 30 min, and total protein was measured at 540 nm on the Zeiss PMQ II spectrophotometer. Bovine serum albumin was used as a standard.

Specific activity. A unit of activity was defined (Spiegelman and Coursen, 1975) as that amount of enzyme which catalyzed the disappearance of 1 nM of OPCA per minute. Specfic activity was expressed as units of enzyme activity per mg of mycelial protein.

RESULTS

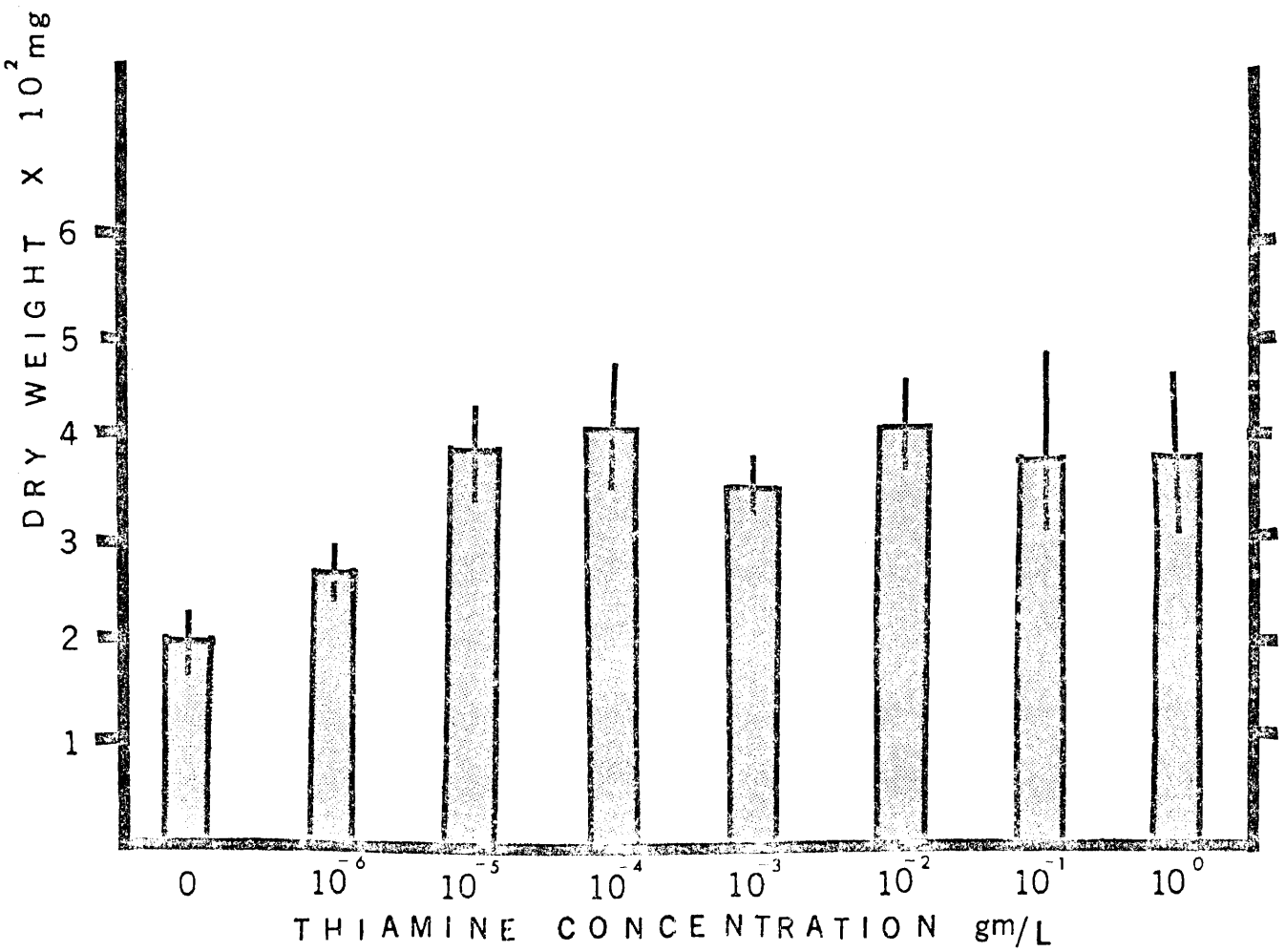
Selection of growth conditions. Several growth conditions were tested for their ability to shorten the lag phase and increase the slope of the exponential phase in the formation of mycelial mats. It was believed that these changes could lead to a reduction in the time required for such mats to reach stationary phase of growth (dry weight and DNA) and thereby reduce the time lag noise in the physiological age of the mycelial mat. In these experiments, aging mats are defined as those which are not growing as measured by the DNA concentration. Hence, the faster such mats stop growing, the greater the duration of the aging period.

Conidia of A. ornatus were preincubated in a defined liquid growth medium or a phosphate buffer for 10 hrs to stimulate germination. This procedure was found to be unsuitable in conjunction with the spore spray inoculation technique. The conidia seemed to become entangled with their germ tubes and clumped together. The larger clumps blocked the oriface of the sprayer. The further development of the smaller clumps on the media led to an uneven development of the mycelial mat.

Various thiamine concentrations were tested for their ability to promote a high rate of growth (Fig. 1). After 96

Figure 1

Total dry weight of mycelial mats of A. ornatus after 96 hrs of growth on a defined agar medium containing serial concentrations of thiamine. The range includes from 10^{-6} to 1 gm/L thiamine, plus a control defined medium without thiamine. Each bar represents the mean dry weight of 10 separate mycelial mats. The vertical lines indicate the variation between replicate mats.

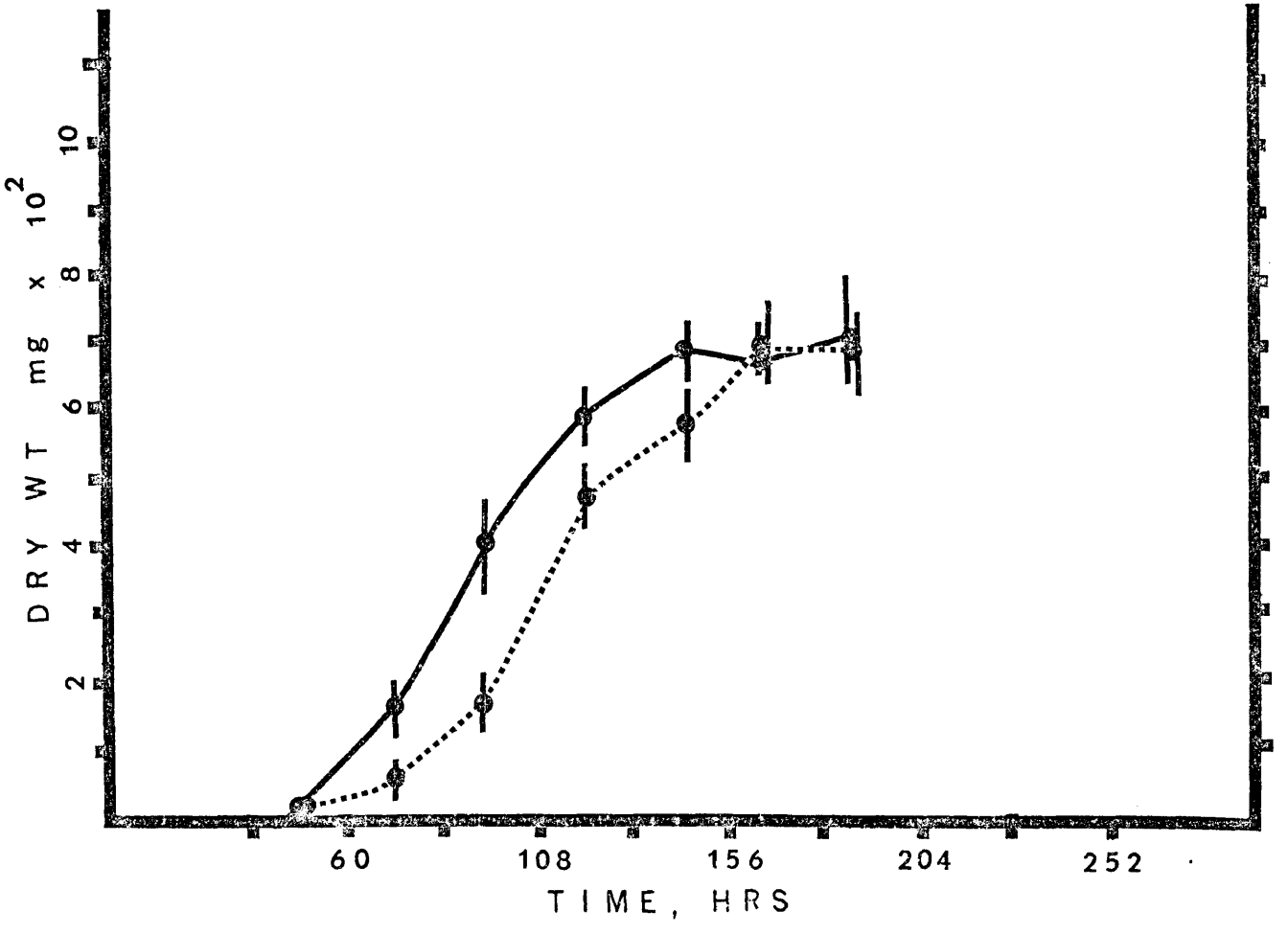

DRY WEIGHT X 10^2 mg
1
2
3
4
5
6
0
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
10^{0}
THIAMINE CONCENTRATION gm/L

hrs incubation in the dark on membrane covered agar plates containing the defined medium, mats had an average dry weight of 200 ± 15 mg. The addition of thiamine to the defined medium resulted in a substantial increase in dry weight. A concentration of 10^{-6} gm/L produced mats with an average dry weight of 275 ± 25 mg, while a concentration of 10^{-5} gm/L produced mats with an average dry weight of 400 ± 50 mg. This was an increase of 37.5% and 100% respectively over mats grown in the absence of thiamine. Further increase in thiamine concentration had no additional effect in dry weight. The strain used in these experiments showed dense sporulation, but concentrations above 10^{-3} gm/L caused an uplifting of the mycelium from the cellulose membrane covering the agar.

The growth of the fungus incubated in the dark on membrane covered defined agar medium containing 10^{-5} gm/L thiamine was measured through 185 hrs (Fig. 2). This concentration appeared to promote early increases in dry weight. At 72 hrs, mats grown on a medium containing thiamine had a dry weight of 155 ± 15 mg, while those grown without thiamine had a dry weight of only 60 ± 10 mg. This represents an increase of 158% in dry weight over mats grown without thiamine. The overall result was an apparent shift of the entire dry weight curve to the left. The average dry weight maintained at stationary phase (700 ± 70 mg) was reached at 144 hrs in mats grown on a medium containing thiamine, but

Figure 2

Total dry weight vs age of the mycelium of A. ornatus grown on a defined medium with (●—●) or without (●···●) the presence of 10^{-5} gm/L thiamine. Each point represents the mean dry weight of at least 6 separate mycelial mats. The vertical lines indicate the variation between replicate mats.


DRY WT mg x 10^2
2
4
6
8
10
60
108
156
204
252
TIME, HRS

was not acheived by mats grown without thiamine until 168 hrs. However, it is difficult to tell whether the onset of the aging phase was reached approximately 24 hrs sooner by the mats grown on a medium containing 10^{-5} gm/L since the DNA concentration was not measured.

The dry weight and DNA concentrations of cultures grown on growth chambers on defined liquid medium was measured throught 252 hrs under light or dark incubation, and compared to cultures grown on membrane covered defined agar medium (Figs. 3 and 4). Cultures grown on liquid medium exhibited an extended lag phase in comparison to those grown on agar medium. Mats grown on liquid medium were still in lag phase at 228 hrs when incubated in the light but this was reduced to 108 hrs when incubated in the dark, while mats grown on a defined agar medium had a lag phase duration of 84 hrs when incubated in the light, but only 60 hrs when incubated in the dark. The degree of conidiation evident in mycelial mats grown under various conditions can be seen in plates 1 through 3. Dense conidiation was observed on cultures grown on liquid medium when incubated in the light or dark (Plate 1) and on cultures grown on agar medium when incubated in the light (Plate 2). The green-gold tinge of the conidia was usually evident during early lag phase. A mat grown on defined agar medium and incubated in the dark is shown in Plate 3. Conidiation was sparse and was not evident until 108 hrs, at which time the mat was in exponential phase.

Figure 3

Total dry weight (●——●;●····●) and total DNA (●– –●;●· ·●) vs age of the mycelium of A. ornatus grown in growth chambers in the light (●——●;●– –●) or dark (●·····●;●· ·●). Each point represents the mean of at least 6 separate mycelial mats and the vertical lines indicate the variation between replicate mats.

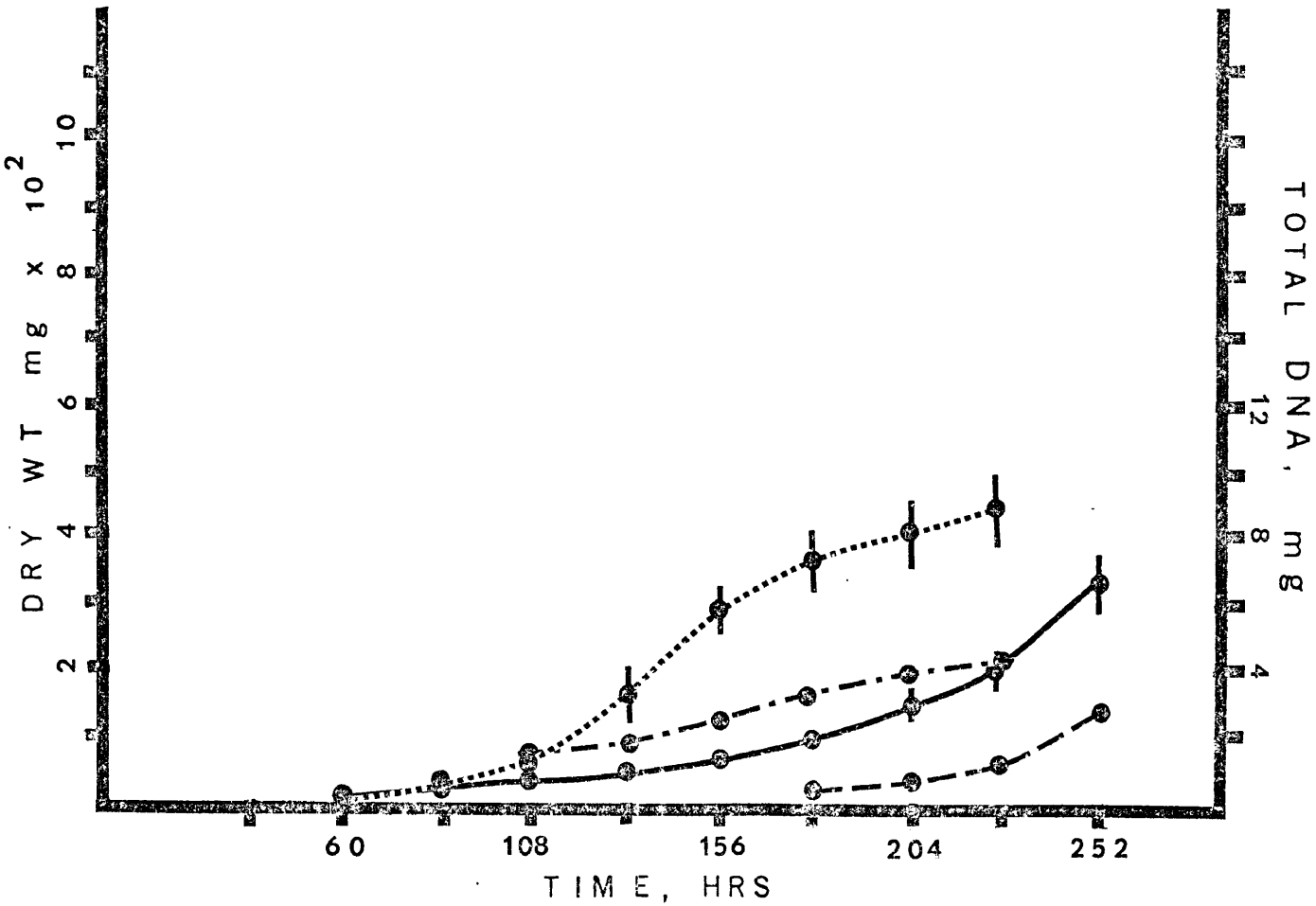
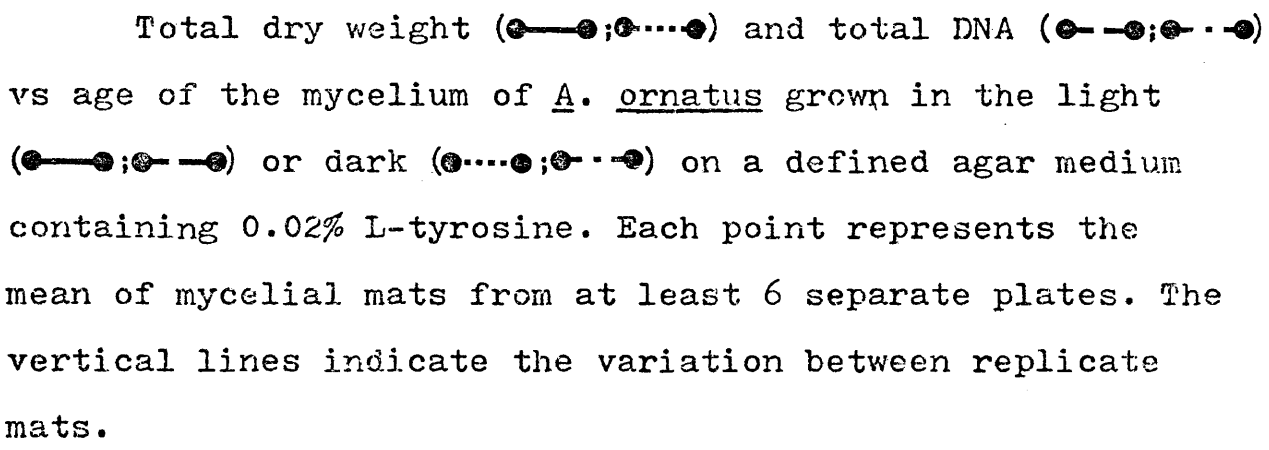

DRY WT mg x 10^2
2
4
6
8
10
TOTAL DNA, mg
4
8
12
60
108
156
204
252
TIME, HRS

Figure 4


Total dry weight and total DNA vs age of the mycelium of A. ornatus grown in the light or dark on a defined agar medium containing 0.02% L-tyrosine. Each point represents the mean of mycelial mats from at least 6 separate plates. The vertical lines indicate the variation between replicate mats.

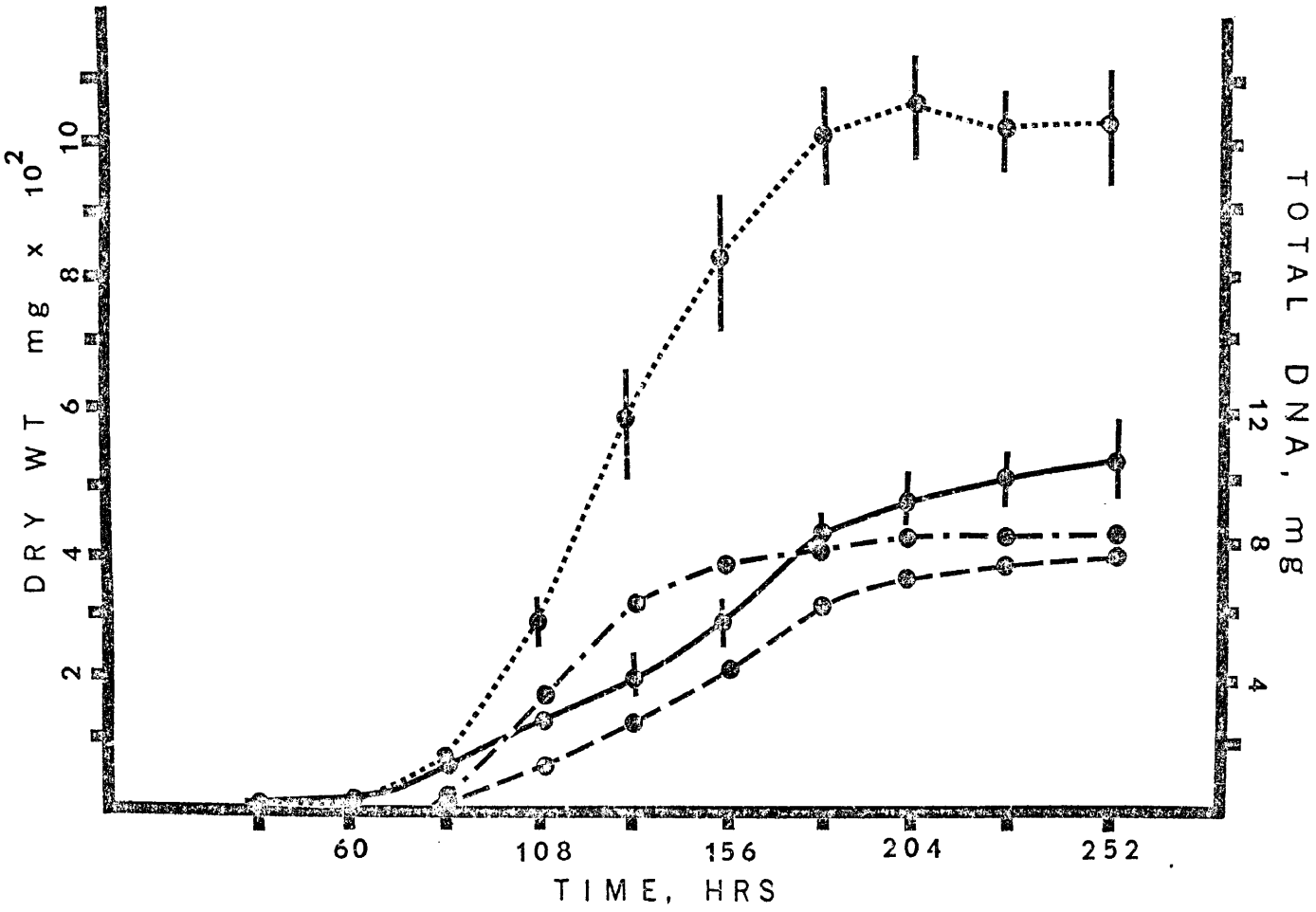

DRY WT mg x 10^2
2
4
6
8
10
TOTAL DNA, mg
4
8
12
60
108
156
204
252
TIME, HRS

Plate 1

A densely conidiating mat of A. ornatus grown on a cellulose xanthate membrane in a 100mm diameter glass growth chamber and floated on 2L of a defined liquid medium containing 0.02% L-tyrosine.

Plate 2

A densely conidiating mat of A. ornatus grown on a cellulose-xanthate membrane placed on 50 ml of a defined agar medium containing 0.02% L-tyrosine contained in a 90 mm diameter glass Petri plate and incubated in the light.

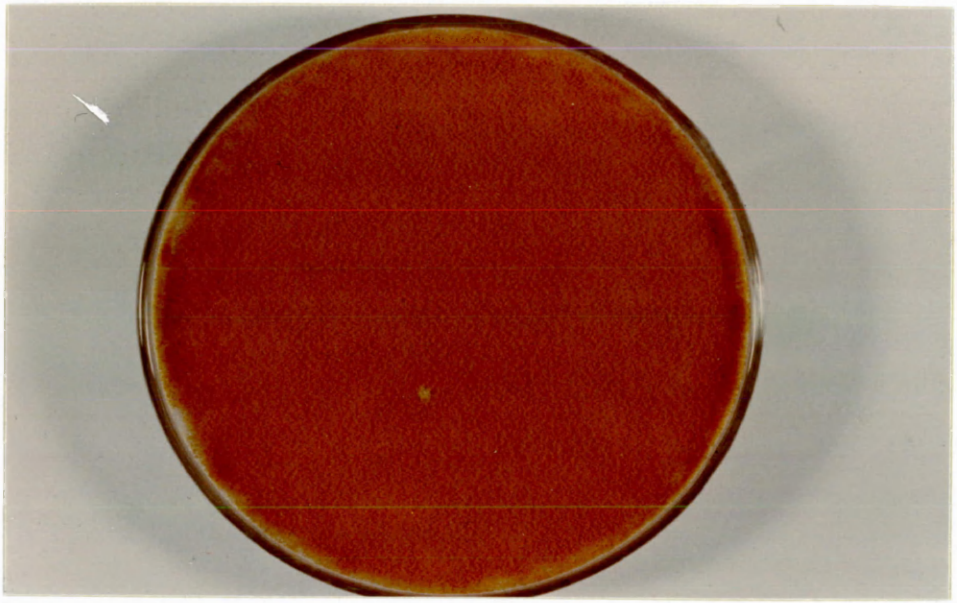
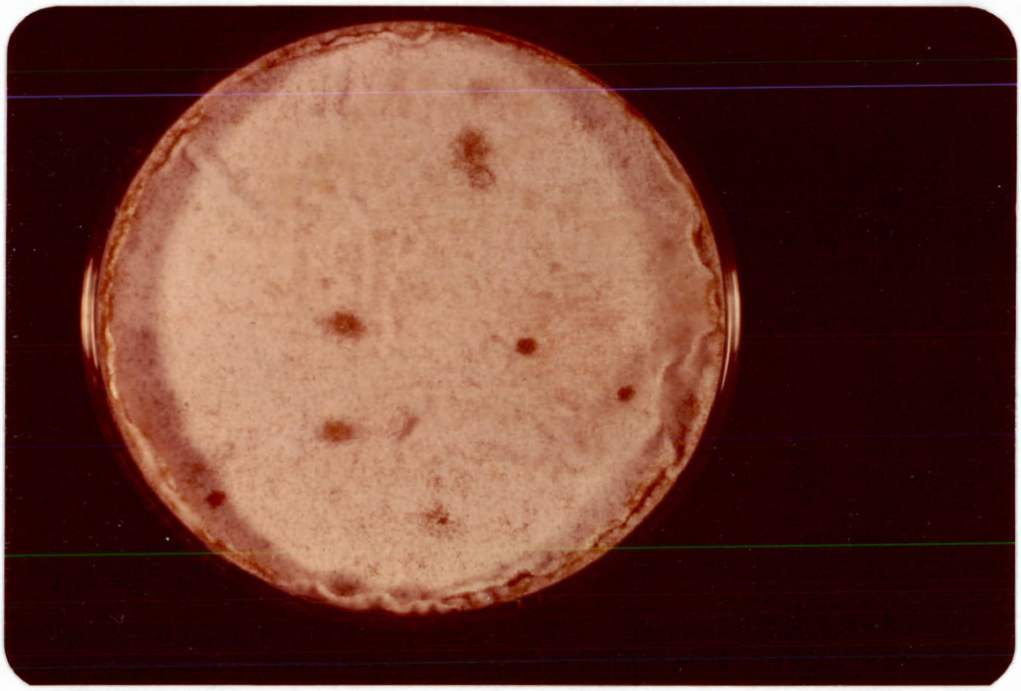


Plate 3

A sparsely conidiating mat of A. ornatus grown on a cellulose-xanthate membrane placed on 50 ml of a defined agar medium containing 0.02% L-tyrosine contained in a 90 mm diameter glass Petri plate and incubated in the dark.



Growth of A. ornatus under the selected conditions. Under the growth conditions examined, it appeared that the system best suited for studying the aging process in A. ornatus was obtained by growing mycelial mats in the dark on cellulose-xanthate membranes overlying a defined agar medium without the addition of thiamine. This combination of growth conditions provided short lag and exponential phases and achieved a stationary phase in dry weight and DNA with only sparse conidiation. Thiamine was not included in the growth medium due to increased wrinkling of mats at later stages which could interfere with the uptake phenomena. Consequently, all subsequent data were obtained from aging cultures prepared in this manner.

The majority of conidia sprayed on the cellulose-xanthate membrane covering a defined agar medium germinated within 10 hrs after the spray inoculation. A thin interwoven network of hyphae was evident by 24 hrs and at 36 hrs, a complete mycelial mat was formed. Between 60 and 156 hrs only very light conidiation occurred, and uniformily covered approximately less than 10% of the mat. This gave the mat a general off-white appearance, as formed by the vegetative thallus (Plate 3). By 132 hrs, the mat had thickened sufficiently to allow it to be peeled off the membrane as a complete unit. Membranes supporting fungal mats for 252 hrs did not show a loss of integrity or weakness, but some signs of etching were evident. Some uplifting or wrinkling of the mycelium off the membrane was observed at 252 hrs. After 276

hrs, the membrane showed signs of weakening and the mycelium began to adhere to the membrane. Wrinkling increased with additional conidiation on the undersurface of the wrinkles, and small, dark pigmented areas appeared on the underside of the mat.

The length of dark incubation at 24°C required for the onset of aging in A. ornatus was determined by measuring the dry weight and total DNA at 24 hr intervals, beginning at 36 hrs (Fig. 4).

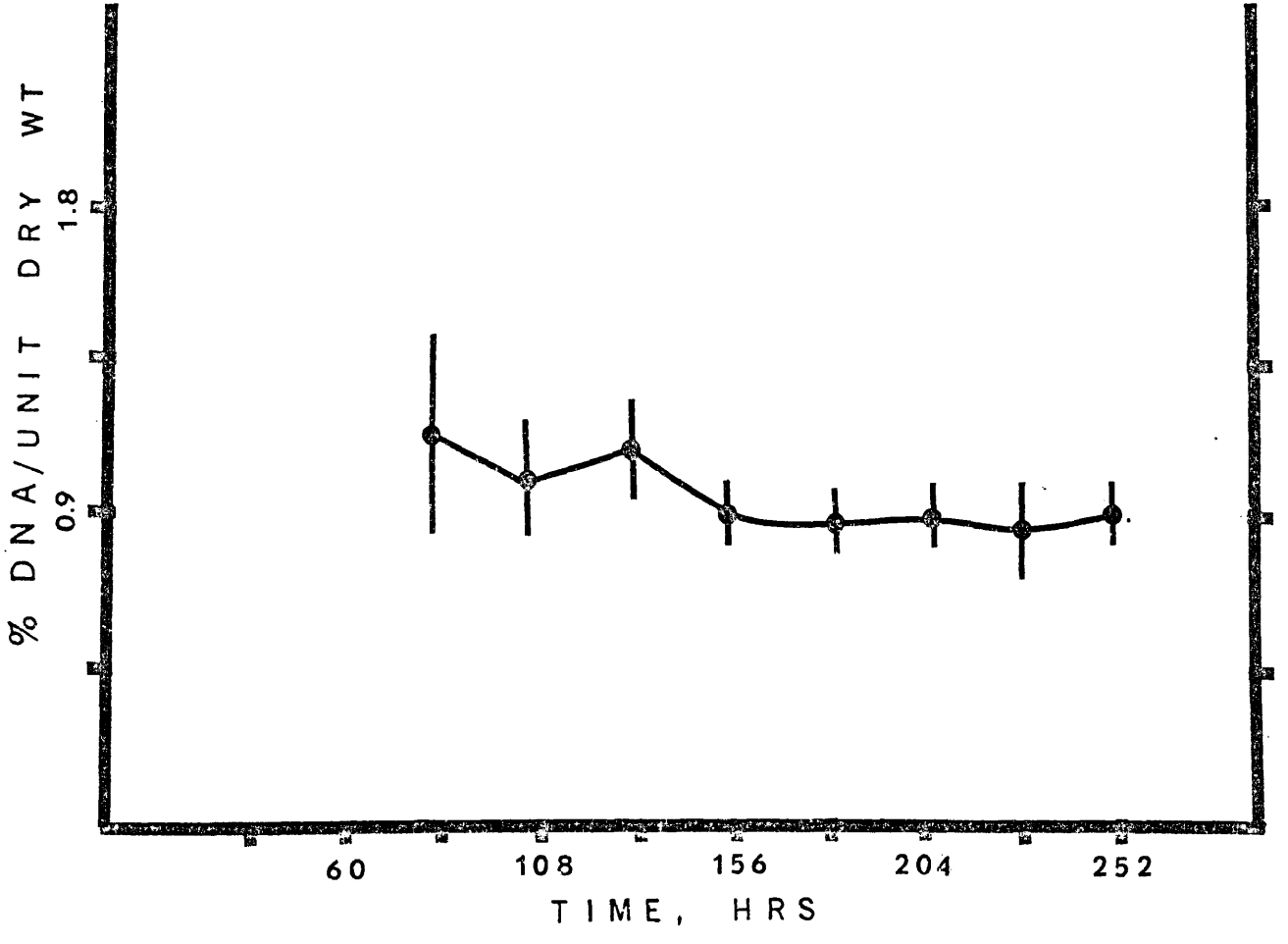
The dry weight showed a lag phase from 0 to 84 hrs at which time the average dry weight was 75 ± 25 mg. This was followed by an exponential phase leading to an average dry weight of 950 ± 100 mg at 180 hrs. No further increases in average dry weight were evident throughout the remainder of the experiment.

The total DNA obtained from the above mats showed a similar curve. Because early growth provided only a limited supply of fungal material, the DNA could not be calculated until 84 hrs. However, an exponential phase was evident from 84 to approximately 132 hrs as the total DNA increased from an average of 0.7 ± 0.1 mg to 6.0 ± 0.4 mg. The DNA gradually entered the stationary phase between 132 and 180 hrs and maintained a plateau of approximately 8.0 mg DNA through 252 hrs.

A graphic plot of the % DNA/unit dry weight perhaps better illustrates the attainment of a stationary growth phase (Fig. 5). Initially, large fluctuations in % DNA were

Figure 5

Percent DNA/unit dry weight vs age of the mycelium of A. ornatus grown on a defined agar medium in the dark with 0.02% L-tyrosine. Each point represents the mean of at least 6 separate mycelial mats. The vertical lines indicate the variation between replicate mats.


% DNA/UNIT DRY WT
1.8
0.9
60
108
156
204
252
TIME, HRS

evident. At 84 hrs, the % DNA averaged 1.2 ± 0.4% which gradually decreased to an average of 0.9 ± 0.1% at 156 hrs. The large fluctuation was expected at early stages because the total dry weights at these points were low and any small deviation in DNA levels in a given sample would result in a large % change. After 156 hrs when dry weight had substantially increased, a definite DNA plateau was reached and maintained throughout the remainder of the experiment.

The glucose and pH levels of the defined medium during the growth of A. ornatus were also determined and are shown in Fig. 6. Both showed a slow but steady decline throughout the duration of the experimnet. Glucose concentration gradually decreased for the first 108 hrs from an initial concentration of 40 gm/L to an average of 36 ± 1 gm/L after which a slightly greater depletion of glucose from the medium was evident. Approximately 20% (8 gm/L) of the initial glucose concentration remained in the medium after 252 hrs of uptake by A. ornatus. The pH declined a total of 1.9 units, reaching a pH of 2.9 at 252 hrs.

Cyclic AMP - discovery, verification, and purification. Cyclic AMP was initially detected in 4 separate samples obtained from 156 hr mycelial mats (Table 1). The average concentrations in the crude extracts was 11.0 ± 4.0 pm/mg of fresh mycelial weight. Extracts which were incubated for 15 min in the presence of phosphodiesterase had an average cyclic AMP concentration of 1.8 ± 0.6 pm/mg of fresh weight.

The crude extract was purified by collecting 5 ml

Figure 6

Glucose concentration (●——●) and pH (●····●) of the medium vs age of the mycelium of A. ornatus grown in the dark. Each point represents the means of at least 6 separate mycelial mats. The vertical lines indicate the variation between replicate mats.

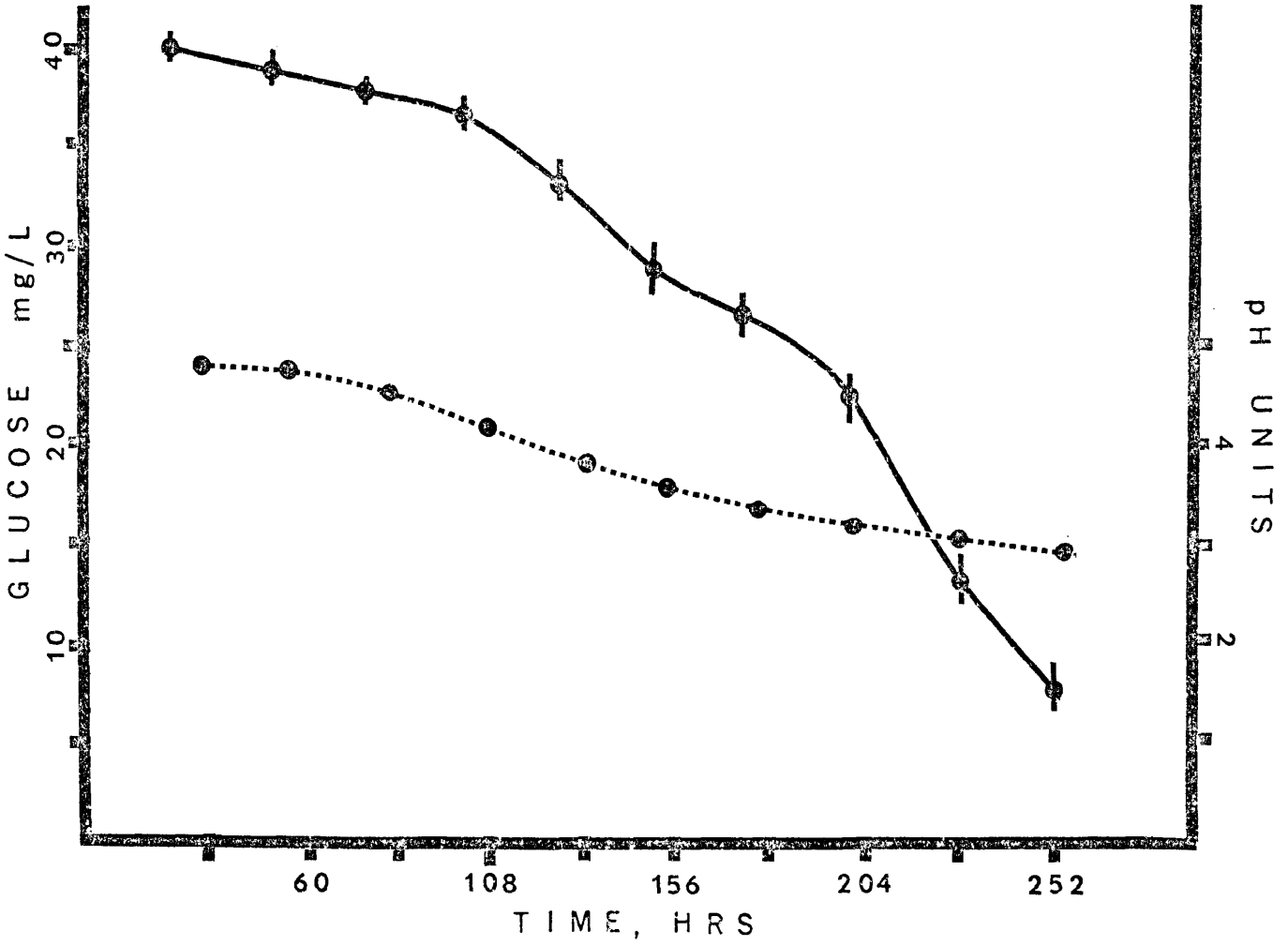

GLUCOSE mg/L
10
20
30
40
pH UNITS
2
4
60
108
156
204
252
TIME, HRS

Table 1

Endogenous concentration of cyclic AMP with or without phosphodiesterase incubation in crude extracts obtained from mycelial mats of A. ornatus.

Sample	pm cyclic AMP/mg fresh wt
extract	
1	7.5 ± 0.3%
2	15.0 ± 0.3%
3	10.2 ± 0.3%
4	10.8 ± 0.3%
average	11.0 ± 0.3%
extract w/phosphodiesterase	
5	2.4 ± 0.3%
6	1.2 ± 0.3%
average	1.8 ± 0.3%

Crude extracts (#1-6) were adjusted to a pH of 7.5 with KOH powder before assay. Phosphodiesterase (0.5 units) was incubated at 30°C for 15 min in samples 5-6 before assay.

fractions obtained by passage through a Dowex-50 column. To determine when the active fractions were eluded from the column, a standard cyclic AMP solution was passed through a Dowex-50 column and 5 ml fractions were analysed for activity by measuring the absorbance at 264 nm (Fig. 7). For all experimental analysis of the extracts, fractions 9 through 21 were collected and combined since these contain the majority of the active material.

The endogenous concentration of cyclic AMP present in 156 hr crude extracts with and without phosphodiesterase and the endogenous concentration after purification by passages through a Dowex-50 column are shown in Fig. 8. The crude extract levels have already been discussed. The combined fractions of the partially purified extract after the first passage through the column, showed an average concentration of 84 $\pm$ 10 pm/mg fresh mycelial weight. This was an increase of 663% of concentration over the levels found in the crude extracts. The second passage through the column showed an increase of endogenous levels to an average of 95 $\pm$ 9 pm/mg of fresh mycelial weight. Since this represents only a 13% increase in concentration over the level determined for the single passage through the column, it was subsequently eliminated from the purification processes in all remaining studies of cyclic AMP analysis.

Age-related changes in endogenous cyclic AMP levels. To determine if there were any changes in endogenous levels

Figure 7

Distribution of cyclic AMP in consecutive 5 ml fractions eluted from a Dowex-50 column previously charged with a solution of commercially prepared cyclic AMP. Open bars indicate fractions normally collected and combined when columns were charged with extracts obtained from A. ornatus.

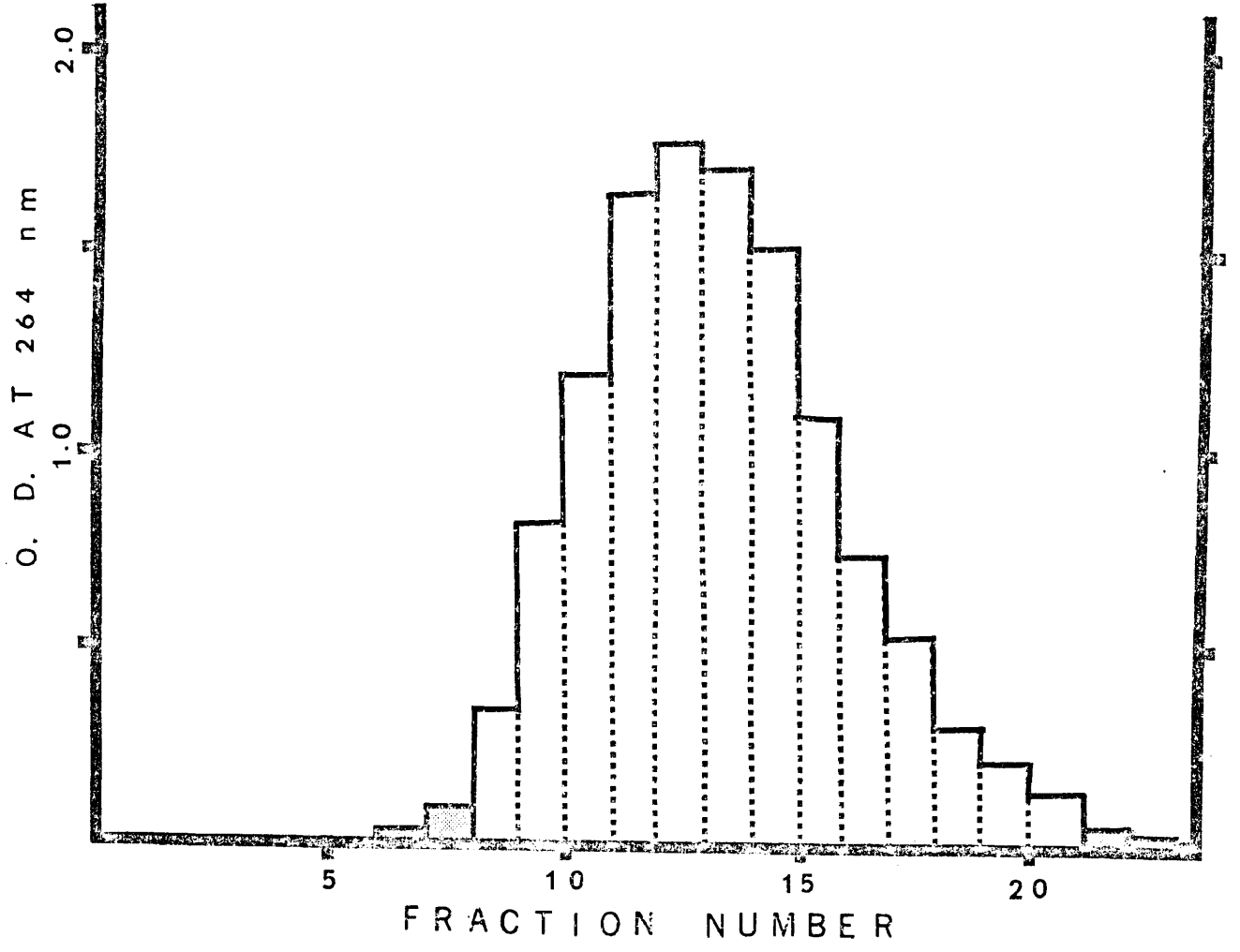
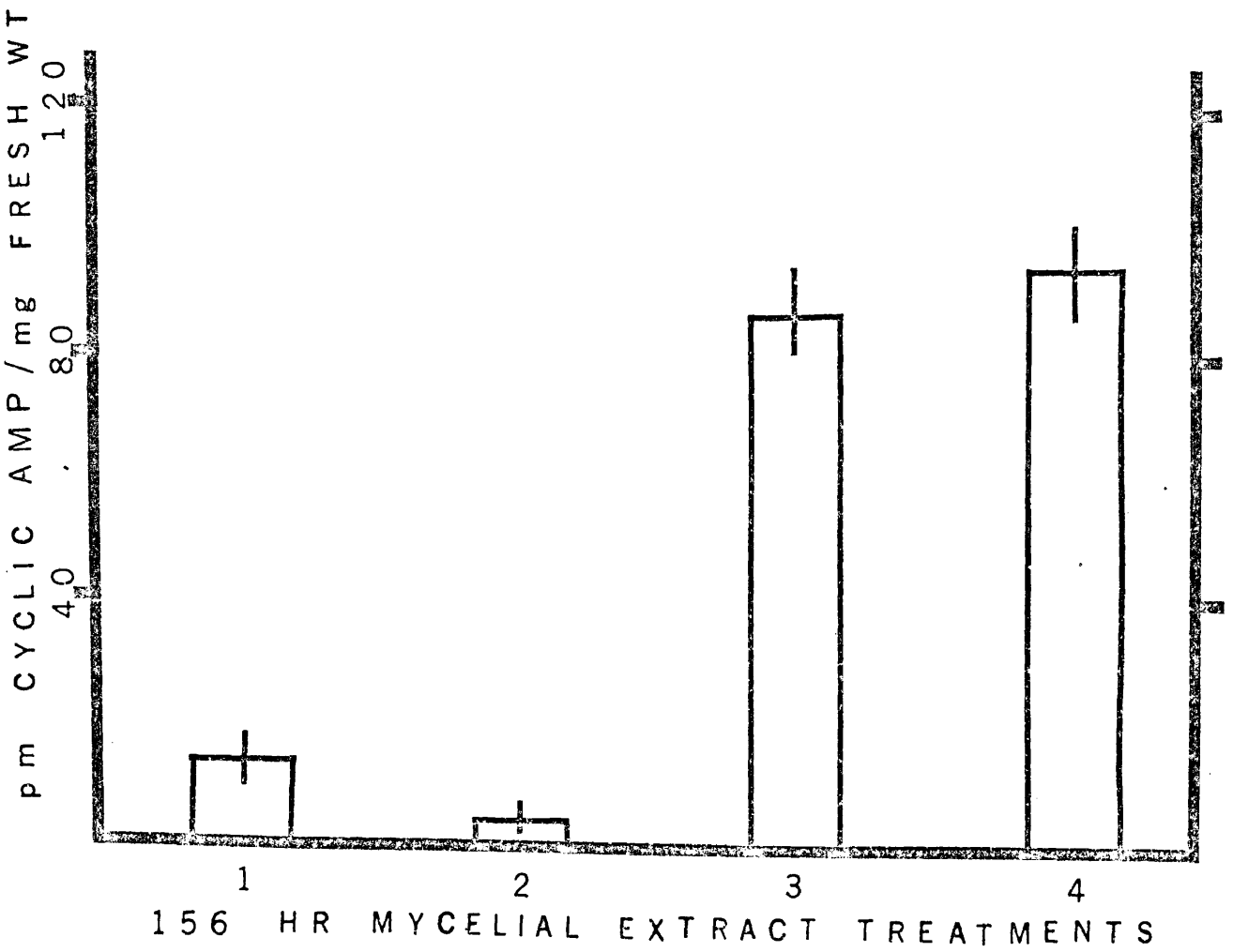

O. D. AT 264 nm
2.0
1.0
5
10
15
20
FRACTION NUMBER

Figure 8

Endogenous concentration of cyclic AMP present in 156 hr mycelial mats of A. ornatus. Each bar represents a different treatment of the crude extract. Data points for the crude extract with (2) or without (1) phosphodiesterase, extract eluded from a single passage through a Dowex-50 column (3), and extract eluded from two consecutive passages through the Dowex column (4), represent the means obtained from 4, 2, 6, and 6 separate mycelial mats respectively. The vertical lines indicate the variation between replicate mats.


pm CYCLIC AMP / mg FRESH WT
40
80
120
1
2
3
4
156 HR MYCELIAL EXTRACT TREATMENTS

of cyclic AMP in aging cultures of A. ornatus, analyses were made on mycelial mats of 156, 204, and 252 hrs which was the stationary phase of growth as determined by DNA concentration (Fig. 9). Endogenous concentrations of cyclic AMP progressively decreased from an average of 84 ± 10 pm/mg of fresh mycelial weight at 156 hrs to 43 ± 6 pm/mg of fresh weight at 204 hrs to 32 ± 5 pm/mg fresh mycelial weight at 252 hrs. A posteriori tests indicate a significant difference between the means ($\mu_3 < \mu_2 < \mu_1$) at the 99% confidence level, where μ_1= 156 hr, μ_2= 204 hr, and μ_3= 252 hr samples.

Age-related changes in OPCA carboxylyase induction. A concentration of 0.1% L-tryptophan was added to the defined induction medium in order to induce OPCA carboxylyase activity. Mats were induced for 6 hrs at 3 separate ages. The results are shown in Fig. 10. An average specific activity of 10.0 ± 1.5 units was obtained with 156 hr mats. The ability to be induced declined with time reaching an average of 3.2 ± 0.4 units at 204 hrs, and an average of 2.2 ± 0.6 units at 252 hrs. A basal activity of OPCA carboxylyase was present at all 3 ages and also showed a corresponding decline, dropping from an average specific activity of 2.25 ± 0.25 units/ mg mycelial protein at 156 hrs, to 1.8 ± 0.4 units at 204 hrs and 1.4 ± 0.4 units at 252 hrs.

A posteriori tests of induced levels show a significant difference between the means ($\mu_3 = \mu_2 < \mu_1$) at the 99% confidence level, and a significant difference between the

Figure 9

Concentration of endogenous cyclic AMP present in aging cultures of A. ornatus. Each bar represents the mean cyclic AMP concentration obtained from 6 seaparate mats at each age (156, 204, and 252 hrs). The vertical lines indicate the variation between replicate mats.

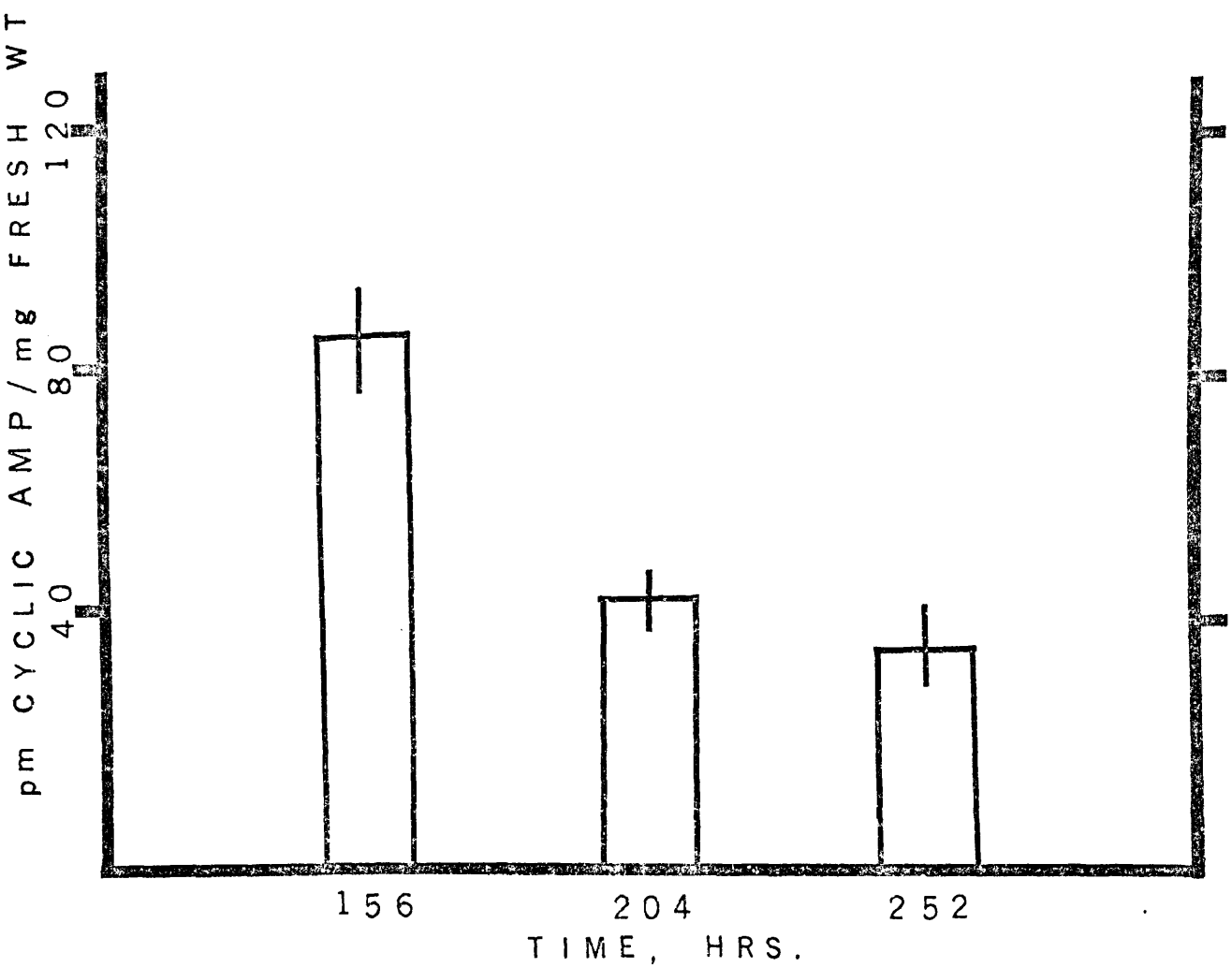
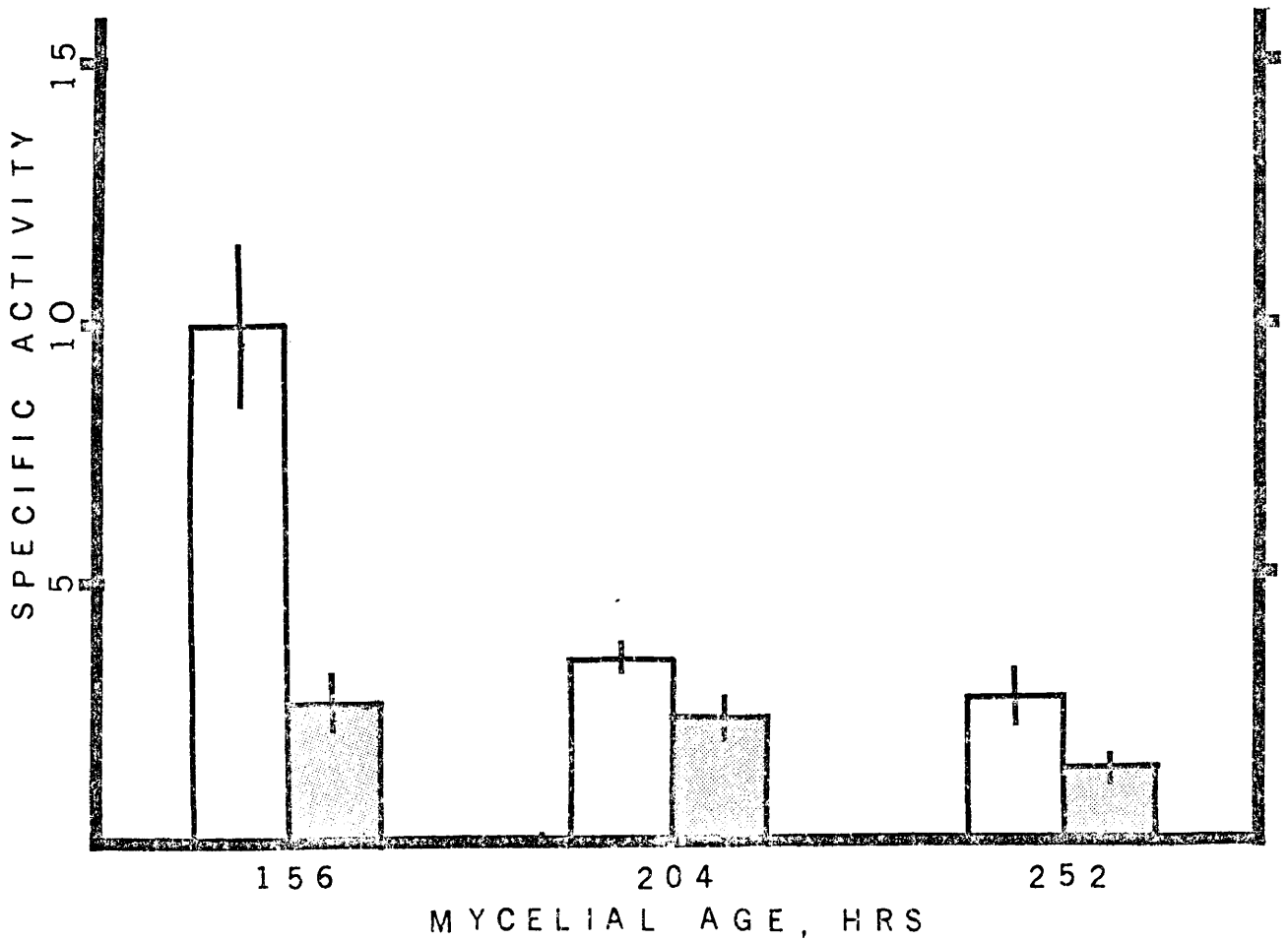


Figure 10

Specific activity of OPCA carboxylyase present in tryptophan induced and un-induced mycelial mats of aging cultures of A. ornatus. The shaded bars represent the OPCA carboxylyase activity of mycelial mats which were transferred to a defined medium plus 0.1% tryptophan for 6 hrs. The open bars represent the enzyme activity of mats transferred to a defined medium lacking tryptophan for a similar length of time. Each bar represents the mean OPCA carboxylyase activity obtained from at least 6 separate mats. The vertical lines indicate the variation between replicate mats.


SPECIFIC ACTIVITY
5
10
15
156
204
252
MYCELIAL AGE, HRS

means ($\mu_3 < \mu_2 < \mu_1$) at the 85% confidence level, where μ_1 = 156 hr, μ_2 = 204 hr, and μ_3 = 252 hr samples. A posteriori tests of the basal activity show a significant difference between the means ($\mu_3 < \mu_2 < \mu_1$) at the 99% confidence level, where μ_1 = 156 hr, μ_2 = 204 hr, and μ_3 = 252 hr samples.

Cyclic AMP - OPCA carboxylyase interaction. Since it had been demonstrated in other organisms that cyclic AMP levels may influence enzyme induction (Pastan and Adhya, 1976), it was felt that a change in endogenous cyclic AMP levels in A. ornatus may be responsible for the loss in the induction of OPCA carboxylyase evident in Fig. 10. Accordingly, aging mycelial mats were preinduced with cyclic AMP prior to induction with tryptophan and subsequently assayed for OPCA carboxylyase activity (Fig. 11). No apparent difference exists in OPCA carboxylyase activity between mycelial mats of the same age which had been induced on the cyclic AMP - tryptophan (A/T) medium and those which were induced on defined medium containing the tryptophan without cyclic AMP (D/T; T). This was true for mycelial mats at each age - 156, 204, and 252 hrs old, where OPCA carboxylyase activities showed a decline from an average of 10 $\pm$ 1.5 units to 3.2 $\pm$ 0.4 units and 2.2 $\pm$ 0.6 units respectively. Results of a T-Test analysis are shown in Table 2 and indicate that a significant difference exists between induced and non-induced samples at all ages.

Since exogenously added cyclic AMP apparently did not effect OPCA carboxylyase activity, it was felt necessary to

Figure 11

Specific activity of OPCA carboxylyase present in tryptophan induced and un-induced mycelial mats of aging cultures of A. ornatus preinduced in the presence or absence of cyclic AMP. Open bars (☐) represent the specific activity of mycelial mats which were incubated 15 hrs on a defined medium containing 10^{-3} M cyclic AMP and then transferred to a defined medium containing 10^{-3} M cyclic AMP and 0.1% tryptophan for 6 hrs. Shaded bars (■) represent the specific activity of mycelial mats which were incubated 15 hrs on a defined medium lacking cyclic AMP and then transferred to a defined medium containing 0.1% tryptophan for 6 hrs. Striped bars (▨) represent the specific activity of mats which were not preinduced, but were transferred to a defined medium containing 0.1% tryptophan for 6 hrs. Cross-hatched bars (▩) represent the specific activity of mycelial mats which were not preinduced, but were transferred to a defined medium lacking tryptophan for 6 hrs. Each bar represents the mean specific activity obtained from at least 6 separate mats. The vertical lines indicate the variation between replicate mats.

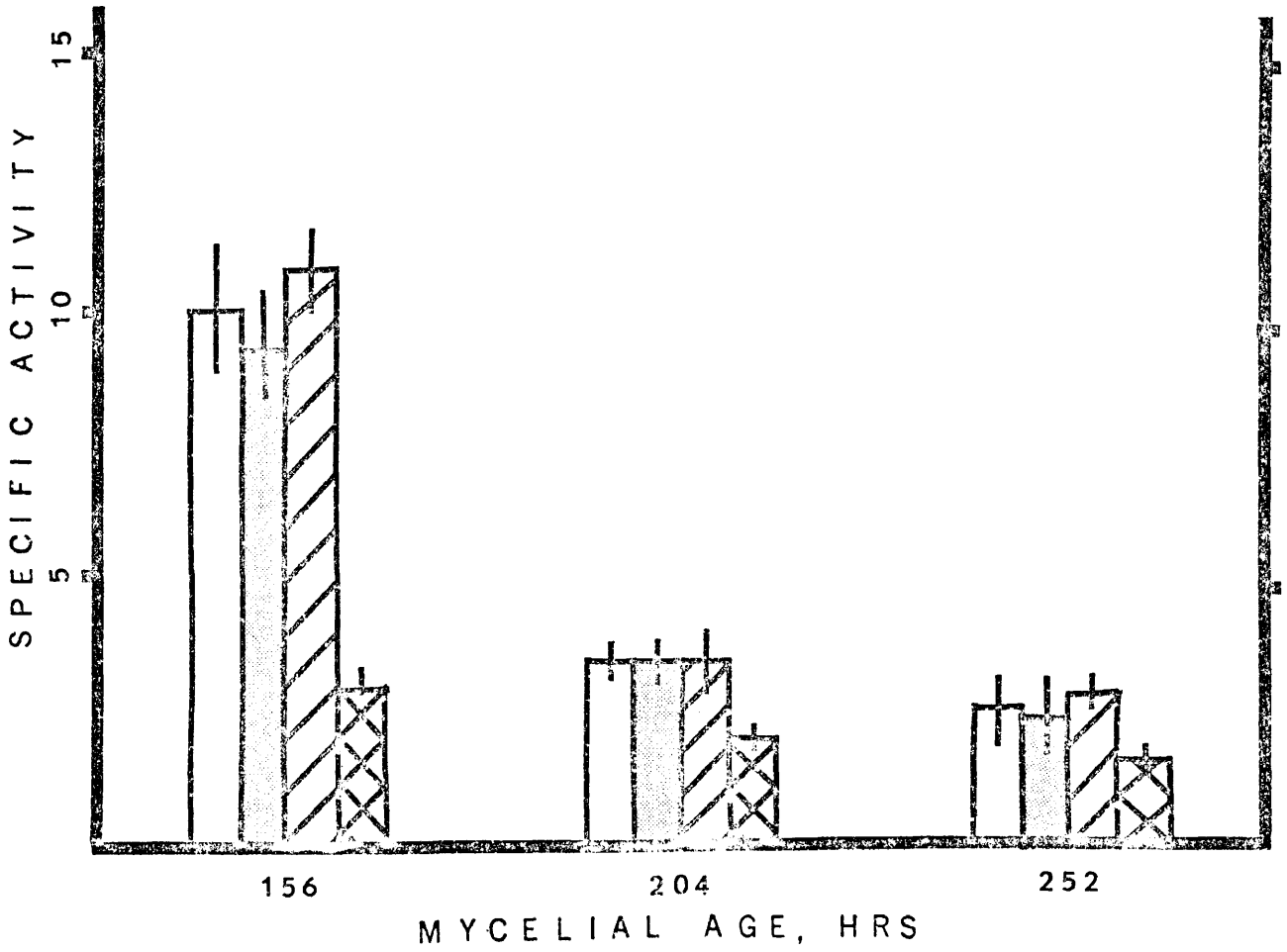

SPECIFIC ACTIVITY
15
10
5
156
204
252
MYCELIAL AGE, HRS

Table 2

T-Test analysis of induced vs un-induced (basal) specific activity of OPCA carboxylyase in crude extracts at three separate ages.

age of extract	% confidence level
156 hrs	99.9%
204 hrs	99.9%
252 hrs	98.0%

The % confidence level at each age represents the significant difference of the mean OPCA carboxylyase activities between induced and basal levels.

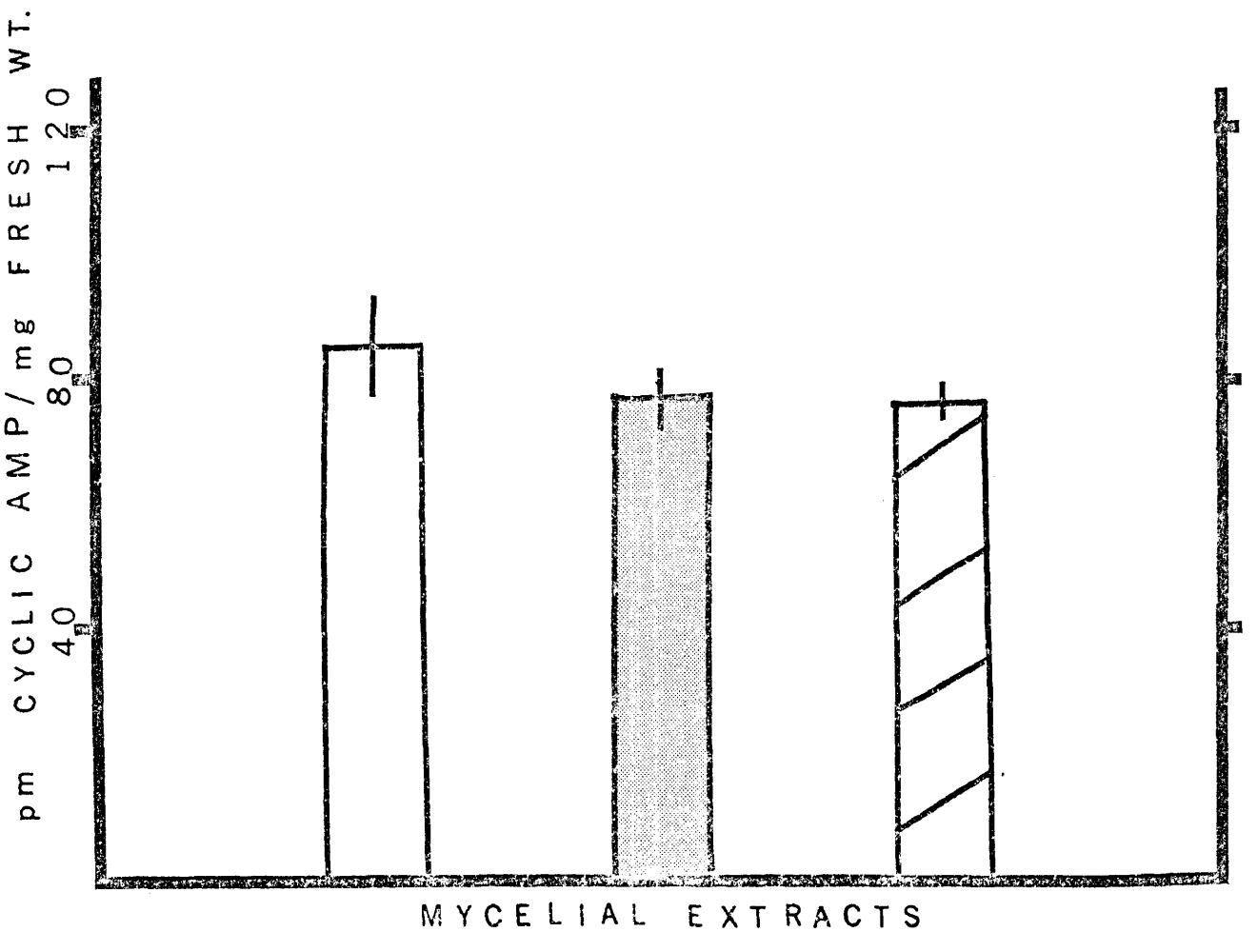
See Fig. 10 for the OPCA carboxylyase specific activity ranges with respect to induced and basal levels at all three ages.

determine whether the added cyclic AMP actually raised the endogenous cyclic AMP concentrations, and whether tryptophan induction had any effect on the endogenous cyclic AMP level. Mycelial mats which were 156 hrs old, were preinduced with 10^{-3}M cyclic AMP for 21 hrs and assayed for endogenous cyclic AMP as indicated earlier. The results are presented in Fig. 12. The 156 hr purified extract had an average activity of 84 $\pm$ 10 pm/mg fresh mycelial weight, whereas an average cyclic AMP activity of 77 $\pm$ 5 pm/mg fresh mycelial weight was obtained for mats incubated on 10^{-3} M cyclic AMP for 21 hrs.

Similar tests were run on 156 hr mycelial mats which were induced for 6 hrs by 0.1% L-tryptophan but not exposed to exogenous cyclic AMP (Fig. 12). An average cyclic AMP activity of 75 $\pm$ 2 pm/mg fresh mycelial weight was determined. A T-Test analysis between control 156 hr purified extract and extracts incubated 21 hrs on 10^{-3} M cyclic AMP, or extracts induced 6 hrs on 0.1% L-tryptophan indicate significant differences in activities at a confidence limit of 80% and 90% respectively.

Figure 12

Concentration of endogenous cyclic AMP in 156 hr mycelial mats of A. ornatus incubated in the presence or absence of exogenously added cyclic AMP and in the presence or absence of L-tryptophan. Open bar (☐) represents the concentration of endogenous cyclic AMP present in mycelial mats grown on a defined agar medium for 156 hrs. The shaded bar (▩) represents the concentration of endogenous cyclic AMP present in mycelial mats which were grown on a defined medium for 135 hrs and transferred to a defined medium containing 10^{-3} M cyclic AMP for 21 hrs. The striped bar (▨) represents the concentration of endogenous cyclic AMP present in mycelial mats which were grown on a defined medium for 150 hrs and transferred to a defined medium containing 0.1% tryptophan for 6 hrs. Each bar represents the mean concentration of cyclic AMP obtained from at least 6 separate mates. The vertical lines indicate the variation between replicate mats.


pm CYCLIC AMP/mg FRESH WT.
40
80
120
MYCELIAL EXTRACTS

DISCUSSION

Although asexual sporulation was completely inhibited in A. ornatus when grown in the dark on complex liquid or agar medium, this effect was never observed when the organism was grown under the same conditions on a defined liquid or agar medium containing tyrosine as reported by Schwemmin (1960). In his study, he reported that the addition of tyrosine to the defined medium would have the same effect on conidiation as was observed on complex medium when the cultures were incubated in the dark. Complete inhibition of conidiation would be desirable as it would reduce possible developmental interference in age-related studies. Dense conidiation occurred in cultures grown on liquid medium whether incubated in the light or dark and in cultures grown on agar medium incubated in the light. Cultures grown in the dark on agar medium showed very sparse conidiation, but it was never totally absent (Plates 2 and 3). The conidiation of mats grown in the light was expected (Schwemmin, 1960). Additionally, mats grown in the light exhibited less vegetative growth. This could possibly be caused by an accumulation of a substance which inhibits phosphorylation of glucose (Hill, 1976). Dense conidiation by mats grown in the dark on liquid defined medium containing tyrosine was not

expected. It is possible that the use of a liquid growth medium may have altered the metabolic or biochemical pathways in operation. Scott, et al. (1973) has reported lower cyclic AMP levels in liquid grown Neurospora crassa cultures and it has been demonstrated that cyclic AMP plays an important role in the conidiation of fungi (Uno and Ishikawa, 1973a; 1973b).

The biochemistry of conidia may differ from that of the mycelium (Cochrane, 1958). Studies by Galbraith and Smith (1969) in A. niger have shown that changes in the enzyme activities of the tricarboxylic acid and glyoxalate cycle have been attributed to conidiation, but these change only occurred during conidiogenesis. However, unpublished data from our laboratory (Cleary, 1975) failed to demonstrate any differences in OPCA carboxylyase activity between conidiating and non-conidiating cultures. Conidiating strains have been used previously in aging studies (Nagasaki, 1968; Spiegelman and Coursen, 1975). Therefore all biochemical studies that were run on A. ornatus were done on cultures grown on defined agar medium plus tyrosine in the dark which yielded only sparsely conidiating mats.

Before an association of certain biochemical events with the aging phenomenon can be made, some criteria of aging in the employed growth system must be found. Gottlieb and Van Etten (1966) examined several biochemical possibilities in an attempt to establish conditions which mark the onset of aging. Total DNA is a direct representation of

events associated with karyokinesis. With attainment of a constant total DNA level, it may be assumed that nuclear division has ceased. the DNA curve (Fig. 4) and the % DNA/ unit dry weight (Fig. 5) indicate that nuclear division has ceased by 156 hrs in cultures of A. ornatus grown in the dark on a defined agar medium. Since all biochemical events depend on what is recorded on the DNA template, the onset of aging as defined in these studies may begin once the amount of DNA is constant and nuclear division has ceased.

Because 156 hrs has passed before the stationary aging phase is reached, the age of the cells could vary as much as 156 hrs. This could be assumed if each cell maintained a physiologically independent existence after cytokinesis. Yanagita and Kogane (1963) have shown that the spore spray technique leads to a clonal mode of aging. Because of hyphal anastomoses and rapid transport of materials between the joining hyphal strands which occur in ascomycetous filamentous fungi, the fungal mat is composed of masses of cells which appear to be the same physiological age (Burnett, 1968). As the age of the entire mycelial mat increases, the average age of the cells composing the mycelium increases.

To our knowledge, this represents the first report of the presence of endogenous cyclic AMP in A. ornatus. The occurrence of endogenous cyclic AMP in this organism was demonstrated on 30 different occasions throughout these investigations. The low level of cyclic AMP upon incubation of the crude extract with phosphodiesterase, which breaks the

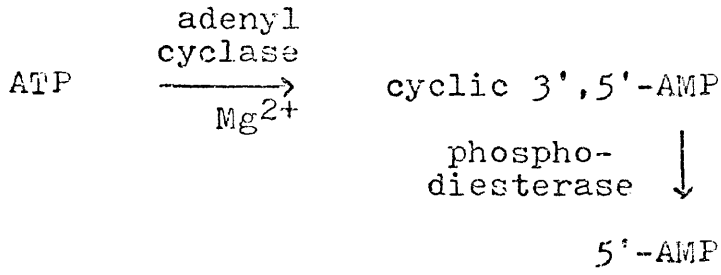
3' sugar phosphate bond, confirms the presence of the cyclic nucleotide.

Possible binding "enhancement factors" or excessive salt concentrations in the crude fungal extract which could possibly interfere with the binding assay (Weinryb, 1972) were eliminated with the passage of the sample through a Dowex-50 ion exchange resin. This passage eliminated inhibiting factors as there was a ten-fold increase in cyclic AMP concentration over the values obtained for the concentration in the crude extract. Since only a 13% increase in cyclic AMP concentration was gained in a second passage of the eluate through the Dowex-50 column, the second passage was eliminated in further investigations. Of other nucleotides that may be present in the extract, only non-cyclic GMP is reported to be eluted with the Dowex-50 cyclic AMP fractions (Schultz, et al., 1974). According to Steiner, et al., (1972), only ATP and cyclic GMP are likely to interfere with the competitive protein binding technique employed. This commercial kit has been used successfully in the determination of cyclic AMP levels in other fungal cells (Tsuboi and Yanagishima, 1975; Uno and Ishikawa, 1974). In those experiments a less exacting purification technique was employed than the one used here.

The results shown in Fig. 9 indicate an apparent age-related effect on cyclic AMP in A. ornatus. Increasing levels of cyclic AMP were reported in E. coli during stationary phase, but this was attributed to a depletion of glucose

from the medium (Wayne, et al., 1975). In scenescent human fibroblasts, no change of cyclic AMP concentration was noted (Haslam, et al., 1974), yet various plant tissues showed a decrease in cyclic AMP levels with age (Kessler, et al., 1974).

The cellular cyclic AMP concentration apparently represents a balance between two enzymes. Adenyl cyclase catalizes the formation of cyclic AMP from ATP and phosphodiesterase hydrolyses it to 5'-AMP (Robison, et al., 1971).



(after Robison, et al., 1971)

Adenyl cyclase and phosphodiesterase have been found in fungi (Flawia and Torres, 1972; Polya, et al., 1975). Recently, adenyl cyclase has been shown to have a 20-fold age-related decrease of activity in erythrocyte membranes of normal male CD rats (Pfeffer and Swislocki, 1976). This would lead to a lower production of cyclic AMP. The suggestion is that biochemical changes which somehow regulate endogenous cyclic AMP levels would cause an indirect control of many cellular processes, perhaps including aging which would represent a feedback regulation. Some indication of this was presented by Abou-Sabbe, et al.,(1975) who demonstrated in E. coli, that various limiting biochemical sources

react with the membrane-bound adenyl cyclase system, stimulating or inhibiting synthesis of cyclic AMP, and thus indirectly control the rates of a metabolic process, in this case inducible enzyme synthesis. In S. fragilus (Sy and Richter, 1972), adenyl cyclase was strongly influenced by growth conditions such as carbon source and rate of aeration. A similar case has been presented in mammalian systems particularly with respect to regulation of adenyl cyclase which in turn regulates with phosphodiesterase, cyclic AMP concentration (Turner and Bagnara, 1971). Also, hormones have been implicated as instrumental in aging mammalian systems since Adelman has noted age-related alterations in rat hepatic enzymes which are hormone mediated (Adelman, et al., 1972; Adelman and Freeman, 1972).

The ability to induce OPCA carboxylyase with tryotophan in a conidiating strain of A. ornatus has been previously established (Spiegelman and Coursen, 1975). In their studies, the localization of the enzyme was not known. It may have been in the hyphae, in the conidia, or associated with the processes leading to the differentiation and maturation of the conidia.

The strain used in the present experiments showed only very sparse conidiation, yet OPCA carboxylyase activity was comparable to that reported in their studies. Hence, the levels of activity observed suggest that the activity is primarily in the vegetative hyphae and that the conidiation processes are not a necessary influence on the induction of

OPCA carboxylyase. A 5-fold increase of induced absolute specific activity is observed in the sparsely conidiating strain over the dense conidiating strain at early stationary phase when maximum inducibility is evident. However, the same increase is found in comparing the basal levels. Therefore, the relative increases of the induced enzyme activities over the basal level are the same for the two strains. Unlike the dense conidiating strain which showed a gradual decrease in specific activity with time, the sparsely conidiating strain showed an initial sharp decrease followed by an asymptotical leveling off in the induction process.

Cyclic AMP's role in A. ornatus is unknown at present. Results shown in Fig 9 and 10 indicate that as the level of endogenous cyclic AMP declines, so does OPCA carboxylyase activity. It has been noted that OPCA carboxylyase is an inducible enzyme and that cyclic AMP is a mediator of inducible enzyme synthesis in prokaryotes (Pastan and Adhya, 1976). A similar relationship might exist in fungi. Evidence of a possible cyclic AMP-inducible enzyme relationship has been reported in fungi (Van Wijk and Konijn, 1971; Wiseman and Lim, 1974; Uno and Ishikawa, 1974). With this in mind, the addition of the enzyme inducer, tryptophan, to the medium could cause a change in endogenous cyclic AMP levels. Results shown in Fig. 12 fail to indicate any substantial change in endogenous cyclic AMP concentration upon the addition of tryptophan. Although it has been reported that

the addition of certain limiting nutrient sources may provoke a change in cyclic AMP concentrations (Abou-Sabbe, et al., 1975), there is no information available which would indicate that tryptophan reacts with this system.

Alternatively, if cyclic AMP and enzyme inducibility are related phenomena in A. ornatus, the addition of cyclic AMP to the induction medium could be expected to stimulate a rise in OPCA carboxylyase activity, if the peak activity is not already present. Results indicated in Fig. 11 do not show a change in OPCA carboxylyase activity with the addition of cyclic AMP at any age. However, it may be that the exogenous cyclic AMP did not penetrate into the cell. Some indication of this is presented in Fig. 12, where the cyclic AMP addition to the medium did not cause a rise in endogenous cyclic AMP concentration at 156 hrs.

Reports on the ability of naturally occurring exogenous cyclic AMP to penetrate fungal membranes have been conflicting. Tsuboi and Yanagishima (1975) indicated an 8-fold increase of the intracellular levels in S. cerevisiae when a 10^{-3} M concentration of exogenous cyclic AMP was added for a 15 hr incubation period. Uno and Ishikawa (1973b) demonstrated that cyclic AMP could be incorporated in FIS^{c} and FIS^{+} strains but not FIS^{-} strains in C. macrorhizus. Scott and Solomon (1975) noted that growing cultures of N. crassa were impermeable to cyclic AMP. Interestingly, Tsuboi, et al. (1972) showed that 10^{-5}M and 10^{-4}M cyclic AMP reversed glucose repression in S. cerevisiae, but 10^{-3}M had no effect. It is

not known why there is this discrepency, but in general, phosphorylated compounds have difficulty in penetrating cell membranes (Robison, et al., 1974) and rapid destruction by intracellular (Robison, et al., 1974) or extracellular (Wold and Suzuki, 1974; Polya, et al., 1975) phosphodiesterases is possible.

Frequently, derivatives such as dibutryl cyclic AMP (dbcAMP) have been used because of their greater membrane penetration ability or greater resistance to the action of phosphodiesterase, and can produce similar effects as those of the natural nucleotide. In M. racemosus, dbcAMP was found to raise endogenous levels of cyclic AMP (Paznokas and Sypherd, 1975) and promote yeast bud formation (Larsen and Sypherd, 1974). Tsuboi et al. (1972) demonstrated in S. cerevisiae that dbcAMP was even more effective in reversing glucose repression on sporulation than natural cyclic AMP. However, in A. niger, dbcAMP failed to imitate the action of cyclic AMP in promoting adhesiveness of conidia (Wold and Suzuki, 1973b).

Alternatively, methyxanthin derivitives such as theophylline and caffeine have been used successfully in obtaining cyclic AMP-mediated responses in fungi (Uno and Ishikawa, 1973b; Wold and Suzuki, 1973a). These derivatives presumably act in raising endogenous cyclic AMP levels by inhibiting cyclic AMP-dependent phosphodiesterase. However, in S. cerevisiae, caffeine appears to act independently of cyclic AMP concentrations (Tsuboi and Yanagishima, 1973).

Caffeine promoted sporulation in an acetate medium in S. cerevisiae, whereas theophylline and cyclic AMP did not, and caffeine failed to raise intracellular cyclic AMP levels (Tsuboi and Yanagishima, 1975). Additionally, theophylline failed to produce the same responses of cyclic AMP in A. niger (Wold and Suzuki, 1973b). Obviously, there are several drawbacks in the use of dbcAMP, caffeine and theophylline, as their precise mechanisms of action are not known. It was for this reason that the naturally occurring nucleotide was used in this experiment.

It is possible that the depletion of the glucose from the incubation medium (Fig. 6) may have been responsible for the onset of the stationary phase in cultures grown on agar defined medium in the dark during which these aging phenomenon were measured. However, at the onset of the stationary phase in these cultures, 65 ± 2% (26.5 ± 2 gm/L) of the initial glucose concentration remained in the medium and 20% (8 ± 2 gm/L) remained at the conclusion of the experiment.

Since the glucose was present in the induction medium, the possibility exists that a "glucose effect" may be in operation on endogenous cyclic AMP concentration and enzyme induction. In comparing Fig. 6, 9, and 10, it is noted that as the concentration of glucose decreases within the medium so does cyclic AMP concentration and OPCA carboxylyase induction. It is possible that the observed reductions in activity are simply due to a depletion of the carbon source (glucose) from the medium.

According to Pastan and Adhya (1976), glucose is inhibitory on cellular cyclic AMP concentrations. The higher the concentration of glucose in the medium, the lower the endogenous levels of cyclic AMP. This is contrary to that observed in the present experiments, which indicated that the decrease of cyclic AMP in A. ornatus with time was not a function of glucose. This phenomenon was also noted by Sy and Richter (1972) in stationary yeast cultures and in A. niger (Zonneveld, 1976) even after total glucose depletion from the medium.

In the case of enzyme inducibility, Pastan and Adhya (1976) also noted in their review article that glucose was inhibitory on E. coli's ability to synthesize inducible enzymes. Again, this is contrary to the observations made in the present experiments. As the concentration of the glucose in the medium decreased so did the ability of A. ornatus to induce OPCA carboxylyase. Mats at all ages were always transferred to an induction medium containing 4% glucose. Therefore, if there is a glucose effect, it should be the same for each age.

The results shown in Fig. 6 also indicate a pH drop of the defined agar incubation medium from an initial level of 4.8 to 2.9 after 252 hrs. It is possible that the decrease in cyclic AMP concentration and the loss of enzyme induction activity may have been due to the drop in pH of the medium. However, cyclic AMP and OPCA carboxylyase were measured after 156, 204 and 252 hrs. At 156 hrs, the pH level was 3.5

units. Therefore, between the initial and final measurements of the nucleotide and enzyme levels, the pH dropped 0.6 units. More significantly, between 156 hrs and 204 hrs the decrease in the pH was only 0.2 units, and between these points the cyclic AMP concentration decreased 49% and the OPCA carboxylyase activity decreased 68%.

In addition, the 156, 204, and 252 hr mats were transferred to fresh defined agar medium before the measurement of the OPCA carboxylyase activity. There was no difference in activity whether the transfer to a fresh medium was for 6 or 21 hrs. Also similar levels of endogenous cyclic AMP were observed in the 156 hr purified extracts taken directly from the old incubation medium and purified extracts which came from mats which had been transferred to a fresh defined medium for 21 hrs.

If decreases in the ability to produce cyclic AMP and to induce the synthesis of OPCA carboxylyase are age-related phenomenon, as suggested in these studies, then an understanding of how age may lead to the death of an organism is suggested. In E. coli, Pastan and Perlman (1972) proposed a model whereby cyclic AMP stimulates gene transcription by its effect on the operon system. Cyclic AMP combines with a receptor protein (CRP) which causes an allosteric change in the CRP, enabling it to bind to DNA. The complex binds close to the appropriate promoter which produces a change in DNA so that RNA polymerase can bind. Transcription begins in the presence of nucleotide triphosphates.

Additional regulation controls have been proposed. Because of differential inducibility of β-galactosidase and tryptophanase on various carbon sources in E. coli, Botsford (1975) suggested the importance of the transport of catabolites in determining the mediation of enzyme induction. The catabolites could somehow alter the CRP in addition to cyclic AMP in a unique way to fit each particular operon promoter region. Cyclic GMP has been implicated in transcription inhibition by interfering with the cyclic AMP-CRP interaction in E. coli (Piovant and Lazdunski, 1975; Haggerty and Schief, 1975). However, in the yeast M. racemosus, cyclic GMP did not fluctuate in response to the cyclic AMP ability to influence morphology, nor did exogenous supplies seem to alter the cyclic AMP effects (Orlowski and Sypherd, 1976).

Cyclic AMP is an important metabolic regulator. Its role in enzyme induction has been established (Pastan and Adhya, 1976) and enzyme induction has been shown to be age-related. Cyclic AMP's presence, as well as a need to be rapidly produced is of utmost importance if an organism is to make physiological adjustments. Deficiencies in an organism's ability to regulate the cyclic AMP levels by interference in any of the aforementioned mechanisms could be detrimental. Although these studies were unable to establish any direct involvement of cyclic AMP in the loss of enzyme induction with age, the loss of the ability to induce

enzymes, such as OPCA carboxylyase in A. ornatus, illustrates an organism's inability to make adjustments to the environment. It is possible that other important metabolic processes may also be impaired. Consequently, a point may be reached when an organism can no longer maintain itself and dies.

In conclusion, this study has examined several methods to produce a satisfactory aging system. To our knowledge, these experiments represent the first demonstration of cyclic AMP in A. ornatus. The data presented here have suggested that the decreases in cyclic AMP concentration and OPCA carboxylyase activity are age-related phenomena and that a "glucose effect" does not appear to be present. It was also observed that the enzyme inducer, tryptophan, did not cause a rise in endogenous cyclic AMP concentration and that the addition of exogenous cyclic AMP to the induction medium did not effect OPCA carboxylyase activity. In conjunction with this, it was found that exogenous cyclic AMP did not cause a change in endogenous cyclic AMP concentrations.

The mechanisms which are responsible for the observed responses, and a possible relationship between OPCA carboxylyase and cyclic AMP are not clear. The isolation of a non-conidiating strain and the development of a growth system which would maintain the concentration of essential nutrients in the medium, and would reduce the time lag "noise" for the onset of aging would be helpful in reducing the possibility of non-age-related effects.

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