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Some Characteristics of Enzyme Induction in Aging Cultures of Aspergillus ornatus

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SOME CHARACTERISTICS OF ENZYME INDUCTION IN AGING CULTURES OF *ASPERGILLUS ORNATUS*

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ABSTRACT

The phenomenon of enzyme induction in aging cultures of Aspergillus ornatus was examined under various conditions of culture and induction. Under standard conditions of culture on phosphate buffered defined medium and 6 hours induction with 0.1% tryptophan, the specific activity of the inducible enzyme o-pyrocatechuic acid carboxylyase decreased 50% with age while the uninduced level of that enzyme remained constant. Increasing the induction time to 10 hours allowed the specific activity of the enzyme from older mats to reach the level of the younger mats, which were not affected by the increased time of exposure to tryptophan. This effect was also achieved by increasing the concentration of the inducing substance, tryptophan. The effectual concentration of tryptophan, that is, that body of inducer that actually enters the cell, is not different between the ages used but the incorporation of labeled tryptophan into de novo protein synthesis is depressed from 20-35% in older mats. Maintaining the pH of the culture medium as growth of the organism proceeded was achieved with citrate buffered defined medium. A constant pH in the growth medium delayed the aging effect, as measured by a reduction in enzyme inducibility, but an aging effect is nonetheless apparent independent of a possible "pH effect".

SOME CHARACTERISTICS OF ENZYME INDUCTION IN AGING CULTURES OF *ASPERGILLUS ORNATUS*

INTRODUCTION

Notwithstanding the genetic machinery, a living organism develops, ages and dies as a result of, (or in spite of) its immediate molecular environment. Therefore, regulation in influx, utilization and efflux of such molecules is fundamental to all biological processes. Availability of utilizable substrates, minerals, vitamins, trace elements, concentration of oxygen and presence of inhibitory materials or supra-optimal ions affect, on a primary level, all cellular functions.

Transport of biologically important molecules across the semi-permeable plasma membrane into the interior of the cell for assimilation is possibly the first site for regulation. Materials can be selectively permeable based on their size, lipid solubility and external to internal concentration ratios. In the absence of passive diffusion, transport of selected biological molecules may be facilitated or made possible by way of constitutive (Benko et al., 1967) or inducible (Cohen and Monod, 1957; Pall, 1969) permeases. Permeases are subject to regulation via feed-back mechanisms when accumulation of carried substrate reaches a critical level (Robertson et al., 1972), by the level of de novo protein synthesis in the cell (Gross and Ring, 1969) or by the presence or absence of the appropriate charged t-RNA molecules (Alim and Ring, 1976).

Ion fluxes across the membrane are also subject to regulation, the classic example of this being the sodium-potassium exchange pump (Hodgkin and Keynes, 1955). The sodium pump maintains a low internal concentration

of Na^+ in spite of high external concentration by exchanging internal Na^+ for external K^+. Not only are ion concentrations subject to regulation in this manner, but the internal/external concentration ratio finally established affects many other activities. A system illustrating this effect has been described in yeast cells (Armstrong, 1972). Cells actively utilizing a carbohydrate substrate transport K^+ from the medium so that the internal concentration doubles in as little as 15 minutes. In exchange for this cationic influx, H^+ ions are exported in a 1:1 ratio. The presence of internal K^+ allows protein synthesis and DNA replication to proceed normally. However, under unbuffered culture conditions, the continual H^+ efflux greatly acidifies the medium without affecting the internal pH and eventually depresses general cation influx including that of K^+. Thus, what is a relatively simple cation exchange may affect the workings of the entire cell.

Once internalized, these biological materials are metabolized and, just as their entry was governed by physical or chemical mechanisms, they, also, become regulatory agents for subsequent metabolic events. This is particularly true for the effecting molecules in enzyme induction.

Classic enzyme induction, as unraveled by Jacob and Monod (1961) and more lately by Bertrand et al. (1975), is the enhancement of the synthesis of an enzyme in response to an effector (inducer) or reasonable analog of that effector. Therefore, not only is enzyme induction completely dependent on inducer availability and concentration but also on all those factors which would affect protein synthesis as well, such as temperature, pH, presence of necessary co-factors and other regulatory features, e.g. feedback and repression controls.

Because of the cooperative interplay of enzyme induction with all

other aspects of cellular physiology, the phenomenon becomes a very useful tool for studying the changes that occur, on the cellular and subcellular levels, with aging of that cell. Not only is enzyme induction useful in that it encompasses so many other cellular features, but, because induction is a measure of cellular response to the environment (adaptation), we now have a tool for measuring changes with age in the adaptability of the cell to a given environmental stimulus.

Aging will be defined here as that process an organism undergoes in its life span after the onset of cellular maturity, or, in the case of simpler organisms, after nuclear proliferation and cellular differentiation have ceased. Many general theories have evolved to explain the inevitable decline in general vigor associated with the aging process leading to eventual death; several will be presented here in three major categories.

The first is that of programmed cell death as originally described by August Weissman in 1889, who stated that selection pressures would weed those individuals from a population who are no longer valuable contributors, i.e. those older, non-reproducing members. Later investigators believe the selection pressures are more indirect, that is, those deleterious genetic defects which lead to aging do not show up phenotypically until after reproductive maturity therefore there is no opportunity for natural selection to remove those features from the gene pool (Wilson, 1974). In support of the programmed death theory is the work of Leonard Hayflick (1970) who found that cultured human fibroblasts divide approximately 50 times and then die even though cultural conditions would permit indefinite divisions.

Agreeing there is a genetic basis for cellular aging but arguing

against programmed senescence is Leslie Orgel, creator of the error catastrophe theory (Orgel, 1963). He maintains errors in DNA replication occur through mutations which eventually accumulate in the cell. This is particularly devastating when the error is in the production of an enzyme involved in any of the facets of protein synthesis. Obviously, this could result in transcribed templates from potentially normal DNA being translated into abnormal or possibly fatal proteins. However, there is little solid experimental evidence to support this otherwise attractive theory (Lewis and Tarrant, 1972; Bjorksten, 1974).

The third major category of theories of cellular aging is that of crosslinkage. The proponents of this theory argue that DNA is extremely stable especially in its double stranded form making an error accumulation theory unlikely. However, the presence of crosslinking molecules, some of which are natural by-products of metabolism, between or within macromolecules such as DNA could easily disrupt DNA replication and consequently, all cellular functions. Other macromolecules could be just as easily affected--large aggregates of protein such as collagen would seriously disrupt organismal function (Bjorksten, 1974).

A related theory, the free radical theory, states that free radicals produced as a natural consequence of cellular function are dangerous to the cell and could produce symptoms normally associated with aging. Oxygen is the most common generator of free radicals during mitochondrial oxidation (Demopoulos, 1973). Lipid peroxidation within cells due to the presence of free radicals is the phenomenon that could result in serious cytological consequences including: (1) altered membrane permeability; (2) cholesterol deficiency; (3) disruption of membrane-bound enzymatic activities; and, (4) accumulation of potentially harmful by-products of

the peroxidation (Gordon, 1974). Current experiments with vitamin E, butyl hydroxytoluene (BHT) and other antioxidants on the effect of these products on the aging process have produced a range of effects from increased life span in mice (Harmon, 1968), to no effect or shortened life span in another strain of mice (Tappel et al., 1973).

Interestingly, a free radical aging effect has also been described in fungi. An early worker (Pratt, 1924; see Parks and Robertson, 1969) described "staling agents," free radical products of the fungi themselves that shortened average germtube length. More recently, (Parks and Robertson, 1969) another fungal metabolite had been shown to induce vacuolation, a symptom of aging in fungi, upon exposure of juvenile mycelia to stale culture medium filtrate.

More specific effects of aging on biological systems include changes in chemical composition (Gottlieb and Van Etten, 1964, 1966; Van Etten and Gottlieb, 1965; Van Etten et al., 1966) and in membrane lipid content (Grinna and Barber, 1972). Sullivan and Debusk (1974) proposed membrane glycoproteins might be reasonable targets for producing devastating effects on entire cellular function, but other authors, (Bertrand et al., 1975) have found no deterioration of membrane structure or function with age.

A decrease in respiration has been noted with age, sometimes with a corresponding shift in respiratory enzymes (Van Etten et al., 1966; Özelkök and Romani, 1974). If this phenomenon is generally true, then it is not surprising that structural mitochondrial changes also occur (Sohal, 1975) with increasing age of the organism as well as defective production of mitochondrial ribosomes (Rifkin and Luck, 1970).

As protein synthesis is the single most important factor in cellular function, it is logical to look there for signs of deterioration with age,

regardless of whether that deterioration is a result of accumulated somatic mutations, preprogramming, DNA cross linkage or other mechanisms.

In Neurospora, the ratio of an active enzyme (glutamic dehydrogenase) to immunologically cross reacting material (CRM) decreases dramatically with age indicating mistranslation of the protein and not decreased synthesis of the enzyme molecules (Lewis and Holliday, 1970). In expanding and aging leaves of Populus, ^{14}C- photosynthetate was incorporated into a TCA soluble pool at constant rates during aging but the TCA insoluble proteins incorporated less ^{14}C as the leaf aged (Dickmann and Gordon, 1975). Rat liver microsomes also show a decreased capacity for protein synthesis when from older rats, but interestingly, the ribosomal fractions from the same tissues show comparable protein synthesizing abilities with time. This data indicates the existence of some sort of chemical factor, apparently retained by microsomes during their preparations, which has the ability to regulate protein synthesis. In fact, upon fractionation and separation of the microsomes on sephadex gel, and testing each fraction for its effects on a cell-free protein synthesizing system, two factors were found in the young and old liver microsome preparations. One factor activated protein synthesis and was more abundant in the younger microsomes while the other factor, more abundant in the older preparation, inhibited protein synthesis and appeared to work at the site of aminoacylation of t-RNA. The nature and site of action of the activator were not determined but the administration of glucocorticoids to the animal before use induced a higher ratio of activator to inhibitor (Goswami, 1977).

Enzymes, as specialized proteins, can be affected by age in at least two ways. Either actual synthesis of the catalyst is affected quantita-

tively or qualitatively, or the activity of the enzymatic process is changed due to a post-translational modification of any of the factors necessary for that activity.

Measurement of the Kreb cycle enzymes in a mycobacterium showed a decrease in activity of all enzymes in the pathway except isocitrate lyase which increased nearly five-fold. This data indicates some regulatory mechanism is involved and that a general depression of the synthesis of all proteins has not occurred (Murthy et al., 1973). In one study of filamentous fungi, most of the enzymes tested showed two pH optima while aging. Generally the more acid conditions were optimum for enzymes in the older systems. Isozymes or a conformational change in the enzyme would explain this phenomenon (Nagasaki, 1968). Gershon and Gershon (1970), using immunological techniques postulated the existence of two populations of enzyme molecules in preparations from older nematodes, one active and one inactive. This work is often cited as support for Orgel's error catastrophe theory of aging. However, Reiss and Rothstein (1975) further purified the same enzyme, tested it immunologically and while they confirmed the existence of the CRM previously reported, they concluded the preparation consisted of a population of partially active molecules. The basis of this conclusion lies in the fact that the enzyme prepared from young and old nematodes bound to the same degree in an affinity column.

Another mechanism behind changes in enzyme activity with age has been recently reported (Wu, 1977). The observations in this report indicate the activities of two liver enzymes decrease during young adulthood and, upon the onset of aging, increase and revert to a neonatal regulation in response to the stimulating agents c-AMP and glucagon. The

author explains this phenomenon as the inability of the older cell to maintain the more sophisticated state of enzyme regulation that young adults possess; thus with age it loses this control and reverts to a more basic, neonatal type of regulation.

Enzyme induction, which incorporates the phenomenon of protein synthesis and response to regulatory factors is also affected by the aging process. Again, the age effect may be seen at the level of protein synthesis or at the level of regulation of the induction. Quite a bit of work has been done in determining age effects on the adaptive response of enzymes to stimulation. One earlier study, while not finding significant differences in the levels of enzyme activity in response to inducer, did strongly suggest that age differences in experimental animals is significant and should be treated seriously (Correl et al., 1965). Other studies, though, have found some very interesting changes in enzyme induction with age. Aged, fasted rats respond to glucose with significantly lower levels of glucokinase activity than fasted younger rats. In younger rats, glucose kinase levels reached the pre-fasted level 8 hours after re-feeding. In older rats, 24-48 hours are required after feeding before the enzyme activity reaches its initial level. In addition, the decreased enzyme activity of the older rat can be raised to the level of the younger animals by administering high concentrations of insulin which is, under physiological conditions, a normal regulator of glucokinase (Adelman, 1970).

Fungi have proven to be an excellent tool for the study of aging eukaryotic cell systems. The material is readily subcultured and "ages" relatively rapidly--7 to 10 days. The spore spray technique of Yanagita and Kogané is extremely valuable in this work. Spores are sprayed onto

prepared petri plates, germinate and eventually anastomosize creating a uniform fungal mat of one uniform physiological age (Yanagita and Kogané, 1963). One possibly undesirable characteristic of fungi is their propensity for sporulation; germinating conidial contamination in a so-called aged system could alter the average age of the mat. This problem has been reduced by using a species of fungi with photo-sporulation control. Aspergillus ornatus, Raper, a filamentous ascomycete, displays this property. Upon addition of tyrosine to the medium and exposure to light, this fungus will readily sporulate. But, in the dark, growth is entirely vegetative (Schwemmin, 1960).

In addition, this species of fungus is an excellent choice due to its phylogentic proximity to Neurospora crassa, which is widely used in genetics studies. Most of the enzyme induction work using Neurospora is probably applicable to Aspergillus ornatus (Hutter and DeMoss, 1967).

In particular, a considerable amount of work has been done on the inducible enzymes of the tryptophan catabolic pathway in Neurospora and A. niger. Tryptophan enters the cell and induces tryptophan pyrollase, kynureninase (Matchett et al., 1968) and o-pyrocatechuic acid carboxylyase to form the final product, catechol (Subba Rao et al., 1967). Work accomplished in this laboratory has not only proved the existence of o-pyrocatechuic acid carboxylyase in A. ornatus but that the specific activity of the induced enzyme decreases with age from six times the basal level to only two times the basal level (Spiegelman and Coursen, 1975). The study presented now attempts to further characterize the age-related adaptations of that inducible enzyme to varying environmental conditions including length of induction time, concentration of inducer (and subsequent internal compartmentalization of that inducer), and environmental

pH effects on the responsiveness of the system.

MATERIAL AND METHODS

ORGANISM AND MEDIA

Stock cultures of Aspergillus ornatus Raper, originally supplied by Dr. D.J. Schwemmin, were maintained in the dark at 4°C on 6-12 mesh silica gel particles (Davidson Chemicals, Baltimore, Md.) saturated with a 5% (w/v) powdered milk solution containing ground mycelial fragments (Perkins, 1962). Subculturing from stocks was performed weekly by shaking several crystals of silica gel onto 1.5% agar slants of complex medium containing per liter, 3 gm Bactotryptone, 40 gm glucose and 20 gm Bacto Malt extract (Schwemmin, 1960). Experimental mycelial mats were grown in the dark at 22°C in 9 cm glass petri plates containing 50 ml defined medium. For composition of defined medium, see Appendix I. The pH was adjusted to 4.8 or 5.0 with 5.0 M HCl and the medium autoclaved 15 minutes (Schwemmin, 1960). Some experiments were conducted using citrate buffered medium in which case the above medium was augmented with 15g/l Na Citrate. All the above chemicals were supplied by Fisher Scientific Co., Fair Lawn, New Jersey.

GROWING PROCEDURES

Slants inoculated from subcultured slants were dark grown for 72 hours, exposed to light for 24 hours and returned to the dark an additional 72 hours. This procedure produced dense sporulation. Spores were harvested by gently scraping the surface of the slant with a sterile loop containing a .01% solution of sodium lauryl sulfate. After scraping, the conidial suspension was decanted into a sterile centrifuge tube and spun at 12,000 rpm for 10 minutes at 20°C in a RC2-B Sorvall refrigerated centrifuge with SS-34 fixed angle rotor (Ivan Sorvall Inc., Norwall, Conn.).

The spore pellet was washed twice with 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, New York).

The resultant spore suspension was sprayed using a glass atomizer (#422550 Kontes Glass Co.) powered by air pumped through a $HgCl_2$ trap on to 50 ml of defined medium in petri plates previously covered with sterile 0.4 μ polycarbonate membranes (Nucleopore Corporation, Pleasonton, Ca.). In order to provide for continuous and even coverage each plate was sprayed with two bursts of 1 second duration with the plate rotated 180° between bursts. After spraying, the plates were immediately covered and placed in a dark incubator at 22°C.

DNA EXTRACTION AND ASSAY

Mats were removed from membranes at 24 hour intervals from 96 hours up to 264 hours after spraying, dried at 55°C for 48 hours in ceramic crucibles and weighed. One hundred mg of dried fungal material were placed in 75 ml glass disrupter flasks (VWR#34007-066, VWR Scientific, Baltimore, Md.) with 50 gm .45-.50 mm glass beads (VWR#34007-146) and 30 ml of cold 5% trichloracetic acid (TCA). The material was homogenized for 120 seconds under continuous gas-liquid CO_2 cooling in a Braun MSK mechanical cell disrupter. Eighteen ml of the homogenate were centrifuged at 8000 x g at 4°C for 10 min, and the pellet was resuspended and stored at 4°C in 10 ml of 5% TCA for 8 hours. Centrifugation and resuspension into 5% TCA were performed three more times after 16, 2 and 2 additional hours incubation in 5% TCA. Two one-hour incubations in a 3:1 (v/v) 75% ethanol-ether solution followed after the last incubation in cold TCA. The suspension was finally sedimented at 8000 x g and the pellet resuspended in 2 ml 10% TCA and boiled for 30 minutes. The boiled solution

was then sedimented at 12,000 x g in the Sorvall centrifuge for 10 minutes and the supernatant fluid decanted--0.5 ml of the fluid being analyzed for DNA with 0.05 ml 5% cysteine-HCl and 5.0 ml 60% H_2SO_4. A light pink color developed after one hour at room temperature, the degree of color change being determined by the Spectronic 20 colorimeter at 490 nm (Stumpf, 1947).

GLUCOSE AND pH DETERMINATION

Glucose levels remaining in the defined medium after periods of fungal growth on that medium were measured by boiling 1 ml of 3,5-dinitrosalicylate reagent with 0.5 ml 1.5% agar solution (diluted 9:1 with water) for 5 minutes. After boiling, the solutions were cooled and 18 ml double-distilled H_2O added. The optical density of the solutions were read at 540 nm on the Spectronic 20 colorimeter (Clark, 1964). The pH was measured using a pH meter, Corning model M-10, by the immersion of the combination electrode (Corning #476050) into the melted agar. Glucose and pH determinations of unused agar defined medium were also made as controls.

INDUCTION AND MEASUREMENT OF O-PYROCATECHUIC ACID CARBOXYLYASE

Induction of the enzyme o-pyrocatechuic acid carboxylyase (OPCA carboxylyase) was performed by removing mycelial mats at 7 and 10 days of age and floating those mats on 10 ml of defined medium plus tryptophan to final concentrations of 0.01%, 0.10%, and 1.0%. Some experiments were conducted using .01M OPCA (K and K labs) in defined medium as the induction medium and some experiments using 0.1% tryptophan in citrate buffered medium. Floating mats were subsequently placed in a dark incubator for the duration of exposure to trypotophan which, depending on the experiment, ranged from 2 to 10 hours. Some experiments required mat

exposure to tryptophan plus defined medium for 20 to 60 minutes, after which the mats were rinsed in defined medium and reincubated in 10 ml defined medium without tryptophan, so that the total incubation time equalled 6 or 10 hours.

After incubation for the required period of time, 3 gm fresh weight of each mat was placed in a 75 ml glass disrupter flask with 15 ml 0.005M phosphate buffer (pH = 7) and 50 gm glass beads and was disrupted in the MSK tissue homogenizer for 60 seconds while being continuously cooled by a gas-liquid CO_2 stream to inhibit deactivation of the enzyme. After disruption, the homogenate was poured off into a centrifuge tube and spun for 10 minutes at 4°C at 10,000 x g in the Sorvall centrifuge. The resultant supernatant fluid (about 5 ml) represented the crude enzyme solution. A 0.8 ml sample of the supernatant fluid was added to the reaction mixture consisting of 1.0 ml citrate-phosphate buffer (pH = 5.2, 3.3 or 2.9 depending on the experiment), 0.2 ml double-distilled H_2O and 0.2 ml .01M OPCA. An inactivated blank was prepared by substituting the 0.2 ml distilled H_2O with 0.2 ml 5% $HgCl_2$. The reaction was allowed to proceed 18 minutes in a constant temperature water bath at 30°C and was stopped with the addition of 1 ml of 1N HCl. OPCA was extracted with 10 ml ethyl acetate and the phases were separated at 3100 rpm for 2 minutes in a clinical centrifuge (International-Cl, Needham Heights, Mass.). The disappearance of OPCA compared to that of the inactivated blank was measured at 320 nm on a Zeiss PMQII Spectrophotometer (Subba Rao et al., 1970).

Protein determination of the crude enzyme solution was performed using a modification of the biuret method of Gornall (1949). To 1 ml of the homogenate supernatant was added 4 ml of biuret reagent. After 30

minutes, the samples were centrifuged at 16,000 x g in the Sorvall centrifuge so as to sediment out the cloudy precipitate, and the protein was measured at 540 nm on the Spectronic 20 colorimeter against a standard of bovive serum albumin.

SPECIFIC ACTIVITY

One unit of specific activity of OPCA carboxylyase is defined as that amount of enzyme which catalyzes the disappearance of 1nmole of substrate per minute per mg mycelial protein (Spiegelman and Coursen, 1975).

TRYPTOPHAN UPTAKE

Tryptophan uptake by the mycelium was measured by cutting disks from mats with a #14 cork borer and floating these disks on 0.7 ml of .01, .10 or 1.0% ^{14}C labeled tryptophan solution in defined medium. Each drop of 0.7 ml contained 0.7 uCi activity from a stock solution of tryptophan of specific activity 56.8 mCi/nmole (tryptophan, L-(side chain-3-^{14}C) from New England Nuclear, Boston, Mass). Disks were maintained for the duration of the 10, 30 or 60 minute exposure in a dark incubator at 22°C and were gently shaken at intervals to insure even distribution of labeled material. After exposure, 4 smaller disks cut with a #2 cork borer were removed from the floating disks and were washed three times in defined medium at 22°C. After washing, two disks were deposited directly into 10 ml scintillation fluid (PCS, Amersham/Searle, Arlington Heights, Illinois) and the two other disks into 250 µl cold 5% TCA. Twenty-four hours later, the residual disks, (TCA insoluble fraction) were removed, washed three times in distilled water, and placed in 10 ml of scintillation fluid. In addition, 200 of the 250 µl of TCA were pipetted out and swirled into solution with 10 ml of scintillation fluid. This represents the TCA soluble fraction. All samples

were counted 50 minutes or within 1.0% error in a Beckman LS-3133T Liquid Scintillation counter. As most of the samples were heterogeneous mixtures, counts per minute could not be converted to disintegrations per minute therefore the problem of quenching was usually ignored and only direct comparisons between experimental replicates of the same fractions were made. At one time, the TCA soluble fraction was converted to DPM in order to make an approximation of velocities and the Michaelis-Menton constant for comparison with reported values.

RESULTS

MAINTENANCE AND GROWTH

Maintaining stock cultures of Aspergillus ornatus on silica gel has been very successful with no apparent morphological changes occuring in the fungus during the year it has been stored in this manner. Neither, apparently, is the valuable sporulation control this organism possesses affected by long periods of storage on a desiccant material. The subcultures on complex medium have consistently yielded vegetative mycelia that sporulate only after exposure to light within the 240 hour culture period.

Sprayed mycelial mats produced by the spray spore technique on defined medium plus tyrosine, were also very satisfactory in regard to sporulation control but showed a degree of light sensitivity much greater than previously suspected. Keeping developing mats in a dark incubator was evidently not sufficient; since small amounts of light could enter the incubator even when exercising reasonable caution when entering the incubator. But by covering the plates before placing in the dark incubator and by not inspecting the plates before actual use, excellent control was achieved.

GROWTH OF THE ORGANISM

The growth of the organism is represented graphically in Figure 1, a growth curve depicting total DNA and dry weight of the mat against time in hours. DNA increases steadily up to 144 hours when it levels off reaching a plateau of 7.5-8.5 mg by 168 hours which it maintains until 240 hours. After 240 hours of growth, sporulation was occasionally observed on the underside of the mat where uplifting sometimes occurred thus raising the level of total DNA. For this reason all experiments

Figure 1. Total dry weight and DNA in mg of the mucelial mat of Aspergillus ornatus vs. time. Each point represents the mean of three replicates from three separate experiments. The vertical lines represent the standard error.

DRY WEIGHT (mg x 10⁴)
2
4
6
8
10
12
96
120
144
168
192
216
240
264
HOURS OF GROWTH
TOTAL DNA (mg)

were conducted at 168 and 240 hours (7 and 10 days) before sporulation could possibly interfere with results.

Dry weight increases steadily up to 1100 mg at 216 hours reaching a stable level of 1150 mg at 240 and 264 hours, 72 hours after the DNA plateau had been reached.

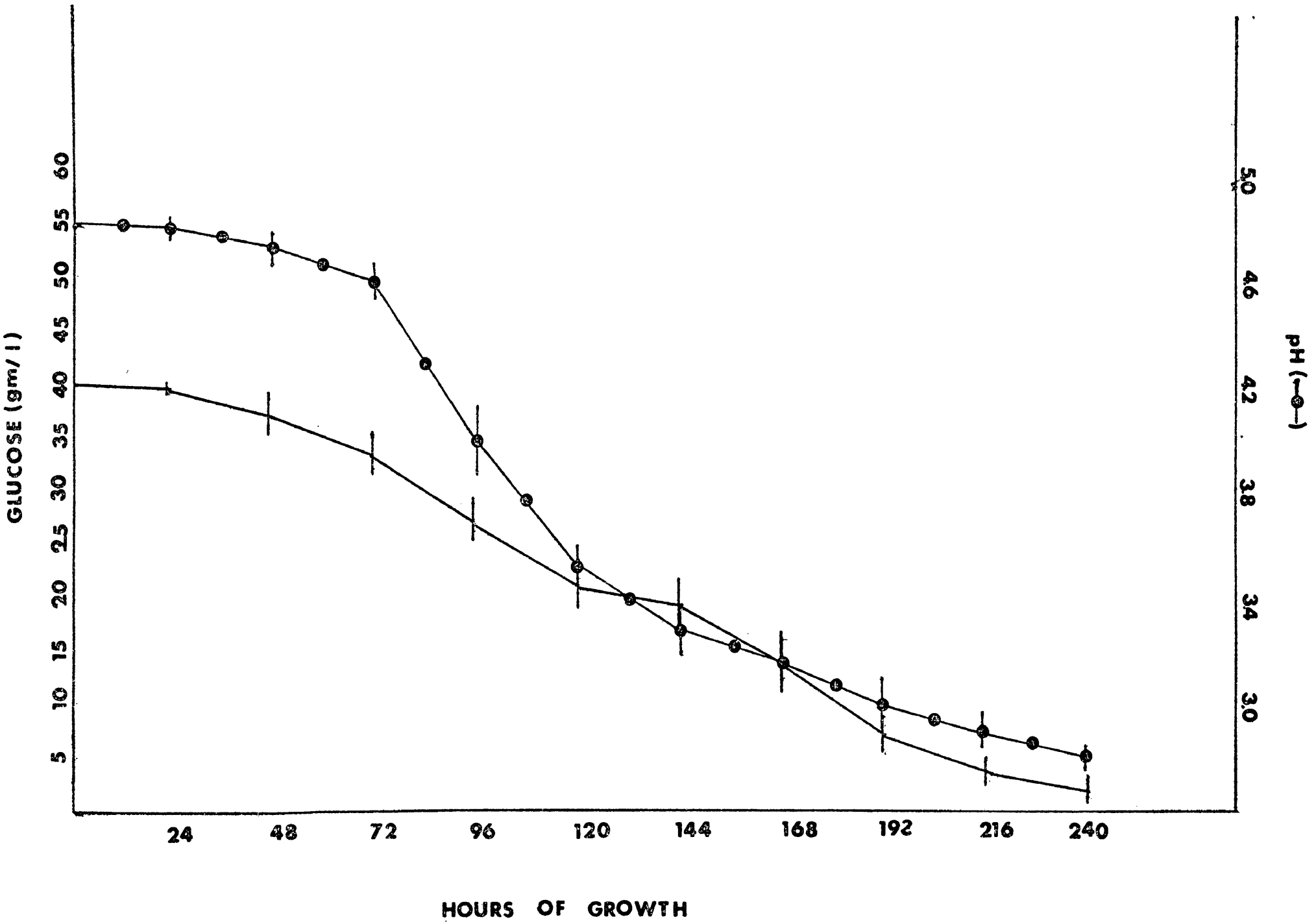
Measurement of glucose concentrations remaining in medium used to support the growth of the fungus reveals that the glucose concentration steadily decreases from 40 gm/1 to 3 gm/1 over a 240 hour period (Figure 2). The slope of the decrease is almost constant through time and doesn't show a greater utilization of glucose during the periods of greatest weight gain as described in Figure 1, indicating even after the organism has ceased karyokinesis and weight gain, it is still utilizing glucose for respiratory purposes.

The pH of the defined medium without citrate buffer (Figure 2) also decreases steadily from an initial value of 4.8 to 2.9. Most of the drop in pH occurs before 7 days, that is, before cell division ceases, and the drop amounts to only an additional 0.5 units, on the average, from 7 to 10 days. However, mats from the same spray series have at times shown a difference of only 0.2 units from 7 to 10 days of growth.

OPCA CARBOXYLYASE INDUCTION

Mycelial mats of Aspergillus ornatus, when placed on induction medium of 0.1% tryptophan in defined medium for 6 hours typically show approximately a 50% decrease in specific activity of the induced enzyme with age while the levels of uninduced enzyme activity remain relatively stable (Spiegelman and Coursen, 1975). To determine if this aging effect is dependent on the concentration of the inducing substance, 7 and 10 day mats were exposed to varying concentrations of tryptophan for 6 hours. The

Figure 2. Concentration of glucose remaining in the medium and pH of that medium after supporting the growth of mycelial mats of Aspergillus ornatus through time. Each point is the mean of at least three separate determinations. The vertical lines represent the standard error.


GLUCOSE (gm/l)
5
10
15
20
25
30
35
40
45
50
55
60
pH (—●—)
3.0
3.4
3.8
4.2
4.6
5.0
24
48
72
96
120
144
168
192
216
240
HOURS OF GROWTH

results are shown in Figure 3.

The specific activity of OPCA carboxylyase on 0.01% tryptophan was not significantly different from the normal basal level. The specific activity obtained using 0.1% tryptophan was 9-10 units at 7 days and 6.5 units at 10 days while induction on 1.0% inducing medium produced a specific activity in the older mat equaling that of the younger mat (9.7 units and 10.5 units, respectively). Plotted on a semi-log scale, the increase in specific activity with logrithmically increasing concentrations of tryptophan in a 10 day mat, is linear. The curve plateaus at the higher inducer concentrations in the 7 day mat.

The results of an attempt to determine optimum induction periods on 0.10% tryptophan inducing medium are shown in Figure 4. An induction period of 6 hours appears to be the minimum time necessary for full expression of the enzyme (11.5 units activity) at 7 days while the 10 day mats under similar conditions show only 60% of the 7 day activity. The younger mats maintain essentially the same level of enzyme activity under longer incubation times of 8 and 10 hours, while the older mats continue to show an increase in specific activity with longer exposures to tryptophan until they approach maximum activity (10-11 units activity) with 10 hours of induction. (9-11.5 units of specific activity is considered to be maximum expression of the enzyme since at no time was this value exceeded). Again, a linear increase in enzyme activity in 10 day mats was noted with increasing induction times.

Although 6 hours was shown to be the minimum time on 0.10% tryptophan necessary for maximum expression of the enzyme, the possibility remained there might be two phases in this 6 hour time period--an induction phase followed by a period of incubation. This was tested by placing 7

Figure 3. The effect of tryptophan concentration on the induction of o-pyrocatechuic acid carboxylyase in aging mycelial mats of Aspergillus ornatus. Zero percent represents the basal level. Open bars represent 7 day mats, lined bars 10 day mats. Each bar represents the mean of at least three replicates from three separate experiments. The vertical line is the standard error. A T-test determined significant differences (>99% chance, $H_1 \neq H_2$) between ages in which vertical lines do not overlap. Specific activity equals nmoles substrate converted per minute per mg mycelial protein.

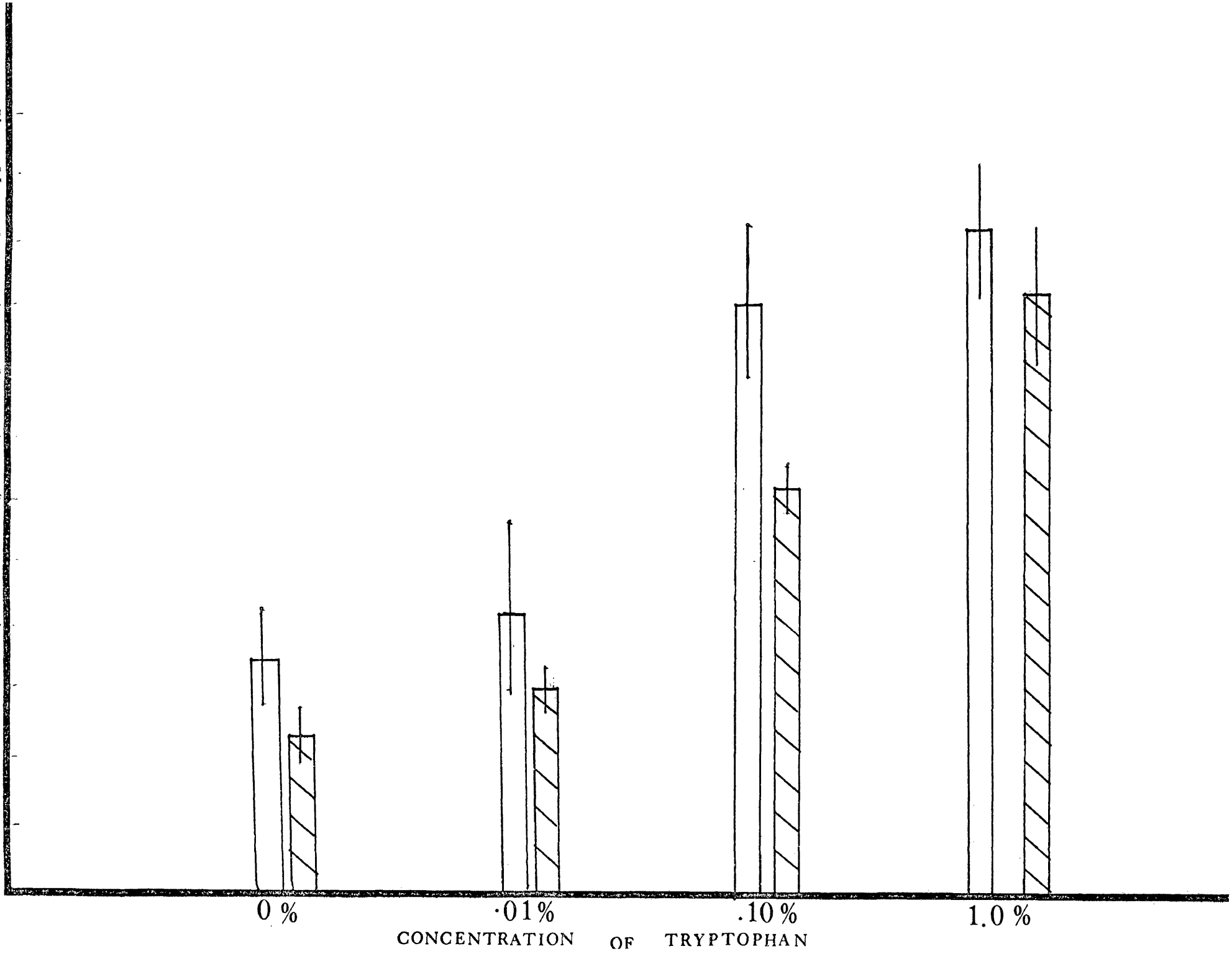
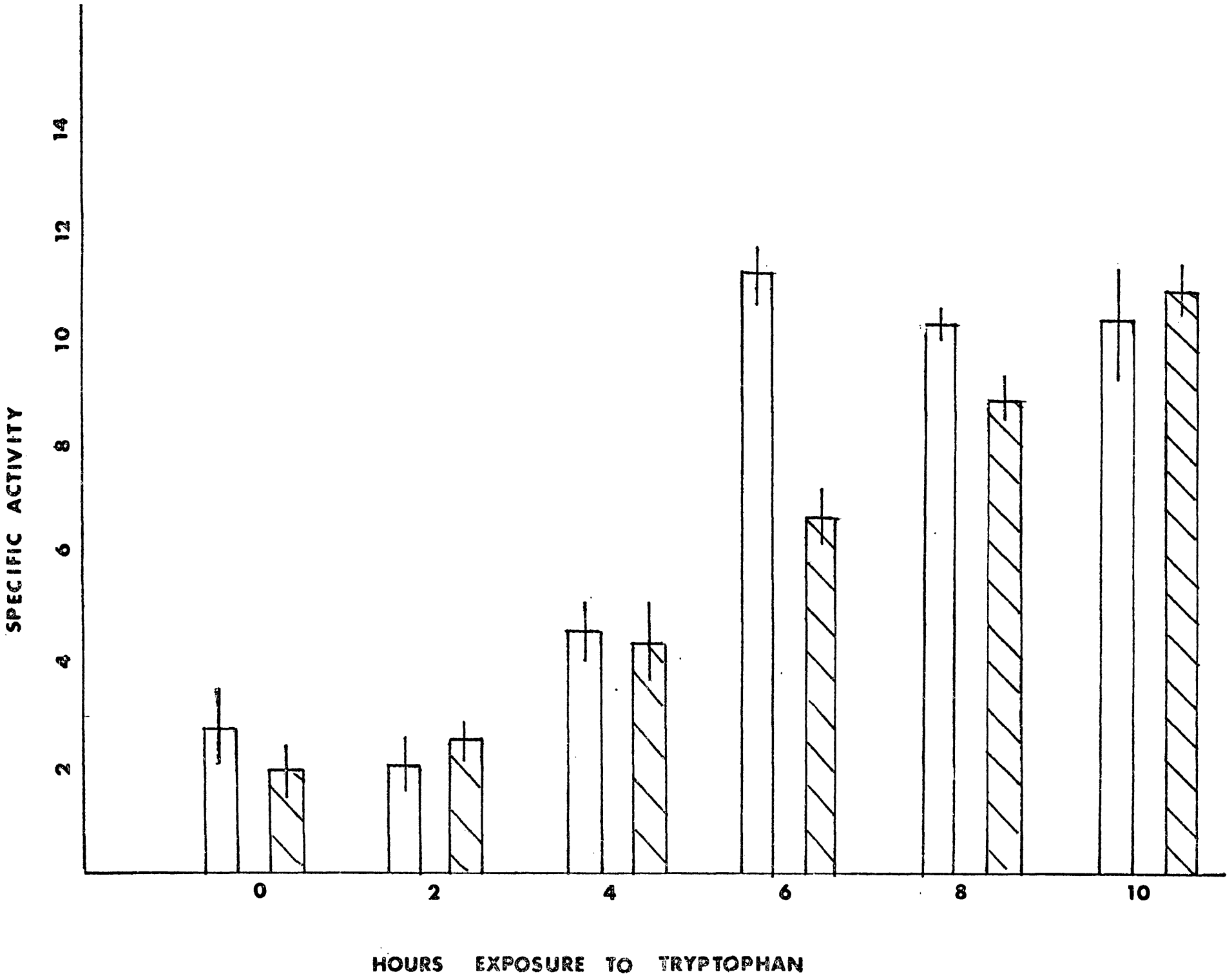

1
2
3
4
5
6
7
8
9
10
11
12
0 %
·01%
.10 %
1.0 %
CONCENTRATION OF TRYPTOPHAN

Figure 4. The effect of the duration of the induction time of o-pyrocatechuic acid carboxylyase in aging mycelial mats of Aspergillus ornatus by 0.1% Tryptophan. Zero hours represents the basal level. Open bars represent determinations on 7 day mats and lined bars on 10 day mats. Each bar is the mean of at least two replicates of three separate experiments. The vertical line is the standard error. A T-test determined the only significant difference between ages are those in which the vertical lines do not overlap (>99% chance, $H_1 \neq H_2$). Specific activity equals nmoles substrate converted per minute per mg mycelial protein.


SPECIFIC ACTIVITY
2
4
6
8
10
12
14
0
2
4
6
8
10
HOURS EXPOSURE TO TRYPTOPHAN

and 10 day mats on 10 ml of liquid defined medium containing 0.10% tryptophan for periods of time ranging from 20-60 minutes. Mats were then rinsed and placed on 10 ml defined medium lacking tryptophan for a period of time such that the induction and incubation time equalled 6 or 10 hours. The results are shown in Figure 5. Evidently, for a 7 day mat, 40-60 minutes of induction with 0.1% tryptophan is great enough to allow maximum activity of OPCA carboxylyase provided an incubation time of at least 5 hours follows. Ten day mats not only require a longer induction time of 180 minutes, but also a longer incubation time of 7 hours in order to reach the same maximum acitivity as younger mats.

The data in Figure 5 lead to a suggestion that tryptophan itself is not the primary inducing substance but rather that it may be one of the tryptophan catabolites further along the pathway. Indeed, Figure 6 illustrates that 0.01M OPCA, with only 1 hour of induction and no incubation period, promotes OPCA carboxylyase activity to the same degree as 6 hours of exposure to 0.1% tryptophan.

TRYPTOPHAN TRANSPORT

All data in this section involving counts per minute were normalized, i.e. raw uptake value using 0.10% tryptophan were multiplied x 10 and values using 0.01% tryptophan x 100. This adjustment counteracts competition from unlabeled tryptophan in the medium and was necessary because the amount of labeled material added to varying concentrations of unlabeled tryptophan was constant.

Tryptophan transport into the vegetative mycelium was determined at 7 and 10 days of age in order to explore the possibility of a correlation between internal concentrations of inducer and enzyme activity. Table 1 shows the results of these experiments. No differential uptake into the

Figure 5. The effects of the induction time and incubation time on o-pyrocatechuic acid carboxylyase in aging mycelial mats of Aspergillus ornatus by 0.1% tryptophan. The induction times are marked in minutes on the x-axis and additional incubation time was such that total time on new medium equalled six or 10(*) hours. The open bars represent determinations on 7 day mats, the lined bars on 10 day mats. Each bar is the mean of at least two replicates from three separate experiments. The vertical line is the standard error. A T-test determined values between ages are significantly different if vertical lines do not overlap (>99% chance, $H_1 \neq H_2$). Specific activity equals nmoles substrate converted per minute per mg mycelial protein.

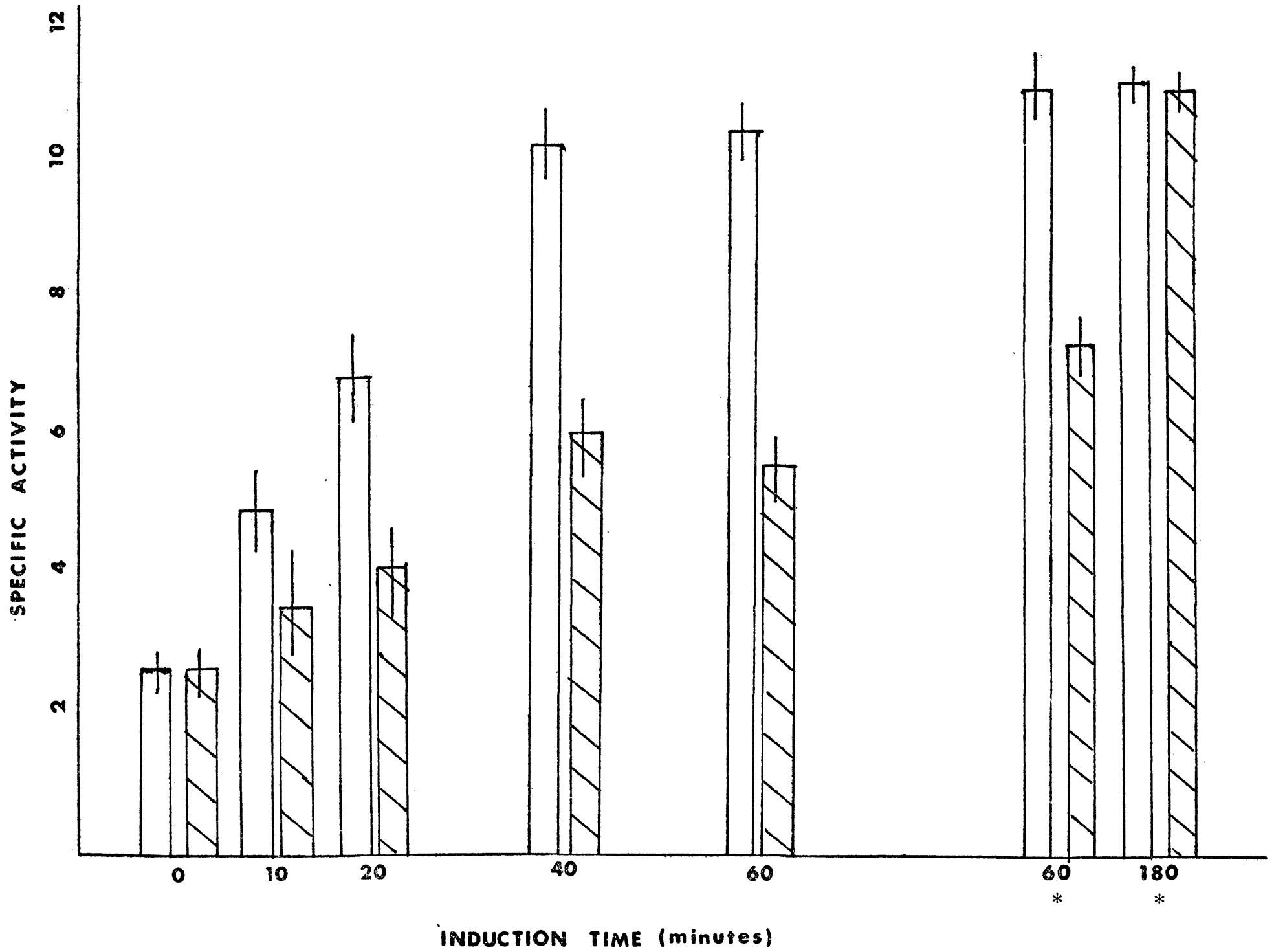

SPECIFIC ACTIVITY
12
10
8
6
4
2
0
10
20
40
60
60
*
180
*
INDUCTION TIME (minutes)

Figure 6. The effect of o-pyrocatechuic acid (OPCA) and tryptophan on the induction of OPCA carboxylyase in aging mycelial mats of Aspergillus ornatus. Specific activity of mats exposed to 0.1% (.005M) tryptophan six hours is compared to specific activity of mats exposed to .01M OPCA one hour. Open bars represent determinations at 7 days, lined bars at 10 days. Each bar represents the mean of two replicates from three separate experiments. The vertical line is the standard error. A T-test determined values between ages are significantly different if vertical lines do not overlap (>99% chance, $H_1 \neq H_2$). Specific activity equals nmoles substrate converted per minute per mg mycelial protein.

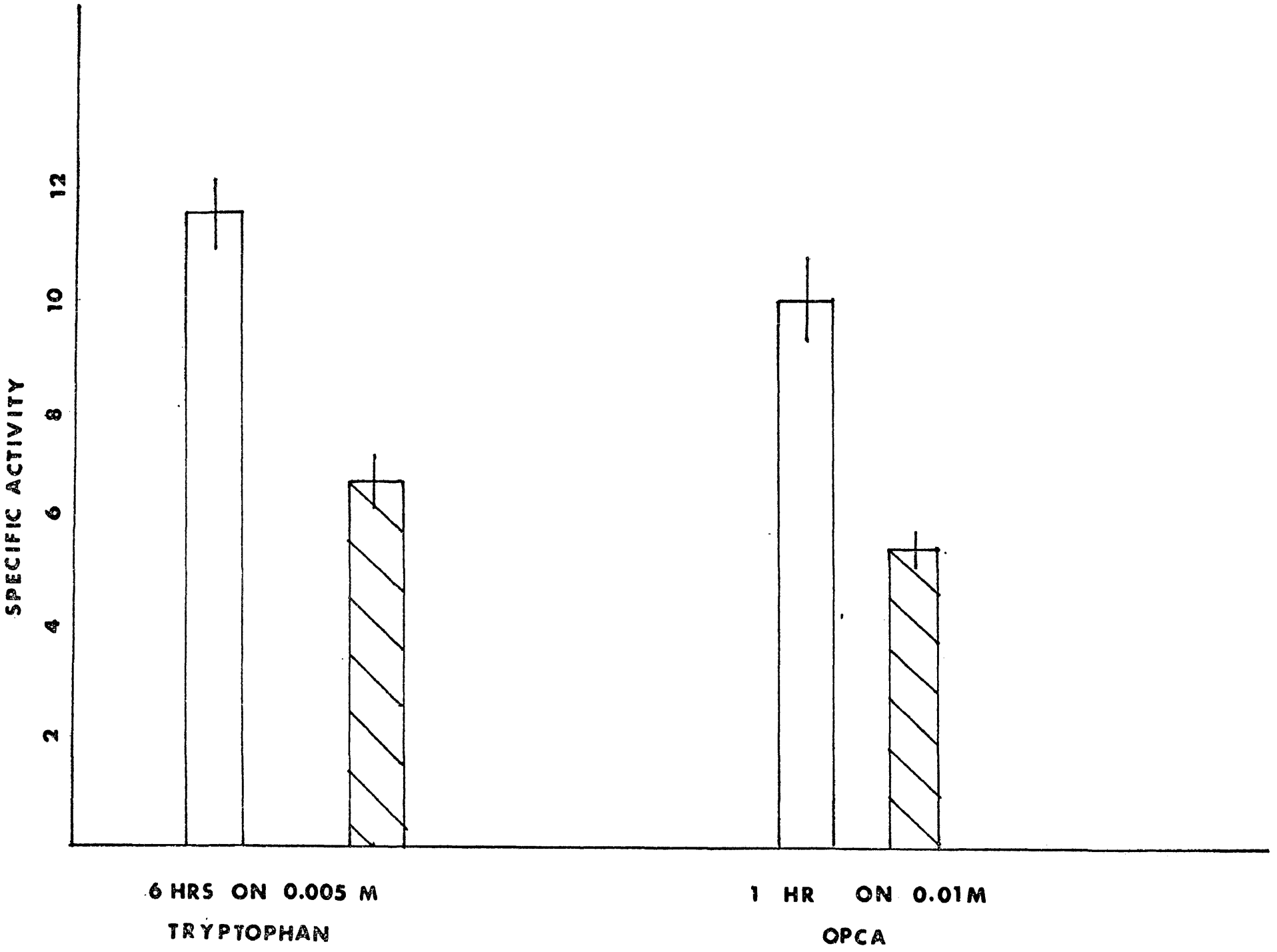

SPECIFIC ACTIVITY
2
4
6
8
10
12
6 HRS ON 0.005 M
TRYPTOPHAN
1 HR ON 0.01M
OPCA

TABLE 1. ^{14}C PRESENT IN THE WHOLE MAT, TCA SOLUBLE FRACTION AND TCA INSOLUBLE FRACTION IN AGING MYCELIAL MATS OF ASPERGILLUS ORNATUS AFTER 60 MINUTES EXPOSURE TO LABELED TRYPTOPHAN (SPECIFIC ACTIVITY 56.8 mCi/MMOLE). EACH VALUE IS THE MEAN OF TWO REPLICATES FROM FOUR SEPARATE EXPERIMENTS FOLLOWED BY THE STANDARD ERROR. A T-TEST DETERMINED WHICH VALUES BETWEEN AGES ARE SIGNIFICANT (>99% CHANCE, $H_1 \neq H_2$). IN THOSE CASES OF SIGNIFICANT DIFFERENCES BETWEEN AGES, THE PERCENTAGE DIFFERENCE BETWEEN THE VALUES IS GIVEN.

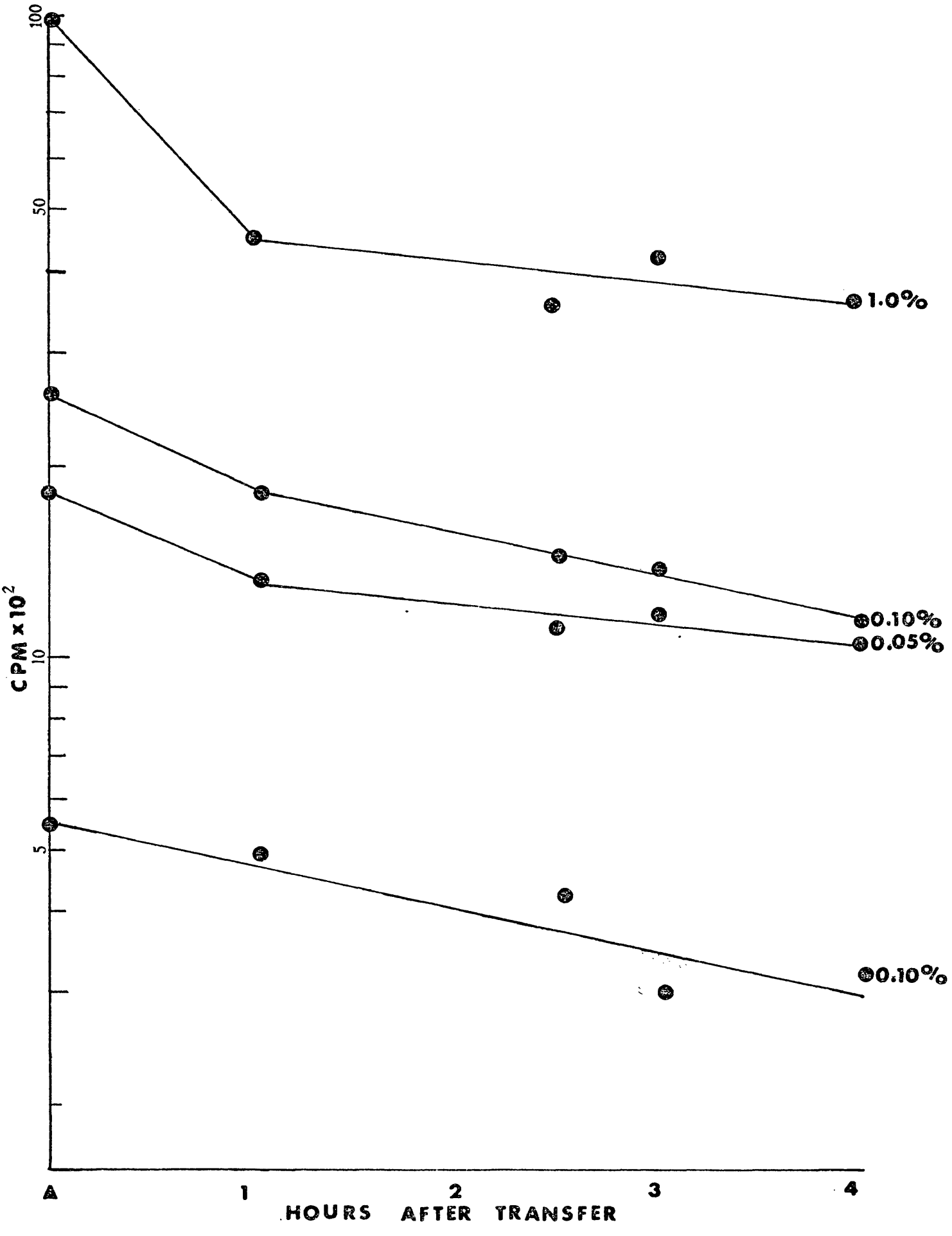
TABLE 1.

.01% tryptophan	whole mat	TCA soluble	TCA insoluble
7 day	10,344 ± 897	8,008 ± 525	1,956 ± 113
10 day	10,019 ± 1,050	7,532 ± 920	1,264 ± 90
	Δ = NS	Δ = NS	Δ = 35%
.10% tryptophan	whole mat	TCA soluble	TCA insoluble
7 day	43,333 ± 3,895	27,650 ± 882	4,455 ± 144
10 day	39,781 ± 4,564	30,945 ± 1,057	3,468 ± 165
	Δ = NS	Δ = NS	Δ = 23%
1.0% tryptophan	whole mat	TCA soluble	TCA insoluble
7 day	214,360 ± 9,904	163,250 ± 18,050	17,580 ± 1,575
10 day	238,283 ± 17,662	177,766 ± 5,988	23,133 ± 876
	Δ = NS	Δ = NS	Δ = NS

whole mat occurred, nor was uptake into the TCA soluble fraction different between 7 and 10 days of age. However, it is significant that the only difference found in any of the fractions occurs in the TCA insoluble fraction and that that difference shows a depressed tryptophan incorporation into protein in the older mats. A 35% decrease in tryptophan incorporation into protein was observed at 0.01% tryptophan and a 23% decrease with 0.1% tryptophan. Interestingly, at 1.0% tryptophan, the only concentration of the inducer (Figure 3) shown to allow maximum expression of the enzyme in 10 day mats, incorporation into protein at 10 days is equal to that at 7 days.

In order to check the possibility that observed incorporation of radioactive tryptophan into the mat might be an exchange diffusion phenomenon, #14 size disks were exposed to labeled tryptophan for 60 minutes and from each a smaller #2 sized disk was removed, washed and counted. The remaining larger disk was washed and replaced on .7 ml unlabeled defined medium plus the appropriate concentration of tryptophan and smaller disks were cut, washed and counted from it every hour up to 4 hours. If exchange diffusion were the only phenonemon being measured, one would expect an exchange of the labeled tryptophan back into the medium and the internal count rate to drop, eventually to very low levels. (Cohen and Monod, 1957). Although the internal count rate did drop over the four hour incubation period (Figure 7) the drop was niether rapid nor substantial after the first hour of incubation. The greater drop of internal label within that first hour is possibly due to some cross exchange of tryptophan across the cell membrane thus contributing to the overall uptake process. The greater the concentration of labeled tryptophan used, the greater the slope of the loss of label within

Figure 7. Rate of leakage of ^{14}C from mycelial mats of Aspergillus ornatus into unlabeled defined medium. Points on the y-axis are CPM of internalized ^{14}C-tryptophan into the whole mat (specific activity 56.8 ci/mole) after 60 minutes exposure to various concentrations of tryptophan. Points read off the x-axis (time) represent CPM of internalized ^{14}C-tryptophan remaining in mat after exposure to unlabeled defined medium. Points are a single determination. Point marked by Δ represents the transfer of the disk to unlabeled medium.


100
50
10
5
CPM x 10²
1.0%
0.10%
0.05%
0.10%
A
1
2
3
4
HOURS AFTER TRANSFER

the first hour. The loss of internal label during a 60 minute period may represent re-equilibration of internal to external concentrations of tryptophan when the internal concentration is greater than maintained by the permeability barrier. After equilibration, a drop in internal levels of the side chain-3-^{14}C tryptophan with time into unlabeled medium would be expected due to normal metabolism and excretion of tryptophan by-products including, very possibly, $^{14}CO_2$ from the side chain. Other factors also suggest the likelihood that actual transport is taking place such as the adherence of the data to a hyperbolic Mechaelis-Menton curve (Figure 8, inset 1).

In order to compare approximate Km and Vmax values with those reported in the literature, tryptophan uptake into the TCA soluble fraction in counts per minute was converted to μmoles/min. Since (1), only an approximation of the kinetics values were desired, and, (2) probably 70-80% of tryptophan uptake enters the TCA soluble pool, and, finally (3) the TCA soluble fraction was the only homogenous fraction counted in the scintillation counter, the TCA soluble values were used rather than those for uptake into the entire mat. The Lineweaver-Burke plot in Figure 8 indicates the approximate Km is 5000 μ and the maximum velocity, 450 μmoles/min/disk.

EFFECT OF pH ON THE INDUCTION PROCESS

In order to determine the effect of the drop in environmental pH on the enzyme induction process, mats were grown on defined medium supplemented with 50mM citrate. The effect of added carbon substrate on glucose utilization appears to be minimal while pH maintenance is fair (Figure 9). Apparently, in response to the added citrate, the fungus is producing metabolites which initially acidify the medium (from 5.0 to

Figure 8. Lineweaver-Burke plot of tryptophan incorporation into TCA soluble fraction of mycelial mats of Aspergillus ornatus. Points represent the mean of two replicates from at least two separate experiments. Km = 5000μM, Vmax = 450 μmoles/min. Inset 1 is a Michaelis-Menton curve of the same data. Inset 2 is CPM of internalized ^{14}C-tryptophan against time of exposure to label.

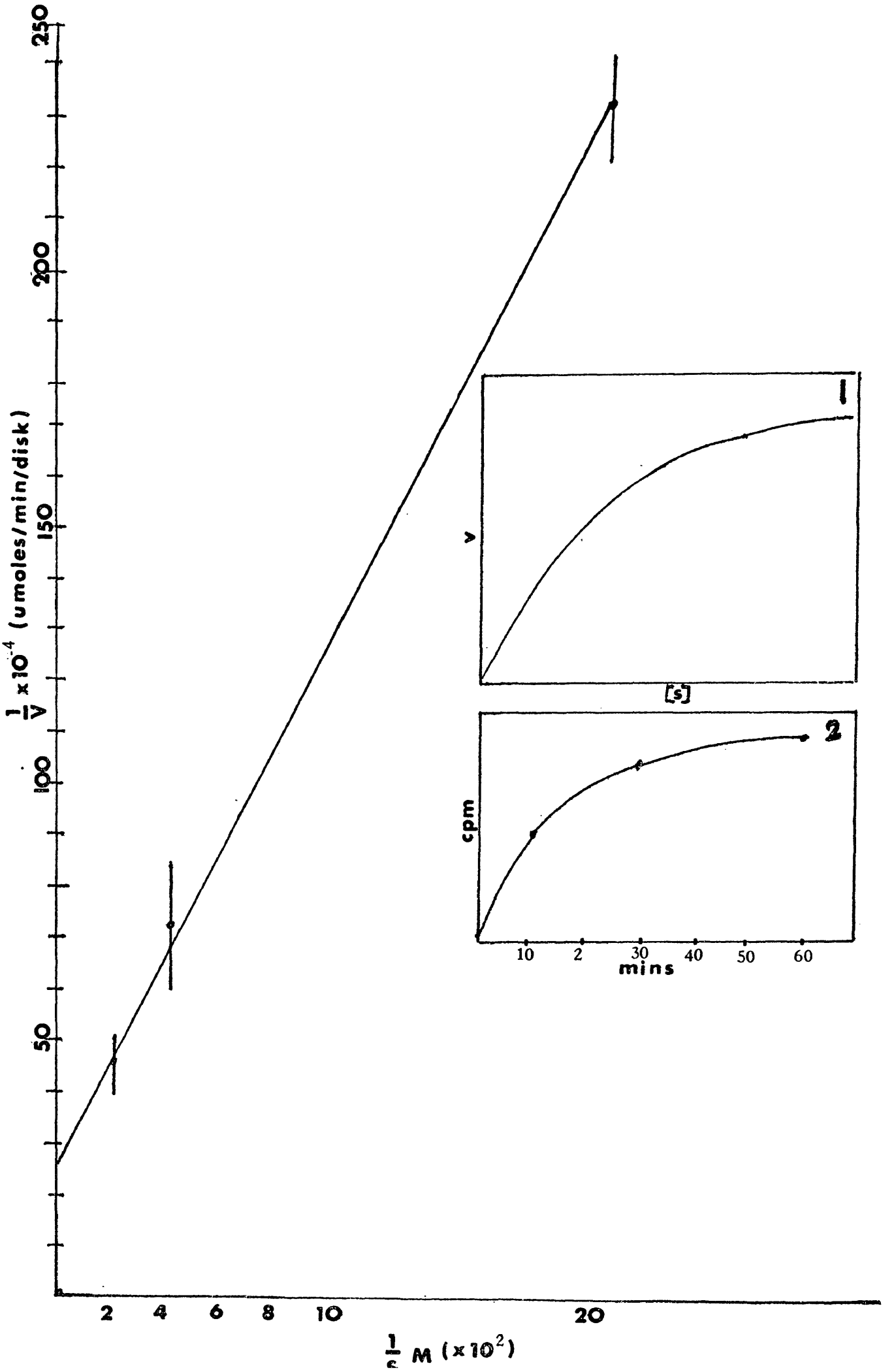
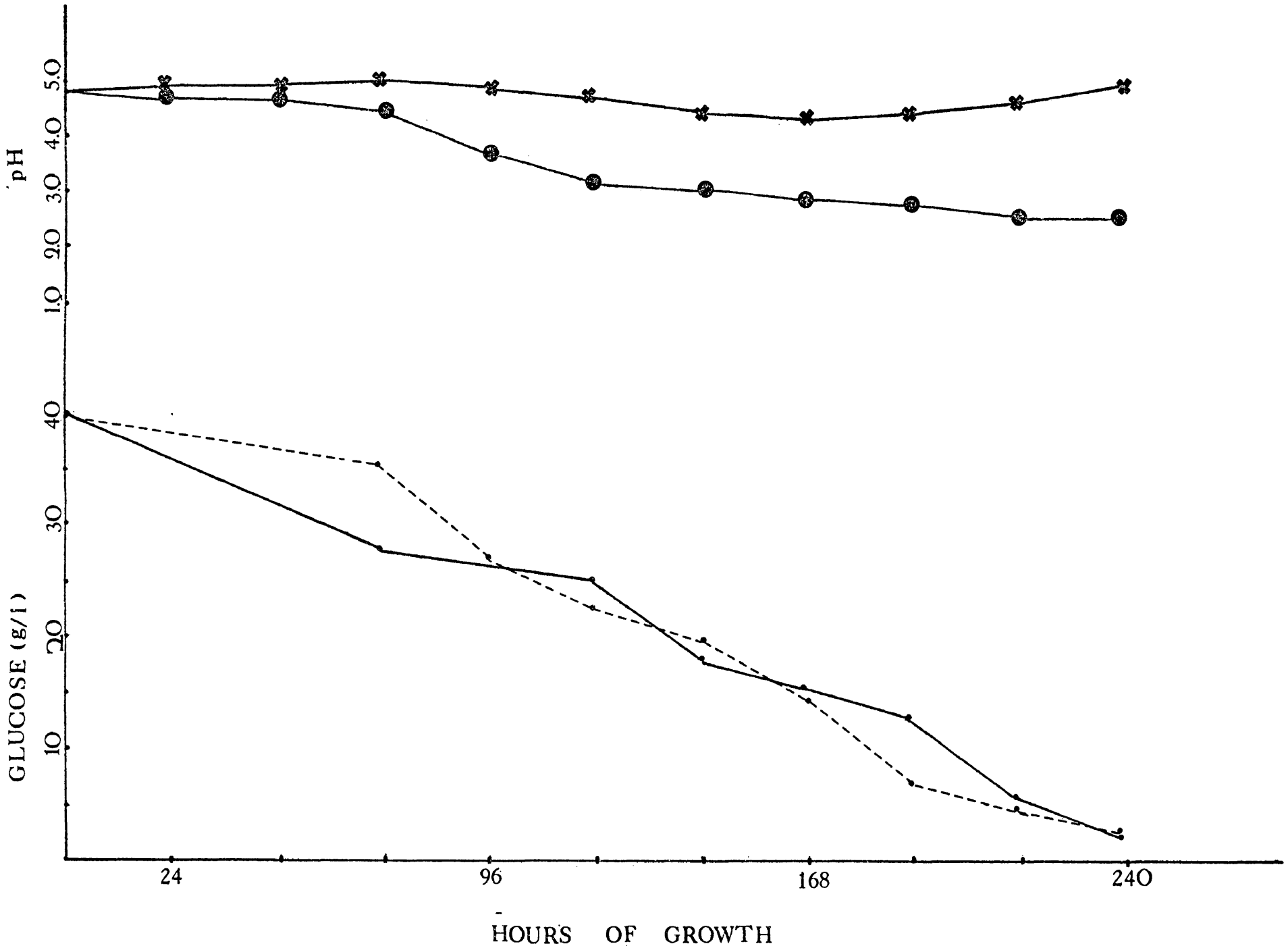

250
200
150
100
50
$\frac{1}{V} \times 10^{-4}$ (umoles/min/disk)
2
4
6
8
10
20
$\frac{1}{s}$ M ($\times 10^2$)
1
v
[s]
2
cpm
10
2
30
40
50
60
mins

Figure 9. Concentration of glucose and pH of the medium after the growth of mycelial mats of Aspergillus ornatus on different buffered mediums. (———) Glucose in g/l remaining in 50 ml of citrate buffered medium. (----) Glucose in g/l remaining in 50 ml defined medium without citrate. (—X—X—X—) pH of 50 ml citrate buffered medium against time of fungal growth on that medium. (—•—•—•—) pH of 50 ml defined medium without citrate against time of fungal growth on that medium. Points are a single determination except glucose (in defined medium without citrate) which has been previously described.


pH
5.0
4.0
3.0
2.0
1.0
GLUCOSE (g/l)
40
30
20
10
24
96
168
240
HOURS OF GROWTH

4.3) but, after 7 days, make it more basic (from 4.3 to 4.9).

Enzyme levels, (defined here as the difference in OPCA levels between mean induced and mean basal levels divided by mean mg protein) in mats grown on defined medium without citrate are 5.7 at 7 days and 3.1 by 10 days (Figure 10). Partial control of the pH drop with citrate buffered medium results in an enzyme level of 4.8 at 7 days and a comparable 4.3 at 10 days. The possibility remains, however, that maintaining a relatively constant pH in the medium delays the aging effect. One of the limitations using the petri dish containers is the small volume which may result in exhaustion of glucose by 240 hours of fungal growth. By growing mats on membrane covered media in 400 ml glass culture dishes however, glucose is no longer a limiting factor and mats may be aged much longer than 240 hours. Using this system, the enzyme level at 7 days is 6.0, at 10 days 5.9 and at 14 days, 0.94. (Figure 10).

Table 2 is a compilation of all the pH data including: 1) the pH values that occur in the medium using defined medium, defined medium plus citrate buffer in petri dishes and defined medium plus citrate in culture dishes, 2) the pH values in the actual crude fungal homogenate ground in phosphate buffer or in water, 3) the pH values in the 10 ml of induction medium (defined medium with or without citrate plus the appropriate concentration of tryptophan). This is the material the mat was exposed to during the actual induction process, and, 4) the reactions of the enzyme systems to varying pH's of the reaction mixture. The table also includes notations as to whether or not the pH changed with age and whether or not the specific activity of the enzyme changed under the same conditions.

Figure 10. The effect of different cultural conditions on the induction of o-pyrocatechuic acid carboxylyase in aging mycelial mats of Aspergillus ornatus. Enzyme levels (mean OPCA units of induced mats--mean OPCA units of uninduced mats/mean mg protein), of mats grown on 50 ml defined medium without citrate in petri dishes  , mats grown on 50 ml defined medium with citrate in petri dishes  , and mats grown on culture dishes of 400 ml defined medium with citrate  . Each bar represents a mean value of two replicates from two separate experiments.

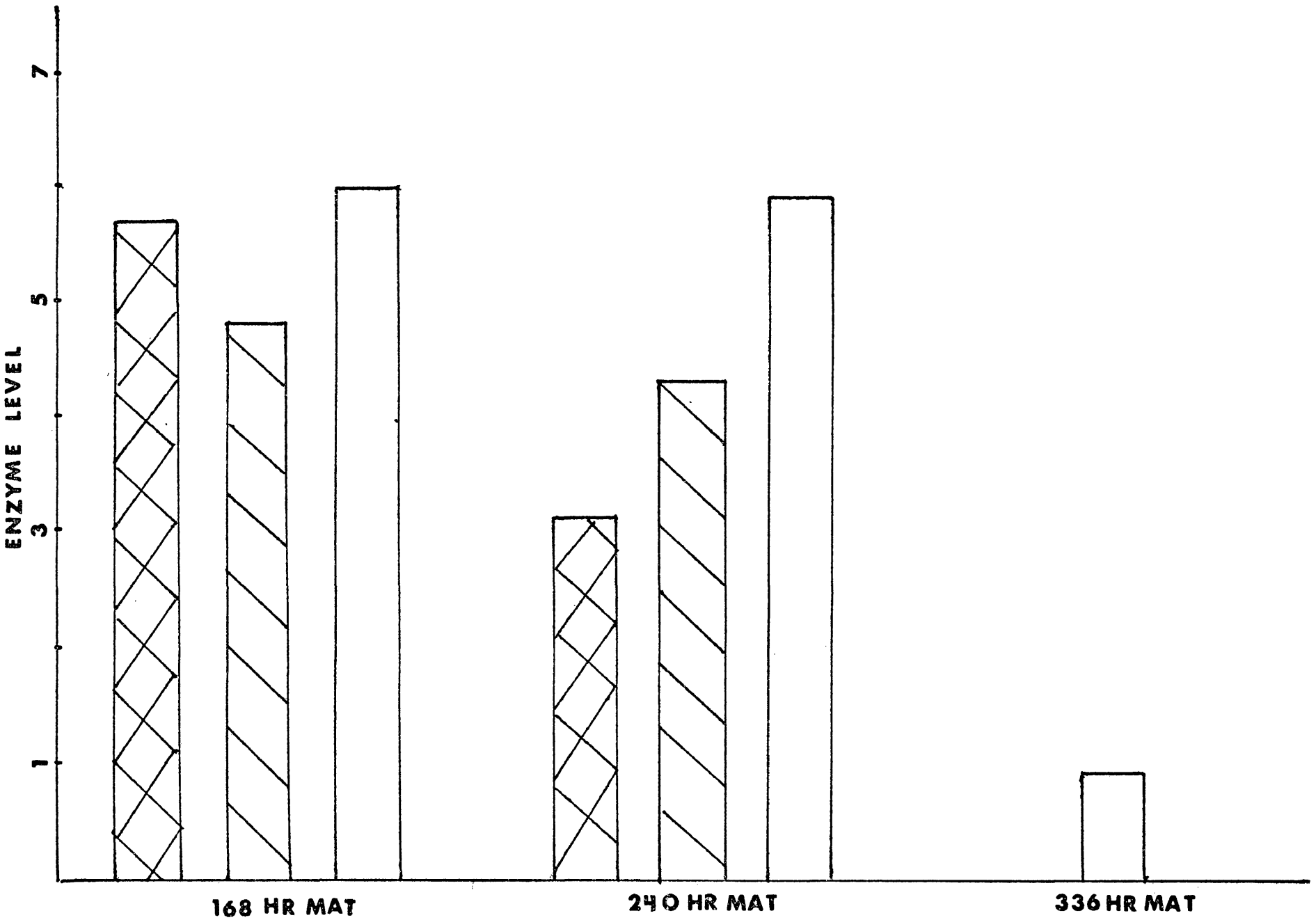

ENZYME LEVEL
1
3
5
7
168 HR MAT
240 HR MAT
336 HR MAT

TABLE 2. COMPILED pH AND ENZYME ACTIVITY DATA OBTAINED FROM EXPERIMENTS WITH AGING MYCELIAL MATS OF *ASPERGILLUS ORNATUS*. I. MEAN pH VALUES OF DEFINED MEDIUM WITH OR WITHOUT CITRATE DURING FUNGAL GROWTH. II. MEAN pH VALUES OF FUNGAL HOMOGENATE GROUND IN BUFFER OR WATER. III. MEAN pH VALUES OF SPENT INDUCTION MEDIUM, EITHER WITH OR WITHOUT CITRATE FROM MATS OF VARIOUS AGES. IV. DIFFERENT pH VALUES OF REACTION MIXTURE AND NOTATION OF ENZYME RESPONSE. DM = DEFINED MEDIUM WITHOUT CITRATE, CM = DEFINED MEDIUM WITH CITRATE, ✓ INDICATES A CHANGE IN THE pH OR ENZYME ACTIVITY BETWEEN AGES, X INDICATES NO CHANGE OCCURRED WITH AGE, NA = NOT APPLICABLE.

TABLE 2.

Item	$\bar{X}$ pH from 168 hr mats	$\bar{X}$ pH from 240 hr mats	$\bar{X}$ pH from 336 hr mats	Δ pH ?	Δ activity ?
Used Medium					
Defined medium (DM)	3.3	3.0	NA	✓	✓
Citrate buffered medium (CM).					
CM in 400 ml	4.2	4.7	NA	✓	X
Culture dish -- top	4.9	4.9	4.8	X	✓
bottom	5.0	4.9	4.9	NA	NA
Fungal Homogenate					
Grinding in .05m PO_4 buffer, pH = 7.0	7.0	7.0	NA	X	✓
Grinding in H_2O, pH = 7.0	8.4	8.4	NA	X	NA
Used Induction Medium					
DM grown mats on DM induction medium	3.5	3.1	NA	✓	✓
DM grown mats on CM induction medium	4.5	4.4	NA	X	✓
CM grown mats on DM induction medium	3.6	3.6	NA	X	X
CM grown mats on DM induction medium	4.8	4.8	NA	X	X
CM culture dish grown mats on DM induction medium	3.6	4.1	5.3	✓	✓
Reaction Mixture Adjusted to Various pH Values					
5.2	NA	NA	NA	NA	no activity
3.3	NA	NA	NA	NA	no activity
2.9	NA	NA	NA	NA	no activity

DISCUSSION

Aging, as defined earlier in this paper, is that period of time in the life span of an organism after cellular proliferation and nuclear division have ceased. Accordingly, the total DNA content within that organism should remain constant during that period. As indicated under Results, this period of time in mats of Aspergillus ornatus under the described growth conditions is from 168 to 240 hours, 7 to 10 days (Figure 1). During this time, protein levels remain constant, dry wieght increases for an additional 72 hours after the DNA levels become constant, glucose utilization proceeds at a constant rate and pH levels of the growth medium drop an additional .2 to .4 units indicating the organism is continuing normal metabolism in spite of the non-cycling state of the cells. Experiments were not conducted after 240 hours of growth on 50 ml medium due 1) to the possible onset of new spoulation and 2) to the near exhaustion of glucose in the medium.

The phenomenon of enzyme induction is used in this work as a parameter for the occurance and degree of cellular aging. A progressive decrease in the ability of an organism to adapt to environmental conditions (presence of an inducing substance) with age has been described in many systems (Ratha and Kanungo, 1977; Correll et al., 1965; Gregerman, 1959; Adelman, 1970) including Aspergillus ornatus (Spiegelman and Coursen, 1975). However, the level of the aging effect has not been established. Individual steps in enzyme induction include permeation of the inducing substance into the cells, effector conversion into the appropriate form if necessary, interaction of the effector with the receptor on the genome, transcription of mRNA, in eukaryotic organisms--post-transcriptional processing of mRNA and transfer of mRNA to cyto-

plasmic ribosomes, translation of enzyme protein and final modification of the protein such as polymerization (Yagil, 1975). Higher systems have another level for regulation of enzyme induction, and therefore another site for an aging effect--that of hormonal interactions. In aging rat liver, the increased time necessary for glucokinase levels to achieve full activity can be overcome with increasing levels of insulin indicating that faulty hormonal regulation may be at the base of the aging effect (Adelman, 1970). However, in salivary gland tissue of the same aged rats, tissues which are not subject to any obvious hormonal effects, incorporation of ^{3}H-thymidine into newly synthesizing DNA is at a much slower rate than in younger rats. The rate decrease is linearly proportional to the age of the animals (Adelman, 1972). In A. ornatus, the presence of cyclic AMP, a possible intercellular hormone, has been proven and also shown to decrease with age but the implications of this fact related to decreased enzyme induction are not clear (Unger, 1975).

The presence and action of the inducing substance is obviously fundamental to the induction process. In A. ornatus, logrithmically increasing concentrations of tryptophan in older mats increased the specific activity of OPCA carboxylyase linearly (Figure 3). But in younger mats, increasing the concentration beyond the lowest activating dose tested had no significant effect. The data indicates the possibility that the enzyme synthesizing system is saturated in younger mats at an inducer concentration 10 fold less than that necessary for older mats. The concentration of inducer necessary for a constant rate of enzyme activity is dependent on the concentration of the enzyme itself, or, on the rate constant. In addition, the concentration of inducer

needed for activation of a constant rate of protein synthesis is dependent on the frequency of translation of the protein and the concentration of active mRNA from translation (Yagil, 1975). Changes in any of these factors could explain the need for increased inducer (substrate) concentration in the aged mycelial mat. Indeed, the concentration of mRNA has been postulated to be the rate limiting component for induced enzyme synthesis in at least one bacterial system (Marr and Marcus, 1962).

Exposing mats to a given level of tryptophan (0.10%, the lowest activating level tested) for increasing lengths of time results in increasing levels of enzyme activity, the 10 day mats reaching a peak 4 hours after the 7 day mats. The rate of change of enzyme activity, while constant in 10 day mats, increases in the younger organism. Again, the rate of enzyme activity is dependent on inducer, enzyme and mRNA concentrations and the frequency of translation. The phenomenon of sluggish enzyme activity in older mats, (overcome by increasing reactant concentration) has been reported by other authors dealing with enzyme induction (Adelman, 1970, 1972; Lee, 1956).

The separation of the minimum 6 or 10 hour induction time, depending on the age of the mats, into, for example, a 60 minute induction time followed by 5 hours of incubation (Figure 5), suggests a sequential induction phenomenon. This suggestion is strengthened by induction of full levels of OPCA carboxylyase with one hour exposure to OPCA (see Fig. 6). Sequential induction is defined as the progressive induction of enzymes in a catabolic pathway each enzyme of which is induced solely by the preceeding product (Ornston, 1970). Sequential induction provides for economy of newly synthesized protein but is not often

seen in its extreme form. More than one enzyme may be induced by the same metabolite-coincident induction, or an enzyme may be induced by a metabolite several steps in advance of the creation of the primary substrate. In Neurospora, tryptophan, kynurenine, and n-formyl kynurenine all induce kynureninase within 10 minutes of exposure-coincident induction. However, through studies with various mutants and inhibitors, N-formyl kynurenine, the metabolite directly preceding kynurenine, was discovered to be the inducer of physiological significance (Turner at al., 1970). Strict sequential induction is probably not the phenomenon observed in A. ornatus, either. What has limited our ability to detect OPCA carboxylyase induction by tryptophan in under 4 hours is probably the small enzyme activity involved.

It is conceivable, since sequential induction is probably a large component of the overall process, that the age related effect lies entirely in the formation of the primary metabolite, OPCA. However, not only is the overall time necessary for full enzymatic expression greater in the ten day mat, the actual induction component of that time is three times as great as in the 7 day mat. This indicates the age, or pH, effect is on the induction process as well as on the time required for tryptophan catabolism to OPCA.

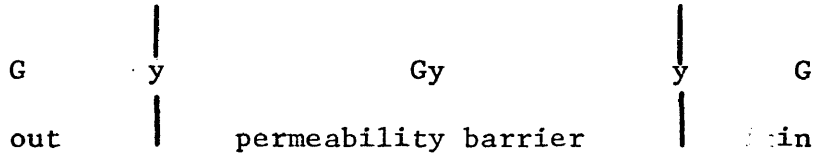
An obvious question concerning the differential tryptophan concentration required for maximum enzyme activity is whether or not uptake into the mycelium is different between 7 and 10 day mats. Amino acid transport into fungal systems has been studied by several workers in Penicillium griseofulvum (Whitaker and Morton, 1971), Neurospora crassa (Zalokar, 1969; Pall, 1968; Lester, 1965; Wily and Matchett, 1966, 1967; Matchett et al., 1967), Penicillium chrysogenum (Benko et al.,

1967) and in Claviceps sp. (Robertson et al., 1972). In general, amino acid uptake into vegetative mycelium or germinated conidia is dependent on some transport system, either active or facilitative. Specifically, tryptophan transport across the cell membrane of Neurospora has been shown to be mediated by an energy dependent, stereo specific transport system, the characteristics of specificity being an uncharged side chain and an α amino group next to a carboxyl (Wiley and Matchett, 1966). The system is under genetic control and the gene has been indentified Lester, 1965). Pall (1968) has described two transport systems for Neurospora; Transport System I which corresponds to previously reported data and, Transport System II which appears to have all its activity in older, carbon starved cultures apparently acting in a scavenger capacity.

Most of the Neurospora work, with the exception of that of Pall (1968) has been performed on germinating conidia with excellent agreement among authors of a Km for the tryptophan uptake system of about 50 μM. However, in our work, the approximate Km based on uptake into the TCA soluble fraction (Whitaker and Morton, 1971) is 5000 μM. The 100 fold differnece in these figures quite possibly is due to the difference in experimental conditions and medium. In our work, relatively thick mats of one piece were laid on liquid medium so that only the underside of the mat was exposed to tryptophan. In further justification of such a high Km, other fungal systems have values reported from 1000 μM (Benko et al., 1967) to 2000 μM (Whitaker and Morton, 1971).

The leakage observed from mycelial mats after 60 minutes exposure to ^{14}C-labeled tryptophan is consistent with other reported transport systems. Rate of ^{14}C entry is apparently at equilibrium with rate of

^{14}C exit after 60 minutes exposure to labeled tryptophan (Figure 8, inset 2) and slow exchange of the label into unlabeled medium after that time is possibly simple exchange of labeled for unlabeled amino acid or a result of internal concentrations being greater than the selective permeability barrier created by the permease system can maintain. This latter possibility is described by:



(from Cohen and Monod, 1957)

Carbon-labeled leucine into Penicillium griseofulvum also shows this appearance of label into the external medium after removal of the organism from the radioactive source (Whitaker and Morton, 1971). These authors attributed the observed slow leakage in the leucine system after equilibration to a comparatively weak binding of the amino acid to hypothetical binding sites or the existence of an osmotically sensitive expandable free amino acid pool.

Whatever the actual mechanism for tryptophan uptake in A. ornatus, exogenously supplied tryptophan undoubtedly accumulates into various cellular fractions of the mycelium and becomes available for metabolism. Early work of Zalokar (1960) on the kinetics of amino acid uptake in Neurospora indicated the existence of two metabolic pools of exogenously supplied amino acids. Transported amino acids enter an intermediate pool, are diverted to an expandable pool and proceed into areas of protein synthesis. Later work with Neurospora confirms the existence of at least one TCA soluble free amino acid pool from which exogenously supplied tryptophan is drawn into the cellular machinery. These workers

(Matchett and DeMoss, 1963) stated, on the basis of their work with mutants enabling them to distinguish between exogenously and biosynthetically synthesized tryptophan, that originally external tryptophan from a TCA soluble pool is selectively oxidized by the trypotophan catabolic cycle while biosynthetically generated tryptophan is used preferentially for protein synthesis. It is also the internal free amino acid pool that regulates the uptake system by repression/depression of the labile protein elements of the transport system (Matchett et al., 1967), and the induction of tryptophan catabolic enzymes (Matchett and DeMoss, 1963).

Since it is the free amino acid pool (TCA soluble fraction) that regulates uptake and contains the effectual concentration of inducer for tryptophan catabolic enzymes in Neurospora, it is reasonable to postulate a direct link between tryptophan uptake and subsequent OPCA carboxylyase induction in A. ornatus (see Hutter and DeMoss, 1967). Uptake into the TCA soluble pool is also a logical site for a possible age related effect.

Several authors, working with alkaloid production in Claviceps spp., have reported a correlation of internal substrate/inducer (tryptophan) concentrations and alkaloid biosynthesis (Krupinski et al., 1975; Robertson et al., 1972; Bu'lock and Barr, 1968). One worker quantified internal tryptophan concentrations and determined an actual critical concentration of internal TCA soluble tryptophan necessary for initiation of alkaloid production. (Řeháček et al., 1970). However, for OPCA carboxylyase activity in aged mats of A. ornatus, the correlation of internal tryptophan levels and enzyme induction is not direct. The reported Km for the enzyme is .30 mM (Subba Rao, 1967) but, in our

system, the enzyme is not induced at internal tryptophan concentrations below 6.0 mM. In addition, no differential uptake of tryptophan into whole mats or the TCA soluble pool was noted to correlate with the dramatic decrease in enzyme induction with age. Tryptophan is apparently entering the cell at a rate independent of the age of the mycelium but the ability of the cell to utilize the substrate is impaired. It is interesting that the only difference with age found in any of the label incorporation fractions was in the TCA insoluble pool (Table 1), that of newly synthesized insoluble proteins. Based on the method of incorporation measurement, it is possible disks of the same radius from older mats are thicker and therefore provide for greater sample quenching of counts but, the fresh weights of the disks between ages are not significantly different. This is true even though the total dry weight of a 10 day mat is greater than a 7 day mat probably because mycelial material for disks was taken from a flat area of the mat, (not convoluted), and therefore is an area of the mat that on the average, would weigh less than a disk of equal radius from a convoluted portion of the mat.

Impairment of the cellular protein synthesizing machinery with age has been previously reported (Dickmann and Gordon, 1975; Goswami, 1977) and is a phenomenon which could seriously disrupt all cellular functions including that of enzyme induction. A decreased rate of <u>de novo</u> enzyme synthesis with age in response to standard doses of inducer would explain the response of the enzyme to increasing concentrations of tryptophan and induction times. It is also interesting that the only concentrations of tryptophan tested that promoted equal enzyme activity in the differently aged mats (1.0%) is the only concentration

that promotes euqal trypotophan incorporation into protein. Unfortunately, the degree of protein synthesis depression in older mats (23% with 0.10% tryptophan) is not great enough to account for the closer to 50% decrease in specific activity between 7 and 10 day mats.

This data however, while interesting, can not be used as solid evidence for an explanation of age related depressed enzyme activity in A. ornatus. Tryptophan incorporation into mycelial proteins is not necessarily related to tryptophan incorporation into de novo OPCA carboxylyase synthesis. In addition, the experiment only measures incorporation of tryptophan from an exogenous supply into mycelial protein. It is conceivable that with age, a shift to a greater dependence of tryptophan from biosynthetic supplies for protein synthesis is occuring with an end result of equal protein output between ages not measurable in this experiment.

The overall conclusions therefore, from the uptake studies on aging mats is that the transport system is not impaired with age but that the cells' ability to catabolize internal substrate material is--possibly as a result of depressed de novo protein synthesis. Although most reports indicate impairment of the transport system with age (Zalokar, 1960; Matchett et al., 1967; Robertson et al., 1972; Langheinrich and Ring, 1976) at least one other (Bertrand et al., 1975), does not.

An environmental factor that can have a major effect on the enzyme induction process is pH, which might modify the induction itself, the synthesis of the enzyme or on the catalytic activity of the enzyme. The pH drop of 2 units in the defined growth medium in our system could be producing effects interfering with aging phenomena.

The pH of the growth medium can at least be partially controlled

by the addition of 50mM citrate buffer. Growth of the fungus is morphologically unchanged and the photosporulation control maintained. The fungus does not appear to be utilizing the citrate as a carbon source, at least, glucose utilization proceeds at a rate comparable to the non-citrate system (see Figure 9).

However, biochemical changes do appear to result from the buffer addition, in particular the specific activity of OPCA carboxylyase with age. Citrate, whether as a chemical or a buffer affects enzyme induction in 50 ml systems such that the activity of a 10 day mat is equal to that of a 7 day mat. It is possible that this fact indicates environmental pH is completely responsible for the decreased enzyme response in this organism over time, or it is possible that controlling the pH shifts the rate of the aging effect so that it takes longer to achieve a measurable significant difference in activity levels. Unfortunately, fungal mats of A. ornatus can not be allowed to grow more than 240 hours on a 50 ml petri dish as the glucose supply is limiting. But, by spraying spores on membrane covered 400 ml glass culture dishes of citrate buffered defined medium, mats grow normally and can be maintained for 336 hours (14 days) at which time 25g/l glucose still remain in the medium. The results of testing enzyme levels in this and the petri dish system are shown in Figure 10 and indicate that an aging effect on enzyme induction is present independent of a pH effect. Possibly the observed result of high levels of H^+ in the medium enhances whatever mechanism results in the age related effect or mimics that mechanism.

If acidic environments amplify the aging effect, it would be helpful to know at which level of cellular function in enzyme induction low

external pH has the most effect. Table 2, a compilation of pH data related to enzyme induction as measured, will be used in making this determination. In reading the notations beside entries in the table as to whether or not pH and/or enzyme induction changes occurred with age, it can be seen the only events of a lowered pH and a correspondingly lowered level of enzyme induction are the changes that occur in the growth medium prior to induction, and the changes in non-citrate induction medium during the 6 hour mat exposure time. However, in using citrate buffered induction medium, the pH does not change but enzyme induction of a mat grown on non-citrate medium decreases typically with age. Therefore, it can be concluded that a pH change during the actual induction process is incidental to the enzyme activity. What remains is the apparent impairment of cellular function in the presence of high external H^+ ion concentrations that result in lessened enzyme activity upon induction.

Apparently (by measuring pH of crude fungal homogenate ground in water) internal pH does not change either with age or as a result of high external concentrations of H^+. It is not unusual for living organisms to have homeostatic mechanisms for maintaining internal pH values; in Valonia, changes in the external pH have no effect on internal levels short of using such an extreme level so as to injure the cell (Hoagland and Davis, 1923). In N. crassa the exact relationship between internal and external pH values is not known (Slayman, 1965). In further support of a change in internal pH not being responsible for the observed decrease in specific activity, the pH of the cell free enzyme reaction mixture was dropped to equal that of the growth medium at the time the mat was sacrificed. Theoretically, this

could have produced "old" enzyme activity in young mats or could have pointed out a change in pH optimum with age. However, no activity at all was detected; the pH levels used were apparently too low to support enzymatic activity.

If the consequence of the pH phenomenon becomes evident prior to actual induction, then what is that effect as related to enzyme induction? In yeast, a H^+-K^+ cation exchange has been described. Evidently the organism extrudes succinic and carbonic acids in exchange for K^+ uptake into the cell (Conway and Brady, 1949). Eventually, however, the external pH reaches such a low level as to depress K^+ influx and enhance efflux into a K^+ free medium (Rothstein and Bruce, 1958). Low levels of intercellular K^+ in E. coli result in disturbances of cellular functions such as protein synthesis, possibly at the level of transfer of charged amino acids to the growing peptide chain (Lubin and Ennis, 1964). Also in E. coli, the intercellular K^+ and Na^+ concentrations have been shown to be a function of external cation concentrations, including H^+, and the age of the culture. As metabolic products accumulate and the external pH drops through time, K^+ leaks more rapidly from the cell in apparent exchange with external Na^+, normally kept at low internal concentrations (Schultz and Solomon, 1961). In Neurospora, as the external p_H drops, the internal negative potential increases resulting in a more rapid K^+ leak into the medium.

If a cation exchange or pump is affected by low external pH in Aspergillus, one might expect (as a consequence of changing cation levels) amino acid transport systems to be affected. Generally, such systems depend on a Na^+ exchange (Meister, 1973) but at least one tryptophan transport system has been shown to be independent of exter-

nal Na^+ concentrations (Grahame-Smith and Parfitt, 1970).

Perhaps internal cation levels as directed by external pH are responsbile for cellular impairment prior to the induction process. A cell has a certain response mechanism to environmental conditions, including those of cation concentrations. Possibly a young cell would respond to an external pH of 2.9 in the same fashion as an old cell. But more likely, there is a degree of adaptation involved and in young mats, the ability to adapt to a harsh environment is still greater than that of an old mat. It is known the cation exchange phenomenon is energy dependent. In a "less energetic," older organism, unfavorable internal/external cation ratios could result more easily in response to environmental conditions than in "vigorous," younger organisms. In our system, even though the pH in external medium does continue to fall an additional mean of .4 units from 7 to 10 days, that drop is probably not significant. In a single spray series, the drop has been as low as .2 units yet the resultant decrease in enzyme induction with age is typical of other spray series. Therefore, the significant pH difference is more likely from 4.8 to the 3.3-2.9 range and the response of an older mat, exposed three days longer to these conditions may be more impaired than the response of the younger mat to the same pH.

In controlling the pH of the growth medium, the adaptive response of the cell to inducer is freed of the normally concomitant response to external H^+ ion concentrations; consequently a measurement can be made of the one function. Eventually, under constant pH conditions, the cellular machinery is impaired regardless. Whether the age-related disfunction in enzyme induction is a result of the same

mechanical reasons as when the disfunction was pH related or is a mimic of that reason is not known.

In conclusion, as a consequence of age and pH the degree to which OPCA carboxylyase in Aspergillus ornatus can be induced is diminished. Under increasing concentrations of inducer and time of exposure to that inducer, the enzyme level of an old mat, even at a low environmental pH, can be raised to the level of the younger mat. This data suggests the enzyme induction system is acting more slowly, possibly as a result of a quantitative or qualitative change in the enzyme itself or any of the components of the protein synthesizing system. Indeed, labeled tryptophan incorporation into protein (which decreases with age) suggests depressed enzyme synthesis might be responsible for the aging effect. However, the apparent degree of depression of protein synthesis is not great enough to fully account for the 50% decrease in enzyme activity with age.

A drop in external pH produces effects in the enzyme induction process which enhance or mimic age-related effects. High external H^+ concentrations in other systems result in a cation imbalance and ultimately in cellular disfunction. This disfunction is conceivably at the protein systhesis level as discussed previously.

The age-related depression of enzyme induction is not seen at the level of tryptophan regulation of catabolic enzyme induction; that is, the regulatory pools of tryptophan are equal between ages. However, the response of the system to standard regulatory doses of inducer is impaired.

Other considerations to be made concerning the level of the age-pH consequence on enzyme induciton in A. ornatus are, (1) the concen-

tration of repressor molecule--the rate of enzyme synthesis varies inversely to the first power of repressor concentration (Sadler and Novick, 1964) and, (2) the possibility that increasing cell death within the theoretically homogenous fungal mat is resulting in an average decrease in activity. That is, living cells are responding fully and dead cells, not at all (Novick and Weiner, 1957).

This study does not provide hard evidence for any of the general theories of cellular aging described in the introduction. It does support those theories dealing with quantitative or qualitative changes in protein synthesis, for whatever mechanical reason, and further confirms the postulate that aging organisms have a decreased ability to respond and adapt to such environmental demands as external pH or changing conditions which normally induce adaptive enzyme response.

Appendix I

Composition of Defined Medium (from Schwemmin, 1960)

tyrosine	0.2 gm
KH_2PO_4	1.0 gm
$MgSO_4 \cdot 7H_2O$	0.5 gm
NaCl	0.1 gm
$CaCl_2$	0.1 gm
$(NH_4)_2$ tartrate	5.0 gm
glucose	40.0 gm
trace element solution	1.0 ml
agar	15.0 gm
double-distilled H_2O	1000.0 ml

Composition of Trace Element Solution

$Na_2B_4O_7 \cdot 10\ H_2O$	88 mg
$CuSO_4 \cdot 5H_2O$	393 mg
$FeSO_4 \cdot 6H_2O$	910 mg
$MnCl_2 \cdot 4H_2O$	72 mg
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	37 mg
$ZnSO_4 \cdot 7H_2O$	8800 mg
double-distilled H_2O	1000 mg

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