THE CITRIC ACID FERMENTATION BY *ASPERGILLUS NIGER*: REGULATION OF GROWTH, CELL ADHESION, AND CITRIC ACID ACCUMULATION BY ZINC AND ADENOSINE 3', 5'-CYCLIC MONOPHOSPHATE

by

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To My Wife, My Parents, and My In-Laws

ABSTRACT

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The citric acid fermentation by Aspergillus niger was studied in submerged shake flask cultures with a minimal salts medium and sucrose as carbon source. The object of the investigation was to elucidate both the general biology of the fungus under these experimental conditions, as well as the physiological mechanism of citric acid accumulation. Although high sucrose (14%) concentrations were most conducive to acidogenesis, low sucrose (0.8%) was employed here as this condition was considered to represent a more "natural" state than did high sucrose.

The flasks were inoculated with unclumped conidia which then germinated into proliferating hyphae. Concomitant with germination and hyphal extension was a continuous process of aggregation of conidia and germlings, so that the eventual mode of growth was in the form of aggregates of hyphae, termed pellets. The degree of aggregation was interpreted to reflect the adhesiveness of the cells. Cell-cell interactions are probably important in the control of growth and differentiation in the fungal thallus, so it is considered that the regulation of cell adhesiveness is an important aspect in the development of the organism. Clumping reduced the number of conidia which germinated, which suggests that clumping may also be a mechanism of autoinhibition of germination.

Cyclic AMP, GMP, glucagon, Fe^{+3}, and EDTA enhanced conidia clumping, while L-epinephrine, concanavalin A, methanol, and snake venom inhibited. The effect of snake venom was very complicated, and depending upon the experimental conditions it either increased

or decreased the number of pellets actually formed. Zinc antagonized the stimulation of conidia clumping by cyclic AMP and EDTA. Zinc also increased the adhesiveness of vegetative hyphae, while Fe^{+3}, Mn^{+2}, and Ca^{+2} were comparatively ineffective. ADP had no influence on conidia aggregation, but it increased the adhesiveness of hyphae. Under certain physiological conditions (see below) cyclic AMP decreased the adhesiveness of the hyphae. The implications of these results in the regulation of cell adhesion are discussed.

The citrate fermentation was characterized by biphasic dynamics, there being a growth phase, or trophophase, and an acidogenic phase, or idiophase. The two phases are considered on the basis of the results to represent distinct, alternate differentiated states. The trophophase-idiophase condition was controlled by the concentration of the zinc ion. At high zinc concentrations the cultures were maintained in the trophophase and citric acid did not accumulate, but at low zinc concentrations the cultures responded to zinc deficiency and differentiated into the acidogenic idiophase. Iron, manganese, and calcium were without significant effect.

"Handling" the pellets in a certain way (agglomerating) or treating them with snake venom either maintained the cultures in the trophophase or caused the reversion of the idiophase cultures back to the trophophase. This result is interpreted as being a manifestation of "neoplastic transformation" of the fungus cells. ADP similarly favored the trophophase over the idiophase. Since this putative transformation occurred under the normally acidogenic low zinc conditions, it is considered that the function of zinc in the fermentation is regulatory, rather than permissive.

Concanavalin A inhibited aggregation and stimulated both growth and citrate production. Phytohaemagglutinin did not affect aggregation but did enhance growth and acidogenesis. These findings suggest that sugar moieties on the cell exterior play a role in the control of growth and development of this fungus.

Cyclic AMP had a significant influence on the rate of growth and the adhesiveness of the cells, and on the rate of citrate accumulation. However, the response of the mold to cyclic AMP varied with the phase in the fermentation. In the trophophase added cyclic AMP enhanced growth and adhesion, and proliferating cells contained high endogenous levels of the nucleotide. But after the transition to the idiophase was initiated added cyclic AMP reduced the growth and adhesion, and once the full idiophase was realized added cyclic AMP stimulated acidogenesis. Thus, cyclic AMP apparently serves to amplify all the physiological phenomena which are characteristic of the phase the cells are in, regardless of whether it is the trophophase or the idiophase. Since the phase "decision" was controlled by the concentration of the zinc ion, it is contended that cyclic AMP may be the agent of zinc in affecting the physiology of the organism.

EDTA and to a lesser extent ferrocyanide simulated many of the effects of cyclic AMP, hinting that the mechanism of action of cyclic AMP may be linked to the metabolism of cations, probably zinc. Evidence is presented which argues that the sites which control acidogenesis reside in zinc ion receptors located on the cell exterior. Cultures in the acidogenic idiophase contained low intracellular, but comparatively high extracellular levels of cyclic AMP. It is therefore suggested that

in the acidogenic idiophase it is the exogenous cyclic AMP which is significant in affecting the physiology, and that this may be accomplished by the nucleotide interfering in some way with the metabolism of zinc at these putative control loci on the cell surface. That is, in effect, zinc is the "agent" of cyclic AMP in affecting physiology in the acidogenic idiophase.

It is postulated, then, that zinc and cyclic AMP may be partners in the general regulation of growth and development of this organism, and in particular the regulation of citric acid accumulation. In the trophophase intracellular cyclic AMP may be the thallogenic agent of zinc, while in the idiophase zinc ions (located on control loci on the cell exterior) may be the acidogenic agents of cyclic AMP. Based on that assumption it is proposed that the characteristic of citric acid accumulation evolved because of the ability of the acid to solubilize zinc and other minerals from biologically unavailable complexes in the soil. A scheme is described which explains how the fungus may be able to control its own supply of inorganic nutrients by using zinc and cyclic AMP to regulate growth and acidogenesis.

This organism was found to possess several of the enzymes pertaining to cyclic AMP metabolism. The presence of adenyl cyclase and two cyclic AMP binding proteins is shown, as well as two apparently distinct phosphodiesterases, one intracellular (I-PDE) and the other extracellular (E-PDE). The I-PDE had a Km for cyclic AMP of 2.5 mM, a pH optimum of 7.5, did not require but was activated by magnesium ions, and was inhibited by EDTA. The E-PDE had a Km for cyclic AMP of 18 μM, and a pH optimum of 3.5 in either citrate or acetate buffer. There was

no activity at pH 7.5. Preliminary evidence suggests that the activity of the E-PDE may be regulated by an endogenous inhibitor.

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GLOSSARY OF TERMS

Adhesion - refers to the ability of cells (conidia and hyphae) to adhere to each other or to some foreign surface, with no implication intended as to the mechanisms by which this is effected.

Conidia-Cyclic AMP Transition Point (C-CTP) - the number of inoculating conidia which, at a specific time, defined the response of the organism to exogenous cyclic AMP. At conidia densities greater than the C-CTP cyclic AMP inhibited growth, and at conidia densities less than the C-CTP cyclic AMP enhanced growth.

Differentiation - the sum of processes by which the specific acquisition of specific metabolic consequences (or loss thereof) distinguishes daughter cells from each other or from the parental cells (Smith and Galbraith, 1971).

Fermentation - is used in a colloquial sense to refer to the microbiological production of secondary metabolites, including citric acid. There is no implication of anaerobiasis intended.

Filamentous Growth - hyphae individually dispersed throughout the medium.

Idiophase - the phase in the fermentation in which secondary metabolites are synthesized. Cell proliferation does not occur (Bu'Lock et al., 1965; Bu'Lock, 1967).

Pellet Growth - a globose aggregate of hyphae.

Primary Metabolites - those metabolites directly involved in catabolism and anabolism.

Respiration - the oxidation of primary metabolites, with oxygen as the terminal electron acceptor.

Secondary Metabolism - that aspect of metabolism dealing with the synthesis and degradation of secondary metabolites.

Secondary Metabolites - the term was first proposed by Bu'Lock (1961) and is defined by Weinberg (1970) as "natural products that have a restricted taxonomic distribution, possess no obvious function in cell growth, and are synthesized by cells that have stopped dividing".

Specific Activity (of enzymes) - see Methods.

Specific Activity (of mycelia in terms of their acidogenic ability) - μmoles citrate produced per gram dry weight of mycelia.

Time-Cyclic AMP Transition Point (T-CTP) - the time in the citrate fermentation at which, at a specified conidia concentration, the response of the mycelia to added cyclic AMP changed. In the period prior to the T-CTP cyclic AMP enhanced growth, but in the period following the T-CTP cyclic AMP inhibited growth.

Trophophase - the phase in a fermentation in which cell proliferation takes place. Nutrients are assimilated in a more or less constant ratio, and secondary metabolism does not occur (Bu'Lock et al., 1965; Bu'Lock, 1967).

ABBREVIATIONS

ADP - adenosine 5' diphosphate

AMP - adenosine 5' monophosphate

ATP - adenosine 5' triphosphate

C-CTP - Conidia-Cyclic AMP Transition Point

Con A - conconavalin A

cpm - radioactive counts per minute

CTP - Cyclic AMP Transition Point

Cyclic AMP (cAMP) - adenosine 3',5'-cyclic monophosphate

2',3'-cyclic AMP - adenosine 2',3'-cyclic monophosphate

Cyclic GMP (cGMP) - guanosine 3',5'-cyclic monophosphate

DB-cyclic AMP - N^6-2^1-O-dibutyryl adenosine monophosphate

dpm - radioactive disintegrations per minute

DTT - dithiothreotol

E-PDE - extracellular phosphodiesterase

GDP - guanosine 5' diphosphate

GMP - guanosine 5' monophosphate

GTP - guanosine 5' triphosphate

I-PDE - intracellular phosphodiesterase

PHA - phytohaemagglutinin

SV - snake venom

T-CTP - Time-Cyclic AMP Transition Point

TLC - thin layer chromatography

INTRODUCTION

Introduction

Industrial microbiology is considered to have originated in ancient times with the production of fermented beverages and foodstuffs, but until the time of Pasteur, there was no understanding as to the nature of the fermentation process. In 1856 Pasteur was engaged by the French wine industry to investigate the souring of wines, and in the subsequent studies he and his students discovered that fermentation was a characteristic of living organisms, and that "spontaneous generation" did not occur (Thinman, 1971). These studies were extended to many different bacteria and filamentous fungi, and in the process established that many different and potentially useful products were excreted into the medium by these organisms. The development of the chemical and food industries at around the turn of the century created a demand for many of these products, and this led to the industrial production of ethanol and lactic acid (Hastings, 1971). World War I saw the development of the yeast-glycerol fermentation, and the acetone-butanol fermentation by Clostridia, while Fleming's discovery of penicillin in 1928, coupled by the demand generated by World War II created the antibiotic industry. Continued research on these subjects has disclosed a host of useful microbial transformation products, and engineering advances have allowed their large scale and economical production (Prescott and Dunn, 1959; Rainbow and Rose, 1963; Perlman, 1969; Turner, 1971). Finally, the world today is faced with an expanding population, a limited agricultural potential, and an increasingly polluted environment, and may yet have to look to

industrial microbiology for the production of food.

The citric acid fermentation played an important role in many of these developments, and it has the reputation of being one of the most important and successful achievements in the field of industrial microbiology. In 1922, about 90% of the world supply of citrate was obtained from citrus fruits (Prescott and Dunn, 1959), but now almost all is obtained from Aspergillus niger fermentation of cheap sugar sources, and the total dollar value of the citric acid business is in the range of $100 million (Lockwood and Schweiger, 1967). Studies on the citrate fermentation by A. niger began as early as 1893, and as they were often of a pioneer nature they have contributed greatly to the general understanding not only of mycological fermentations, but also of the physiology of fungi. Furthermore, the excellent success obtained served to firmly establish the economical feasibility of mycological fermentations, thereby providing a firm base for the entire industry. For example, when the great and immediate need for penicillin arose in World War II, this problem was tackled with confidence and skill arising from knowledge pertaining to the already well established citrate fermentation.

In spite of these achievements, however, neither the cause, nor the reason for citrate accumulation is known. Most investigations have centered on establishing the optimum conditions for production in laboratories, and the translation of these results to an industrial scale. Other studies have been concerned with the metabolic origin of citrate; most of these took place before the

elucidation of the glycolytic pathways and the tricarboxylic acid (TCA) cycle. Less information is available on the physiological causes of citrate accumulation, probably because a successful fermentation had been achieved before the science of biology had advanced sufficiently to tackle this matter realistically. There is a large literature available on the citric acid fermentation, which has been reviewed by Von Loescke (1945), Foster (1949), Johnson (1954), Cochrane (1958), Prescott and Dunn (1959), Perlman and Sih (1960), Martin (1963), Lockwood and Schweiger (1967), and Rehm (1967).

Filamentous fungi are well known for their ability to synthesize a wide variety of secondary metabolites (see Glossary), many of which are of large commercial and theoretical interest (Turner, 1971; Weinberg, 1970; Perlman, 1969). Citric acid is normally a primary metabolite, but under certain conditions, it is excreted in large amounts into the growth medium, and as such becomes a secondary metabolite. Many different fungi possess this ability, but only certain strains of A. niger have been used commercially (Prescott and Dunn, 1959).

The mechanism of extracellular citric acid accumulation by A. niger is a problem which has intrigued investigators for many years. Some of the factors which favor production are: high-yielding strains, some of which have been obtained from lower-yielding soil isolates by mutation; low trace metal levels produced by chelating agents or by the use of pure water and chemicals, as well as a proper trace metal balance; high oxygen tension during the later part of

the fermentation; high sucrose and low nitrogen and phosphate levels; a low pH, as oxalate and gluconate may be produced under more alkaline conditions; a small, hard, smooth, and very compact type of pellet structure; and highly vacuolated, and somewhat distorted hyphae accompanied by the absence of reproductive structures (Perlman and Sih, 1960). Low molecular weight alcohols also enhance production, and these seem to render the mycelia more tolerant to otherwise inhibitory concentrations of trace metals (Moyer, 1953). Of course, a certain minimal amount of trace metals are required to achieve sufficient mycelia mass to synthesize any acid at all, but excess ions favor continued growth at the expense of acidogenesis. The fermentation is a two-stage process, with an initial period of active growth without accumulation followed by a stationary growth phase when accumulation occurs (Shu and Johnson, 1948a). As changes in the factors which favor accumulation will greatly affect the yield, the response is determined by environmental as well as genetic information. Citrate production takes place under unfavorable growth conditions, and it has been assumed that it results from abnormalities in the metabolism of the mold.

The acid is synthesized from glucose via the Embden-Meyerhof-Parnas (EMP) pathway, pyruvic dehydrogenase, and citrate synthase, with oxaloacetate being formed by pyruvate carboxylase, and under normal conditions the TCA cycle is presumed to operate (Meyrath, 1967; Ahmed et al., 1972). Abnormalities in the TCA cycle or respiratory chain caused by mutations or trace metal deficiencies should result in improper operation of the respiratory mechanism. Then active

citrate production caused by high sucrose levels in mycelia with reduced citrate oxidation capacity might result in the pile up of citrate in the cells and the subsequent spill over into the medium. The classical approach at a mechanism has been, therefore, to try to demonstrate lesions on the TCA cycle by comparing enzyme activities under accumulating and non-accumulating conditions. Ramakrishnan et al. (1955) and Usami et al. (1971) have demonstrated increased activities of citrate synthase and reduced activities of aconitase and isocitric dehydrogenase, which is the expected result, and which correlated properly with growth and citrate accumulation. However, other work has not corroborated this (La Nauze, 1966; Ahmed et al., 1972), and indeed mitochondria isolated from accumulating mycelia display respiratory control and can oxidize citrate as well as other TCA cycle intermediates (Ahmed et al., 1972). But aside from this important question it is clear that a mechanism must eventually explain at a more fundamental level how the enzyme patterns and physiological orientation change under accumulating and non-accumulating conditions.

One of the problems associated with the citrate fermentation as well as many other mycological fermentations, is that they do not appear to be subject to tightly coupled metabolic regulation as in other biological systems (Demain, 1968). Concepts developed with mammalian cells and Escherichia coli have implied that the glycolytic pathways should be inhibited by their end products. For example, it is well known that phosphofructokinase (PFK) is the pacemaker enzyme of the EMP pathway, and it is subject to strict inhibition by ATP

and citrate (Racker, 1965; Krebs, 1972). This control could possibly be lacking or at least "modified" in the A. niger citrate fermentation. The ubiquitous and omnipotent biological regulator adenosine 3',5'-cyclic monophosphate (cyclic AMP or cAMP) functions in some tissues as an activator of glycogenolysis and glycolysis (Robison et al., 1968); it overcomes the citrate and ATP inhibition of PFK in muscle and liver fluke (Monsour and Setlow, 1972). It was therefore reasoned that if citrate accumulation is a result of the loss of glycolytic control, then cyclic AMP may play a role in the process. Addition of cyclic AMP to the growth medium of citrate accumulating cultures by the author was rewarded with a large stimulation in the rate of citrate synthesis. This result hinted that the causal site for citrate accumulation may be associated with the regulation of PFK. However, further investigations into cyclic AMP physiology and recent advances in cyclic nucleotide research, have suggested that the effect of cyclic AMP on the mycelia may be considerably more profound.

It has been reasonably well established that cyclic AMP is involved in the regulation of growth and differentiation in at least some biological systems (see Schultz and Gratzner, 1973). It was discovered in this study that the nucleotide appears to play a similar role in A. niger. These developments shed new conceptual light on the citrate fermentation: whereas this phenomenon had previously been regarded to be a pathological condition, stemming from nutritional deficiencies and natural or artificially induced mutations, it now seems that perhaps it should be considered to be

a normal differentiated state, a natural and beneficial response to adverse environmental conditions, akin to sporulation. The crucial and puzzling role that trace metals play in this fermentation is now explicable in view of the accumulating evidence that calcium ions operate in a regulatory capacity in the growth and differentiation of several organisms (Whitfield, 1973; Cameron and LeJohn, 1972). There is evidence that iron, manganese, and zinc may also play a similar role in regulating secondary metabolism (differentiation) in bacteria, Bacillus sp., and fungi, respectively (Weinberg, 1970). There is also evidence that cyclic AMP and calcium metabolism are closely linked (Rasmussen, 1970; Whitfield, 1973), which raises the possibility that a similar connection may exist between cyclic AMP and these trace elements.

It is the purpose of this dissertation, therefore, to present evidence to support the view that the citrate fermentation is a "natural" differentiated state, which occurs in response to nutrient deficiencies. In these studies, the deficient nutrient was zinc; evidence and arguments are presented which assign to zinc a regulatory role in this organism, controlling growth and differentiation. It is shown that cyclic AMP is an important regulator of growth, aggregation, and perhaps differentiation. It is interpreted that cyclic AMP is not an initiator of information pertaining to these conditions; rather cyclic AMP operates as an amplifier of information. Cyclic AMP stimulates growth and aggregation under growth oriented conditions, where intracellular levels of the nucleotide are high and the extracellular levels are low. Cyclic AMP inhibits

growth and aggregation under conditions of zinc deficiency, and instead stimulates citrate accumulation, the alternative state to growth. In this case, intracellular cyclic AMP levels are low but extracellular levels are high. The regulatory signal governing the physiological response to cyclic AMP is the zinc ion. Preliminary evidence and arguments are presented which link cyclic AMP and zinc metabolism, and based on this tentative assumption a teleological explanation for citrate accumulation is presented which hypothesizes that citrate accumulation has positive evolutionary advantage because of the trace metal solubilizing properties of the acid.

HISTORICAL

THE DEVELOPMENT OF THE CITRATE FERMENTATION

The production of citric acid by fungi was discovered by Wehmer in 1893, and this is regarded to have introduced the first phase in the history of this subject. He named his organism Citromyces, but it is now believed to have been a member of the Penicillia (Raper and Thom, 1945). Because Wehmer apparently had designs on a commercial fermentation, some of his information pertaining to the best accumulating conditions is scanty. Nevertheless, several papers published between 1893 and 1912 (see Foster, 1949) established that high sugar concentration should be used, that a "small quantity of mineral salts" (NH_4NO_3, KH_2PO_4, and $MgSO_4$) could be added (Wehmer, 1894), and that neutralization with $CaCO_3$ was essential for high yields. Wehmer's earlier work on the oxalate fermentation (1891) had demonstrated that oxalate should be trapped by alkaline cations in order for production to continue. He therefore believed that $CaCO_3$ was necessary not only for neutralization of the acid, but also to "fix" the citrate and thereby prevent its re-utilization. Neutralization was later found to be detrimental to citrate production when A. niger was used, as this favored oxalate formation at the expense of citrate, and the citrate is not re-utilized as long as sucrose is present in the medium (Wang, 1940; Gardiner et al., 1956).

Wehmer was unable to establish a commercial fermentation, and economic considerations aside, he attributed this failure to the difficulty of selection of proper organisms, the degeneration of the organisms, and contamination problems (Wehmer, 1910). These, especially

the first two, have proved to be important characteristics of the fermentation. It is significant that neither Wehmer, nor the early workers who followed him discovered that A. niger accumulates citrate (Zahorsi, 1913; Thom and Currie, 1916), and in fact is much more prodigious in this respect than are the Penicillia. The black Aspergilli were considered at that time to be oxalate producers. In retrospect, it seems that this may have been due to high levels of trace metals in the medium used, as well as to the relatively alkaline conditions. Both these factors are now known to be detrimental to citric acid production by A. niger.

Wehmer (1891) also conducted the classical studies on the process of oxalate formation by fungi. These studies were important from the point of view of mycological biochemistry and physiology in that they established one of the most important characteristics of fungi: that the physiological response varies enormously with the cultural environment (Wehmer, 1893a,b). This concept illustrated the need for detailed systematic investigations of all mycological processes, and has proved to be, aside from strain selection, the most important aspect of the citrate fermentation.

The second phase of the history of the citrate fermentation was begun by Currie in a classical paper published in 1917. He discovered that certain strains of A. niger were capable of growing well in surface cultures at a low initial pH, and that under these conditions citrate accumulated. This disproved Wehmer's earlier assumption that neutralization was necessary, a view that had dominated the field at that time. Neutralization with $CaCO_3$ was probably

required for Wehmer's Penicillia, however, as these organisms are less tolerant to acid.

Currie's other major contribution was the recognition that the minor constituents of the media were important in the accumulation process, and that the media previously used were too complex. He defined a synthetic medium consisting of sucrose, NH_4NO_3, KH_2PO_4, and $MgSO_4$, and established optimum concentrations for each of these. The pH was adjusted to 3.4 with HCl, which he proved was a superior acid to any other for this purpose. This has been confirmed by others (Foster, 1949).

Shortly thereafter, Currie left the laboratories of the U.S. Department of Agriculture, where the work had been carried out, and moved to the Chas. Pfizer Co. Inc. in New York State. This company, probably using Currie's knowledge, introduced the first commercial process in the world in 1923 (Ward, 1970). Maze and Perrier (1904a,b) had earlier reported that the majority of citrate was produced by surface cultures of pennicillium after the growth was completed. Similar results were also obtained by Currie (1917) and later Doelger and Prescott (1934); these workers also found that the optimum concentrations of the medium constituents were such that growth was limited. Molliard (1922) proved that phosphate deficiency was one of the most important parameters. These preliminary studies firmly entrenched another requisite principle of the citrate fermentation: that citrate accumulation is incompatible with growth, and occurs only under conditions unfavorable to growth. Without this knowledge the industrial fermentation would not have been possible (Lockwood and Schweiger, 1967).

As has been true of all studies of the citrate fermentation, Currie was plagued with irreproducibility of results. This may have been due to his mistaken belief that his chemically defined medium was all that was required for fungal growth; as with Wehmer, he was unaware of the extreme importance of trace metals in influencing the course of fermentation. The conventional wisdom at this time was that trace metals were not required for growth (Pfeffer, 1895). There is no doubt that Currie's glassware, tap water, and chemicals were contaminated with varying amounts of trace metals.

It is a curious aspect of this subject that, excluding the trace metal function and the submerged fermentation technique, Currie's paper in 1917 established practically every important characteristic of the fermentation that is known today. As we shall see, the role of trace metals in fungal growth had been completely misinterpreted by the scientific community, so Currie can hardly be blamed for missing the point. Later advances depended upon the elucidation of the role of ions, and the advancement of engineering techniques for the achievement of submerged processes.

By the decade of the 1930's the citric acid fermentation had become a relatively stable business, although great skill was required to effect large yields. However, all the commercial and laboratory fermentations were carried out in shallow pans with surface cultures, and this method had great influence on the industry. Hastings (1971) states that although the surface culture method was very good for the production of citrate, it was "a sad misfortune in another direction, because it colored the thinking of those who were

about to start on fermentation's greatest adventure, the production of penicillin". Penicillin is produced in small amounts in surface cultures, but the yields are very much higher in submerged cultures. Kluyver and Perquin (1933a) appear to have been the first to conduct submerged fermentations using agitation, and this method was employed in the kojic acid fermentation by Aspergillus flavus (Kluyver and Perquin, 1933b). A group at the U.S. Department of Agriculture used this technique and was influential in introducing the concept of change from surface to submerged fermentations (Ward, 1972). In the late 1930's two stirred aluminum fermentors, equipped with air spargers, were installed at the Color and Farm Waste Division of the Bureau of Chemistry and Soils, on a site where the Pentagon now stands. Gluconate was produced by submerged fermentation (bubbled air) (May et al., 1934), and later a 140 gallon rotary fermentor was established at Ames, Iowa (Wells et al., 1937). A submerged fermentation for lactate by Rhizopus was described (Ward et al., 1938), and this is believed to be the basis for commercial production by Miles Laboratories. Finally, in collaboration with Florey and Chain of the British Government (Hastings, 1971), a submerged procedure for penicillin production was initiated, and after 1943 essentially all the antibiotic was produced in this way.

Submerged procedures now dominate industrial microbiology, and fermentors of 50,000 gallons capacity, equipped with agitation and aeration devices are employed (Blakebrough, 1967; Lockwood and Schweiger, 1967; Aiba et al., 1965). The advantage of the submerged culture technique is that it is much more rapid, much larger volumes may be used, and there is lower labor cost, simpler operation, and

less problems of contamination. From a scholastic point of view, this procedure lends itself more easily to the study of physiology, because the mycelia are more homogeneous in nature, especially when the growth is in the filamentous form (Smith and Galbraith, 1971).

Continuous cultures are not yet used to any great extent in mycological fermentations, partly because of the problem of maintaining the culture in the proper physiological state associated with the production of the product. For example, most secondary metabolites, including citric acid, are produced only by non-growing mycelia (Lockwood and Schweiger, 1967). Other problems are associated with physiological degeneration and contamination. Continuous cultures are now being used for the production of beer, and perhaps penicillin, and are likely to be of great importance in the future (Lockwood and Schweiger, 1967; Turner, 1971).

A submerged citrate fermentation was introduced in an important paper by Perquin in 1938, and this reoriented many citric acid workers away from the shallow pan method. He used shake flask cultures, and rigorously established many of the conditions which influence citrate accumulation under these conditions. There had been other attempts at a submerged process, but these had proved unsuccessful (Karrow and Waksman, 1947; Prescott and Dunn, 1959). This technique was taken up and studied in shake flask replacement cultures first by Szucs (1944), and then by Karrow and Waksman (1947). Shu and Johnson (1948a) used shake flasks and defined many of the important parameters which control the process when the entire fermentation is carried out in the growth medium in a one-step fermentation. Snell and Schweiger (1949), of Miles Laboratories,

first patented the use of the paddle fermentor, and Martin and his colleagues at the NRC Laboratories in Ottawa carried out extensive studies on ferrocyanide-treated beet molasses using a tower fermentor (Martin and Waters, 1952; Steel, Lentz and Martin, 1955; Martin, 1957) and later used a paddle-agitated fermentor (Clark and Lentz, 1963). The tower fermentor consists of a long, narrow, thin column filled with medium. The medium is inoculated with pre-grown mycelial pellets, and agitation is brought about by bubbling air or oxygen up from the bottom of the tower. This technique does not seem to have found much use, although it is being studied (Suzuki et al., 1966; Akbar et al., 1967; Cejkova et al., 1967; Leopold et al., 1967; Orlov et al., 1968; Horitsu, 1971). The author is unaware as to what extent submerged fermentations are used for the production of citric acid; the intense commercial competition and secrecy associated with this process precludes publication of industrial techniques. Turner (1971) states that the submerged process is not used for the industrial production of citric acid, but it is apparently used by Miles Laboratories (Lockwood and Schweiger, 1967).

Other than the submerged technique, the little progress made in the two or three decades following Currie's work was not due to a lack of diligence or interest by investigators, but rather to two important facts: that the citrate fermentation is extremely sensitive to small amounts of trace metals, and that there are large strain differences in the response to these metals. The great surge of interest in industrial microbiology in the 1940's, along with the

production of purer chemicals and the discovery and use of ion exchange resins to purify the media, resulted in rapid elucidation of many of the pertinent problems still to be solved.

The trace metal nutrition of fungi had been one of the favorite research topics for mycologists since the classic work of Pasteur's student Raulin in 1869 on the growth requirements of A. niger (reviewed by Foster, 1939; 1949). Raulin discovered that zinc, iron, and a wide range of other nutrients were required for the growth of this organism. However, later workers were able to grow fungi in media consisting only of C, N, O, Mg, S and P. This inconsistency led to the widespread belief that Raulin had misinterpreted his results, and that the agents in his medium were acting as stimulants of growth rather than requirements. In 1895 Pfeffer proposed the "chemical stimulant" theory, which supposed that heavy metals were actually metabolic poisons, and the increased growth which attended their presence was an abnormal reaction to the poison. This point of view held sway until Steinberg, starting in 1919, showed that these ions were in fact required for growth. The French workers Bertrand and Javallier had discarded Pfeffer's theory some years earlier, but Steinberg crystallized the concept (Foster, 1939).

Steinberg's work, which extended over three decades, stimulated renewed interest in the effects of trace metals on mold metabolism. Many different laboratories, particularly those of Bernhauer, Chrzaszcz, Butkewitsh and Wassiljew (reviewed by Von Loescke, 1945; Foster, 1939, 1949) tested the effects of ions,

especially zinc, on organic acid production in surface cultures. Zinc generally proved to be unfavorable for organic acid synthesis, resulting instead in increased efficiency of sugar utilization, and therefore growth, at the expense of the organic acid accumulation. The citrate fermentation seemed to be somewhat of an exception, as zinc increased yields in some strains of A. niger, although it decreased yields in other strains. Less was known about iron, copper and manganese, but high concentrations of iron were believed to be detrimental to the fermentation under some conditions. In spite of these conflicting results, the cumulative work of the twenties and thirties clearly indicated that trace metals, particularly zinc, were important in organic acid fermentations, including citrate. In 1939 Foster was able to state: "Since organic acids are waste products of mold metabolism and result from the break-down of carbohydrates, it is natural to conclude that the heavy metal nutrition of the causative organism is influential in the accumulation of acids. It is obvious that the sensitivity of A. niger to metallic ions may be a matter of great importance in large scale mold fermentation processes".

The trace metal situation was clarified somewhat by a group at the University of Wisconsin (Shu and Johnson, 1949b; Johnson, 1954) which, among others (Tomlinson et al., 1950; 1951) carried out some of the most careful and precise studies on the subject (see below). The use of ion exchange resins to purify the media was a significant factor in these experiments (Perlman et al., 1946). It was shown that the optimum concentration of each element varied not

only with the strain of organism, but also with the concentration of other nutrients present (Johnson, 1954). Thus iron, for example, either stimulated or inhibited citrate accumulation depending only upon the zinc concentration. This concept is known as "ion antagonism" (Foster, 1949). Perlman (1949a) and Shu and Johnson (1947) proved that sufficient quantities of trace elements could be carried over with the conidia from the conidiation medium into the fermentation medium to seriously retard the progress of the fermentation. Perlman (1949a) showed that young conidia were better than old conidia, and that the temperature of storage was important, five degrees centigrade being better than twenty-five degrees centigrade. These studies, and others like them, pointed out many sources of error and offered clear explanations for many of the anomalous results which had been reported, thus establishing a firm base for further research.

As the importance of trace metals for citrate accumulation became known, the problem in a commercial context became focused on production of citrate at the lowest possible cost, and the scale-up of laboratory conditions to an industrial level. Of prime importance was to obtain a cheap source of sugar, such as beet or cane molasses, suitable for fermentation, and this entailed the removal of contaminating metals. Sjolander (1945) and Karrow and Waksman (1947) had proved that low yields often obtained from technical grades of sugar were due, in part at least, to inhibiting ions, by showing that the addition of the ash from these carbohydrate sources resulted in reduced yields. Other workers have confirmed

the inhibitory influence of trace metals in sugars (Gardiner et al., 1956; Noguchi and Johnson, 1961; Millis et al., 1963; Sanchez-Marroquin et al., 1970). The tactics eventually employed oriented around removing the trace metals from sugars by use of ion exchange resins and chelating agents, or the desensitization of the mold to these trace elements.

Ion exchange resins have been used by many investigators (Sjolander, 1945; Perlman et al., 1946a; Karrow and Waksman, 1947; Woodward et al., 1949; Perlman and Sih, 1960; Noguchi and Johnson, 1961; Millis et al., 1963; Sanchez-Marroquin et al., 1970; Chang and Terry, 1973), and this technique appears to be the most common in industrial fermentations (Lockwood and Schweiger, 1967). The use of ferrocyanide to remove contaminating ions was first suggested by Mezzadroli (1938), and was apparently used by the Germans in World War II (B.I.O.S., 1946). Other workers have also used this agent (Perlman, 1943; Gerhardt et al., 1946; Bernhauer et al., 1949; Clement, 1952; reviewed by Perlman and Sih, 1960). Much of the work connected with the use of ferrocyanide has been carried out by Martin, Clark and their colleagues at the NRC laboratories in Ottawa, and among other things, they confirmed the variability of Chatham beet molasses with respect to high citrate yields, and the subsequent need to conduct preliminary experiments in order to determine the optimal ferrocyanide concentration (Martin and Waters, 1952). Other methods used to eliminate inhibitory ions have been treatment with aluminum hydroxide (Shu and Johnson, 1949b); treatment with specific precipitants including morpholine (Sjolander, 1945; Schweiger and

Snell, 1949), quaternary ammonium or cyclic-ammonium compounds (Nebe, 1940), small amounts of milk powder (Szucs, 1949), camphor or tannic acid (Wai, 1955); other chelating agents such as EDTA, CDTA, and DTPA (Choudhary and Pirt, 1966; Qadar and Abdullah, 1971) but none of these, including ferrocyanide, appear to have found commercial application (Lockwood and Schweiger, 1967).

A significant advance in the technology of the citric acid fermentation was the discovery by Moyer (1953) that the addition of low molecular weight alcohols and esters resulted in increased yields of citrate in both surface and submerged cultures. Production of itaconic acid and ethylene oxide dicarboxylic acid, but not kojic or gluconic acids, were also increased by other strains of the Aspergilli and Penicillia (Moyer, 1953). The effect appeared to be to render the mycelia much less sensitive to normally inhibitory levels of trace metals, thereby eliminating the need for purification of the media. Other effects were to increase the tolerance of the fermentation to large pH changes, and to limit growth and sporulation of the mold. The increase in citrate production could not be accounted for by metabolism of the alcohols, for methanol was not metabolised, and although ethanol could be utilized and even converted to citrate when the mold was grown on acetate (Bernhauer and Bockl, 1932; Foster, 1949), in Moyer's experiments the alcohols did not increase citrate accumulation until they had begun to exert a toxic effect on growth (Moyer, 1953). Either germinated or ungerminated conidia could be used, and the most potent methanol concentration varied with the inoculum load. According to Lockwood and Schweiger (1967) alcohols have not been employed commercially in the citrate

fermentation.

The most common and obvious means of overcoming the trace metal problem has been to select strains which are insensitive to trace metals. In addition, mutagenic agents are often able to produce high-yielding progeny (see below). Undoubtedly industrial concerns are continuously seeking to improve their strains in this way. Another interesting technique apparently used by Miles Laboratories exploits the fact of ion antagonism (Lockwood and Schweiger, 1967). Copper is used as an antagonist of iron for aconitase, an enzyme which requires iron as a cofactor (Schweiger, 1961). Organic ion antagonists are also available (Lockwood and Schweiger, 1967).

The technology relating to the citrate fermentation is a jealously guarded secret, so there is a paucity of recent literature on the subject. It has been reported that certain classes of lipids are able to increase yields (Millis et al., 1963), and there are commercial designs in this finding (University of Melbourne, 1960). The fluoride ion has also been reported to enhance accumulation of citrate (Takami, 1967; 1968).

Almost all of the results presented here have been a result of utilitarian interest, and so generally the physiological significance of the findings has been disregarded. The following sections deal more extensively with this aspect.

SECONDARY METABOLISM AS A DIFFERENTIATED STATE

Before continuing, it is relevant to this discussion to consider briefly advances which have been made recently in the

understanding of the physiology and dynamics of other mycological fermentations. It has become increasingly clear (Bu'Lock, 1967; Weinberg, 1970) that fungi excrete two kinds of secondary metabolites (SM): one type is growth-linked, and is produced coincident with growth; the other, which is far more common, is independent of growth, and is synthesized only after growth has ceased. Several studies (see Demain, 1968) have demonstrated that profound changes occur in physiology during the "growth" phase and the "producing" phase. In addition to this, the gross similarity between the production of SM and differentiated structures in fungi (Smith and Galbraith, 1971), bacteria (Weinberg, 1970), and plants and animals (Luckner, 1972) have caused many authors to consider the "producing" phase to be a "differentiated" state. The point is that cells which are growing (mitosis or hyphal extension of the meristematic apex) do not participate in the synthesis of the particular SM or differentiated structure, and as a corollory, the reverse is true; the two types of cells differ in morphology, respiration, metabolic intermediate flux, macromolecular composition and synthesis, and enzyme make-up.

This concept is relevant to the "philosophical" appraisal, and therefore the understanding of the citric acid fermentation, because this phenomenon has previously been regarded to be a "pathological" condition induced by nutritional deficiencies, or genetic and physiological defects. Some of the arguments in support of this belief are presented below. For a comprehensive discussion of this aspect of secondary metabolism see Woodruff (1966), Demain (1968), Smith and Galbraith (1971), and for mathematical models see

Megee et al. (1970) and Pirt (1969).

The classical studies on metabolic phasing are those of Borrow et al. (1961, 1964) on the gibberellic acid fermentation by Gibberella fujikuroi, and these may be regarded as defining a general case (Bu'Lock, 1967). They described an initial period of rapid exponential growth during which nutrients were assimilated in a constant ratio, and the composition (6% fat, 16% carbohydrate, 13% phosphorous-containing compounds) and morphology (filamentous, non-vacuolated) of the mycelia remained unchanged (balanced phase). This period, during which no gibberellic acid was synthesized, continued until one of the nutrients was depleted. Oxygen limitation appeared to cause a transition from exponential to linear growth (1964). In glucose-limited fermentations cell proliferation ceased, mycelial autolysis took place, and no gibberellic acid was produced. When nitrogen was exhausted in the presence of high glucose cell proliferation ceased, but the other nutrients continued to be assimilated until the glucose was exhausted (storage phase). There was a large increase in dry weight which was accounted for by increased fat and carbohydrate synthesis; the fat, observable as oily droplets in the hyphae, could amount to as much as 45% of the dry weight, and the carbohydrate as much as 32%. In phosphorous and magnesium-limited fermentations proliferation continued until the subsequent exhaustion of either glucose or nitrogen, and during this "transition" phase the carbohydrate content of the mycelia increased, while in magnesium-limited fermentations the fat content also increased. In phosphate-limited fermentations the uptake of both magnesium and potassium ceased and reserves of metaphosphate were used; preliminary results indicated

that cell proliferation continued until this phosphate reserve was exhausted. The "maintenance" phase followed the storage phase: all the nutrients had been assimilated and no further changes occurred in the composition or appearance of the mycelia; this period continued until glucose was depleted, and then autolysis took place. Gibberellic acid was not synthesized during the "balanced" phase but was produced at a linear rate in the storage and maintenance phases; that is, in all cases acid was not produced until cell proliferation had ceased. Synthesis terminated upon glucose exhaustion, or in some cases for unexplained reasons apparently associated with aging of the mycelia. The cessation of cell proliferation was accompanied by vacuolation of the hyphae.

Becker (1963, 1964) has studied several industrial-type antibiotic fermentations, and has defined cytochemical and morphological criteria of metabolic phasing which agree with the general results of Borrow et al. Vacuolation of the hyphae is normally associated with older parts of the hyphae, and rarely with the young, growing tips (Robertson, 1965; Park and Robinson, 1967).

Taber (1964), Taber and Tertzakian (1965), and Taber and Siepmann (1965) conducted similar studies on the Claviceps fermentations, and discussed the difficulties inherent in defining and measuring growth in filamentous organisms. Defining growth as "polymeric non-extractable mycelial residue" (experimentially expedient) they distinguished a phase of true cell replication, a phase of "primary shunt products" such as polyols and oligosaccharides, and a phase of "true" secondary metabolism. This is contrasted with Borrow's "balanced" phase of constant inoculum replication and mycelial

consistency. Bu'Lock (1965, 1967) points out, however, that true balanced growth is seldom actually observed because of variations in filament branching and macromolecular synthesis, and further argues that the accumulation of triglyceride and carbohydrate inside the hyphae and SM outside the hyphae are merely different manifestations of the same phenomenon of metabolic rearrangement following growth termination. He therefore introduced the terms "trophophase" (nutrient) to describe the "growth" phase, and "idiophase" (peculiar) to describe the "accumulating" phase.

The trophophase-idiophase concept has been further supported by studies with continuous cultures. Bu'Lock et al. (1965) were able to control the extent to which malonyl CoA was converted to patulin (putative enzyme induction) by regulating the growth rate, but could not achieve the full idiophase until the dilution rate was zero. On the other hand, Pirt and Righalato (1967) concluded that a slow growth rate was optimal for penicillin production in continuous culture, and a rapid trophophase followed by slow growth in the idiophase best in batch cultures.

It is not clear how growth termination initiated by nutrient depletion causes the pronounced changes which occur in the idiophase. Foster (1947) introduced the term "shunt metabolism" to describe the synthesis of antibiotics, and this has been extended to other systems (Foster, 1949; Tanenbaum, 1965; Bu'Lock, 1967; Woodruff, 1966; Demain, 1968; Weinberg, 1970). During trophophase, the primary metabolites in the glycolytic pathways and the TCA cycle are kept in low, balanced concentrations, and feed-back mechanisms consistent with growth and energy demand apply. However, upon nutrient depletion,

which precludes growth there is a rapid build-up of these metabolites, which may become injurious to the cell; to overcome this the organisms have evolved species-specific metabolic shunts to eliminate them ("detoxication" theory, see Weinberg, 1970; Luckner, 1972), and thus the formation and excretion of the various SM. This may be effected by "activation" of pre-existing enzymes (eg. citrate activation of acetyl CoA carboxylase) or induction (or "expression", see Mitzenberg, 1972) of appropriate enzymes. Enzyme induction kinetics, and experiments employing protein synthesis inhibitors suggest that de novo enzyme synthesis does occur. As an example, Bu'Lock et al. (1965) and Bu'Lock (1967) have concluded that with Penicillium urticae the enzymic conversion of 6-methylsalicylic acid to gentisyl derivatives and patulin occurs by "sequential" enzyme induction. Other examples are presented in Demain (1968).

The analogy between the idiophase and conidiation or sporulation in filamentous fungus cultures has been noted by many authors (Bu'Lock, 1961; Woodruff, 1966; Schaeffer, 1969; Smith and Galbraith, 1971), prompted by the casual observation that they possess many common features. Like idiophase, it is well known that sporulation and other forms of microbial differentiation occur after the phases of rapid vegetative growth (Klebs, 1909; Hawker, 1957; Schaeffer, 1969), and with Aspergillus nidulans (Carter and Bull, 1969) and A. niger (Galbraith and Smith, 1969) this is brought about by nutrient exhaustion. Similar teleological explanations have been ascribed to secondary metabolism and sporulation. Smith and Galbraith (1971) comment upon this: "of particular interest to this review are the connections being recognized between typical examples of secondary

metabolite formation and the development of specialized structures, such as spores, conidia, and sclerotia, which prolong survival under adverse conditions." As with secondary metabolism, maximum conidiation occurs with A. niger (Ng et al., 1973) and Pennicillium chrysogenum (Righelato et al., 1968) in chemostats at a growth rate between zero and that necessary for purely vegetative growth.

Widespread changes occur in the mycelial enzyme composition of A. niger (Smith and Anderson, 1973) particularly macromolecular degrading enzymes, during the transition from the growth phase to the differentiating phase. Mycelia of A. niger grown in media on which they will eventually conidiate have a different pattern of glycolytic enzymes from the mycelia grown on non-conidiating media; these differences are observed long before the differentiation is actually manifested. Changes in metabolic flux and concentrations occur upon growth termination, and are considered by some authors to be sufficient to induce differentiation (Wright, 1968; Wright and Gustafson, 1972). The depletion of endogenous reserves may actually trigger the final stages of morphogenesis (Wright, 1970) and help to achieve a sequential order of events. These arguments are reminiscent of those advanced to explain induction of enzymes for SM (Bu'Lock, 1965).

Both conidiation (Turian, 1969; Smith and Anderson, 1973) and SM (Finn, 1965; Arnold and Steel, 1958) are known to be oxidative processes or at least to require oxygen. Recent studies on A. niger (Smith and Valenzuela-Perez, 1971; Smith et al., 1971; Valenzuela-Perez and Smith, 1972; Smith and Ng, 1972) have demonstrated the presence of both the EMP and hexose monophosphate pathway (HMP) during growth and conidiation, but differences in the contribution to glucose catabolism:

the EMP dominated in the growth phase, and the HMP in the conidiation phase; this was in agreement with work on other fungi (see Smith and Anderson, 1973; Smith and Galbraith, 1971). Similar studies have been carried out in a few mycological fermentations. Bu'Lock et al. (1965), studying the patulin fermentation with P. urticae observed a period of intense respiration and HMP activity during the trophophase, and slower respiration and EMP dominance in the idiophase. During the citrate fermentation, the EMP appears to dominate (Cleland, 1953; Shu et al., 1954). Similar results were obtained with the itaconic fermentation with Aspergillus terreus (Bentley and Thiessen, 1957a). Therefore, although sporulation and SM are similar in nature, they would appear to be different in this respect, at least for the examples cited.

The general incompatibility between growth and differentiation in developing mammalian systems is well known (Green and Todaro, 1967; Rutter et al., 1973). In culture, "normal" mammalian cells grow and divide in a monolayer on the culture vessel, but this mitosis stops when the culture becomes confluent (Stoker, 1967; Wolstenholme and Knight, 1971), or when nutrients (serum factors) become depleted (Wolstenholme and Knight, 1971). Malignant cells on the other hand are less fastidious about serum factors, and do not become contact-inhibited, and thus are able to grow to much higher cell densities. Similarly, malignant cells are considered to be "less differentiated" than are non-malignant cells (Paul, 1969).

Weinberg (1970) has pointed out the similarity between the growth of mammalian cells and SM and sporulation in fungal and bacterial cultures (Monod, 1949); the cells are relatively undifferentiated

during mitosis, but after growth has stopped differentiation may occur. Typical differentiated products which may loosely fit the definition of SM include collagen, cell pigments (melanin), myosin, keratin (Bullough, 1969), spores and fruiting bodies, and the various structures associated with specialized cells. Consider also the well known Dictyostelium discoidium growth cycle: growth (mitosis) occurs during the vegetative phase, but following nutrient (bacteria) depletion, the amoebae aggregate into a slug, and eventually differentiate into a fruiting body; growth does not take place after aggregation (Bonner, 1971; Newell, 1971; Garrod and Ashworth, 1973). The amoebae are facultative anaerobes during the growth phase, but are obligate aerobes during the differentiation phase. Such examples are very common in biological systems, and may be found in any discussion on developmental biology.

Tumor cells are also characterized by an increased rate of aerobic glycolysis (Warburg, 1930) and lactate synthesis, a pattern not in accord with the well known Pasteur effect, which assumes glycolytic control at the level of phosphofructokinase (Racker, 1965, 1973; Koobs, 1972; Krebs, 1972). This hints at a kind of similarity between the synthesis of SM and the growth of cancer cells, for although the cancer cells are highly mitotic while idiophase cells are not, a lot of glycolytic control is implied in both cases. There is oblique evidence that oxygen may be involved in some way with the differentiated state in eucaryotic organisms, including fungi (Turian, 1969; Smith and Galbraith, 1971). High oxygen levels are also required for secondary metabolism in fungi, including the citrate fermentation (see below).

One further point need be made with respect to the general knowledge available on secondary metabolism. Weinberg (1970) has compiled a large list of examples of SM which are sensitive to trace metals, and implied that there may be a "unitary function" of trace metals in secondary metabolism. There are many similar features among the examples listed, and these are summarized below.

1) The "key" active metal may either promote or inhibit the production of SM, depending on the organism and the SM.
2) Only three trace metals seem to be important: manganese for Bacillus sp., iron for other bacteria and Actinomycetes, and zinc for fungi and Actinomycetes. In some cases more than one metal is effective, and then each metal is effective alone. Calcium and magnesium are unable to substitute for any of the active metals.
3) Although the metals are required for growth (10^{-7}M), the concentration which influences SM is one to three orders of magnitude higher. Therefore, the SM response to metal occurs independently of growth. In the range above which the metal stimulates growth, and below which it is toxic (about 10^{-3}M), the yield of the product varies linearly with the log of the concentration of the key metal.
4) It has been mentioned previously that SM production may cease in aged cultures, even though the mycelia are viable and are in the same cultural environment which had been favorable to SM. If culture longevity is important then it is assumed that the same "key" metal which affects SM should also affect culture longevity. This has been shown in a number of cases.

These discussions seem to indicate that the idiophase in at least some mycological fermentations may be considered to be a differentiated state, analogous to sporulation. Since SM normally occurs in the absence of sporulation (Weinberg, 1970), perhaps it is even an alternate condition. Furthermore, if the idiophase is a differentiated state, and if Weinberg's generalizations hold true, this implies a profound role for trace metals (zinc for fungi) in the regulation of growth and differentiation in the systems cited. Evidence is accumulating that calcium may play such a role in other eucaryotic cells (Whitfield, 1973), including aquatic fungi (Cameron and LeJohn, 1972; LeJohn, 1973; LeJohn et al., 1974). Calcium has also been shown to be required for fruiting but not growth in some fungi (Baser, 1951, 1952; Lu, 1973). Little reference has been made here to the citrate fermentation. In the following pages this subject will be examined with respect to some of the concepts developed here.

THE CITRATE FERMENTATION: GENERAL INFORMATION

THE METABOLIC ORIGIN OF CITRIC ACID IN A. NIGER

The metabolic origin of citric acid in A. niger is a subject which attracted the attention of investigators for many years, and although many different theories were proposed, most are only of historic interest (Von Loeske, 1945; Foster, 1949; Johnson, 1954; Prescott and Dunn, 1959; Perlman and Sih, 1960). Claisen and Hori (1891) were the first to suggest that biological citrate formation arose by a condensation of acetate and oxaloacetate. Later, Raistrick and Clark (1919) and Chrzaszcz and Tiukov (1930) postulated this

mechanism for molds, and eventual work on the TCA cycle in animals gave powerful support to this possibility (Krebs, 1943). This mechanism was again taken up by Weinhouse and Lewis (1949) and Lewis and Weinhouse (1951), who showed that surface cultures of A. niger producing citrate with acetate as the carbon source converted radioactive acetate into citrate; in addition this work provided evidence for a C_2-C_4 condensation as well as for the presence of the TCA cycle in A. niger.

Although it was agreed that citrate was formed from a four carbon compound, the origin of this compound was not clear (Foster, 1949). There were considered to be two possibilities, either a condensation of two acetate moities (Thunberg-Weiland Reaction; Thunberg, 1920), or a condensation of pyruvate with carbon dioxide (Wood-Werkman Reaction; Wood, 1946). El-Kerdany (1947) proved that $^{13}CO_2$ was incorporated into citrate by surface cultures of A. niger, and the same results were obtained with $^{14}CO_2$ (Foster et al., 1941), while Foster and Carson (1950) and Martin et al. (1950) confirmed this with $^{14}CO_2$ in submerged cultures. However, Foster and Carson (1950) also determined that acetate labelled in the methyl group was converted into citrate by sucrose-grown submerged replacement cultures in a manner consistant with a mechanism involving the condensation of three C_2 moities. In an attempt to resolve this question Bomstein and Johnson (1952) studied radioactive acetate incorporation into citrate by high-yielding glucose-grown submerged cultures. They concluded that the acid was formed by a C_2-C_4 condensation, and that most of the C_4 compound was produced by CO_2 fixation, although

a C_2-C_2 condensation could still account for some of the citrate produced. Continuing this work with glucose 3,4-^{14}C as substrate, Cleland and Johnson (1954) finally showed clearly that glucose was split into two C_3 fragments, followed by decarboxylation of one and carboxylation of the other; these two products condensed to form citrate. In other experiments these workers also demonstrated that labelled CO_2 was incorporated into the terminal carboxyl group of citrate, as predicted by a C_3-C_1 condensation, rather than a C_2-C_2 condensation.

Final proof of this mechanism was provided by enzymatic studies. Stern and Ochoa (1949) first demonstrated citrate synthesis from oxaloacetate and acetate by cell free extracts from pigeon muscle, and Ramakrishnan and Martin (1954) reported the same result for A. niger. The Ottawa group also showed that most of the enzymes of the TCA cycle were present in mycelia of A. niger grown under non-citrate accumulating conditions (Martin, 1954; Ramakrishnan, 1954). Recently, Ahmed et al. (1972) have demonstrated that citrate accumulating mycelia similarily contain all the TCA cycle enzymes; these cultures also contain tightly coupled mitochondria capable of oxidizing NADH and all the intermediates of the TCA cycle.

The presence of the EMP pathway in fungi was first indicated when Lynon and Hoffman-Walback (1948) obtained aldolase preparations from P. notatum mycelia, and the results of Cleland and Johnson (1954) indicated that under citrate accumulating conditions the EMP was the major pathway of glucose dissimilation, while Shu et al. (1954) concluded that this scheme accounted for 78% of the glucose catabolized.

The French workers Bertrand and De Wolf (see Results, Section I) proved the presence of several of the enzymes of this pathway in A. niger, and Smith et al. (1971) have confirmed this and also demonstrated some of the enzymes of the HMP.

Finally, Collins and Kornberg (1960) provided a mechanism for citrate accumulation from acetate by demonstrating a glucose-repressible glyoxylate cycle in A. niger. The radioactive labelling patterns that had been obtained earlier by Lewis and Weinhouse (1951) were consistant with a mechanism involving the combined operation of the glyoxylate and TCA cycles (Kornberg, 1959). It may therefore be concluded from these studies and many others that the metabolic machinery possessed by citrate accumulating strains of A. niger conforms to the well established patterns of the EMP pathway and the TCA cycle.

THE PRINCIPLE OF EXCLUSION BETWEEN GROWTH, CITRATE ACCUMULATION AND CONIDIATION

There is a large body of evidence which demonstrates that as with other secondary metabolites, citrate is not accumulated by growing cultures (Lockwood and Schweiger, 1967; Meyrath, 1967). Maze and Perrier (1904a, b) reported that high yields of citrate were obtained after the growth was essentially complete in surface cultures, and Currie (1917) showed that there was little acid accumulation during the first two or three days following a conidia inoculation; when a "vigorous mycelial felt" had formed, there was a rapid linear rise in acid production which continued until the sucrose was exhausted. A similar pattern has been described by others (Porges, 1932; Doelger

and Prescott, 1934).

Perquin (1938) and Karrow and Waksman (1947) recognized a growth phase and an accumulating phase in submerged cultures, and this was clearly shown by Shu and Johnson (1948a), and confirmed by others (Martin and Waters, 1952; La Nauze, 1966; Taeufel and Behnke, 1966a). Kotyschew and Tschesnekov (1927) showed that in submerged cultures no citrate was formed as long as the mycelia were taking up nitrogen from the medium, and this was confirmed by Pornar et al. (1970). Finally, large amounts of citrate may be produced in replacement cultures containing no nitrogen or phosphate (Perquin, 1938; Szücs, 1944; Karrow and Waksman, 1947) so as to prevent cell proliferation. It may therefore be concluded that the citrate fermentation displays the typical trophophase-idiophase dynamics. In addition to this, there is a general incompatibility between growth and citric acid accumulation, and once a certain minimal mycelia mass has been attained almost all "manipulations" which augment citrate production also inhibit growth (see results).

There is also a large body of evidence which indicates that citrate accumulation occurs only to the exclusion of conidiation. Currie (1917) clearly showed that citrate was accumulated by surface cultures only in the absence of significant conidiation. This was confirmed by Doelger and Prescott (1934) and Moyer (1952a), and Lockwood and Schweiger (1967) comment upon the point. Gardiner et al. (1956) found a consistent correlation of both restricted growth and conidiation with high citrate synthesis in several high yielding mutants. Filamentous fungi rarely conidiate in submerged cultures, (Smith and Galbraith, 1971) presumably because of a lack of sufficient

oxygen, but the lack of "reproductive structures" was one of the criteria listed by Snell and Schweiger (1949) which were required for citrate accumulation to occur. This appears to be a general characteristic of the secondary metabolism of fungi (Weinberg, 1970). These observations argue that citrate accumulating strains of A. niger are able to exist in at least three mutually exclusive differentiated states associated with growth, secondary metabolism, and conidiation.

FUNGI WHICH ACCUMULATE CITRIC ACID

Citric acid accumulation is an extremely wide-spread phenomenon in fungi, especially in the Aspergilli and Penicillia, and under the appropriate conditions nearly all strains excrete small amounts. Other fungi possess this property as well, but only selected strains of A. niger produce high yields, and are therefore of industrial importance (Foster, 1949; Prescott and Dunn, 1959; Perlman and Sih, 1960).

STRAIN VARIABILITY AND PHYSIOLOGICAL DEGENERATION

A well known characteristic of filamentous fungi is the tendency to "natural variation" and "physiological degeneration" (Foster, 1949), and this has been epitomized in the famous remark of Albreck von Haller two centuries ago when he described them as a "mutatable and treacherous tribe" (Foster, 1949). In 1876 Pasteur recognized the variation which may occur with respect to fermentation ability, and termed it "la polymorphisme physiologique", and Foster refers to it as an "axiom". The citric acid fermentation is similarly subject to strain variability (Bernhauer and Bockel, 1932), as are other mycological fermentations

(Weinberg, 1970). Currie (1917) states that although most of his cultures produced citric acid, the "cultures which cannot be distinguished on morphological grounds give quite different results under the same conditions". Bernhauer (1928a) found that some strains were good citrate producers, some were good gluconate producers, and some were intermediate. Consecutive transfer of cultures through a highly acidic medium led to increased acid-forming powers (Bernhauer et al., 1929; Doelger and Prescott, 1934; Bernhauer, 1941), probably because of selection of acid-tolerant variants (Foster, 1949). Acid tolerance does not seem to be the only factor involved, however, as more citrate was produced when the medium was partially neutralized; under these conditions the rate and amount of citrate synthesis was increased, but not the percentage yield (citrate/sucrose) (Frey, 1941; Chrzaszez and Peyros, 1935; Karrow and Waksman, 1947). More recently, over 400 strains of A. niger were tested for citric acid production on 23 different culture media; the best strain converted sugar in 75% yield in 60 l. fermentation (Sanchez-Marroquin et al., 1963).

Strain variants have been obtained artificially by exposure of spores to mutagens. Methods used include U.V. light (Snell and Schweiger, 1949; Horri et al., 1950; Gardiner et al., 1956; Imshenetskii et al., 1959; Millis et al., 1963; Sanchez-Marroquin et al., 1969; Chang and Terry, 1973), X-rays (Diller et al., 1946; Diller et al., 1950; Quilico et al., 1949; Gardiner et al., 1956), radium salts (Nakazawa et al., 1939) and ethyleneimine (Sanchez-Marroquin et al., 1969; 1970) or multiple-step mutations (Gardiner et al., 1956). Ilezuk (1968) obtained 131 morphological mutants from conidia of 24 strains of

A. niger by U.V. radiation. He found no correlation between color of conidia and the ability to produce citrate, but among the mutants, 55% were higher-yielders than the parent. The progeny obtained are often poorer accumulators than the wild type, but on occasion are much better.

The most common explanation for the manifestation of strain variability induced by natural or artificial means is the "sensitivity" to trace metals. Quilico and Dicapa (1932) surmised that the effect of iron on citrate produced depended upon the strain used; in one case they obtained a decrease, in another an increase. Perlman et al. (1946), using five strains of A. niger in surface culture on two media highly purified by ion exchange resins, concluded that the optimum concentration of a given element varied with the strain. Perlman (1947) tested 75 strains of A. niger in submerged culture and found considerable variation among them; some were less affected by metal ions than others. Gardiner et al. (1956) described several high-yielding mutants as showing restricted mycelial development, retarded sporulation, and a lack of sensitivity to heavy metals; they hypothesized that the mutant may be "deficient in certain metal-dependent enzymes associated with growth..". Millis et al. (1963) used trace metal sensitivity as a screening technique for the isolation of high-yielding mutants. Sanchez-Marroquin et al. (1970) showed that their several mutants were all less sensitive to the adverse effects of heavy metals. Although it is undoubtedly true that most (if not all) high-yielding mutants and good natural strains are less sensitive to trace metals, there have been only limited attempts at characterization of the mutations (Gardiner et al., 1956; Chang and Terry, 1973).

Citrate accumulating cultures will sometimes, with no apparent change in growth morphology, lose their acid-forming ability (Yuill, 1953a, b; Perlman et al., 1946a), and once this happens it may be impossible to recover the capacity, even by repeated spore inoculations (Yuill, 1953a; Perlman et al., 1946a). This phenomenon, known as "physiological degeneration" is common to all fungi, and is a great source of trouble to commercial concerns and scientists alike. Foster says pessimistically that "all investigations dealing with specific metabolic functions of a fungus sooner or later encounter physiological degeneration manifested by progressive loss of the function of particular interest". Steel et al. (1955) reported how their cultures suddenly lost the ability to produce large amounts of citrate, and instead displayed increased growth and changes in pellet morphology.

The danger of completely losing the culture can be overcome by periodical re-isolation of single spores (Perlman and Sih, 1960), storage at low temperatures (Chrzaszez and Zakorny, 1938; Perlman, 1947; 1949), and avoidance of "foggy" patches of sterile mycelium (lacking spores) when mass transfers are made (Yuill, 1953a). Sub-culturing repeatedly in synthetic media often leads to degeneration (Chrzaszez and Zakorny, 1938; Bernhauer et al., 1941). Spores are best stored under a layer of mineral oil, in sterile soil at low temperatures, or by freeze drying (Yuill, 1953a; Bernhauer et al., 1941; Mahrita and Hesseltine, 1958).

Natural variation and physiological degeneration in asexual fungi like A. niger have been attributed to the formation of heterokaryons (Foster, 1949). These are formed when hyphae and germ tubes

intertwine and fuse, a fusion bridge forms between them (anastomosis), and nuclei which are the same mating type, but genetically different, pass into the recipient hyphae, but do not combine. The resultant "cytoplasmic hybrid" now generally manifests the characteristics common to both types of nuclei.

There have been several unsuccessful attempts to increase yields of citrate by the use of heterokaryons, the theory being that the increased ploidy (gene dosage) in the hybrid should result in greater synthesis of the acid. Heterokaryons of Aspergillus fonsecaeus produced intermediate or lesser amounts of citric acid than their parents (Ciegler and Raper, 1957; Raper and Fennell, 1953; Yuill, 1951), and attempts to enhance or stabilize them have failed (Yuill, 1953b; Ciegler and Raper, 1957). Chang and Terry (1973) have recently examined this question with diploids and heterokaryons of A. niger. Auxotrophs obtained by U.V. treatment produced yields varying from 30% to 100% of the prototrophic parent, and increasing the ploidy tended to restore the resultant diploids or heterokaryons to the original capacity of the parent, but not beyond. Even crosses of high-yielding auxotrophs had the same results. These workers concluded that the citrate accumulating property is inherited through the nucleus, and the ploidy is not an important factor; the increased yields obtained from crosses of low producing auxotrophs could be explained by gene complementation.

OXYGEN REQUIREMENT

Oxygen is required not only for growth, but also for the secondary metabolism of almost all filamentous fungi (Finn, 1954; Arnold and Steel, 1958). Perquin (1938) studied the citrate

fermentation in submerged cultures and showed that although oxygen inhibited growth in the first 24 hours, it stimulated citrate production at later stages. Pure oxygen was more effective than ordinary air. Shu and Johnson (1948a) found that cultures produced more citrate when agitated on a rotary shaker at a high rpm than when agitated on a low one, which they concluded was due to increased oxygenation. Extending these studies, Shu (1953) determined that the rate of oxygen utilization in shaken flask fermentations was directly proportional to the rate of citric acid formation, and substitution of oxygen for air resulted in higher rates of synthesis. Martin and Waters (1952) describe how interruption of oxygenation even for a few minutes during the first 24 hours delayed the onset of citrate production, or eliminated it altogether. Interruption of oxygenation at any time in the fermentation curtailed subsequent acidogenesis. Clark and Lentz (1961) found that oxygen stimulated citrate accumulation in the first half of the fermentation, and acid production was correlated with oxygen consumption; at later stages there was no relationship between oxygen and citrate synthesis. Lockwood and Schweiger (1967) remark that for both the citrate and the itaconate fermentations, interruption of aeration leads to curtailment of acidogenesis although there is no requirement for efficient oxygenation during the period of acid production, when growth has terminated. Horitsu (1971) found that the oxygen pressure must be kept low during the early stages of the fermentation when the conidia were germinating, and high during the following phases of growth and citrate production. Respiration and citric acid production of the mycelia increased while the growth of the fungus decreased with increasing oxygen pressure.

MORPHOLOGY

Numerous workers studying the submerged fermentation have noticed a distinct and consistent correlation between the microscopic and macroscopic morphology of the mold and the ability to produce citrate. Snell and Schweiger (1949) described a particular morphological type which they claimed was essential for citric acid accumulation to occur: 1) abnormally short, stubby, forked, bulbous mycelium, 2) numerous, swollen, oval to spherical-shaped cells, well distributed throughout the mycelial structure, 3) mycelial structures all showing granulation, and numerous vacuoles or refractive bodies, 4) absence of normal reproductive bodies (vesicles or sterigmata), 5) mycelia aggregated into compact aggregates or colonies having a granular appearance and sizes under 0.5 millimeters in cross section and averaging 0.1 millimeters. The most important factor in controlling this morphology was iron, which had to be maintained at levels below one ppm.

Ferrocyanide also caused the mold to grow in the form of roughly spherical aggregates, or pellets (Clements, 1952), and a gross pellet morphology similar to that of Snell and Schweiger was reported by Martin and Waters (1952) to be necessary for high yields. Steel et al. (1954) carried out rigorous experiments to show that the pellet formation was a function of the ferrocyanide concentration, the pH, and the number of conidia used. The morphological form could vary between the filamentous and "retarded" type; the former was characterized by excessive growth and always yielded poor citrate results. Manganese has been reported to cause the filamentous type of growth (Clark et al.,

1966). This gross pellet type of morphology has been reported to correlate with other treatments conducive to citrate accumulation, and it may be a general feature of the phenomenon (Section IV).

Currie (1917) and Doelger and Prescott (1934) demonstrated that the surface to volume ratio was important not only in the growth of the mold, but also in the actual production of citrate, which was favored by mats as thin as possible. This could be attributed to a "mass transfer" effect (Aiba, 1965; Blakebrough, 1967), which relates to rate of exchange of nutrients and products between the mycelia and the medium. This discovery had led to the standardized use of shallow pans for surface culture fermentations (Foster, 1949; Perlman and Sih, 1960). Conceivably the small type of pellet which favors accumulation in submerged culture could relate to this as well, as the large pellets which are sometimes formed tend to reduce yields (Steel et al., 1954); Moyer et al., 1953; Section IV).

The importance of a pellet inoculum in influencing citrate production in submerged cultures was demonstrated by Steel et al. (1955). As discussed by Martin (1955) too small an inoculum reduced both the rate of citrate synthesis and the final yield, while too high an inoculum, although effecting a higher initial rate, finally inhibited accumulation as the density of pellets in the fermentor became so great that aeration and agitation were greatly reduced. Millis et al. (1963) showed that a conidia inoculum also had an important effect on the fermentation cultures, there being an optimum concentration. The number of conidia used to inoculate has an important bearing on the pellet morphology (Section IV). Doelger and Prescott (1934) reported that there was also an optimum conidia concentration for citrate production

in surface cultures. If too high a number were used some of the conidia did not germinate, and this in some way had an inhibitory influence.

TEMPERATURE

Doelger and Prescott (1934) demonstrated that the temperature of the fermentation was an important factor in the citrate fermentation, as excessively high temperatures reduced yields. Citrate production increased from 8°C to 27°C, but above 30°C "citrate production will decrease and a greater amount of titratable acidity will be due to the formation of oxalic acid". Kovats (1946) found that first growing the mycelia at 28°C to 30°C, and then carrying out the fermentation at 20°C, resulted in higher yields than if the entire procedure was at the higher temperature. Perlman (1947) showed that transferring an actively accumulating culture from 25°C to 35°C resulted in rapid utilization of sucrose but curtailment of citrate accumulation; when the temperature was returned to 25°C citrate production resumed. Yamada and Hidaka (1964) determined 26.5°C to be the most favorable overall temperature for citrate accumulation, as growth was optimal at 37°C but acidogenesis greatest at 22°C; shifting from the higher temperature in the growth phase to the lower temperature in the producing phase gave the best results. This temperature effect may relate, in part at least, to the sensitivity of the organism to trace metals, as the adverse effects of certain ions on citrate production was accentuated as the temperature was increased from 25°C to 35°C (Tomlinson et al., 1951; Kito et al., 1953). It may also relate to the conformation of the lipid membrane (Section VII).

THE CITRATE FERMENTATION: COMPOSITION OF THE MEDIUM

A wide variety of media have been used in studies on the citrate fermentation (Perlman and Sih, 1960). This discussion will be limited as much as possible to those studies carried out in synthetic media purified by ion exchange resins (post W.W. II) Publications before the war should be considered to be, in certain respects, less reliable due to the lack of understanding of the role of trace metals in the fermentation.

Trumpy and Millis (1963), working with a high yielding mutant (derived from Wis 72-4) have carried out the most comprehensive studies on the optimal medium composition. Although medium composition is highly empirical, there is no doubt that their findings will be applicable to many studies. This medium was (g/l): sucrose, 140; $MgSO_4 \cdot 7H_2O$, 0.15; KH_2PO_4, 0.3; NH_4NO_3, 1.4; trace metals (mg/l) Fe^{++}, 2.0; Cu^{++}, 0.3; Zn^{++}, 0.1. They observed no manganese effects at concentrations 1 mg/l and less, although the Wisconsin group (Shu and Johnson, 1948b; Noguchi and Johnson, 1961) and the NRC group (Clark et al., 1966) working with the parent strain, observed highly adverse affects from 2 ppb manganese.

CARBON

Herzog and Polotsky (1909) obtained citrate from glucose, and a variety of hexoses, pentoses, and disaccharides. Later, Arnelung (1927) showed that citrate could be formed by A. niger from C3, C4, C5, and C6 sugars as well as from disaccharides of hexoses. This has been confirmed by Bernhauer (1928c, d) and Molliard (1924).

As is common to all organic acid fermentations (Foster, 1949),

citric acid accumulation is favored by high sucrose levels, both in surface cultures (Wehmer, 1894; Currie, 1917; Porges, 1932; von Loescke, 1945) and in submerged cultures (Shu and Johnson, 1948a). The amount of sugar utilized and growth obtained increases with increasing sugar concentration (Porges, 1932), until growth becomes nutrient limited, presumably, and then only citrate production increases (Shu and Johnson, 1948). Concentrations of from 10-20% appear to be optimal (Johnson, 1954); higher levels of sugar inhibit growth, and lower levels favor production of mycelia without citrate. However, the efficiency of conversion of sucrose (product formed/sucrose utilized) to both citric acid (Shu and Johnson, 1948a) and cell mass (Foster, 1949) varies inversely with the sucrose concentration, which has also been shown for the fumaric acid fermentation by Rhizopus (Foster and Waksman, 1939).

Carbon dioxide at low concentrations does not have any influence on citrate production, while at higher concentrations growth is inhibited (Prescott and Dunn, 1959). This is a somewhat unexpected result, as CO_2-fixation is an important step in the pathway from glucose to citrate (Cleland and Johnson, 1954; Henderson and Lamonds, 1966).

NITROGEN

There is some disagreement with regard to the best nitrogen source in the citrate fermentation, although it is generally agreed that the nitrogen supply should be kept low so as to restrict excessive growth (Currie, 1917; Doelger and Prescott, 1934; von Loescke, 1945; Karrow and Waksman, 1947; Foster, 1949; Johnson, 1954; Trumpy and Millis, 1963). Some workers claim that NH_3NO_3 is the most favorable

medium constituent (Doelger and Prescott, 1934; Shu and Johnson, 1948a), others claim that only nitrate salts should be used (Porges, 1932; Bernhauer, 1928e; Agnihotri, 1966), while still others suggest that only ammonium ions are best (Currie, 1917; Snell and Schweiger, 1949). An extensive bibliography may be obtained from von Loescke, 1945. Although urea has been used successfully (Karrow and Waksman, 1947) organic sources such as amino acids (Agnihotri, 1966; Hezuk and Galeziewska, 1968), peptone (Bernhauer, 1928e), and yeast extract (Karrow and Waksman, 1947) have generally proved unsatisfactory, resulting in increased growth at the expense of citrate production. Some of these results may probably be explained by the presence of contaminating trace metals, as amino acids are excellent metal chelators.

There has not been sufficient work published to discern whether the ammonium-nitrate controversy has any basis in physiology, or whether it is merely a reflection of strain differences and other unrelated cultural effects. Tomlinson and Campbell (1950) suggest that the favorable results obtained with $NaNO_3$ by Porges (1932) may have resulted from trace metal impurities in the chemicals. Using surface cultures and ion-exchange purified media, they observed a large drop in acid production after a switch from $NaNO_3$ to NH_4NO_3, which could be overcome by increasing the manganese content (Tomlinson et al., 1951). They were unable, however, to demonstrate any chemical impurities in the salts. There appeared to be an interaction between ammonium nitrate and manganese. It is possible that the different results obtained may be a pH effect; citrate accumulation requires a low pH and the rate of

synthesis is best at about pH 3.0. Higher pH's favor oxalate and gluconate production. Therefore, $NaNO_3$ would tend to increase the pH as the nitrate radical is incorporated into the mycelium, and the NH_4Cl would have an opposite effect. A similar situation is believed to operate in the oxalate fermentation by A. niger (Foster, 1949). NH_4NO_3 would probably first lower the pH and then later raise it again, as it has been shown with G. fujikuroi that ammonium was always utilized before nitrate (Borrow et al., 1964). Schweiger and Lockwood (1967) of Miles Laboratory claim that "by proper balance of ammonium salts, mineral acids and alkali metal nitrates, the fermentation can be controlled to give only citric acid...." Batti (1967) and Shepard (1963) have both used NH_4OH to adjust the pH.

One further possible explanation should be borne in mind, in accord with the "differentiation" possibility of the citrate accumulating conditions. Conidiation in submerged cultures of A. niger is repressed by ammonium but not nitrate ions (Smith and Galbraith, 1971, Smith and Anderson, 1973), and the ammonium ion has been suggested as a candidate for a "center formation" regulator during aggregation of D. discoidium (Bonner, 1971). That is, ammonium ions may play a role in differentiation in fungi.

In some fermentations e.g. (Gibberellic acid) nitrogen deficiency is sufficient to bring about secondary metabolism, but this does not appear to be the case with the citrate fermentation, at least for the production of large quantities of acid (Johnson, 1954). Shu and Johnson (1948a) reported an optimal concentration for ammonium nitrate; amounts either more or less inhibited both growth and citrate

production. In another study using purified media and an excess of all other nutrients except manganese, they showed that the total growth was roughly proportional to the amount of ammonium nitrate added. Increasing ammonium nitrate caused small increases in citrate synthesis, but once a certain low mycelial mass was achieved a further increase in ammonium nitrate had little influence on total citrate production and only low amounts of citrate were produced. Similar results were obtained by Karrow and Waksman (1947) in replacement cultures. In another study, Trumpy and Millis (1963) found some interaction between ammonium nitrate and the other media constituents, but concluded that growth restriction by nitrogen limitation was necessary, but not sufficient to cause large amounts of citrate accumulation.

MAGNESIUM SULFATE

As with nitrogen limitation, magnesium sulfate deficiencies appear to be necessary but not sufficient to induce significant citrate accumulation (Johnson, 1954). Currie (1917) showed that certain low levels were necessary for growth and acid synthesis to occur, but beyond the optimal concentration (0.15 g/l), both growth and acidogenesis were repressed, with no change in the specific activity of the mycelia. Shu and Johnson (1948a) observed little effect on growth and specific activity at concentrations from 0.25 g/l to 2.0 g/l. Shu and Johnson (1948b) obtained growth roughly proportional to the salt concentration provided that nitrogen did not then become limiting, but only small amounts of citrate were produced. Snell and Schweiger (1949) report that $MgSO_4$ (up to 0.08%) resulted in increased yields, rate, and

efficiency of citrate production. On the other hand, Karrow and Waksman (1947) reported a sharp reduction in citrate yield by $MgSO_4$ in phosphate limited replacement cultures. Trumpy and Millis (1963) found that increasing magnesium sulfate (0.15 to 0.35 g/l) exerted an inhibitory effect on acid production; this was observed at all ammonium nitrate levels (0.94 to 2.7 g/l), but only at low phosphate levels (<1.0 g/l).

PHOSPHATE

Many fermentations are able to tolerate a variety of inorganic compounds, but in most cases the phosphate concentration is critical, and excesses must be avoided (Demain, 1968). A similar situation appears to be true for the citrate fermentation, and unlike N, Mg, or S deficiency, phosphate limitation alone may be sufficient to produce relatively high yields of citrate (Johnson, 1954).

Molliard (1922) discovered that citrate accumulated in surface cultures when growth was restricted by phosphate deficiency, and this feature has been exploited in many surface culture fermentations (Johnson, 1954; Lockwood and Schweiger, 1967). Working with submerged replacement cultures, Perquin (1938), Szücs (1944, 1948), and Karrow and Waksman (1947) all stressed the importance of a lack of, or a very small amount of assimilatable phosphate. Snell and Schweiger (1949) reported that phosphate limitation (<0.02%) restricted mycelial development and produced the most desirable quantity and character of mycelial aggregates for citrate production. However, Shu and Johnson (1948a) studied a medium deficient in iron and zinc, and found that phosphate levels in excess of that required for good growth were without

effect. Investigating this anomaly, Shu and Johnson (1948b) concluded that in replacement cultures phosphate limitation was essential only if iron and zinc were present in excess. This medium was deficient in manganese, however, and addition of low amounts of manganese to the replacement medium caused a pronounced inhibition of accumulation, even at low phosphate levels, although low phosphate tended to restrict the severity of the manganese inhibition of citrate synthesis. Therefore phosphate deficiency induced citrate accumulation in the presence of zinc and iron, but not manganese. It was not determined whether zinc or iron deficiency could cause citrate accumulation in the presence of manganese.

Sanchez-Marroquin et al. (1970) have found that with several mutants studied in trace metal deficient media, monopotassium phosphate levels as high as 4.0 g/l had no effect on citrate accumulation. These results are in accord with the conclusions of Shu and Johnson (1948b), because the media were trace metal deficient. Lockwood and Schweiger (1967) claimed that although phosphate limitation is required for citrate production by surface cultures, phosphate is without effect in submerged fermentations (iron deficiency induced by copper ions). Trumpy and Millis (1963) found that increasing phosphate (0.25 g/l to 1.0 g/l) had an adverse effect at all magnesium sulfate concentrations, but only at low and not high nitrogen levels. However, they did not observe a significant interaction between phosphate, iron, zinc or copper (manganese was without effect in these studies).

It would appear that high phosphate levels may have adverse effects on citrate production when trace metals are present in excess,

but more work must be carried out to ascertain whether phosphate deficiency alone is sufficient for high yields. The reader will recall Weinberg's (1970) claim that the "key" trace metal was effective alone in influencing secondary metabolism.

Excess phosphate normally results in increased growth and sugar utilization at the expense of SM, but there are other effects as well (Demain, 1968). Martin and Waters (1952), studying the citrate fermentation in ferrocyanide-treated beet molasses in tower fermentors, determined that 0.5 g/l phosphate was required to overcome the toxic effects of ferrocyanide. In later studies (Steel, Lentz, and Martin, 1955), however, they found marked "reduction in acid yield and citric acid ratios with increasing quantities of phosphate supplement", and also "large, irregular, mushy pellets that tended to pack at the bottom of the fermentor and cause an "unstable fermentation". Subsequently, it was shown (Martin and Steel, 1955) that not only did phosphate stimulate sugar utilization, but also it altered metabolism such that gluconate and 5-ketogluconate were produced at the expense of citrate and oxalate. This did not appear to be a pH effect. Sanchez-Marroquin et al. (1970) observed that increasing phosphate led to the production of oxalate and gluconate, with little effect on citrate production. Phosphate ions, therefore, may be able to influence both total growth, morphology, and metabolite flux.

TRACE METALS

It is almost universally agreed that citric acid accumulates in large amounts only when certain heavy metals are limiting (Foster, 1949;

Trumpy and Millis, 1963; Lockwood and Schweiger, 1967), although deficiencies in other nutrients, especially phosphate may lead to citrate production in reduced amounts (Shu and Johnson, 1948b; Johnson, 1954). The only important trace metals appear to be zinc, iron, manganese, and to a lesser extent copper. As discussed above, magnesium is probably not important. Calcium has been used as a neutralizing agent in many surface culture studies to improve yields, but NH_4OH apparently may substitute (Lockwood and Schweiger, 1967). Calcium has also been reported to decrease citrate production (Perlman, Dorrell, and Johnson, 1946), but generally speaking it is without effect. Actually, a calcium requirement has never been established for A. niger (Lilly, 1965).

There is considerable disagreement among investigators regarding the influence of the important metals (Foster, 1939; 1949; von Loescke, 1945; Johnson, 1954; Perlman and Sih, 1960). Strain differences, the use of impure media, ion antagonism, and other factors have contributed to the confusion. One further difficulty lies in the interpretation of the results obtained, as essentially all these researches were conducted to determine empirically the optimum conditions for total citrate production; little attention was paid to the physiological response to the trace metal. For example, Porges (1932a, b) concluded that zinc was beneficial to citrate production because the total yield was increased, but in actual fact this was caused by an increase in the mass of mycelia; the specific activity of the mycelia for citrate production had not really changed.

Another difficulty is associated with the time when the fermentation was assayed for citrate, as meaningful results cannot be

obtained unless the culture dynamics are known. For example, the citrate produced may be re-utilized by the mycelia once the sucrose has been exhausted (Currie, 1917; Wang, 1940), and this must obviously be taken into account. In addition, certain agents may exert different effects at the various phases of the fermentation. Currie (1917) tested the effect of iron and found that in early stages of the fermentation it favored vegetation rather than acidogenesis, but in the later stages the feruginous cultures tended to overtake and surpass the others in acid production. Porges (1932a) obtained similar results with zinc, but Currie (1917) found that zinc favored vegetative development at the expense of citrate.

Shu and Johnson (1948b) conducted the classical studies on the relationship between citrate production and the mineral nutrition of A. niger grown in submerged culture. Their procedure was to purify the medium (aluminum hydroxide precipitation) and then to carry out the fermentation with all nutrients in excess, and only the test substance deficient. In this way it was presumed that the responses could be attributed to deficiencies in the test nutrient. However, as they state, manganese was deficient in all their studies, which perhaps obscures their results to some degree. It was found that citrate was accumulated only when zinc, iron, or phosphate was "deficient". Growth did not occur in the absence of added zinc, and zinc addition resulted in a rise in both growth and citrate yield. The maximum amount of citrate was obtained at a zinc concentration of about 0.5 mg/l, while further increases in the ion level caused additional growth and decreased acidogenesis, with a large reduction in the specific activity

of the mycelia. In the absence of added iron near maximal growth was achieved, but addition of the metal reduced the quantity of citrate produced. There was an interaction between zinc and iron, and the amount of iron giving optimal yields depended upon the zinc content of the medium. The phosphate concentration was also important, and if phosphate was kept low, high citrate yields could still be obtained even at zinc and iron levels which were otherwise inhibitory. Low concentrations of manganese (0.12 mg/l) added to replacement cultures resulted in a severe reduction in citrate production, and as with zinc and iron the adverse effects of manganese were less pronounced at low phosphate levels. These authors concluded that "potassium phosphate, zinc, and iron, affected the yield of citric acid by some means other than their effect on growth".

Tomlinson et al. (1950, 1951) studied Wis 72-4 in surface cultures, and demonstrated that zinc, iron, manganese, and copper were all essential for citrate accumulation to occur, but excessive amounts reduced yields and increased growth. Inhibitory concentrations of manganese resulted in conidiation of the mycelia.

In a later report from the Wisconsin laboratory, Noguchi and Johnson (1961) examined (in submerged culture) a new independently isolated strain of A. niger (N-548) with respect to the role of trace metals in the citrate fermentation. Using media purified by ion exchange resins they obtained similar results as they had in the previous study (Shu and Johnson, 1948b); citrate accumulation did not occur in the presence of manganese, and the best citrate production was obtained in the presence of limited amounts of iron and zinc.

High levels of iron favored vegetation and reduced the amount of citrate synthesized.

Trumpy and Millis (1963) conducted similar comprehensive experiments on the medium composition as had been performed by Shu and Johnson (1948b). These workers, using strain 72-44 derived from Wis 72-4 of Shu and Johnson, concluded that "the most conspicuous feature of these fermentations was the high yield of citric acid at very low concentrations of zinc and the sensitivity of this yield to small additions of zinc". Iron gradually increased growth and simultaneously decreased acid production as the ion concentration was increased. No effect was observed for manganese or copper.

Although Wis 72-4 is one of the most famous citrate accumulating strains of A. niger, others have been used. Sanchez-Marroquin et al. (1970) studied U.V. mutants of an isolate from a Columbian soil in submerged cultures on purified media. They concluded that the highest yields were obtained only when low quantities of heavy metals were present; iron was without effect and zinc tended to increase growth and reduce yields. Another group associated with Miles Laboratories found that excess iron was detrimental to citrate accumulation in submerged cultures (Snell and Schweiger, 1949). Zinc was added to "stimulate the abnormal type of cell structure.....most efficient in citric acid production", but excess zinc caused a severe reduction in yields. Low manganese was also necessary (Lockwood and Schweiger, 1967).

The results of these studies show that although certain minimal balanced amounts of trace metals are required to obtain good growth and citrate production, excessive quantities usually result in decreased

citrate accumulation with either an increase or no change in the amount of growth. Nevertheless, there still remain some inconsistencies regarding the influence of certain metals. Whereas Trumpy and Millis (1963) observed no response to added manganese in their excellent study, this is in contrast to most other reports (Shu and Johnson, 1948b; Tomlinson et al., 1951; Lockwood and Schweiger, 1967). Clark et al. (1966) working with submerged cultures of A. niger NRC-A-1-233 (same as Wis 72-4) in ferrocyanide-treated beet molasses determined that addition of manganese to the fermentation at any time resulted in inhibition of citrate production and filamentous, rather than pellet, type of growth. Horitsu (1971) found that addition of manganese increased growth but decreased acid production. The effect was drastic when added at inoculation, but was insignificant when added 24 hours later. In the present investigation zinc appeared to be the only important ion, and very little response was observed to manganese, iron, or calcium, even when they were tested at high concentrations. It is apparent that in spite of the very many studies which have been carried out, the question of the role of trace metals in the citrate fermentation is still not resolved.

THE PHYSIOLOGY OF THE IDIOPHASE IN THE CITRATE FERMENTATION

As discussed earlier, there is a considerable amount of evidence that the citrate fermentation is biphasic, consisting of a trophophase and an idiophase, and that the idiophase is a differentiated state which is physiologically distinct from the trophophase. It is not clear, however, what physiological changes are implicit in the differentiation to the idiophase. Particularly important with regard to this

is the question of whether the TCA cycle operates during the period of acidogenesis, and there is considerable disagreement on this point.

Using radioisotope tracer techniques, Lewis and Weinhouse (1951) obtained evidence for recycling of the citric acid, and on this basis proposed that the citrate was produced as an "overflow product" of the normal operation of the TCA cycle. Other workers also obtained similar results (Martin et al., 1950; Shu et al., 1954). However, contrary results were obtained by Bomstein and Johnson (1952) and Cleland and Johnson (1954) who found little recycling of the acid, which implied that under citrate accumulating conditions the TCA cycle was not functional, in opposition to the suggestion by Weinhouse and Lewis (1951).

Ramakrishnan et al. (1955) examined this question by studying the changes in the specific activities of TCA cycle enzymes in mycelia when they were induced to accumulate citrate by the addition of ferrocyanide. They found that the commencement of citrate synthesis after 46 hours growth was correlated with an eventual ten-fold rise in the specific activity of citrate synthase and with the complete disappearance of aconitase and isocitric dehydrogenase. Usami et al. (1971) obtained similar results, and found that in mycelia synthesizing citrate in medium with methanol added, citrate synthase activity remained high throughout the culture period while isocitric dehydrogenase remained low. These results are clearly consistent with the idea that the TCA cycle does not operate in the idiophase.

Also in support of this interpretation is the inhibitory effects of certain heavy metals on acidogenesis. Manganese is detrimental to

citrate production in some cases, and this may be related to the overall stimulation of the TCA cycle, and more specifically to the activity of isocitric dehydrogenase (Horitsu, 1971a). Iron also has adverse effects on acidogenesis; this ion is believed to be essential for aconitase activity (La Nauze, 1966; Suzuki et al., 1966) Suzuki et al. (1966) also found that 10 ppm ferrocyanide inhibited aconitase activity, presumably because of the chelation of iron; they concluded that this was the reason for the acidogenic action of ferrocyanide. This finding was in agreement with the earlier report of Ramakrishnan et al. (1955). Both iron (Bertrand and De Wolf, 1956) and manganese (Bertrand and De Wolf, 1955b) have been shown to increase the rate at which citrate is re-utilized once the sucrose in the fermentation medium has been depleted.

On the other hand, different results have been obtained from other studies, and these tend to indicate that the citric acid cycle does in fact operate during the period of citrate synthesis. La Nauze (1966) examined the activities of aconitase and both the NAD and NADP-specific isocitric dehydrogenases in two citrate accumulating strains of A. niger, Wis 72-4, and a high yielding mutant obtained from Wis 72-4. No correlation was found between citrate production and the activities of these enzymes throughout the course of the fermentation, nor was there any significant difference between the two strains. Moreover, although a twenty-fold increase in the iron concentration doubled the activity of aconitase, citrate synthesis was decreased only by 25%. Taeufel and Behnke (1966a) similarily obtained aconitic hydratase and isocitric dehydrogenase activity in the acidogenic phase, and concluded that the TCA cycle was probably not interrupted during the period of citrate accumulation.

Gardiner et al. (1956) showed that their high citrate yielding mutant was not impaired in its ability to utilize extracellular citrate as a carbon source, and although the pathway of degradation of extracellular citrate is not known, there is some evidence that the TCA cycle is involved (Taeufel and Behnke, 1966b, c).

Horitsu and Clark (1966) reported that although 30 ppm ferrocyanide stimulated citrate production by growing cultures it had no influence on NADP-specific isocitric dehydrogenase activity. Higher ferrocyanide concentrations increased citrate accumulation by resting cells, but still had little influence on the activity of this enzyme, and ferrocyanide concentrations up to 125,000 ppm actually slightly stimulated the oxidation by both resting cells and cell free extracts of glucose, cis-aconitate, ∝-ketoglutarate, malate, and oxaloacetate.

In the most recent study, Ahmed et al. (1972) have obtained tightly coupled mitochondria from A. niger during citric acid accumulation which were able to oxidize NADH and intermediates of the TCA cycle, including citrate. Certain enzymes of the TCA cycle (citrate synthase, aconitase, succinic dehydrogenase, fumarase, NAD and NADP-linked isocitric dehydrogenase, and malic dehydrogenase) were found to be functioning, and there was no significant difference in the activities at different stages of the fermentation.

It is therefore still not clear whether the TCA cycle operates under citric acid accumulating conditions. Nor is it clear what other physiological alterations may have occurred during the transition to the idiophase. For example, there may be a modification in the glucolytic patterns, for while it is probable that in the trophophase

glucose is catabolized in both the EMP and HMP pathways (Smith and Galbraith, 1971; Smith and Anderson, 1973), the evidence available indicates that in the idiophase the EMP is the major route of glucose dissimilation (Cleland and Johnson, 1954; Shu et al., 1954). In addition, it is possible that associated with the idiophase there is a mechanism concerned with the specific secretion of citrate from the cells. It is, of course, essential to elucidate the nature of the differentiated change in the idiophase in order to understand the mechanism of citric acid accumulation.

CYCLIC AMP

Cyclic AMP was discovered during the course of investigations into the mechanism of the hyperglycemic action of epinephrine and glucagon (see Sutherland et al., 1962), and since that time this compound has been shown to be involved in a wide variety of diverse biological processes (Robison et al., 1968; Robison et al., 1971a, 1971b; Greengard and Robison, 1972). Below are discussed very briefly some aspects of the biology of cyclic AMP which appear to have relevance to the present investigation.

CYCLIC AMP METABOLISM

The concentration of cyclic AMP at any given time is governed by the relative activities of two enzymes, adenyl cyclase and a cyclic AMP specific phosphodiesterase. Adenyl cyclase catalyses the formation of cyclic AMP from ATP, the other product being pyrophosphate (Rall and Sutherland, 1962). The phosphodiesterase hydrolyses the phosphodiester bond of cyclic AMP, forming 5'-AMP as the product (Butcher and

Sutherland, 1962). The stoichiometrics of these reactions were determined with enzymes obtained from dog liver, but they have not been confirmed with enzymes from microbial sources.

PHYSIOLOGICAL ROLE OF CYCLIC AMP

Soon after the discovery of cyclic AMP it became clear that it mediated the actions of many different mammalian hormones. This led to the proposal that cyclic AMP operated as a "second messenger" in the diverse tissues in which it was found, the primary messenger being the hormone in question (Robison et al., 1971a). It is believed that the hormone exerts its effect by either elevating or depressing the intracellular levels of the nucleotide. This appears to be accomplished by regulating the activity of the membrane-bound adenyl cyclase, rather than by action on the phosphodiesterase (Robison et al., 1971a).

The only microbial system which has been well studied is the regulation of lactose operon in Escherichia coli (Pastan and Perlman, 1970). In this case the synthesis of these enzymes is prevented by glucose, and part of the mechanism involves the maintenance of low intracellular levels of cyclic AMP. Under conditions which allow the operation of operon, cyclic AMP binds to a specific protein; the nucleotide protein complex then binds to the promoter region of the operon and allows transcription to occur.

MODE OF ACTION OF CYCLIC AMP

Two general theories have been proposed to account for the mechanism of action of cyclic AMP in the diverse mammalian tissues in which it operates. One hypothesis considers that the nucleotide

functions by regulating the activity of certain protein kinases, the specificity of the action residing in the nature of the protein kinase and the tissue in question (Kuo and Greengard, 1969). The second hypothesis is that the metabolism of cyclic AMP and calcium are connected, and that cyclic AMP exerts its action through the "mobilization of calcium" (Rasmussen, 1970). A similar scheme has been advanced to account for the control of the proliferation of various types of animal cells (Whitfield et al., 1973).

MATERIALS AND METHODS

MATERIALS AND METHODS

I. MAINTAINANCE OF FUNGUS CULTURES

A. niger NRC-A-1-233, identical to Wis 72-4, was used in these studies. Cultures were obtained from the National Research Council of Canada, Division of Biosciences, Ottawa. Stock cultures were maintained as conidia on agar slants of medium M-1 at 4°C, and were sub-cultured at intervals of one to six months.

Slants were prepared by dissolving the media constituents in separate parts, (see Media), then combining the parts, adding agar, autoclaving, and dispensing into screw cap tubes. The slants were inoculated by loop with conidia, and incubated at 28°C. It was essential to loosen the vial of the tube in order to obtain conidiation; it was also imperative that cultures not be allowed to stand for excessive time at 28°C, as this seemed to lead to degeneration of the cultures. Long term stock cultures were maintained on malt extract agar slants at 4°C, or lyophilized.

II. MEDIA

Three basic media were used in these studies; deviations from this norm are described in the results.

1. Medium M-1

The liquid medium was prepared in four parts as described by Feir and Suzuki (1969). The composition was (g/l): sucrose, 8.8 g; $(NH_4)_2HPO_4$, 1.65 g; KH_2PO_4, 0.1 g; $Na_2HPO_4 \cdot 7H_2O$, 28.8 g; NaH_2PO_4, 13.9 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MnSO_4 \cdot 4H_2O$, 0.001 g;

glass distilled water to one liter. The pH was 6.6 to 6.8, and autoclaved 15 minutes at 121°C, 15

2. Medium M-2 (NON CITRATE ACCUMULATING)

Medium M-2 obtained from Choudhary et al. (1965) was of the following composition (g/l): sucrose, 8.0; NH_4NO_3, 2.5; KH_2PO_4, 2.5; $MgSO_4 \cdot 7H_2O$, 0.25; Trace metals (mg/l): $FeCl_3$, 1.30 (7.9 μM); $CuSO_4 \cdot 5H_2O$, 0.01 (0.38 μM); $ZnSO_4 \cdot 7H_2O$, 0.44 (1.55 μM), glass distilled water to one liter. The pH was adjusted to 3.5 with HCl, and autoclaved as with medium M-1.

The inorganic salts were normally prepared as a 10x or 50x concentrated stock solution, which was stored in the dark and diluted out when used. The trace metal solution was similarly prepared 100x concentrated; the solution was shaken vigorously before use to homogenize the precipitate which had formed.

Chemicals were obtained from the following companies:

Sucrose	Shawinigan (Reagent)
NH_4NO_3	Matheson, Coleman, and Bell (Reagent)
KH_2PO_4	Fisher (primary standard)
$MgSO_4 \cdot 7H_2O$	MacArther Chemical Company (Reagent)
$FeCl_3$	Matheson, Coleman, and Bell (anhydrous reagent powder, sublimed; manufacturers report 0.05% Cu^{++} and 0.05% Zn^{++})
$CuSO_4 \cdot 5H_2O$	Shawinigan (Reagent; manufacturers report 0.003% Fe)
$ZnSO_4 \cdot 7H_2O$	Baker (Reagent; manufacturers report 0.0002% Fe^{+3})

3. Medium M-3 (CITRATE ACCUMULATING)

This is identical to M-2 but is lacking in trace metals. All glassware was washed with 6 N or 12 N HCl, and only 3x glass distilled or Super Q water (Millipore Corp.) was used. Citric acid did not accumulate unless this procedure was followed.

III INOCULUM

A conidia suspension was prepared by gently washing the (hydrophobic) conidia off the agar slants with 5 ml of a sterile 0.05% Tween 80-H_2O solution. Care was taken not to disturb the texture of the agar or the mycelial mat. The suspension was homogenized by "swirling" to break up conidia clumps.

The conidia number was routinely determined from previously prepared standard curve of optical density (Klett-Summerson colorimeter, red filter, 0.5% Tween 80 solution as a blank) versus various conidia suspension solutions. The optical density was related to the conidia number by means of viable plate counts. In experiments where the conidia number was not determined in this manner, the ratio of two slants of conidia (10 ml of suspension) per liter of media was always used. This yielded an inoculum load of approximately 4×10^4 to 6×10^5 conidia per ml of growth medium. The exact conidia number was not determined in the early studies on citrate accumulation. In all experiments the final concentration of Tween 80 in the growth medium was 0.0005%.

IV. CITRIC ACID ACCUMULATION EXPERIMENTS

1. Medium M-3, No Trace Metals (CITRATE ACCUMULATING)

This medium was used in most of the experiments on both citric acid accumulation and aggregation. The whole cell experiments were

performed in 125 ml Erlenmyer flasks washed in 6 N HCl. Each flask received 55 ml of medium and was autoclaved, inoculated, and incubated at 28°C on a rotary shaker at 220 rpm. Cyclic AMP and other effectors were added directly to the medium before inoculation. Deviations from this procedure are indicated in the text.

2. Medium M-2, With Trace Metals (CITRATE NON-ACCUMULATING)

This medium was normally used as a non-citrate accumulating control. Procedures were the same as above.

V. SMALL SCALE ENZYME STUDIES (RESULTS, SECTION V)

Enzyme activities were prepared from mycelia grown under citrate accumulating and non-accumulating conditions as described in Part IV.

The mycelia were separated from the growth medium by filtration through cheesecloth, washed extensively in the homogenizing buffer (4°C) (which varied according to the enzyme under study), and squeezed dry by hand. This washing step was essential to neutralize the acid which seemed to adhere to the cells. Mycelia were suspended in the homogenizing buffer (4°C) and disrupted by hand in an ice bath with a Bellco ground glass tissue homogenizer until the extract appeared completely homogeneous (approximately five minutes). The crushed cell suspension was then centrifuged at 48,000 x g in a Sorvall RC-2B centrifuge at 4°C. The supernatant was decanted and maintained on ice for assay.

VI. LARGE SCALE GROWTH AND PREPARATION OF CELL FREE EXTRACTS (RESULTS, SECTION V)

Large masses of mycelia to be used for enzyme purification were normally prepared in medium M-2, containing 0.8% or 5% sucrose. One

liter of medium was autoclaved in two liter Erlenmyer flask, inoculated, and incubated on a rotary shaker at 28°C and approximately 150 rpm. After two days, the mycelia plus media were dispersed into a container and the pH adjusted to neutrality with KOH. The cells were then filtered through cheesecloth, washed extensively in cold water or buffer, and either lyophilized or used directly. Lyophilized mycelia were stored at -70°C.

If the cells were used without lyophilization they were suspended in 3-5 volumes of buffer (4°C) and homogenized in 30 second bursts in a Virtis "23" Homogenizer or a Sorval Omni-mixer. In either case the homogenizing vessel was immersed in an ice water bath, and the total homogenization time was approximately five minutes. If the cells to be disrupted had been lyophilized, they were first ground to a fine powder in a dry, chilled mortar, and then suspended in 13 volumes of buffer (4°C). Homogenization was then carried out as described above.

Following all disruption, the thick, porridge-like suspensions were centrifuged at 48,000 x g for 30 minutes in a Sorval RC-2B centrifuge at 4°C. The opague supernatant was decanted, and maintained at 1-5°C for further study.

VII. ESTIMATION OF GROWTH

Growth was defined in these studies as dry weight. Mycelia were filtered on a moist cloth, washed in water, lyophilized and weighed. For the estimation of very small weights, the cells or conidia were filtered on previously washed, dried, and weighed Millipore filters (0.45 μ). The cells plus filter were then washed, dried, and weighed.

VIII. ESTIMATION OF AGGREGATION

Growth conditions are described in Section IV-1 of the Methods.

A. Conidia and Germlings

1. Qualitative Assay

Aggregation of conidia and germlings was scored qualitatively by filtering the contents of the flasks through Millipore filters (0.45 μ) and visually observing the clumping patters on the filter. At least five flasks were used per experiment, and the photographs represent typical results. Conidia that are unaggregated appear as a dark homogeneous film on the filter, while those that are aggregated appear clumped so that the white Millipore filter can be seen in the background.

2. Quantitative Assay

This assay is based upon a technique termed "differential filtration". The idea is that when radioactively labelled conidia are filtered through a large pore filter, the unaggregated conidia and germlings pass through the filter, while the large clumps are retained. The radioactivity of the large pore filter is then a measure of aggregation.

Conidia were grown on 2% agar slants of medium M-3 (0.8% sucrose), containing ^{14}C-sucrose (6.7×10^6 dpm per mM). The best procedure seemed to be to transfer the conidia from M-1 agar slants once onto M-3 agar slants, and then transfer the conidia from the M-3 agar slants to the ^{14}C M-3 agar slants.

The Millipore filter assembly was prepared by first laying down a 0.45 μ filter on the sintered glass layer, and then overlaying this with a 61 μ pore size nylon mesh (kindly donated by Dr. H. B. LeJohn: obtainable from Henry Simon Ltd., Stockport, England). The apparatus was set up so that the media and conidia would first have to pass through the nylon mesh before encountering the Millipore filter.

In the aggregation experiments, the radioactive conidia were inoculated into medium M-3, as described in Sections III and IV of the Methods. After a suitable period of growth (five to ten hours), the contents of the flasks were filtered by suction through the Millipore apparatus; the large clumps were retained by the nylon mesh, and the remainder of the conidia were collected by the 0.45 μ filter. The filters were washed with 10 ml of cold medium M-3 (8% sucrose), and then 10 ml of cold water. The nylon mesh was then cut into small squares, placed in scintillation vials along with 10 ml Aquasol, and the radioactivity determined on a Packard Tri-Carb Liquid Scintillation Spectrometer.

As a blank, conidia were inoculated and incubated with the experimental samples on the rotary shaker for one hour, and then filtered and counted. The cpm of these were subtracted from the test samples. In all experiments, the results obtained from the qualitative and quantitative assays agreed exactly.

B. Mycelial Pellets

The vegetative mycelial pellets were counted by the same principle as is used in a haemocytometer. A ruled grid (graph paper)

was placed on a lighted glass support. A square, clear, plastic tray (23 x 34 cm) was placed on top of the graph paper, and oriented so that the lines of the tray coincided with the lines of the ruled grid. Pellets to be counted were then poured into the tray, and arranged by eye so that they were randomly dispersed over the entire surface area. All the flasks corresponding to one test in an experiment were normally counted simultaneously (usually 5-7 flasks).

If the pellet number was small all the pellets were counted directly. If the pellets number was large various random squares were counted, and from the total number of squares under the tray the actual number of pellets calculated. On occasion, the pellets were photographed in the tray with a Polaroid MP-3 Land camera, type 107 film, and the number of pellets determined from the photographs. The flasks containing the pellets could be stored at 4°C for several days without any change in gross morphology or pellet number.

IX. UPTAKE OF RADIOACTIVE NUCLEOTIDES (RESULTS, SECTION VII)

Nucleotide uptake was studied in medium M-3; only Super Q water was used, and all the glassware was washed in 6 N HCl. For the uptake by conidia (eight hours growth), the standard growth conditions as described in Part IV-1 of the Methods were employed. The test nucleotide (10^{-5}M) was added to flasks on the shaker, and after a suitable time, the conidia were collected on a 0.45 μ Millipore filter, washed twice with 10 ml of M-3 containing 10^{-4}M unlabelled nucleotide, and the radioactivity determined in 10 ml Aquasol on the Packard Scintillation Spectrometer.

For the uptake of nucleotides by 22 hour and 45 hour mycelia, the conidia load and medium volume were reduced to one-tenth the regular amount. Conidia (7×10^6) were inoculated into 5 ml of M-3 in 20 ml glass vials, and incubated at 28°C and 220 rpm. At the appropriate time, the test substance was added, and the uptake determined as described above.

The specific activities of the nucleotides were: ^3H cAMP, 3500 cpm per nanomole; ^3H adenosine, 1500 cpm per nanomole; ^{14}C 5-AMP, 9200 cpm per nanomole.

X. CITRIC ACID DETERMINATION

Citric acid was estimated by a coupling of the enzymes "citratase" and "malic dehydrogenase", and following the NADH oxidation at 340 nm.

$$\text{citrate} \longrightarrow \text{oxaloacetate} + \text{acetate}$$

$$\text{oxaloacetate} + \text{NADH} + H^+ \longrightarrow \text{malate} + NAD^+$$

$$\text{citrate} + \text{NADH} + H^+ \longrightarrow \text{malate} + NAD^+$$

Citratase was prepared from Aerobacter aerogenes according to the method of Daron and Gunsalus (1962).

A. aerogenes stock cultures were maintained on trypticase soy agar slants at 5°C, or in a lyophilized state. For the preparation of the enzyme, the organism was grown on the following medium (g/l): trisodium citrate, 9; KH_2PO_4, 2; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.4; H_2O to one liter; adjusted to pH 7.0 with NaOH. The organism was grown in static cultures at 37°C. Cells were collected by Sharples centrifugation after 36 hours, washed twice in cold 0.1 M phosphate buffer,

pH 7.5, and either stored at -20°C or used directly.

Concentrated cell suspensions were prepared in phosphate buffer (4°C), and cells were broken by sonic disruption (Raytheon Sonic Oscellator .10Kc) for 30 minutes. The crushed cell mass was centrifuged for two hours at 200,000 x g in a Spinco untracentrifuge. The supernatant, which contained the enzyme, was dispensed into small amounts and stored at -20°C. The enzyme was stable for several months under these conditions, but freezing and thawing was harmful.

Citrate assays were carried out with the enzyme in this crude form; on some occasions, corrections had to be made for NADH oxidase activity in the extract. The amount of enzyme normally used (0.1 ml) cleaved 0.5 µmole of citrate within 0.5 - 3 minutes.

Citrate determinations were routinely conducted in 3 ml silica cuvettes (1 cm light path) on a Gilford spectrophotometer, Model 2400, with a scale of 2.0 absorbance (A) units. Stock solutions were prepared as follows:

Buffer Mixture: 169 ml of 0.1 M Na-phosphate, PH 7.5
2 ml of 0.2 M $MgCl_2$
75 mg of NADH

Malic Dehydrogenase: 0.1 ml of ammonium sulfate suspension of commercial enzyme diluted in 5 ml cold 0.1 M phosphate buffer, pH 7.5; or sufficient enzyme for an instantaneous reaction. Malic dehydrogenase and citratase were maintained on ice at all times during the assays.

Assay Procedure

The buffer mixture (2.7 ml), malic dehydrogenase (0.1 ml), and citratase (0.1 ml) were added to the cuvette, mixed, and the absorbance

recorded. Then 0.1 ml of the citrate "unknown" sample was added, mixed, and the A change observed. The citrate quantity was determined from a linear standard curve, change of A versus μmoles citrate. A correction of 0.07 A was necessary for dilution of the NADH mixture by the citrate sample. The range of the test was from 0.05 to 0.8 μmole citrate, and 0.5 μmole citrate yielded an A change of about 0.9.

XI. DETERMINATION OF ENDOGENOUS CYCLIC AMP IN THE MYCELIA AND THE GROWTH MEDIA (RESULTS, SECTION VII)

The concentration of cyclic AMP was determined by the method of Gilman (1970), which is based upon competition for binding sites on a cyclic AMP-dependent protein kinase by radioactive and non-radioactive cyclic AMP molecules. Following binding, the nucleotide-protein complex is separated from the assay mixture by filtration through a cellulose acetate Millipore (HAWP 02500) filter, to which the complex binds, and the "unknown" cyclic AMP concentration estimated from an isotope dilution standard curve.

1. Preparation of Cyclic AMP Binding Protein

Cyclic AMP binding protein was prepared according to the method of Miyamamoto et al. (1969) as described by Gilman (1970). Briefly, fresh skeletal muscle was obtained from Canada Packers Co., Winnipeg, and transferred on ice to the laboratory where it was minced and stored at -20°C. The protein was purified from 500 g of tissue through the pH, ammonium sulfate, and DEAE cellulose fractionations, as described by Gilman (1970). The "DEAE II" fraction was used for the cyclic AMP assays, and was stable for at least 18 months at -50°C.

Protein kinase inhibitor was prepared according to the method of Appleman et al. (1966) as described by Gilman (1970).

2. Purification of Cyclic AMP from the Mycelia and Growth Media

The mycelia were grown in 500 ml of medium in two liter flasks as described in Part VI of the Methods. Immediately after removal of flasks from the shaker, the mycelia were collected on cheesecloth, washed once in ice cold water, partially squeezed dry, and within twenty seconds covered with liquid nitrogen. Neutralization of the medium prior to the filtration (Feir and Suzuki, 1969) was omitted to permit the probably inactivation of cellular enzymes involved in cyclic AMP metabolism by acid adhering to the mycelia. The samples were then lyophilized and stored at -70°C. The total time elapsed between the filtration and freezing steps was about two minutes. Only one flask was removed from the shaker at a time, and was shaken by hand until filtration; this minimized changes in pellet-pellet contact and oxygen tension, both of which might influence cyclic AMP levels.

The growth medium filtrate was frozen, lyophilized, and stored at -70°C until subsequent analysis.

For the extraction and concentration of cyclic AMP from mycelia, the lyophilized cells were first ground to a fine powder with a mortar and a pestle. A pre-weighed amount (normally 0.8 g) of the powder was suspended in 15 to 20 volumes of cold 5% trichloroacetic acid (TCA) and homogenized for 15 minutes with a ground glass tissue homogenizer. The homogenate was quantitatively transferred into a graduated cylinder and the volume was recorded. One milliliter of the homogenate was combined with 1.0 ml of 2 N NaOH and saved for subsequent protein analysis. The

remaining homogenate was centrifuged for 30 minutes at 48,000 x g in a Sorval RC-2B centrifuge and for 90 minutes at 200,000 x g in a Spinco Ultracentrifuge to obtain a clear extract. In order to remove the TCA, the clear supernatant was extracted five times with two volumes of water-saturated petroleum ether. The samples were then immersed in a boiling water bath and sparged with air for two minutes to drive off the dissolved ether.

The cyclic AMP was further purified from the TCA-free extract by descending paper chromatography. A portion of the extract (0.700 ml) was spotted in a band (1.5 x 20 cm) along the base of Whatman No. 1 paper. Cyclic AMP markers were spotted on both ends of the band, and paper chromatography carried out in 1.0 M ammonium acetate (pH 7.2) - 95% ethanol (6:15 v/v) for 18 hours. The cAMP marker spots were identified under U.V. light, and the corresponding strip containing the mycelial cAMP was cut out of the paper. The paper strip was cut into small squares and the cAMP was extracted with 30 ml of water. After filtration through a scintered glass filter, the purified cAMP sample was lyophilized in conical bottomed tubes and stored at -70°C until assayed. This chromatography step was essential as the extract contained substances which behaved like cAMP in the assay, but did not co-chromatograph. This procedure yielded recoveries of 71%-91% of ^{3}H cAMP added to the TCA extract before the first centrifugation step.

The lyophilized samples of growth media were suspended in cold 5% TCA, homogenized with a ground glass tissue homogenizer, and the cyclic AMP purified in an identical manner to the mycelial samples.

3. Assay for cAMP

The cAMP-containing samples were dissolved in a small recorded

volume of 50 mM sodium acetate (pH 4.0) and assayed for cAMP according to Gilman (1970). The Millipore filters were dissolved in 10 ml Aquasol and counted on a Packard Tri-Carb Liquid Scintillation Counter. A standard curve was prepared simultaneously with the "unknown" samples, and at least three trials were performed for each sample. Standard curves contained 15 to 18 points, and were linear over the range of 1.5 pmole (2700 cpm) to 22 pmoles (450 cpm).

The assay mixture was as follows:

8 µl binding protein (7.5 g protein)

5 µl ^{3}HcAMP (9,088 cpm; 1.85 pmoles)

5 µl protein kinase inhibitor

32 µl cAMP sample dissolved in 50 mM sodium acetate, pH 4.0.

XII. DETERMINATION OF PROTEIN AND SUCROSE

Protein was estimated by the method of Lowry et al. (1951) with crystalline bovine serum albumin as standard.

Sucrose was estimated by a modification of the anthrone method (Seifter et al., 1950). The anthrone reagent was prepared by dissolving two grams of anthrone powder in one liter of 95% H_2SO_4 (50 ml of H_2O plus 1000 ml of concentrated H_2SO_4). The fresh anthrone reagent (6 ml) was added to 3 ml of sucrose sample (10-150 µg) by allowing the reagent to run slowly down the side of the test tube. Samples were immersed in a boiling water bath for three minutes, and the optical density determined without cooling on a Klett-Summerson colorimeter (red filter).

XIII. ASSAY OF ENZYMES PERTAINING TO CYCLIC AMP (RESULTS, SECTION V)

A. Intracellular Cyclic AMP Phosphodiesterase

1. Paper Chromatography Assay of Purified Enzyme Preparations

Cyclic AMP phosphodiesterase hydrolyses the cyclic phosphodiesterase bond, converting 3',5'-cyclic AMP to 5'-AMP (Sutherland and Rall, 1958). If radioactive cyclic AMP of a known specific activity is used as substrate, the product AMP will also be radioactive and of the same specific activity. Therefore, the reaction rate can be determined by measuring the radioactivity of AMP separated from the cyclic AMP by paper chromatography. The specific activity of this enzyme is defined as the μmoles cAMP hydrolysed per minute per mg protein.

The solvent system used was 1.0 M ammonium acetate (pH 7.2) - 95% ethanol (6:15 v/v). The Rf's of the nucleotides were: ATP, 0.08; AMP, 0.26; adenosine, 0.66; cAMP, 0.50.

Assays were carried out in serum tubes (7 x 50 mm) in a total volume of 100 μl. The assay mixture contained:

KOH-Bicine buffer, pH 7.5	50 mM
$MgCl_2$	7.5 mM
cAMP	2.5 mM
U-^{3}H-cAMP (28 Ci/mmole)	135,000 dpm

The reaction was started by addition of enzyme. The tube was immersed in a 30°C water bath and incubated for 15 minutes. The reaction was terminated by immersing the tube in a boiling water bath for two minutes.

AMP (0.1 μmole in 5 μl) was added to the assay mixture as a

carrier and marker, and 21 µl (1/5 of total) of the mixture were spotted with a Hamilton syringe on Whatman No. 1 paper. Descending paper chromotography was carried out for 18 hours. The spot corresponding to AMP was located under U.V. light, then cut out of the paper and into small squares, which were placed in a test tube. AMP was eluted with 6 ml of water and 4.0 ml of the eluate were transferred into a scintillation vial. Aquasol (15 ml) was added and the vial was shaken to form a homogeneous gel. Radioactivity was then determined on a Packard Tri-Carb Liquid Scintillation Spectrometer.

The dpm values were calculated from an acetone-generated quenching curve constructed under conditions identical to the assay. For all experiments, control runs with boiled enzyme were performed and the control values were subtracted from the radioactivity values of the test assay. Under the assay conditions the reaction rate was proportional to the enzyme concentration, and was a linear function of time. This process yielded recoveries of 90-95% of ^{14}C-AMP added prior to chromotography.

2. Paper Chromotography Assay of Crude Enzyme Preparations

Crude enzyme preparations contained phosphomonoesterase activity which converted AMP to a compound which co-chromatographed with adenosine. Therefore, the cAMP determined was erroneously low. In order to alleviate this problem, reactions were conducted as described in Part 1 only up to the termination of the reaction. Crotalus atrox snake venom (10 µl, 0.1 mg) was then added and the incubation was continued for ten minutes, after which it was terminated by boiling. This step caused the conversion of all the AMP to adenosine. The same procedure

was then followed as described in Part 1, except that the carrier was adenosine in the place of AMP and the radioactivity in the adenosine spot was determined.

3. Dowex Assay

Phosphodiesterase was also assayed using a strong anion exchange resin as first described by Thompson and Appleman (1971). In this procedure, the ^{3}H-AMP produced is further hydrolyzed to ^{3}H-adenosine with snake venom. The anion exchange resin is then added to the reaction mixture to bind the acidic ^{3}H-cAMP and ^{3}H-AMP, but not the neutral ^{3}H-adenosine. Therefore, all the radioactivity remaining in the fluid following treatment with the resin is adenosine.

Dowex I X8-400, obtained from Bio-Rad, was used in this study. The resin was washed extensively in 0.5 N NaOH, then 0.5 N HCl, and finally with distilled water to pH 5.0, and stored as a 33% suspension in water.

Reactions were carried out in an identical manner to that described in Part 1 of this section, only using snake venom as described in Part 2. Following termination of the snake venom action in the boiling water bath, 1.0 ml of the Dowex-water suspension was added to the assay tube. The contents were mixed well and the resin was allowed to settle to the bottom of the tube. The clear supernatant (0.5 ml) was transferred to a scintillation vial and 10 ml of Aquasol were added. The radioactivity was determined as described in Part 1.

The boiled enzyme controls were run with all experiments. This procedure was simple and convenient for enzyme determinations under standardized conditions, but care had to be exercised not to change the

pH and the ionic strength, since they influenced the binding of the charged nucleotides to the resin.

B. Extracellular Cyclic AMP Phosphodiesterase

The assay principle was identical to that described in Part XIII A 1. Reactions were carried out in serum tubes (7 x 50 mm), with a final volume of 100 µl. The reaction mixture consisted of:

K-citrate, pH 3.5	34 mM
$MgCl_2$	20 mM
cAMP	0.060 mM
U-^{3}H-cAMP (28 Ci/mmole)	10^5 cpm

The reaction was initiated by the addition of enzyme, allowed to proceed in a 30°C water bath for 60 minutes, and terminated by immersing the tube in a boiling water bath for one minute. Crotalus atrox snake venom (10 µl, 0.1 mg) was added and the reaction carried out again at 30°C for ten minutes, then terminated by boiling. Adenosine (5 µl, 0.1 µmole) was added, and 38 µl (1/3 of total) were spotted with a Hamilton syringe on Whatman No. 1 paper. Descending paper chromotography was carried out and radioactivity determined in the adenosine spot as described in Part XIII A 1. Control runs with pre-boiled enzyme were carried out under identical assay conditions. The reaction rate was a linear function of time for one hour.

C. Adenyl Cyclase

Adenyl cyclase catalyses the conversion of ATP into cyclic AMP and pyrophosphate (Rall and Sutherland, 1962). The α-phosphate of ATP is the precursor of the phosphate in cyclic AMP. The reaction was assayed in the forward direction using two different techniques, as

described below. The reaction could also be carried out in the reverse direction (not shown). The Gibbs free energy of hydrolysis of cyclic AMP has been reported to be -11.9 Kcal/mole (Greengard, 1971).

1. Paper Chromatography

In this procedure, the ^{14}C labelled ATP substrate is separated from the ^{14}C labelled cyclic AMP product by paper chromatography, and the radioactivity in the cyclic AMP spot determined.

The assay was carried out in serum tubes (7 x 50 mm) in a total volume of 100 µl. The reaction mixture consisted of:

Bicine-KOH, pH 7.5	25 mM
$MgCl_2$	10 mM
Crystalline bovine serum albumin	0.15 mg
ATP	0.1 mM to 5 mM
U-^{14}C-ATP (0.05 mCi/0.115 mmole)	220,000 dpm

The reaction was started by addition of enzyme, allowed to proceed in a water bath at 30°C for 30 minutes, and terminated by immersion in a boiling water bath for two minutes. Cyclic AMP (5 µl, 0.1 µmole) was added to the reaction mixture as a marker and carrier, and an aliquot was spotted with a Hamilton syringe on Whatman No. 1 paper. Descending paper chromatography was carried out in 1.0 M ammonium acetate, pH 7.2 - 95% ethanol (6:15 v/v). Cyclic AMP spots were located under U.V. light, cut out and into small squares, and placed directly into scintillation vials. Ten milliliters of Aquasol or Bray's solution were added and the radioactivity was determined on a Packard Tri-Carb Liquid Scintillation Spectrometer.

In all experiments, pre-boiled enzyme controls were run under

identical conditions. The presence of the chromatography paper in the scintillation vial appeared to have little quenching effect when ^{14}C was used, although it had a large effect when tritium was the radioactive source.

With crude enzyme preparations cyclic AMP (1.0 mM) was included in the reaction mixture to minimize erroneous low results caused by hydrolysis of the product cyclic AMP by phosphodiesterase present in the enzyme preparation.

In some experiments, pyrophosphatase (0.84 units) was included in the assay.

2. ATP Precipitation and Thin Layer Chromatography (Results, Section V, A)

This procedure is similar to that described above, but after the reaction has been completed the ATP is precipitated as a zinc salt, and the cAMP further purified by thin layer chromatography. In the experiment reported in the results (Section V), the following procedures was used:

Assay Buffer	0.15 M	Tris-Cl, pH 7.6
	15 mM	$MgCl_2$
	30 mM	NaF
	3 mM	DTT

The assay was carried out in serum tubes (7 x 50 mm) in a total volume of 310 µl. The reaction mixture consisted of:

100 µl	assay buffer
10 µl	U-^{14}C-ATP (53 mCi/mmole; 10^6 dpm)
200 µl	enzyme extract

The reaction was started by the addition of enzyme and after 30 minutes at 30°C terminated by immersion in a boiling water bath for one minute. Fifty microliters of 1 M $ZnSO_4$ and 50 µl of 1 M Na_2CO_3 were added to the assay tubes, mixed well, and allowed to stand for one hour while the precipitate was formed (Wood et al., 1972). The tubes were centrifuged at 5000 x g for ten minutes and the supernatant was carefully removed. Adenine, cAMP, and ATP (all 0.1 µmole) were added to the supernatant as carriers and 5 µl of the mixture were spotted on silica gel (HF-254; Brinkman Inst.) plates. Thin layer chromatography was carried out in a solvent consisting of isopropanol: ammonium hydroxide:water (7:1:2). The nucleotide spots were located under U.V. light, scraped off the plate, and placed into scintillation vials along wizh 10 ml of Bray's solution. The radioactivity was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer.

D. Cyclic AMP Binding Protein

Cyclic AMP binding protein was assayed as described by Walton and Garren (1970). The assay was carried out in serum tubes (7 x 50 mm) in a total volume of 200 µl. The reaction mixture consisted of:

K-phosphate, pH 6.5	25 mM
$MgCl_2$	7.5 mM
Crystalline bovine serum albumin	0.75 mg
cAMP	50 mM
U-^{3}H-cAMP (28 Ci/mmole)	135,000 dpm

The assay was started by the addition of enzyme, allowed to equilibrate at 30°C for 30 minutes, and then stopped by immersing in ice. Five milliliters of TM buffer (10 mM Tris-Cl, 10 mM $MgCl_2$, pH 8.0,

4°C) were layered over Gelman GN-6 cellulose acetate filters, which had been pre-soaked in the TM buffer, and the assay mixture was transferred to the TM buffer layer. The nucleotide-protein complex was collected on the filter by suction. After washing with TM buffer, the filter was placed in scintillation vials and the radioactivity determined in 10 ml Aquasol on a Packard Tri-Carb Liquid Scintillation Spectrometer. Boiled enzyme controls were run under identical conditions.

XIV. SOURCES OF COMMERCIAL CHEMICALS

The following chemicals were obtained from the Sigma Chemical Company: adenosine, ADP, AMP, ATP, cyclic AMP, 2',3'-cyclic AMP, DB-cyclic AMP, Crotalus atrox snake venom (V-7000), DTT, L-epinephrine, trypsin, U-^{14}C-AMP, U-^{14}C-ATP, and pyrophosphatase.

Other chemicals used were obtained from different commercial sources, as follows: con A from Miles-Yeda Limited, Rehovoth, Israel; ATP and NADH from P-L Biochemicals; malic dehydrogenase from Boehringer and Soehne (Germany); glucagon from Eli Lilly and Company; PHA from Difco; U-^{3}H-cGMP and U-^{3}H-cAMP from Schwartz/mann.

RESULTS AND DISCUSSION

In order to develop the arguments of this thesis in an orderly manner it was considered necessary to divide the work into seven sections. Each section deals with one aspect of the problem, both in terms of results and discussion. The results in Section I suggest that the zinc ion regulates growth and citric acid accumulation, and those in Section II provide supporting evidence for this belief by showing that under special conditions large amounts of growth can occur to the exclusion of citrate synthesis at zinc levels which normally limit growth and allow citrate production. The data in Section III imply that cyclic AMP regulates growth and citric acid accumulation, and those in Section IV that the nucleotide may also control the "adhesion" of conidia and hyphae. The results in Section V prove the presence in A. niger of some of the enzymes pertaining to cyclic AMP, and those in Section VI point out that metal-complexing agents such as EDTA and ferrocyanide can simulate some of the responses to cyclic AMP. Finally, in Section VII it is illustrated how the endogenous levels of cyclic AMP vary under conditions of growth and citric acid accumulation. This Section also presents evidence which allows speculation on the possibility that the metabolism of zinc and cyclic AMP may be connected, and based on this a tentative model is advanced.

Although citric acid production was favored by high sucrose levels, all studies were carried out in low sucrose medium (0.8% or 0.4%) as it was considered that this would tend to represent a more "natural" condition.

SECTION I

CITRIC ACID ACCUMULATING CONDITIONS: THE REGULATION OF GROWTH AND CITRIC ACID PRODUCTION BY ZINC

This Section describes the conditions which were conducive to citric acid accumulation. Previous studies on the composition of the media, and in particular the influence of trace metals have been described in the Historical. It was determined that under the present experimental conditions zinc was the only important metal. The ion seemed to control the "decision" between growth and acidogenesis: at low zinc growth was inhibited and citric acid synthesis occurred; at high zinc the reverse was true, and growth took place at the expense of citrate production.

Citric acid accumulated only under certain conditions which related to the medium composition, and in particular, the presence of zinc. In medium M-1 (high pH, no zinc) acidogenesis occurred, but in relatively small amounts. Citrate was not produced in medium M-2 (containing trace metals), except on occasion for a very small "pulse" late in fermentation (Section VII). Citric acid did not accumulate in medium M-3 (lacking trace metals) when the medium was prepared in ordinary glass distilled water, or when the glassware was washed in lab detergent and rinsed in tap distilled water. However, if the glassware was acid washed, and if pure chemicals (especially sucrose) and triple glass distilled or Super Q (Millipore Corp.) water were used, large amounts of citrate were synthesized in medium M-3. Apparently, there were sufficient trace metals still present as contamininants in M-3 to allow growth and citrate accumulation to occur.

Although high sucrose concentrations are known to favor citrate accumulation, this condition alone was not sufficient to cause citrate accumulation in high yields. While high sucrose levels were very beneficial to citrate accumulation in medium M-3 (Fig. 1), in medium M-2 no citrate was produced after three days in flasks containing as much as 14% sucrose. This indicated that the presence of the trace metals was the critical acidogenic factor.

As discussed in Section III, cyclic AMP stimulated citrate synthesis when added to medium M-3 containing 0.8% sucrose. Fig. 1 illustrates that this was also true at high sucrose concentrations.

Since the only difference between media M-2 and M-3 was the presence of trace metals, experiments were performed to identify the metal controlling acidogenesis. Medium M-3 was prepared, inoculated, and incubated as described in the Methods. After 45 hours, trace metals were added in different combinations to the accumulating cultures, in identical concentrations as were used in medium M-2. Addition of zinc either alone or in any combination resulted in abatement of citrate synthesis within at least five hours (Fig. 2). The other ions did not influence citrate accumulation, although iron appeared to stimulate the re-utilization of citrate after termination of its synthesis by zinc; this re-utilization did not occur until the sucrose levels were very low or nil.

Fig. 3 illustrates the effect of titrating medium M-3 with the M-2 trace metal solution. The metals were added at zero hours, citrate samples taken at 46 hours, and cell weights determined after 72 hours. Citrate accumulated only when the levels of trace metals

Figure 1: Influence of the initial sucrose concentration on citrate accumulation in the presence (X) and absence (●) of cAMP (0.2mM). 500ml of M-3 were inoculated with 7.2 x 10^7 conidia, and incubated at 28°C and 180 rpm. The cAMP was added at zero hours, and the flasks were harvested after 48 hours.

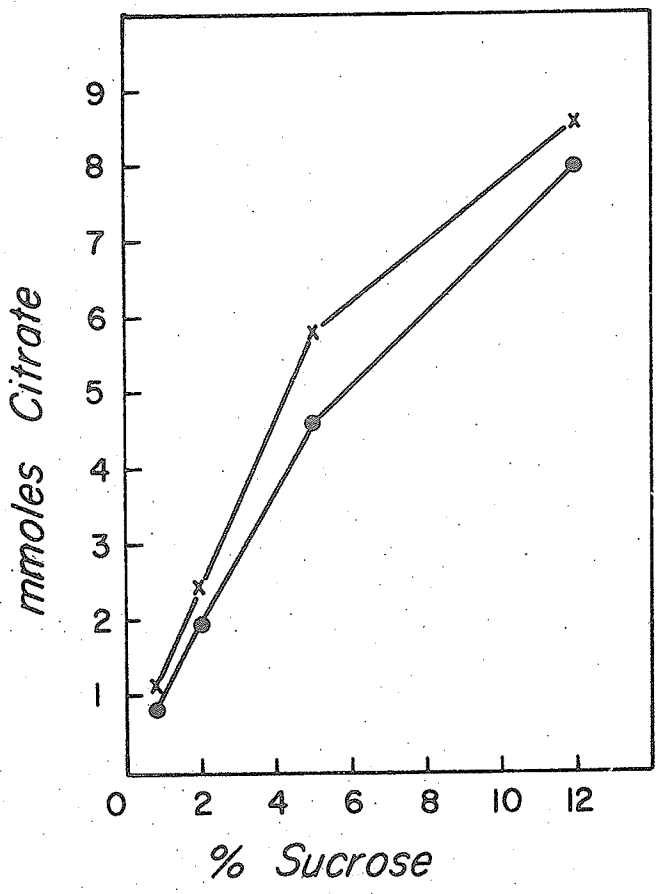

mmoles Citrate
1
2
3
4
5
6
7
8
9
0
2
4
6
8
10
12
% Sucrose

Figure 2: Influence of zinc, iron, and copper on citrate accumulation in medium M-3. The metals were added to accumulating cultures at 45 hours (arrow) in the combinations shown in the Figure. The metal concentrations used were identical to that of medium M-2 and were: iron, 7.90 μM; copper, 0.38 μM; zinc, 1.55 μM. Citrate (✕), sucrose (●).

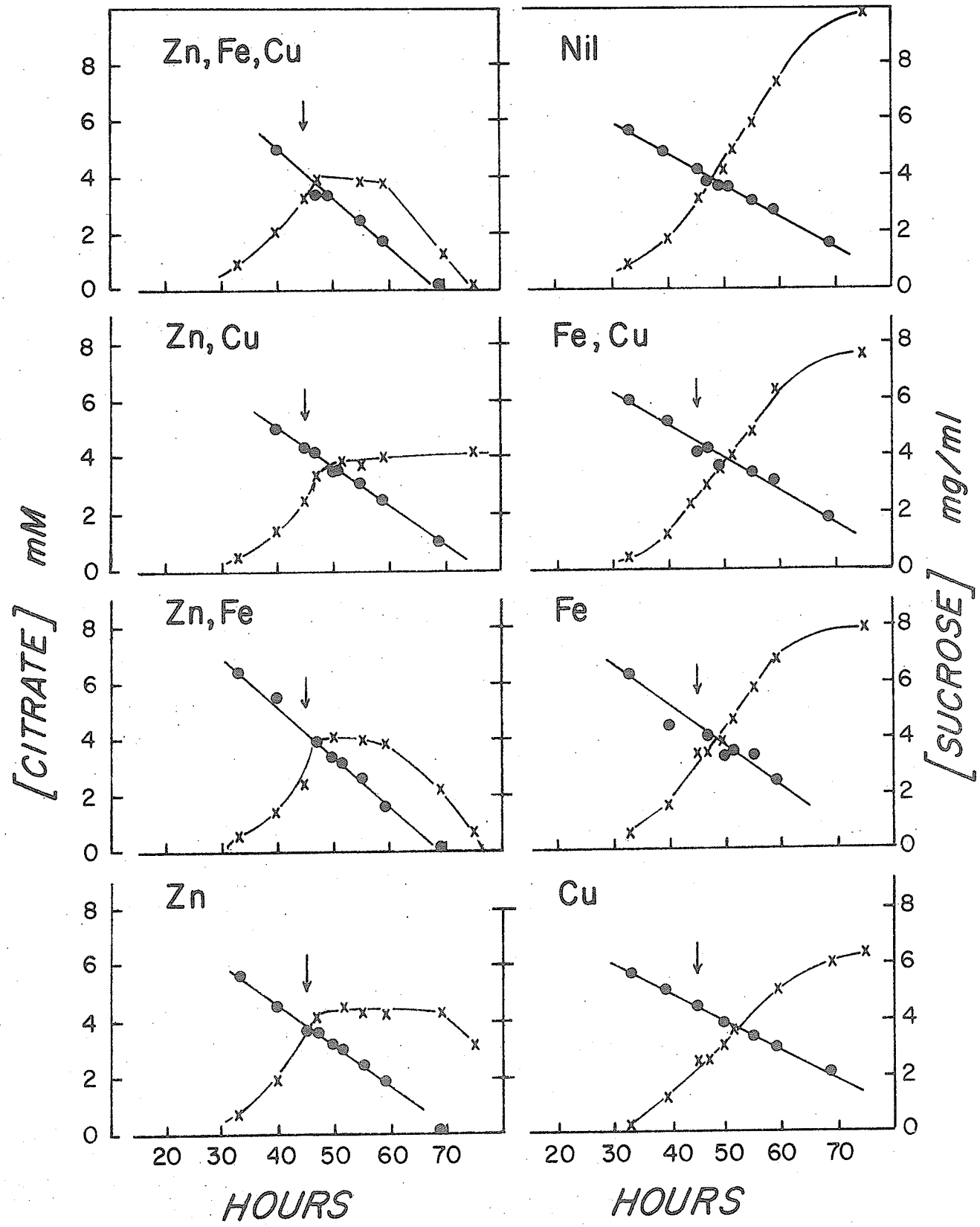
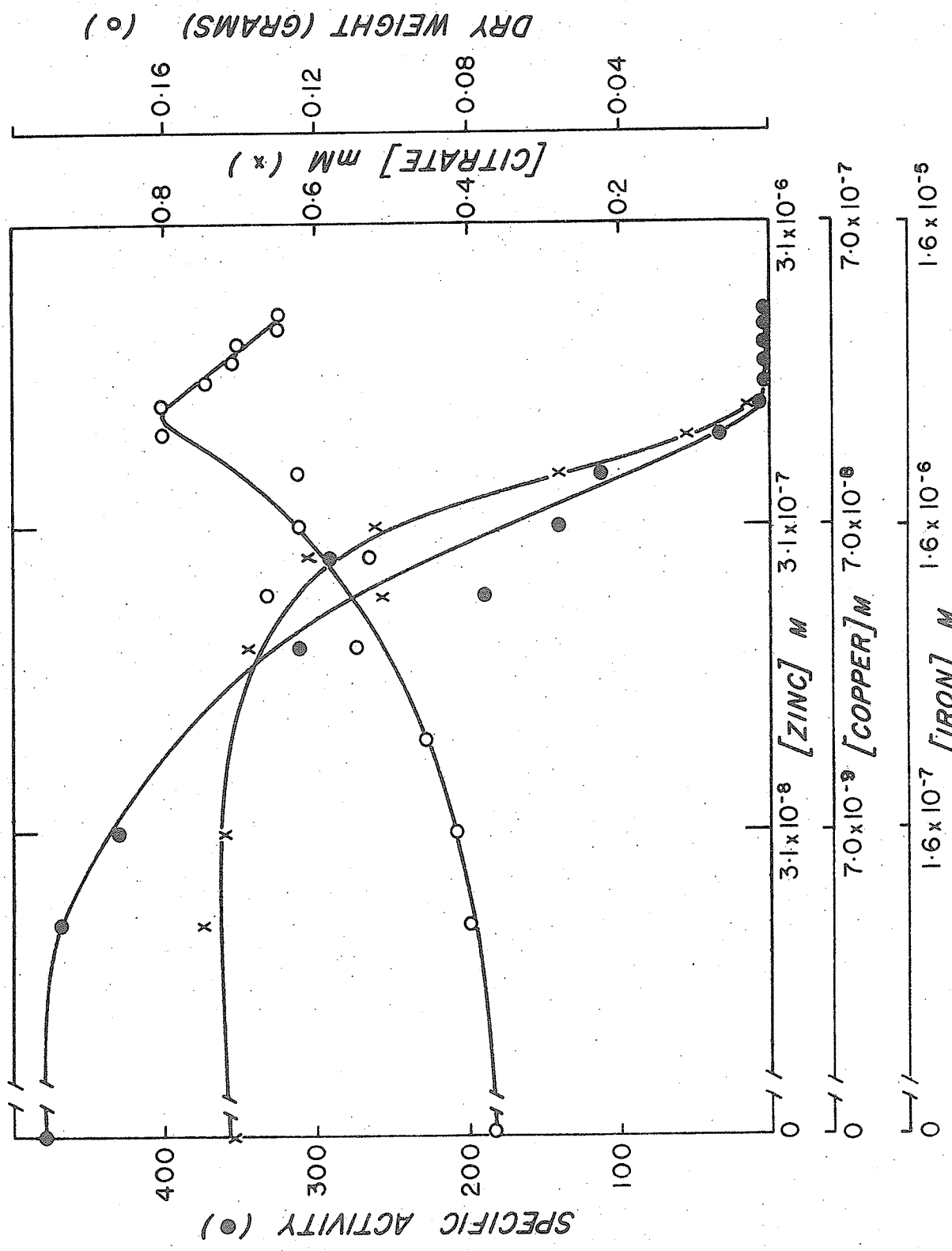

Zn, Fe, Cu
Nil
Zn, Cu
Fe, Cu
Zn, Fe
Fe
Zn
Cu
[CITRATE] mM
[SUCROSE] mg/ml
HOURS
HOURS
0
2
4
6
8
20
30
40
50
60
70

Figure 3: Effect of increasing amounts of M-2 trace metal nutrition on growth and citrate accumulation. The metals were added before inoculation, citrate samples taken after 46 hours, and cell dry weights determined after 72 hours. Specific activity (●) citrate (X), dry weight (O). (6.1×10^6 conidia, n = no. of flasks = 2).


SPECIFIC ACTIVITY (●)
400
300
200
100
[CITRATE] mM (×)
0·8
0·6
0·4
0·2
DRY WEIGHT (GRAMS) (○)
0·16
0·12
0·08
0·04
0
3·1x10⁻⁸
3·1x10⁻⁷
3·1x10⁻⁶
[ZINC] M
0
7·0x10⁻⁹
7·0x10⁻⁸
7·0x10⁻⁷
[COPPER]M
0
1·6x10⁻⁷
1·6x10⁻⁶
1·6x10⁻⁵
[IRON] M

were low. Increasing the levels caused increased growth, but decreased total citrate synthesis and specific activity (μmoles citrate produced per gram dry weight of mycelia).

The same experiment using only zinc is shown in Fig. 4, but in this case, the citrate concentration and dry weights were both determined after 48 hours growth. The effect of increasing zinc concentrations on the mycelial growth and specific activity for citrate production is dramatic, and significantly, qualitatively similar to that observed when all the trace metals were used. There was an average of 3.5mg/ml of sucrose remaining in the flasks containing the highest zinc level and the larger amounts at the lower zinc levels. This result excludes the possibility that the low levels of citrate observed in the zinc replete cultures were due to the re-utilization of the acid by the mycelia (Fig. 2). The zinc concentration causing 50% inhibition of citrate production was about 3×10^{-7}M in Fig. 3, and about 9×10^{-7}M in Fig. 4, so there may have been some interaction between the metals. The higher number of conidia used in Fig. 4, however, could have contributed to the different effective zinc concentration (Section III). The important point is that the pattern was essentially the same in the presence of either the full trace metal solution or zinc ion alone.

Addition of the trace metal solution or zinc ion also had an important influence on the morphology and pigmentation of the mycelial pellets. The high metal pellets were white and large, while the low metal pellets were smaller, contained a bright yellow pigment (YP), and were present in larger numbers. YP is a typical secondary metabolite (Section III), and its appearance usually, but not always,

correlated with citrate accumulation. In the presence of the full trace metal solution (Fig. 3), there was a gradient of white to yellow, from high concentrations to low concentrations of metals. In Fig. 4, zinc concentrations greater than 3.1×10^{-7}M completely inhibited YP formation.

The influence of 10^{-5}M manganese, calcium, iron, and zinc on growth and citric acid accumulation was tested in another experiment, and the results are shown in Table 1. Zinc caused a very large increase in growth and severe inhibition of citrate production, while the other metals had a relatively slight effect. The zinc cultures consisted of large, thick, white pellets, while the control cultures and the cultures containing the other metals contained small, numerous, "flimsy", and very yellow pellets. In another experiment the influence of the same metals on growth and citrate synthesis was tested at 5×10^{-6}M after both 45 and 70 hours growth (Table 2). Again, only zinc had a significant effect, and caused increased growth and decreased citrate accumulation. Note that although most of the growth had been completed by 45 hours, citrate production had still continued until at least 70 hours.

The response of the cultures to high levels of the other medium constituents at various zinc concentrations is shown in Fig. 5. Mycelia were grown in medium M-3 containing 10 times the normal concentration of inorganic salts (NH_4NO_3, KH_2PO_4, $MgSO_4$), and the zinc was added before inoculation. Citrate accumulated, but only to a much reduced concentration even at low zinc levels; there was some inhibition of growth as well. Increasing the zinc concentration resulted in

Figure 4: Effect of increasing amount of zinc on growth, citrate accumulation, and yellow pigment (YP) formation. Zinc was added before inoculation and the flasks harvested after 48 hours growth. Specific activity (●), citrate (x), dry weight (o). (1.4×10^7 conidia; n = 3).

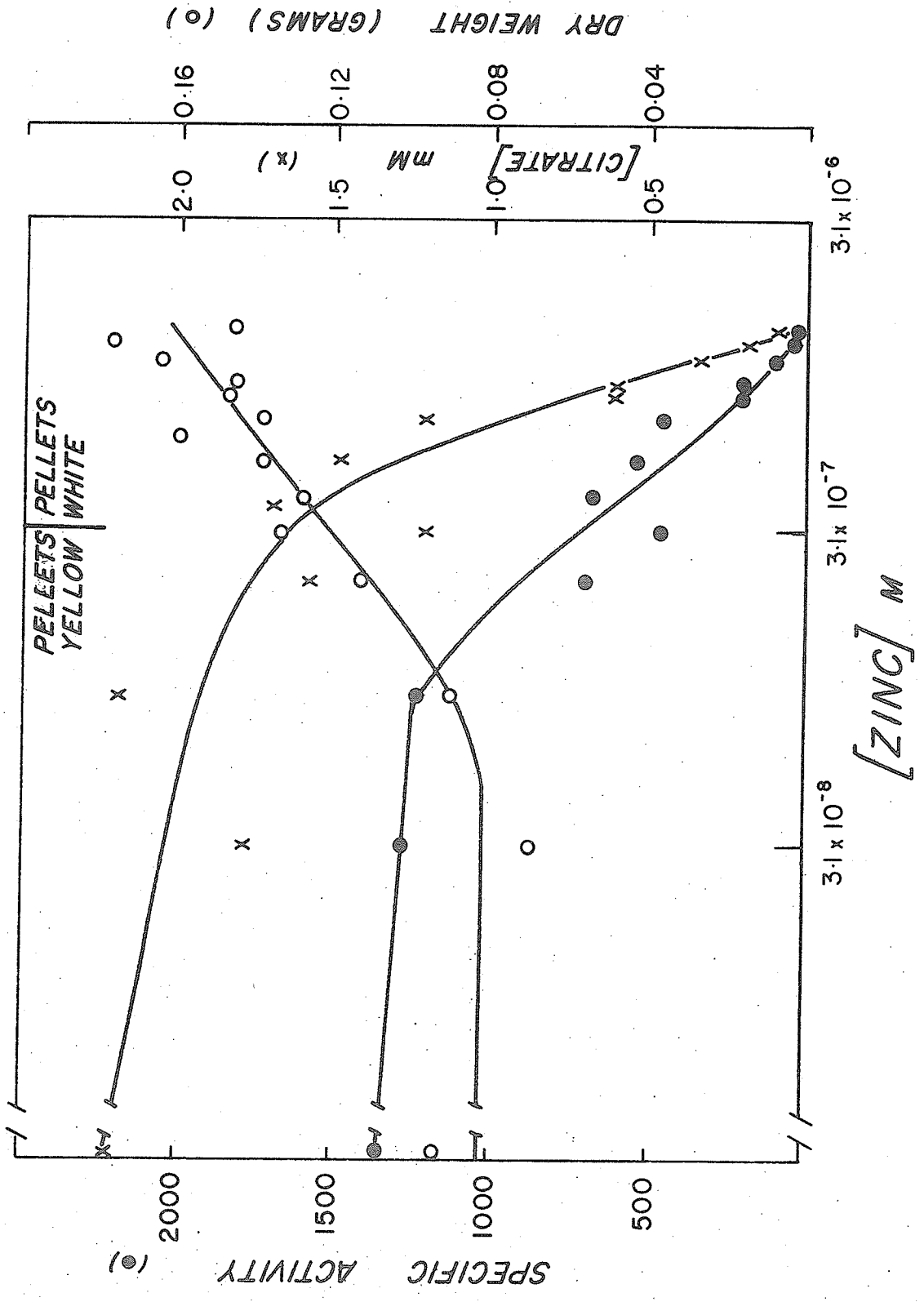

DRY WEIGHT (GRAMS) (○)
0·16
0·12
0·08
0·04
[CITRATE] mM (×)
2·0
1·5
1·0
0·5
3·1 x 10⁻⁶
3·1 x 10⁻⁷
3·1 x 10⁻⁸
[ZINC] M
PELLETS YELLOW
PELLETS WHITE
SPECIFIC ACTIVITY (●)
2000
1500
1000
500

Table 1

Influence of metals on growth and citrate accumulation.

	Dry Weight (mg)	Citrate (mM)	Specific Activity	Specific Activity % of Control
CONTROL (10)*	49.3	2.4	2434	100
$CaCl_2$ (7)	50.9	3.2	3143	129
$MnCl_2$ (7)	64.1	2.8	2184	90
$FeCl_3$ (7)	53.8	2.8	2602	108
$ZnSO_4$ (3)	160.4	1.1	343	14

Metals (10^{-5}M) were added at zero hours, and the flasks harvested at 51 hours. (1.15×10^7 conidia). *(n) = number of flasks.

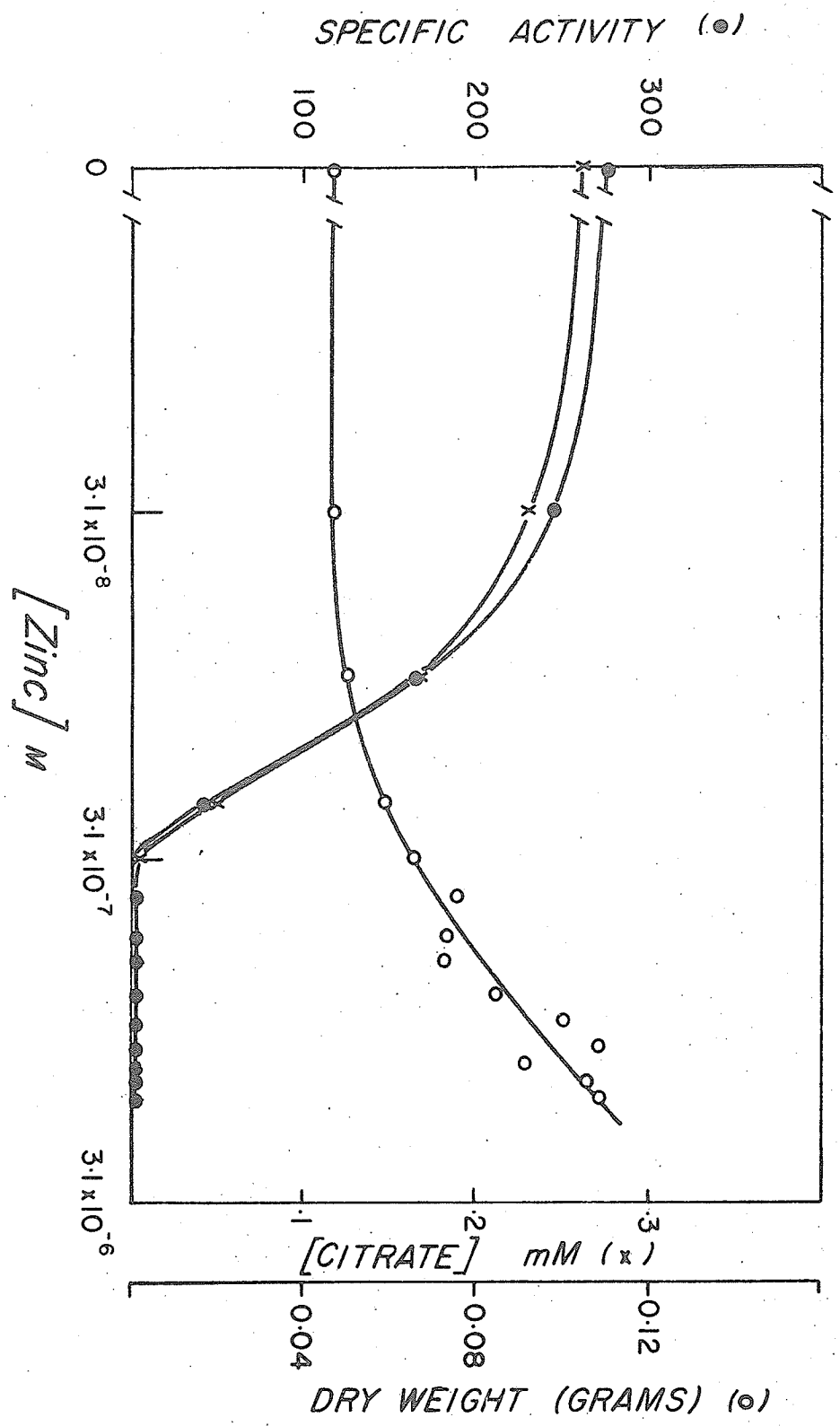
Table 2

Influence of metals on growth and citrate accumulation after 45 hours and 70 hours.

	Time Harvested (hours)	Dry Weight (mg)	Citrate (mM)	Specific Activity	Specific Activity % of Control
CONTROL	45	76	3.9	2570	100
	70	80	7.3	4580	100
$CaCl_2$	45	83	2.9	1750	68
	70	85	9.0	5280	115
$MnCl_2$	45	75	3.4	2260	88
	70	88	8.1	4620	101
$FeCl_3$	45	90	2.6	1440	56
	70	82	8.1	4950	108
$ZnCl_2$	45	175	1.2	343	13
	70	158	1.7	538	12

Metals (5×10^{-6}M) were added at zero hours, and the flasks harvested after 45 hours and 70 hours (9.2×10^6 conidia; n = 3).

Figure 5: Effect of increasing amount of zinc on growth and citrate accumulation in medium M-3 containing 10x the regular inorganic salts. Zinc was added at zero hours and the flasks harvested after 48 hours growth. Specific activity (●), citrate (X), dry weight (O). (8.0×10^6 conidia; n = 5).


SPECIFIC ACTIVITY (●)
100
200
300
0
3·1 x 10⁻⁸
3·1 x 10⁻⁷
3·1 x 10⁻⁶
[Zinc] M
·1
·2
·3
[CITRATE] mM (x)
0·04
0·08
0·12
DRY WEIGHT (GRAMS) (○)

stimulation of growth and inhibition of citrate production. The zinc concentration required for 50% inhibition of citrate synthesis, was 1.2×10^{-7}M as compared to 9×10^{-7}M in the normal medium, so there may be some interaction between zinc and the inorganic salts. Nevertheless, the general pattern of response to zinc was similar to that of mycelia grown in the regular medium (Figs. 3 and 4). Under the conditions of high salts the pellets were white, and somewhat more "slimy" and "sticky" than the cells grown at low salt levels.

Table 3 shows the effect on growth and citric acid accumulation of reducing the inorganic salt constituents of medium M-3 to one-fifth the normal amount. Similar amounts of growth occurred as in the regular medium, and citrate accumulation was still repressed by the presence of trace metals. There was a YP formation with or without trace metals. The addition of zinc alone after 43 hours to cultures grown without trace metals in this same medium resulted in inhibition of citrate production, and an increase in growth (Fig. 6). This result eliminates the possibility that a deficiency in any of the other trace metal or inorganic salt components was the cause for citrate accumulation.

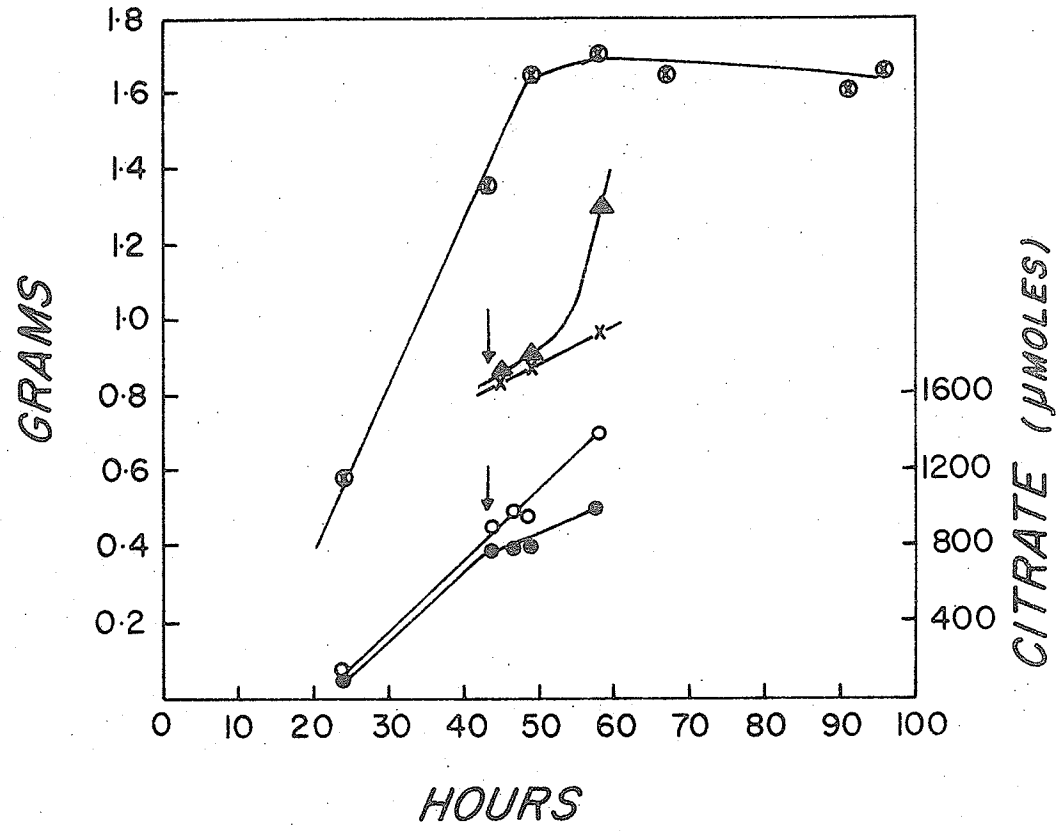
Table 3

Influence of trace metals on growth and citrate accumulation in medium M-3 containing one-fifth the regular amount of inorganic salts.

	Dry Weight (g)	Citrate (mM)
Trace Metals	0.164	trace
No Trace Metals	0.146	4.2

Trace metals (same concentrations as medium M-2) were added at inoculation and the flasks were harvested after 75 hours. (6.3×10^6 conidia; n = 2).

Figure 6: Effect on growth and citrate accumulation of zinc addition (1.55 μM) to medium M-3 containing one-fifth the regular amount of inorganic salts. This experiment was performed in 2-liter flasks containing 500ml of medium and agitated at 160rpm. Zinc was added at 43 hours (arrow). Citrate: zinc added (●), no zinc (O). Dry weight: zinc added (▲), no zinc (x). Growth obtained in the same medium containing the full complement of M-2 trace metals (⊗). (7×10^7 conidia/500ml; n = 1).


GRAMS
1·8
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
CITRATE (μMOLES)
1600
1200
800
400
0
10
20
30
40
50
60
70
80
90
100
HOURS

DISCUSSION

It was concluded from these studies that zinc was the limiting nutrient in medium M-3, since the addition of zinc caused increased growth, even when the inorganic salts were present in one-fifth the normal amount. Citric acid and the other secondary metabolite, YP, accumulated only under conditions of restricted growth; which was directly related to the zinc concentration, and was independent of the other heavy metals and calcium. The other ions must have been present in medium M-3 for growth to occur, but in this semi-purified medium, it is likely that they were present in very low amounts. Increasing the amount of growth three-fold by zinc addition (Table 1) might then be expected to induce deficiency in the other metals, and thereby effect accumulation of citrate and/or YP. That this did not occur argues that zinc is a "special" ion under these experimental conditions, and controls the "decision" between growth and secondary metabolism.

There are insufficient data to interpret the inhibitory action of the high inorganic salts medium, and this question was not pursued further. However, high inorganic salts did not interfere with the basic response pattern of the cultures to zinc.

The exact nature of the role of zinc in the physiology of fungi is not known, but there are indications that it is in some way involved in carbohydrate metabolism (Foster, 1939, 1949). In zinc replete cultures containing other essential nutrients, growth and glucose utilization are fast and efficient; the economic coefficient (sugar consumed/dry weight mycelia) is low, and the respiratory

coefficient (CO_2 produced/dry weight mycelia) is high. That is, the carbohydrate is converted efficiently to CO_2 and cell material. In zinc deficient cultures, however, the carbohydrate oxidation efficiency goes down, as indicated by a rise in the economic coefficient and a drop in the respiratory coefficient. A similar effect is observed by increasing the sucrose concentration (C/N ratio).

Organic acid accumulation is known to be most pronounced when zinc is deficient (Cochrane, 1958) or where sucrose is high (Foster, 1949). This suggests that acidogenesis is related to the efficiency of glucose catabolism, which is in turn a function of the degree of control operating on the EMP pathway. Therefore, a possible site of action of zinc "deficiency" is phosphofructokinase, which appears to be a regulatory enzyme in A. niger (Smith and Ng, 1972). The effect of high sucrose may be in part related to the Crabtree Effect (Koobs, 1972), in which high glucose inhibits respiration and leads to a fermentative type of metabolism conducive to organic acid accumulation.

It is interesting that in these experiments iron and manganese were without significant influence on the citrate fermentation, which is in contrast to most other studies, many of which were carried out with the same strain of the organism. This may possibly relate to the low sucrose concentrations employed here (0.8%); all other investigations were conducted in high glucose or sucrose media (approximately 14%). It is also known that zinc effects on carbohydrate metabolism are less significant at high than at low sucrose levels (Foster and Waksman, 1939). Significantly, zinc is without influence when the fungus is grown on organic acids (Foster, 1949), and has no effect on

the rate of the citrate disappearance from the fermentation medium following sucrose exhaustion (Chesters and Rolinson, 1951). On the other hand, manganese and iron are believed to stimulate the rate of citrate utilization (Perlman and Sih, 1960).

In addition to this role in carbohydrate metabolism, there is evidence that zinc is required for the synthesis of nucleic acids and protein. Zinc stimulated nucleic acid and protein synthesis in A. niger (Bertrand and De Wolf, 1961b), R. nigricans (Wegener and Romano, 1963), and the yeast Rhodotorula gracilis (Cococci and Rossi, 1972); there was no effect on DNA synthesis or oxygen uptake in this latter study. Zinc was required by A. niger for the synthesis of tyrosine, tryptophan (Bertrand and De Wolf, 1960), and phenylalanine (Bertrand and De Wolf, 1961a), and by N. crassa for the synthesis of tryptophan (Nason, 1950). In this present investigation, zinc stimulated protein synthesis (Section VII).

Zinc deficiencies lowered the nucleic acid content in Euglena gracilis (Wacker, 1962) and Nocardia sp. (Webley et al., 1962). This ion also appears to be essential for the synthesis of both nucleic acids and protein in mammalian tissues (Rev. Terhune and Sanstead, 1969). In a recent study, zinc was shown to be required for DNA replication by phytohemagglutinin-stimulated human lymphocytes (Williams and Loeb, 1973). In E. coli, DNA polymerase I (Springgate et al., 1973), RNA polymerase (Scrutton et al., 1971), 5'-nucleotidase, and cyclic phosphodiesterase (Dvorak and Heppel, 1968) have been reported to be zinc metallo-enzymes.

In fungi, zinc deficiency commonly lowers the specific activity

of various enzymes (Cochrane, 1958). Addition of zinc to deficient cultures of A. niger leads to increases in the specific activity of glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Bertrand and De Wolf, 1957), phosphofructokinase glyceraldehyde phosphate dehydrogenase (Bertrand and De Wolf, 1958c), and aldolase (Bertrand and De Wolf, 1958a), but not invertase (Bertrand and De Wolf, 1958b). A zinc-dependent hexokinase has been reported in N. crassa (Medina and Nicholas, 1957). These results may relate to the connection between zinc and carbohydrate metabolism.

In this present study, zinc addition resulted in an increase in the specific activity of pyruvate carboxylase (not shown). With R. nigricans the specific activities of isocitric lyase and malate synthase were increased, as was the acetate induction of malate synthase (Wegener et al., 1967). Zinc caused the increased uptake of glucose and phosphate by Helminthosporium cynodontis (White and Johnson, 1971), as well as the uptake and retension of ammonium-nitrogen (Pugliese and White, 1973); in the latter report the specific activity of glutamic dehydrogenase was about twenty times higher in zinc replete cultures.

In some early studies carried out with surface cultures, zinc was shown to exert an influence on conidiation (Foster, 1949), and on the composition of the mycelium of A. niger (Porges, 1932b; Shultz, 1937; Foster, 1949). Zinc caused an increase in the growth and the fat/mycelia ratio, and a drop in the medium acid/mycelia ratio. Similar results have been obtained with R. nigricans (McHargue and Calfee, 1931). In a recent study on R. gracilis in submerged culture,

growth termination induced by zinc deficiency led to vacuolation of the cells and accumulation of lipid; addition of zinc resulted in further growth and reduced the degree of vacuolation and the lipid content (Cococci and Rossi, 1972).

Zinc also has important effects on the formation of vegetative pigments in A. niger and other fungi. Metz (1930) considered that pigmentation by surface cultures was directly related to the development of the mycelium, and therefore was promoted by zinc, and work with the Penicillia support this view (Foster, 1949). However, different results were obtained here, as YP was synthesized only under the growth limited conditions associated with zinc deficiency. A similar observation has been made regarding the synthesis of the anthroquinone pigment cynodontin by H. cynodontis (White and Johnson, 1971).

The dynamics of citrate synthesis as observed here was also related to the zinc concentration in a similar way. At low zinc levels growth was inhibited and citrate was produced, while at high zinc levels the reverse was true. The same results have been obtained with many organic acid fermentations (Foster, 1949; Romano et al., 1967). Zinc appeared to be the limiting factor in medium M-3, since addition of the ion resulted in the termination of acidogenesis and increased growth, even in media with reduced quantities of the other components. However, the results presented in Section II imply that medium M-3 did in fact contain sufficient quantities of zinc to allow at least twice the amount of growth actually obtained, and with very little citrate accumulation. This argues that zinc may have been operating in a regulatory capacity, and that the relationship between

the ion and the mycelia was not strictly stoichiometric. A similar conclusion has been drawn with respect to calcium and certain lines of cultured cells (Whitfield et al., 1973). For example, normal chicken fibroblasts are not able to proliferate in calcium deficient medium, whereas those infected with Rous sarcoma virus proliferate rapidly (Balk et al., 1973).

The citrate fermentation apparently displays the typical trophophase-idiophase dynamics common to all secondary metabolism (Historical, Sections III, IV, VII). Under these experimental conditions, the trophophase-idiophase "decision" was controlled in part by the zinc concentration. It is likely that the idiophase is a differentiated state (Historical), although the precise nature of the physiological difference between the trophophase and the idiophase is not known. It therefore follows that the zinc ion may be a regulator of growth and differentiation. The information available regarding the role of zinc in growth, primary metabolism, and secondary metabolism (Historical; Weinberg, 1970) does not exclude this possibility.

In the following sections it is shown that cyclic AMP is also involved in the regulation of growth and citrate accumulation, and there is circumstantial evidence that zinc and cyclic AMP are physiologically linked (Sections III, IV, VII). In Section II it is postulated that zinc deficiency leads to the synthesis of a growth inhibitor, which would be an obvious candidate as a regulator of cyclic AMP levels. Other unknown factors also operate to control the extent of growth and secondary metabolism (Section II). One of these may be the degree of

aggregation of hyphae into pellets; metals (possibly zinc) (Section IV, VI, VII) and cyclic AMP (Section IV) seem to be connected with this phenomenon as well.

SECTION II

FACTORS WHICH INFLUENCE THE TROPHOPHASE-IDIOPHASE DECISION IN THE CITRIC ACID FERMENTATION: EVIDENCE FOR THE REGULATORY ROLE OF ZINC AND FOR "NEOPLASTIC TRANSFORMATION" IN ASPERGILLUS NIGER.

Further support for the hypothesis that zinc operates in some way as a regulator of growth and differentiation (as defined as citrate accumulation) in these cultures was obtained from the discovery that snake venom, plant lectins, and pellet "handling", all apparently unrelated to trace metal nutrition, manifested gross changes in the growth, morphology, and citrate accumulating patterns. That these manipulations could effect large increases in growth in zinc deficient cultures suggests that the zinc ion is not simply permissive (stoichiometric), but rather regulatory.

Two basic types of responses were observed: growth stimulation at the expense of citrate synthesis, or stimulation of both growth and citrate production (plant lectins). There were also changes in the final number of pellets produced. The pellet number is considered to be a measure of the "adhesiveness" of the conidia and hyphae (Section IV).

A. SNAKE VENOM

The snake venom used was that of Crotalus atrox (Western Diamondback rattlesnake), obtained from Sigma. Unless otherwise indicated it was added directly to the flasks in powder form before inoculation. Although the venom contained phosphomonoesterase activity, the purpose of the following experiments was simply to demonstrate that its addition could influence the growth, pellet formation, and citrate accumulation of A. niger without changing zinc

concentrations, and not to identify factors responsible for the observed effect.

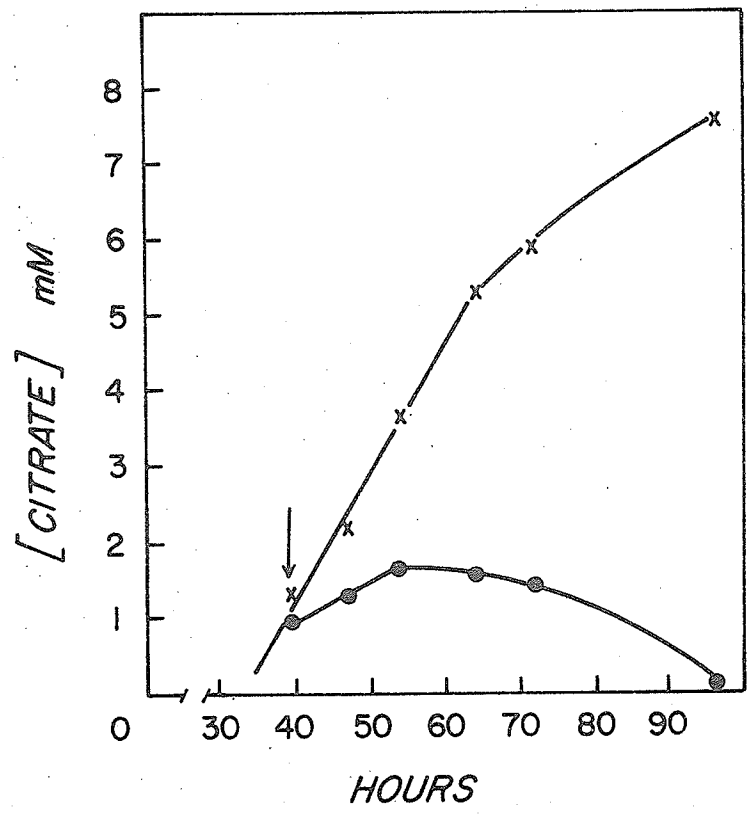
Influence of Snake Venom on Conidia

Snake venom (SV) added to the medium at zero hours induced marked changes in the characteristics of the ungerminated conidia. After 3 1/2 hours incubation, the control conidia were unswollen, clumped in two's and three's, and displayed typical conidia morphology (Section IV). On the other hand, the conidia treated with SV were swollen to about twice the diameter, were completely unclumped, appearing round and translucent, as if devoid of the conidia wall. Furthermore, the conidia plus medium from control flasks were easily filtered through 0.45 μ Millipore filters, and the SV-treated cultures could not be filtered, apparently because the membrane had become clogged by the swollen conidia. Conidia examined after eight hours growth displayed similar characteristics. A possible explanation for these observations was that the SV had partly dissolved the conidia wall, thereby precluding aggregation, and allowing swelling of the spheroplasts. No examination was made of the effect on mature, vegetative hyphae.

Influence of SV on Growth, Acidogenesis, and Pellet Formation

SV added to the growth medium at any time between zero and 39 hours resulted in increased growth and decreased citrate synthesis. Fig. 7 shows the effect of addition of 7.5 mg SV to medium M-3 containing one-fifth the regular amount of inorganic salts. Citrate production was severly curtailed, and there was a 17% increase in growth. Therefore, neither low inorganic salts nor zinc deficiency

Figure 7: Influence of 7.5 mg snake venom, added at 39 hours (arrow), on citrate accumulation in medium M-3 containing one-fifth the normal compliment of inorganic salts. By 96 hours, snake venom mycelia weighed 0.119 gms, while the controls weighed 0.102 gms. Snake venom (n = 3) (●); controls (n = 4) (X). (6.8×10^6 conidia).


[CITRATE] mM
8
7
6
5
4
3
2
1
0
30
40
50
60
70
80
90
HOURS

were sufficient to allow citrate accumulation to occur in the presence of snake venom.

Results obtained from adding SV to the regular medium M-3 are summarized in Table 4. In experiment 1, the SV was dissolved in 30 ml of Super Q water, and dialysed for 18 hours against two 1-liter changes of Super Q water. All the glassware was acid washed. Addition of the dialysed SV to 22 hour cultures resulted in increased growth and decreased citrate synthesis. The SV also had a slight stimulatory effect on hyphal aggregation at this stage, reducing the pellet number. It was concluded that the effect was not due to zinc contamination of the SV, and that the "active" ingredient(s) was non-dialysable.

Table 4, experiment 2 illustrates the results obtained from an examination of the heat stability of the SV. The snake venom was dissolved in Super Q water, immersed in a 95°C water bath for 10 minutes, added to the flasks before inoculation, and the cultures harvested after 47 hours. There appeared to be at least three "factors" present. One promoted growth and was partially heat labile, and another inhibited aggregation and was heat labile. Since the heated SV decreased the pellet number while the untreated SV increased it, there also may have been a third factor which promoted aggregation but was manifested only in the boiled SV, and therefore was heat stable.

Heating the SV enhanced its ability to inhibit citrate production, suggesting that this "effect" was heat stable. However, this may also be a reflection of the clumping of the hyphae. These pellets were large and irregular in shape, and this type of morphology does not

Table 4

Influence of Crotalus atrox snake venom (SV) on growth, citric acid accumulation, and pellet number (aggregation) in medium M-3.

	Dry Weight (g)	Citrate (mM)	Specific Activity	Pellet Number	Conidia Used
Experiment 1*					
Control (n = 4)	0.066	3.4	2575	2793	7.8×10^6
Dialysed SV (n = 2)	0.111	1.2	541	2398	7.8×10^6
Experiment 2					
Control (n = 2)	0.026	3.0	5792	1569	4.5×10^6
SV (n = 3)	0.082	2.8	1700	4574	4.5×10^6
Boiled SV (n = 2)	0.059	1.7	1453	314	4.5×10^6
Experiment 3					
Control (n = 5)	0.035	5.8	8215	2028	4.5×10^6
SV (n = 4)	0.113	1.5	614	1284	4.5×10^6

*Experiment 1: SV (5mg) dialysed and added after 22 hours growth; flasks harvested after 48 hours.

Experiment 2: SV (2.4mg) untreated or heated at 95°C for 10 minutes; flasks harvested after 47 hours.

Experiment 3: SV (5mg) powder added at zero hours; flasks harvested after 48 hours.

seem to be conducive to citrate accumulation (see below, and Section IV). In a similar line of reasoning, the fairly high amount of citrate produced by the cultures containing the unheated SV may be associated with the large number of relatively small pellets which were formed.

The response of the mycelia to the SV seemed to be related to the dose of the agent. When 2.4 mg were added, there was an increase in both the pellet number and dry weight, with little influence on the total citrate produced (Table 4, experiment 2). However, 5 mg SV actually reduced the pellet number by 37%, and had a more pronounced stimulation of growth and inhibition of citrate synthesis (Table 4, experiment 3). Finally, if 10 mg were used, growth and acidogenesis were affected as with 5 mg SV but the mold grew in the form of a few large, irregular clumps (Table 5), not unlike those produced by the heated SV.

In all these experiments, SV inhibited the production of the yellow pigment.

B. CONCONAVALIN A AND PHYTOHAEMAGGLUTININ

The conconavalin A (con A) used was three times crystallized, desalted, lyophilized powder obtained from Miles-Yeda Ltd. Con A, a plant lectin, is a globular protein with a binding specificity towards α-methyl glucopyranoside and sterically related residues (Goldstein et al., 1965). The phytohaemagglutinin (PHA) used was lyophilized powder obtained from Difco. PHA, a glycoprotein and a plant lectin, has a binding specificity towards N-acetyl galactosamine (Rev. by Naspitz and Richter, 1968). These agents were added directly to the growth medium in powder form before inoculation.

Table 5

Effect of snake venom (SV), conconavalin A (Con A), and phytohaemagglutinin (PHA) on growth, citrate accumulation, and aggregation at 45 hours and 69 hours in medium M-3.

Effector	Dry Weight (g)	Citrate (mM)	Specific Activity	Pellet Number
Control - 45 hours	0.076	3.5	2302	--
Control - 69 hours	0.080	7.3	4579	2507
SV - 45 hours*	0.134	0.6	224	--
SV - 69 hours*	0.152	1.9	623	52
Con A - 45 hours*	0.135	6.1	2261	--
Con A - 69 hours*	0.185	8.8	2380	6700
PHA - 45 hours*	0.087	5.9	3390	--
PHA - 69 hours*	0.101	8.1	4000	4583

*10 mg of powder were added at zero hours. (9.2×10^6 conidia; n = 3).

Con A (10 mg) caused large increases in both growth and citrate accumulation by 45 hours in medium M-3, with little change in the specific activity of the mycelia (Table 5). This was in contrast to the effect of SV which increased growth, but inhibited citrate production. The fermentation carried out in the presence of con A was essentially completed by 45 hours, and by 69 hours, only small increases in growth and citrate production had occurred, and all the sucrose had been depleted. The control cultures had become growth limited by 45 hours, but continued to secrete citrate until 69 hours, with some sucrose still remaining. Con A also severly inhibited aggregation, causing a three and one-half fold increase in the pellet number.

PHA (10 mg) evoked a similar, but less intensive response in terms of growth, citrate synthesis, and pellet number. This agent appeared to exert a more pronounced effect on the production of citrate than growth, resulting in increased specific activity at 45 hours. This observation was not further examined.

The con A (5 mg) was potent in the early stages of the fermentation as well, stimulating growth six hours after inoculation of the flasks (control, 0.50 mg; con A, 1.27 mg; 4×10^6 conidia; $n = 5$). The lectin also inhibited the aggregation of the conidia (not shown), which conceivably was translated into an increase in the number of pellets eventually formed (Table 5).

No attempts were made to purify the con A, and it is possible that the preparation may have contained zinc ions. However, con A also increased growth and inhibited aggregation in zinc replete cultures (Table 6). In this case, growth was stimulated at 24 hours,

Table 6

Influence of con A on growth and aggregation in medium M-3 in the presence and absence of zinc.

Additions*	Dry Weight (g)	Dry Weight (%)	Pellet Number	Pellet Number (%)
	Flasks harvested after 24 hours			
Zn	0.025	100	-	-
Zn + Con A	0.047	188	-	-
	Flasks harvested after 47 hours			
Zn	0.188	100	1019	100
Zn + Con A	0.165	88	1950	191
None	0.055	100	1235	100
Con A	0.102	185	2600	211

*10 mg con A, and 1.55 μM zinc (4.5×10^6 conidia; n = 7).

but had become limited by 47 hours, masking the con A effect. Furthermore, if the con A was contaminated with zinc it might have been expected to inhibit, rather than promote, citrate accumulation (Tables 4 and 5).

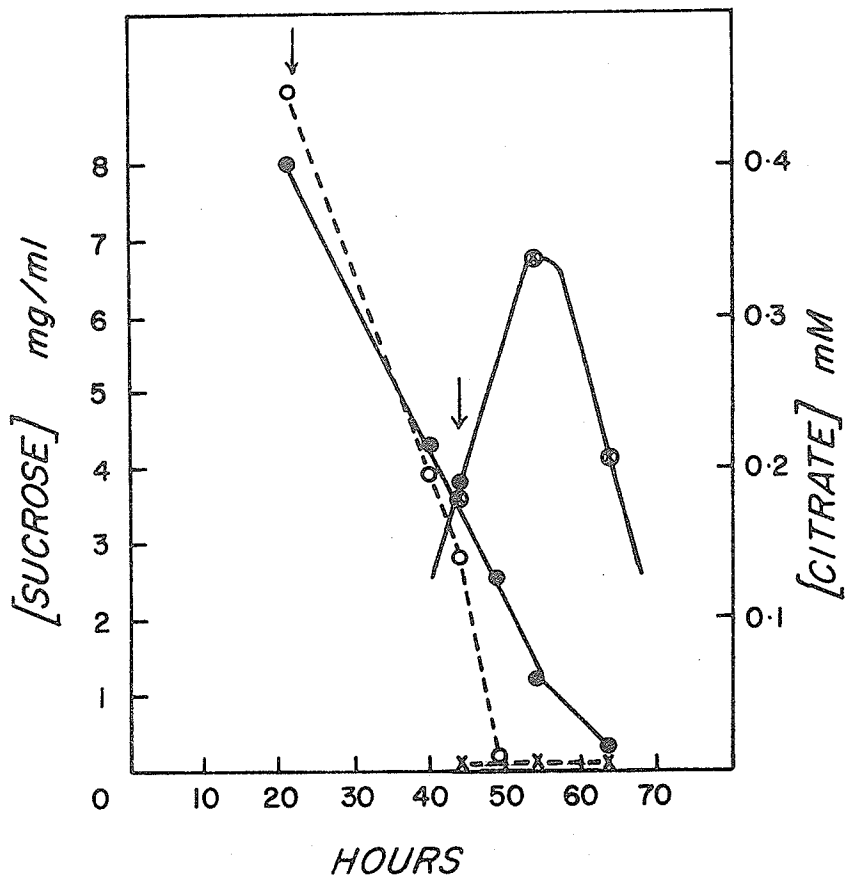
In all these studies, con A inhibited the production of YP, while in the one experiment, PHA had no effect on YP.

C. PHYSICAL HANDLING OF PELLETS

It was not even necessary to add foreign agents to the cultures to influence the growth and secondary metabolism of A. niger. Physical "handling" of the mycelia alone was sufficient. If the pellets were collected on a moist cloth, maintained in air for about 15 minutes, and then returned to the original growth medium, a response similar to that described for SV (10 mg) treatment was obtained. That is, there was a large increase in growth, decrease in pellet number, and inhibition of citrate and YP synthesis.

Fig. 8 illustrates the effect of such a physical "handling" on mycelia grown in medium M-2 containing one-fifth the normal trace metal allotment. Relatively small amounts of citrate were produced by the control cultures, and the sucrose utilization rate was rapid - the standard response in a medium containing zinc. Filtration of the pellets after 22 hours growth was attended by an increased rate of sucrose utilization, and complete inhibition of citrate accumulation. A second filtration at 44 hours caused a similar response. As all the flasks contained significant quantities of trace metals, it is unlikely that this was due to zinc ions accidentally introduced by the treatment.

Figure 8: Effect of pellet "handling" on citrate accumulation and sucrose utilization in medium M-2 containing one-fifth the regular amount of trace metals. Mycelia were collected at 22 hours and 44 hours (arrows). "Handling" (broken line): sucrose (O), citrate (X). Control (full line): sucrose (●), citrate (⊗). (9×10^6 conidia). Control (n = 7), "handling" (n = 3).


[SUCROSE] mg/ml
[CITRATE] mM
HOURS
0
1
2
3
4
5
6
7
8
0·1
0·2
0·3
0·4
10
20
30
40
50
60
70

Preliminary experiments were conducted to identify the parameter responsible for the effect. Cultures were grown in medium M-3 for 22 hours, manipulated in various ways, then returned to the original growth medium and harvested 26 hours later. All the glassware was pre-washed in 6N HCl and all the apparatus was sterilized. In order to ascertain whether the response was related to the contact between pellets, some flasks were drained of almost all the medium, and the pellets allowed to stand for 20 minutes in close contact at the bottom of the flask, but still immersed in the medium. With other flasks, the pellets were physically "handled", i.e. collected on the cloth, rolled into a "ball" with a spatula, maintained this way for 5, 10, and 15 minutes, and then returned to the original growth medium. Control cultures were untreated, except that they were not agitated for 20 minutes. Vacuum was not used in the filtration process so as to prevent the loss of possible volatile compounds.

Only the pellets which had been filtered, gathered and maintained for 15 minutes, displayed the characteristic response: increased growth, and complete inhibition of citrate and YP synthesis (Table 7). The other flasks became growth restricted, accumulated citrate and YP, and were generally indistinguishable from the controls. The 15 minute "ball" did not redisperse into the original, individual pellet structure once agitation was resumed, although by 48 hours a few other pellets had formed.

In other experiments (not shown) the filtered pellets were not rolled together into a clump, but instead were allowed to remain for 15 minutes in a thin contiguous layer on the cloth. Under these circumstances growth was increased and citrate synthesis decreased as

Table 7

Influence of pellet "handling" on growth, secondary metabolism, and morphology.

Treatment*	Dry Weight (g)	Citrate (mM)	Specific Activity	Morphology
Control	0.077	3.4	2207	Yellow pellets
A	0.074	2.8	1890	Yellow pellets
B	0.063	2.9	2301	Yellow pellets
C	0.082	4.0	2440	Yellow pellets
D	0.128	0	--	White clumps

*Pellets were treated after 22 hours growth in medium M-3 (see text), then returned to the original growth medium and harvested 26 hours later. (7.8×10^6 conidia).

Control: Pellets allowed to stand in undecanted, unagitated medium for 20 minutes (n = 4).

A: Most of the medium decanted. Pellets allowed to stand in close contact for 20 minutes, but still immersed in medium (n = 2).

B: Pellets collected on moist cloth, gathered into a "ball" and after five minutes, replaced into the original growth medium (n = 2).

C: Same as B, except after 10 minutes (n = 2).

D: Same as B, except after 15 minutes (n = 2).

above, and the organism grew as large, uniform, and white pellets, but fewer in number than the controls, indicating that some fusion had occurred between the pellets here as well.

Apparently the filtration procedure led to the orientation of the mold into the trophophase rather than the idiophase. This effect was a function of time of "handling", and was not observed in the presence of the growth medium. The influence of time may reflect either the drying of the mycelial mass in the air or some kind of time-dependent interaction among the pellets. Whatever the explanation is, the result was the formation of a permanent bond between the individual pellets.

D. CONIDIA DENSITY

It has been pointed out by Martin (1957) that the number of vegetative pellets used to inoculate their fermenters had an important bearing on both the rate of citrate synthesis and the final yield attained. The same observation has been made when conidia were the inoculating particles, both in surface (Doelger and Prescott, 1934) and submerged cultures (Trumpy and Millis, 1963). An identical result was obtained in the present investigation, as illustrated by the experiment described below.

Flasks were inoculated with conidia at densities ranging from 140 to 1.4×10^7 conidia per flask. Medium samples were taken at various times in the fermentation to determine the rates of citrate synthesis and sucrose utilization. At the time of harvest, all the cultures appeared to be close to the stationary phase of growth, as

both the rates of sugar disappearance and acidogenesis were very low (Table 8).

There are some interesting features of the citrate fermentation illustrated by this experiment. First, although the amount of growth realized at the lower conidia densities was only slightly less than that at the higher levels, the quantity of citrate accumulated was very much lower. Second, although there was adequate sucrose remaining in all the flasks, the mycelia had ceased to either utilize sugar or to synthesize acid. This is an observation common to fermentations of this type (Weinberg, 1970).

The causes of these phenomena are not known, but it is clear that all the flasks contained a similar quantity of zinc. It is postulated below that zinc deficiency may lead to the synthesis of a growth inhibitor, which then turns off the metabolic machinery of the mold.

The difference in the acidogenic ability of pellets formed at high and low conidia densities may also be related to the size of the aggregate. Large, hollow pellets were formed when the conidia number was low, while much smaller pellets arose when the number was high (Section IV). Although the reason is not known, the small pellet morphology is always associated with high rates of citrate accumulation.

E. RANDOM VARIATION

It has been casually observed that filamentous fungi growing in the pellet form fluctuate from flask to flask in the eventual dry weights achieved, and that this is not as pronounced when the growth is filamentous (Pirt and Callow, 1960). It has also been noted that A. niger

Table 8

Influence of the number of inoculating conidia on growth and citric acid accumulation.*

Conidia Used	Harvest Time (hours)	Dry Weight (grams)	Citrate		Sucrose		Specific Activity
			Final (mM)	Rate (mM/hr)	Final (mg/ml)	Rate (mg/ml/hr)	
1.4×10^7	56	0.085	3.4	0.056	3.9	-0.12	2000
1.4×10^6	75	0.074	3.8	0.059	4.7	-0.16	2568
1.4×10^5	98	0.063	2.3	0	5.4	-0.03	1825
1.4×10^4	118	0.067	0.8	0.001	5.0	-0.03	597
1.4×10^3	128	0.070	0.6	0.007	5.2	-0.05	420
1.4×10^2	128	0.050	0.7	0	5.9	-0.05	700

*Medium M-3 (n = 7).

submerged citrate fermentations are subject to variation, even when the experimental conditions are rigorously controlled (Trumpy and Millis, 1963). In the present investigation similar variability was encountered, either among separate experiments, or among flasks within the same experiment.

Table 9 illustrates a fairly extreme case of intra-experimental fluctuation, and the data describe some of the different patterns. In the majority of other experiments, most of the cultures displayed characteristics close to the "average", attaining a mean dry weight, accumulating a mean amount of citrate, and forming a mean number of yellow pellets. This result is typified here by flask 2. Other cultures grew sparsely (flask 9), normally (flask 4), or abundantly (flask 8), but these still produced large amounts of citrate and had high specific activities. Some samples grew normally, but produced only low amounts of citrate (flask 5), and had low specific activities. Finally, some cultures achieved a great deal of growth, but produced only small amounts of citrate, and had very low specific activities (flask 3); these usually had a reduced number of pellets (not shown).

Cultures displaying the growth exemplified by flask 3 are relevant to this discussion, as these characteristics were identical to those produced by mycelia which had been physically "handled" or treated with snake venom. That is, the growth was very abundant and there was a reduced number of large white pellets. Comparatively little citrate was produced. This type of result occurred at a frequency of roughly 5%, although there were cases with varying degrees of severity (flask 1) and it was difficult to score. In other

Table 9

Variability of results within the same experiment in the citrate fermentation.

Flask	Dry Weight (mg)	Citrate (mM)	Specific Activity	Pellet Number
1	107	2.0	930	1777
2	96	2.8	1473	2101
3	128	1.9	729	--
4	93	3.2	1706	2866
5	94	1.6	858	2140
6	79	2.1	1318	2270
7	113	2.9	1306	--
8	107	3.5	1648	--
9	68	2.5	1814	1530
Mean $\pm$ standard deviation	98 ± 6	2.5 ± 0.2	1309 ± 587	2114 ± 311

Flasks were harvested after 48 hours (8.5×10^6 conidia).

experiments (not shown) the increase in growth was even more pronounced and citrate synthesis was totally repressed. It is highly unlikely that these fluctuations were caused by zinc metal contamination of the cultures.

DISCUSSION

The zinc ion was the limiting nutrient in these cultures, and addition of zinc resulted in large increases in growth and the abolition of citrate production (Section I). Nevertheless, the same response could be duplicated by agents and treatments unrelated to zinc, even under conditions of presumed zinc deficiency. Occasionally, zinc-like responses occurred spontaneously, and there was a large degree of variation of results within the same experiment, even under carefully controlled conditions. Evidently, the citrate accumulating state was governed by a balance between the tendency towards growth and the tendency towards secondary metabolism.

It was difficult to ascertain what controls were operating on the mycelia to regulate their growth and secondary metabolism. Certainly the zinc ion was involved, but there were other factors as well. Close examination of citrate accumulating pellets revealed that a "pellet" consisted of a collection of many conidia aggregates, but for unknown reasons only some of the conidia appeared to have germinated. In addition, there is no overt reason why mycelia grown at low conidia densities should cease to metabolise the available sucrose (Table 8). It was obvious that some agent was limiting the growth, and that although it was related to zinc, it did not depend strictly on the

number of zinc ions per unit of mycelia.

One possibility is that the cultures "sense" zinc deficiency, and then limit their growth through the synthesis of a growth inhibitor. A candidate for this putative inhibitor might be the factor(s) which have been described by Park and Robinson (Park, 1961; Park and Robinson, 1964; 1966; 1967), and which may be a common feature of the fungi, including A. niger (Park, 1963). One substance, identified as bikavarin (Cornforth et al., 1971) causes both vacuolation and cessation of extension of growth in tips of fungi growing on agar (Park and Robinson, 1967; Robinson et al., 1969), and its synthesis by submerged and surface cultures rises to a peak as the mycelia enter the stationary phase of growth (Robinson and Park, 1965). Vacuolation is, of course, associated with senescence in fungi (Robertson, 1965; Park and Robinson, 1967), and there is evidence that bikavarin is not effective against hyphae of very young cultures (12 hours). All these observations on bikavarin correlate well with the available information relating to the synthesis of citric acid, and are in accord with the idea that a "growth inhibitor" is involved in some way with the transition of the mycelia from the trophophase to the idiophase.

With the exception of certain sex hormones and bikavarin, very little is known about fungal "hormones". Evidence has been presented recently that compounds of the cytokinin class may function as regulators of differentiation and development in Achlya sp. and Blastocladiella emersonii, apparently by controlling the availability and transport of calcium ions (LeJohn, 1973; LeJohn et al., 1974).

A similar type of mechanism could apply here with respect to zinc.

It has been shown that glucagon and insulin influence the activity of the adenyl cyclase of N. crassa (Flawia and Torres, 1973) and glucagon enhanced the cyclic AMP stimulation of conidia aggregation in A. niger (Section IV). This hints at the possibility that as with animal systems, cyclic AMP levels in fungi may be influenced by "hormones". Since cyclic AMP inhibits the growth of A. niger under conditions of declining growth associated with zinc deficiency (Section III), which also coincides with the synthesis of bikavarin as reported by Robinson and Park (1965), there may be a connection between the two phenomena.

Aside from the question of growth "hormones", it is clear that other factors were involved in the decision between growth and secondary metabolism, and these seemed to be related to the aggregation of the hyphae, and possibly cell-cell interactions within the aggregate. Clumping of the conidia, rind formation on the flasks at the air-liquid interface, and the interaction between the germlings and the rind, produced cultures with pellets of varying sizes and numbers (Section IV). It was noted that the best growth and citrate production were usually obtained when the pellets were small (0.5mm-2mm), uniform, and numerous. Flasks containing fewer and larger pellets usually realized less growth, although the specific activity of the mycelia was unchanged. Since all the cultures seemed to be subject to inhibition by zinc deficiency (with exceptions, see Table 9 and below), it is possible that the latter cultures had become growth inhibited by the degree of aggregation. Fungi grow less rapidly in the pellet mode, displaying cube root kinetics

rather than the exponential growth observed with filamentous cultures (Emerson, 1950; Pirt, 1966; Trinci, 1970). This explanation could explain much of the variability which was encountered in this study with respect to the yields of growth and citric acid.

Con A increased both growth and citric acid accumulation, and inhibited aggregation. This lectin is a potent mitogen in some animal systems (Douglas et al., 1969), and it also stimulates the transformation of lymphocytes into blasts (Perlmann et al., 1970). Con A binds to sugar moieties on mammalian cell surface membranes (Oseroff et al., 1973) and agglutinates transformed cells, or cells treated with trypsin (Inbar and Sachs, 1969a, b). It is likely that these observations will have a bearing on the mode of action of con A on the citrate fermentation. Since the Aspergilli and other fungi and bacteria produce compounds with hemagglutinating properties (Fujita et al., 1972a, b), it is possible that these endogenous lectins play a role in regulating growth and aggregation similar to that suggested here by the con A results.

It was pointed out above that aggregation of the hyphae seemed to lead to a reduction in the total growth and acidogenesis. However, this was apparent only in cultures which were subject to regulation by zinc deficiency. Mycelia which had been grown in the presence of zinc, or zinc deficient mycelia which had been "handled" or treated with SV were highly aggregated, but still grew vigorously and did not accumulate citrate. The same was true for cultures displaying the spontaneous SV-like characteristics (Table 9). Therefore, these types of mycelia were apparently refractive not only to zinc deficiency, but also to the

growth limitation presumably imposed within the aggregate.

It has become apparent that the cell surface and cell-cell contacts play an important role in the growth and development of multicellular organisms (Rutter et al., 1973). Cells which become "transformed" into malignant cells are characterized by a loss of some of the controls which regulate growth. In particular, they are not subject to contact inhibition of growth (Stoker, 1967). They are also able to proliferate rapidly in the absence of calcium ions, whereas normal cells are not (Whitfield, 1973).

Some of the agents and treatments described in this section are likely to be associated with changes in the cell surface of the mold. The SV may modify the surface architecture enzymatically or by some other means, and the plant lectins may accomplish the same purpose by binding to carbohydrate moieties on the cell surface. This suggests the possibility that SV and "handling" induce a form of "neoplastic transformation" in these fungal cells, perhaps by modification of the cell surface. The spontaneous SV-like growth may be spontaneous "transformation". It is noteworthy that both cultured mammalian cells and these "transformed" fungal cells are able to proliferate rapidly in the relative absence of their regulatory ion.

In summary, it is tentatively concluded that the trophophase and the idiophase in the citrate fermentation exist as more or less mutually exclusive states, and the decision between the two conditions is governed in part by zinc ions. However, other unknown factors appear to be involved, and the evidence suggests that the decision is taken at the most profound levels in the cell which control growth and differentiation. Under certain circumstances the mycelia display growth

characteristics similar in concept to some types of cancer cells; they proliferate rapidly and remain relatively undifferentiated. Addition of SV to idiophase cultures seemed to induce a transition back to the trophophase (Fig. 9), and the same result attended "handling" (not shown). This implies that the mycelia were capable of undergoing "transformation" from a unipotential state to a pluripotential state. The sites controlling these responses appear to be in communication with the surface of the cells. If this interpretation is confirmed, then it follows that the mechanism of citric acid accumulation cannot be fully solved until it is understood how eucaryotic cells regulate their growth and development.

SECTION III

REGULATION OF GROWTH AND SECONDARY METABOLISM BY CYCLIC AMP

Under these experimental conditions, the growth and secondary metabolism of A. niger were regulated by the concentration of the zinc ion; high zinc promoted growth and inhibited secondary metabolism, while at low zinc the reverse was true. This section examines the effects on growth and secondary metabolism of adding cyclic AMP to zinc deficient cultures (medium M-3). It was determined that growth was enhanced by the nucleotide early in the fermentation period, but at later stages growth was inhibited and acidogenesis stimulated. The implication of these results is that there may be a connection between cyclic AMP and zinc in the regulation of growth and development in this organism.

A. PHYSIOLOGICAL RESPONSES TO CYCLIC AMP

The number of conidia used to inoculate the cultures had an important bearing upon both the fermentation kinetics and the response of the mycelia to added cyclic AMP. At high inoculation loads (high conidia density) in medium M-3 citric acid appeared in the medium after about 30 hours growth, and continued to increase at a linear rate until the sucrose was exhausted. Addition of cyclic AMP after 16 hours growth resulted in increased rates of citrate synthesis and sucrose utilization (Fig. 9). Addition of cyclic AMP or theophylline after 40 hours growth, also increased citrate synthesis (Fig. 10). Adenosine (1 and 2 x 10^{-3}M) caused a slight stimulation, but 5'-AMP (1 and 2 x 10^{-3}M) had no effect.

Figure 9: Stimulation of citrate production and sucrose utilization by cyclic AMP (10^{-3}M) when added after 16 hours growth. Cyclic AMP cells: citrate production (x) and sucrose utilization (●). Control cells: citrate production (+) and sucrose utilization (o).

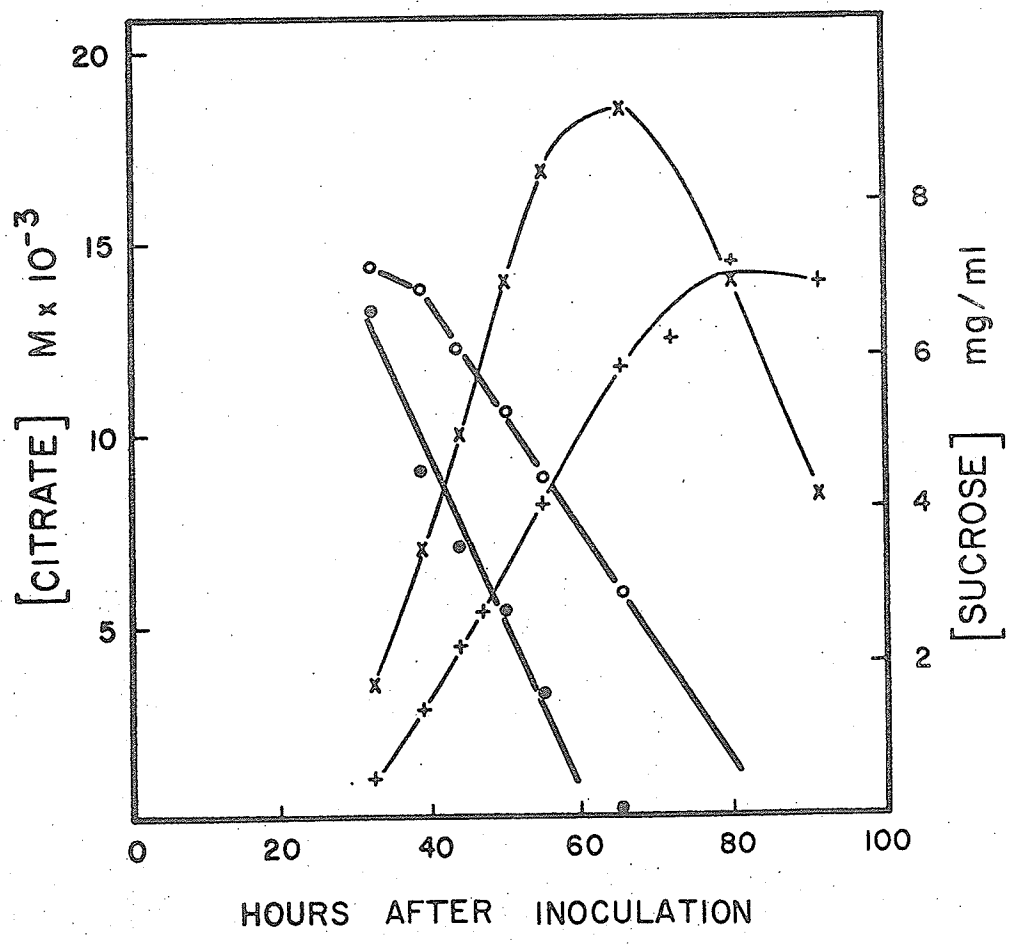
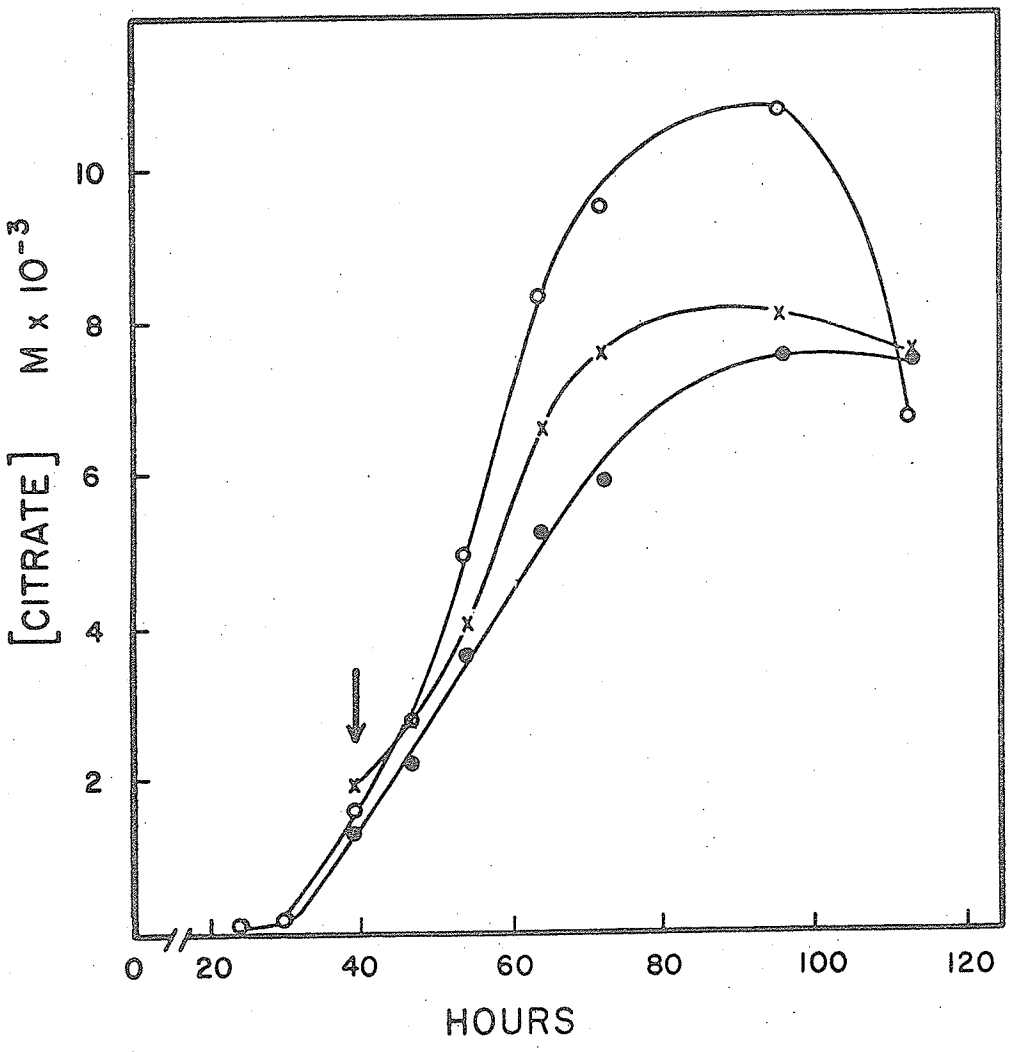

[CITRATE] M x 10⁻³
[SUCROSE] mg/ml
HOURS AFTER INOCULATION
0
20
40
60
80
100
5
10
15
20
2
4
6
8

Figure 10: Stimulation of citrate accumulation by cyclic AMP (1.5×10^{-3}M) and theophylline (1.5×10^{-2}M) when added after 40 hours growth. The data represent the means of 2-4 flasks. The final cell weights were: cyclic AMP, 0.103g; theophylline, 0.092g; control, 0.102g. Cyclic AMP (O), theophylline (X), and control (●). (6.8×10^{6} conidia; n = 2-4).


[CITRATE] M x 10⁻³
10
8
6
4
2
0
20
40
60
80
100
120
HOURS

Cyclic AMP (2.5 mM) added at zero hours to cultures inoculated at high conidia densities (8.2×10^6 conidia per flask) resulted in a characteristic response in terms of growth and citric acid accumulation. After a lag, growth was exponential for a short period, but soon began to decline in rate (Fig. 11). Cyclic AMP stimulated the early growth, but as the growth rate declined the response of the mycelia to the nucleotide changed and growth was inhibited instead. When citric acid eventually began to accumulate, cyclic AMP increased its rate of synthesis. In some experiments, the sucrose utilization rate began to level off late in the fermentation, and such was the case here. Cyclic AMP overcame this effect, and stimulated sucrose utilization.

Mycelia grown at low conidia inoculation loads (low conidia density; 640 conidia per flask) displayed different fermentation kinetics (Fig. 12). Because of the low number of inoculating particles it took longer to accrue the mycelial mass, and consequently the onset of citrate accumulation and YP synthesis was delayed. Cyclic AMP (2.5 mM) added to the cultures at zero hours produced essentially the same response as it had at high conidia densities. There was a large stimulation of the early exponential growth (180% at 28 hours), but as the growth rate began to decline the response to the nucleotide changed, and growth was inhibited slightly (11% by 99 hours). When citrate finally began to accumulate after about 65 hours, cyclic AMP enhanced its synthesis.

At high cell densities the cyclic AMP-treated mycelia usually (but not always) turned yellow before control flasks (about 40 hours),

Figure 11: Influence of 2.5 mM cyclic AMP added at zero hours on growth, citric acid accumulation, and sucrose utilization at high conidia densities. T-CTP (see text) = 14 hours. Cyclic AMP (broken line); control (full line). Log dry weight: cyclic AMP (●), control (x). Citrate: cyclic AMP (○), control (⊗). Sucrose: cyclic AMP (△), control (□). (8.2×10^6 conidia; n = 7).

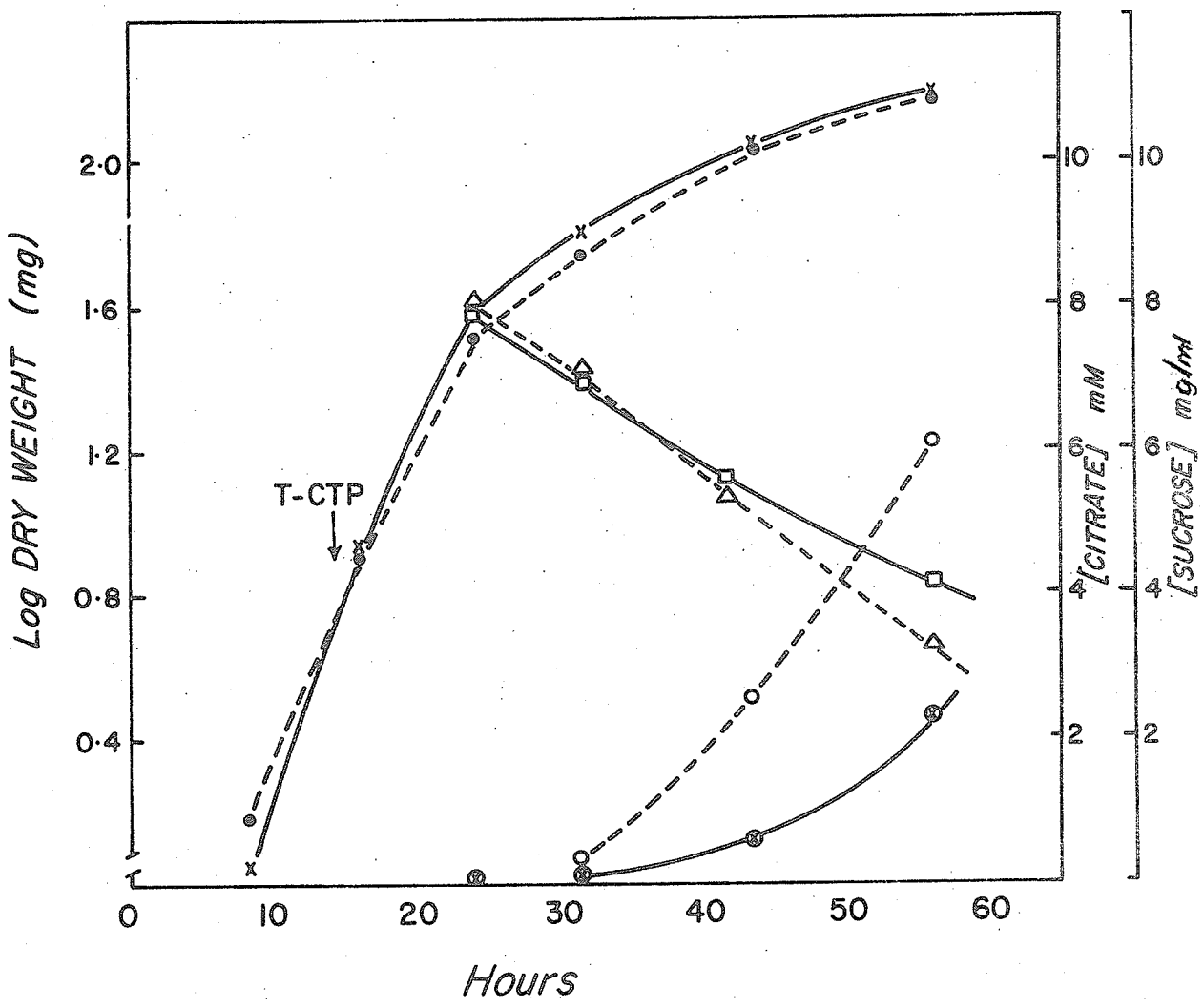
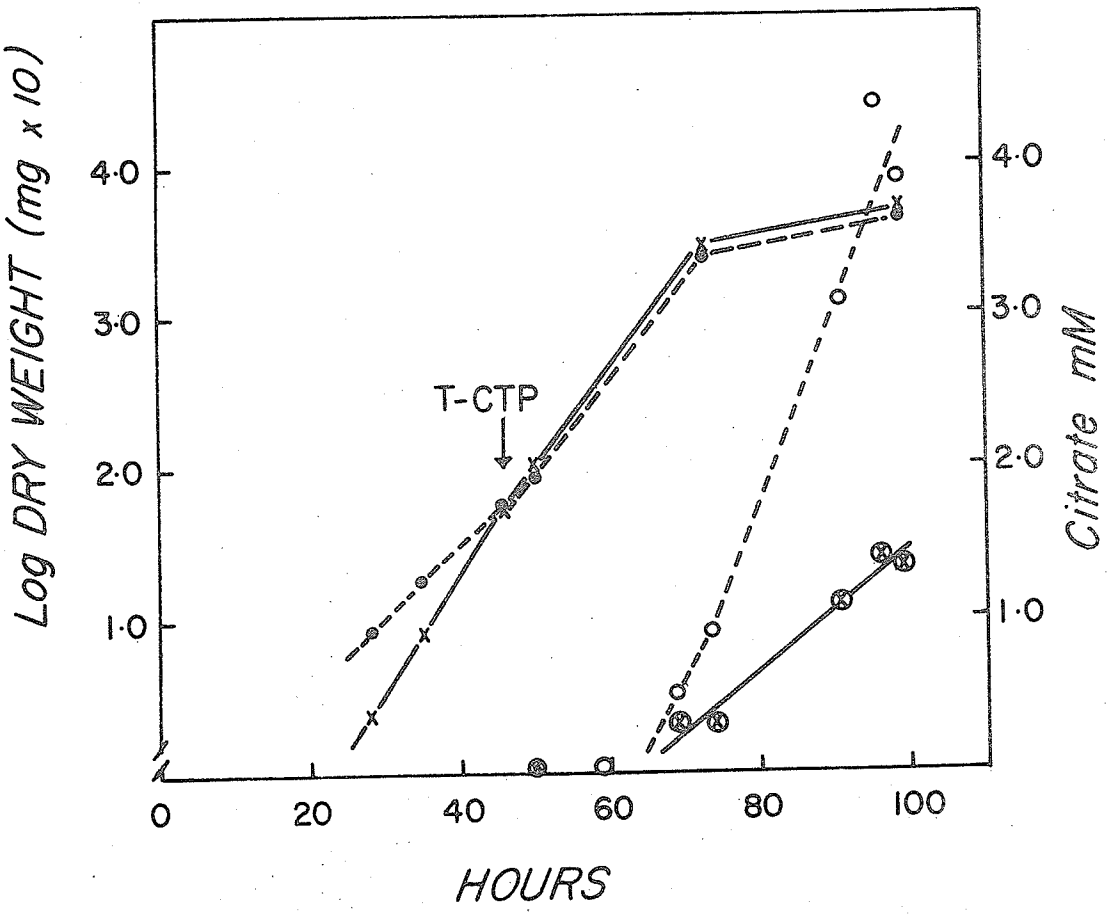

Log DRY WEIGHT (mg)
2·0
1·6
1·2
0·8
0·4
T-CTP
0
10
20
30
40
50
60
Hours
10
8
6
4
2
[CITRATE] mM
10
8
6
4
2
[SUCROSE] mg/ml

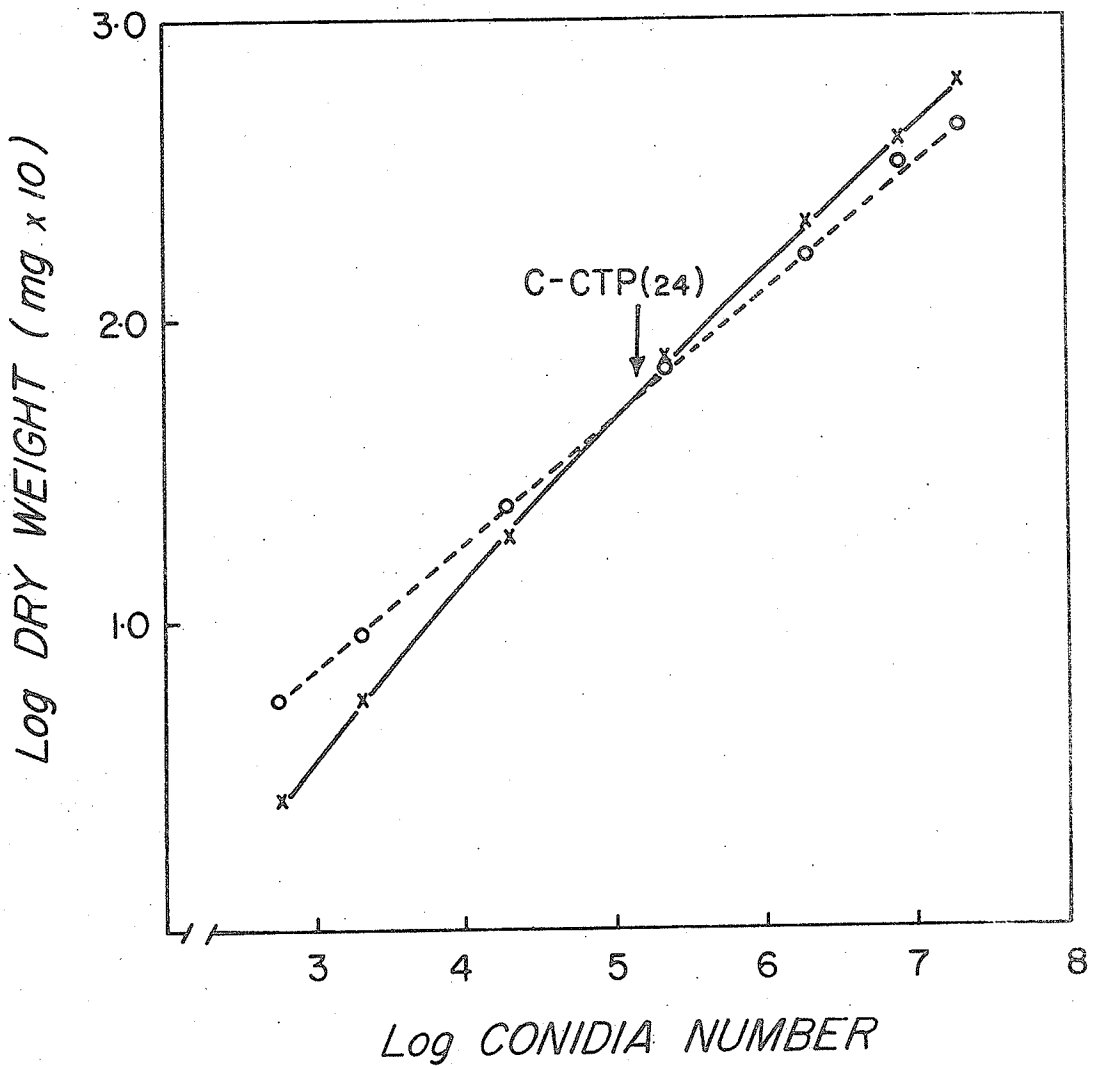
Figure 12: Influence of 2.5 mM cyclic AMP added at zero hours on growth and citric acid accumulation at low cell densities. T-CTP = 46 hours. Cyclic AMP (broken line); control (full line). Log dry weight; cyclic AMP (●), control (X). Citrate: cyclic AMP (O), control (⊗). (640 conidia; n = 7).


Log DRY WEIGHT (mg x 10)
4·0
3·0
2·0
1·0
T-CTP
0
20
40
60
80
100
HOURS
4·0
3·0
2·0
1·0
Citrate mM

but at low cell densities the opposite result was observed. In the experiment described in Fig. 12, after 73 hours growth pellets in six out of 14 control flasks had turned yellow, but none of the cyclic AMP pellets, and by 81 hours, all of the control cultures had turned yellow (6), but only one (out of six) cyclic AMP culture had a slight yellow tinge. Therefore, cyclic AMP stimulated the synthesis of one secondary metabolite (citrate), but not the other (YP); this varied with the conidia number as well as other undefined factors. The most important variable seemed to be zinc, and the "transformed" type of growth described in Section II.

For the purpose of this discussion the "Time-cyclic AMP transition point" (T-CTP) is defined as the time in the fermentation at which the growth response to added cyclic AMP changed from stimulation to inhibition. It is apparent that the T-CTP was an inverse function of the number of conidia used to inoculate; at high conidia numbers the T-CTP was low, while at low conidia numbers the T-CTP was high. Therefore the arithmetic value of the T-CTP must contain reference to the number of conidia used. For example, in Fig. 11 the T-CTP (8.2×10^6 conidia) was 14 hours while in Fig. 12 the T-CTP (640 conidia) was 46 hours. This point is further illustrated in Fig. 13. Flasks were inoculated with different numbers of conidia, in the presence and absence of 2.5 mM cyclic AMP, and harvested after 24 hours growth. Mycelia grown from conidia numbers less than antilog 5.2 were still subject to growth stimulation by cyclic AMP (124% at 210 conidia), but cultures grown at greater conidia densities had

Figure 13: Determination of the "conidia-cyclic AMP transition point" after 24 hours growth (C-CTP = 24). cAMP (broken line); control (full line). After 24 hours, cyclic AMP stimulated growth of cultures seeded with less than antilog 5.2 conidia, but inhibited growth of cultures seeded with higher numbers of conidia. Cyclic AMP (2.5 mM) was added at zero hours. (n = 7).


3·0
2·0
1·0
Log DRY WEIGHT (mg x 10)
C-CTP(24)
3
4
5
6
7
8
Log CONIDIA NUMBER

become growth-inhibited (31% at 2.1 x 10^7 conidia). Therefore, again for ease of discussion, the "conidia-cyclic AMP transition point" (C-CTP) is defined as the conidia number at which, in a specified time, the growth response of the mycelia changed from stimulation to inhibition. In Fig. 13, the C-CTP (24 hours) was 1.6 x 10^5 conidia.

The time related change in the response of the mycelia to added cyclic AMP was not limited to growth, and a similar effect was observed upon aggregation (Section IV). Under conditions of growth stimulation, the nucleotide also increased aggregation, while under conditions of growth inhibition, cyclic AMP apparently promoted de-aggregation. As is discussed in Section IV, the most sensitive stages of aggregation appeared to have been completed by roughly 24 hours at all conidia densities. Consequently the effect of cyclic AMP on de-aggregation was not manifested in cultures inoculated with low numbers of conidia. For example, in Fig. 13 the cyclic AMP flasks contained a mean of 108 pellets, and the controls a mean of 200 pellets. Thus if the CTP was greater than about 24 hours cyclic AMP decreased the pellet number, but if it was less, the nucleotide increased the pellet number.

No attempt was made to ascertain what were the causal agents which controlled the response to the nucleotide, but it seems likely that the zinc ion was somehow involved. It has been demonstrated that zinc controlled growth and secondary metabolism in these cultures (Section I), and that the mycelia behaved as though zinc were operating in a regulatory capacity (Section II). Accordingly, the results from these studies are interpreted in the following way. Early in the

fermentation when the zinc/cell ratio is high, the mycelia are growth oriented and cyclic AMP stimulates growth and aggregation. As growth proceeds the zinc is depleted from the medium and the zinc/cell ratio falls; the cultures then "sense" zinc deficiency and undergo a physiological change (differentiate) such that cyclic AMP inhibits growth and promotes de-aggregation and secondary metabolism. That is, cyclic AMP stimulates growth in the trophophase and acidogenesis in the idiophase, and during the phase transition cyclic AMP inhibits growth. However, the phase decision is governed by zinc. The fact that the T-CTP varied inversely with the conidia number supports this hypothesis, for zinc "deficiency" is likely to be related to the mycelial mass in the culture flasks.

The measurement of endogenous cyclic AMP levels also lends support to this idea (Section VII). Cultures growing in zinc replete medium contained high intracellular levels of cyclic AMP, and growth termination caused by sucrose exhaustion was accompanied by a dramatic fall in the concentration of the endogenous nucleotide. On the other hand, zinc deficient-idiophase mycelia contained considerably lower levels of cyclic AMP, and addition of zinc led to the reversion to trophophase which is accompanied by the attentuation of acidogenesis and a concomitant large increase in the mycelial content of both cyclic AMP and protein. Therefore, not only did added cyclic AMP stimulate growth in the trophophase, but also trophophase mycelia contained high endogenous levels of the nucleotide.

A conceivable inconsistency in these results was the finding that the idiophase mycelia contained fairly low levels of cyclic AMP (Section VII). Since added cyclic AMP inhibited growth and enhanced

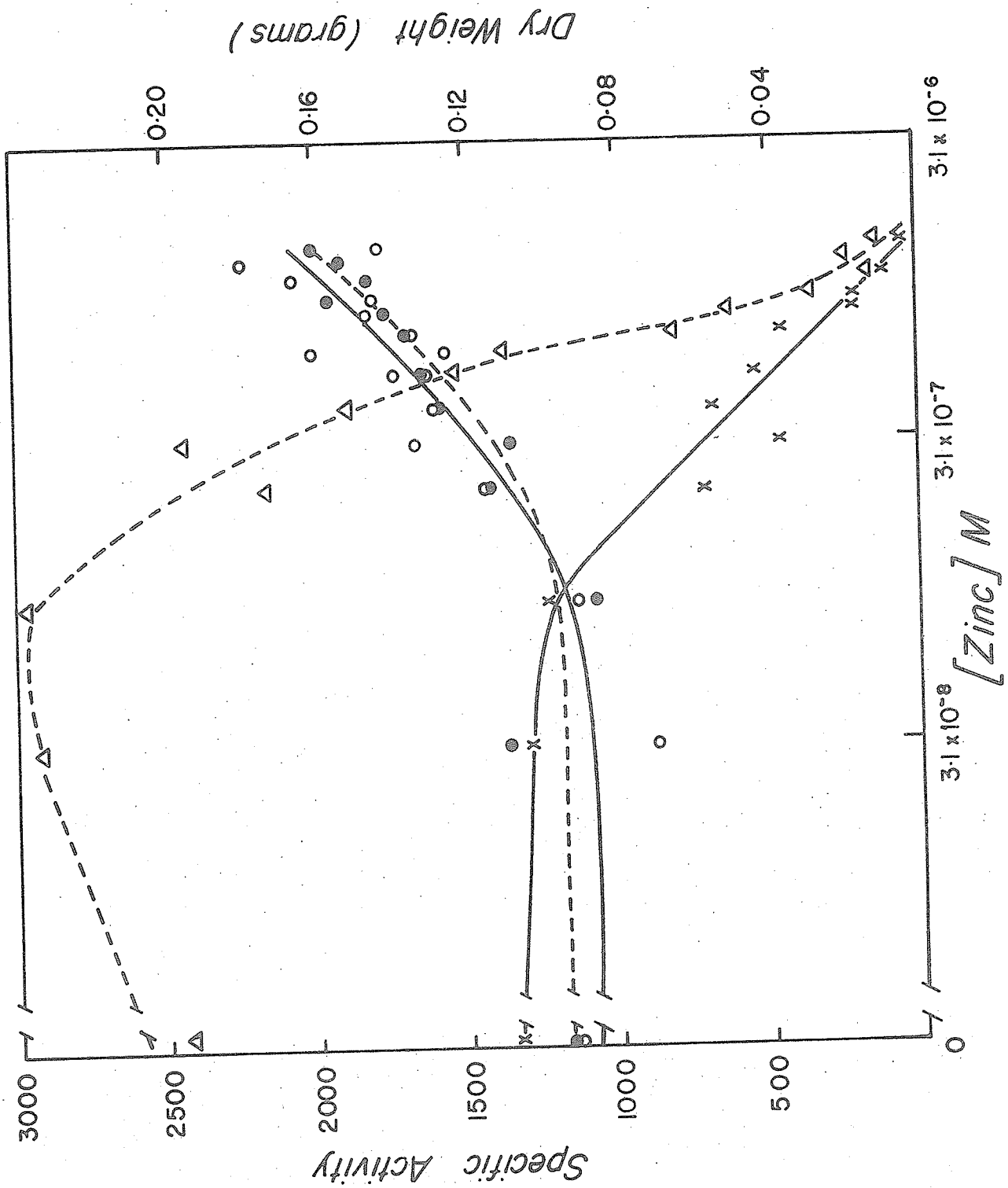
secondary metabolism, the idiophase mycelia might have been expected to be high in cyclic AMP, as were the trophophase cultures, but with the response to the nucleotide changed by the putative differentiation which had occurred. However, these cultures had comparatively high concentrations of extracellular cyclic AMP. Based on this observation and other circumstantial evidence (Section VI, VII) it is considered possible that the extracellular cyclic AMP may be of more importance in regulating secondary metabolism than is the intracellular nucleotide (Section VII).

Additional support for the above interpretation of the results in this section is also provided by the observation that when mycelia were growing in zinc replete medium they were refractive to exogenous cyclic AMP (Fig. 14). At low zinc concentrations growth was limited, and cyclic AMP stimulated acidogenesis. At higher zinc levels more growth was achieved, but at the expense of citrate and YP production, and added cyclic AMP had no effect. That is, the zinc ion defined the differentiated state, and consequently the response of the cultures to cyclic AMP was governed by zinc.

B. SENSITIVITY AND SPECIFICITY TO CYCLIC AMP

There were three different responses of the cultures to exogenous cyclic AMP which were detectable from the studies described in this section. Namely, the nucleotide stimulated "early" growth, inhibited "late" growth, and stimulated acidogenesis. The data presented below demonstrate that all three responses were more or less specific to cyclic AMP, and also were sensitive to relatively low concentrations of the nucleotide. This adds weight to the argument that cyclic AMP is

Figure 14: The relationship between the zinc and cyclic AMP (plus 5 mM theophylline) effects on growth and citric acid accumulation. Zinc was added at zero hours, and cyclic AMP (1 mM) and theophylline were added after 16 hours; the flasks were harvested after 50 hours. Cyclic AMP (broken line): specific activity (Δ), dry weight (●). Control (full line): specific activity (X), dry weight (O). (1.4×10^7 conidia; n = 3).


Dry Weight (grams)
0·20
0·16
0·12
0·08
0·04
3·1 x 10⁻⁶
3·1 x 10⁻⁷
3·1 x 10⁻⁸
[Zinc] M
Specific Activity
3000
2500
2000
1500
1000
500
0

in fact a physiological regulator.

Cyclic AMP concentrations of the order of 10^{-6}M stimulated the early growth (7 hours) of mycelia grown at high conidia densities (Fig. 15). Other adenine and guanine nucleotides promoted growth as well, but not to the extent of cyclic AMP (Table 10). Guanine and adenine are known to stimulate conidia germination in this organism (Tsay and Hanoaka, 1972), and this may have some bearing on these results.

Theophylline was without significant effect on the increase in dry weight of these conidia and germlings. Theophylline, a methyl xanthine and cyclic AMP analogue, is known to be an inhibitor of the phosphodiesterase which breaks down cyclic AMP (Butcher and Sutherland, 1962). It is considered to be supporting evidence that a physiological event is in fact mediated by cyclic AMP, if theophylline either duplicates or potentiates the cyclic AMP effect. In this case it may be that the phosphodiesterase activity was too low for cyclic AMP hydrolysis to be an important factor.

In mycelia grown at conidia densities higher than 4×10^6 the T-CTP was always greater than 24 hours. The subsequent inhibition of growth which was encountered following the T-CTP was sensitive to cyclic AMP concentrations of the order of 10^{-6}M, and Fig. 16 shows the dose response after 31 hours growth. However, although cyclic AMP caused significant inhibition of growth in the 30 hour region of high conidia density fermentations, it did not exert as pronounced an effect at a later period (see below).

Figure 15: Dose response to cyclic AMP: stimulation of growth after seven hours. Each point is the mean of a total of 10 combined flasks from two separate experiments (4.5 and 5.8 x 10^6 conidia). The mean of the control flasks was 0.64 mg.

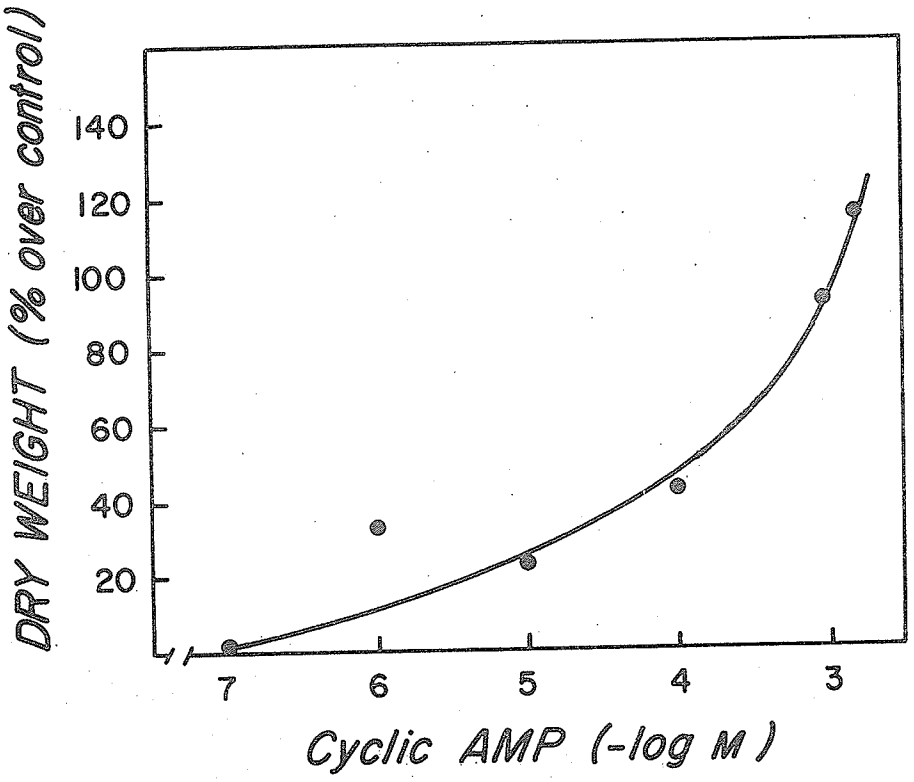

DRY WEIGHT (% over control)
140
120
100
80
60
40
20
7
6
5
4
3
Cyclic AMP (-log M)

Table 10

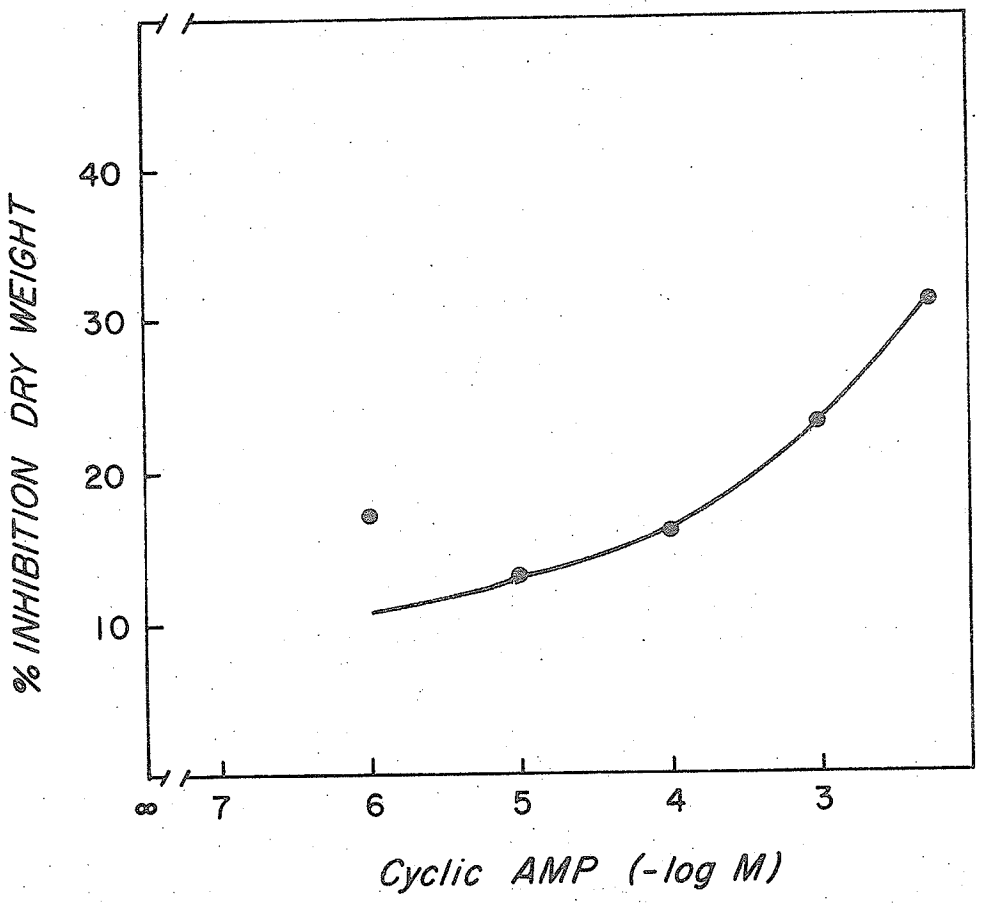
Influence of adenine and guanine nucleotides (all 1.5 mM) on growth after seven hours.

	Dry Weight (mg)	% of Control
EXPERIMENT 1		
CONTROL	0.68	100
cAMP	1.29	190
adenosine	0.98	144
AMP	0.62	91
ADP	0.73	107
ATP	0.94	138
EXPERIMENT 2		
CONTROL	0.41	100
cAMP	0.97	237
cGMP	0.60	146
quanosine	0.85	207
GMP	0.61	149
GTP	0.87	212

Experiment 1 - 5.1×10^6 conidia, n = 5.

Experiment 2 - 4.0×10^6 conidia, n = 5.

Figure 16: Dose response to cyclic AMP: inhibition of growth after 31 hours. Cyclic AMP was added after 11 hours. (8.0×10^6 conidia; n = 15).


% INHIBITION DRY WEIGHT
40
30
20
10
∞
7
6
5
4
3
Cyclic AMP (-log M)

At high conidia densities citric acid accumulation commenced after about 30 hours growth, and except for a short lag period, continued at a linear rate until the sucrose was exhausted. The cyclic AMP stimulation of acidogenesis was observed at concentrations of the order of 10^{-6}M (Fig. 17). The graph depicts the compiled data from five separate experiments. Each point is the mean of at least four flasks and usually six. The point at 10^{-3}M is the mean of 25 flasks and the control line (nothing added) represents 45 flasks. The total number of flasks was 172.

The influence of various adenine and guanine nucleotides on growth and citric acid accumulation at high cell densities is shown in Table 11. The effectors were added after 17 to 19 hours growth and the flasks harvested after 48 to 50 hours. Cyclic AMP reduced growth by 10 to 15%, and caused large increases in the amount of citrate produced. ATP also increased citrate synthesis, probably because it was converted to cyclic AMP in the cells via adenyl cyclase. A 2' and 3'-AMP mixture also increased specific activities, while 2', 3'-cyclic AMP and 5'-AMP did not. This curious result may relate to the idea that the citrate accumulating condition is a differentiated state; both cyclic AMP and 3"-AMP have been reported to induce the formation of fruiting bodies in the Basidiomycete Coprinus macrorhizus (Uno and Ishikawa, 1973). Presumably growth termination results in hydrolysis of nucleic acids, producing 3'-AMP, which then in some way influences differentiation.

The response to adenosine seemed to be somewhat variable: 10^{-3}M adenosine always increased the amount of citrate produced, but on some

Figure 17: Dose response to cyclic AMP: stimulation of the specific activity of citrate accumulating mycelia. See text for further explanation. Cyclic AMP was added after 16-20 hours growth, and the flasks harvested after 48-50 hours. (6-12 x 10^6 conidia).

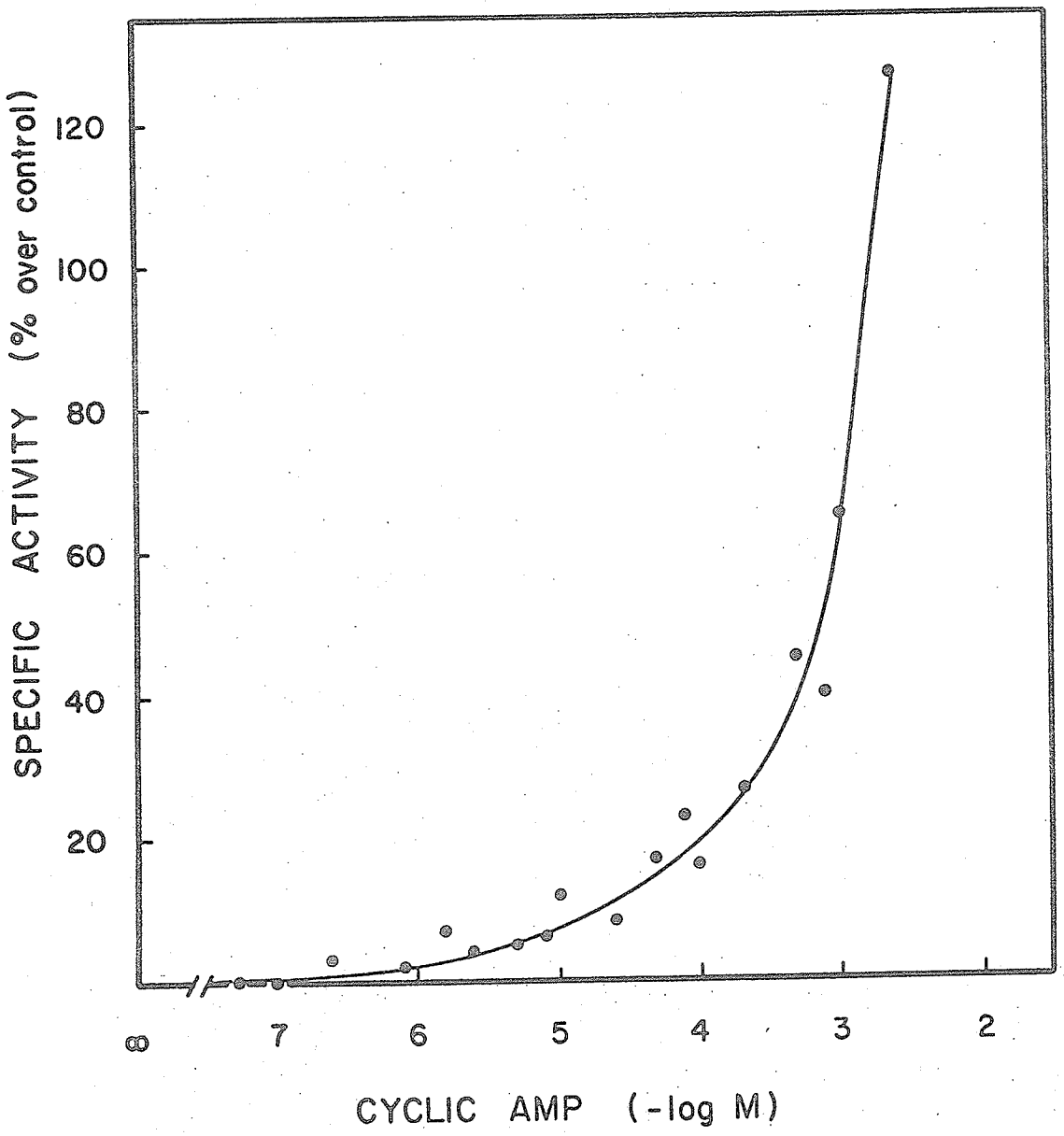

SPECIFIC ACTIVITY (% over control)
120
100
80
60
40
20
∞
7
6
5
4
3
2
CYCLIC AMP (-log M)

Table 11

Influence of various adenine and guanine nucleotides on growth and citrate accumulation.

	Dry Weight (mg)	% of Control	Citrate	% of Control	Specific Activity	% of Control
EXPERIMENT 1 - 8.0 x 10^6 conidia, added at 19 hours						
CONTROL (13)	66	100	92	100	1372±79*	100
1.0mM 3',5'-cAMP (9)	59	89	143	155	2454±195	179
1.0mM 2',3'-cAMP (9)	76	115	108	117	1363±131	99
1.0mM adenosine (9)	82	124	120	130	1481±122	108
1.0mM 5'-AMP (9)	73	110	91	99	1236±105	92
1.0mM ADP (9)	104	156	80	87	792±125	58
1.0mM ATP (9)	69	105	120	130	1864±372	136
1.0mM 2' and 3' AMP (9)	67	100	115	125	1736±122	127
EXPERIMENT 2 - 8.0 x 10^6 conidia, added at 19 hours						
CONTROL (9)	94	100	169	100	1875±158	100
1.0mM cAMP (4)	85	90	263	156	3082±74	163
0.2mM cAMP (4)	81	86	195	115	2374±89	127
1.0mM cGMP (4)	83	88	235	139	2831±349	151
0.2mM cGMP (5)	78	83	162	96	2067±201	110
1.0mM 5'-GMP (8)	92	98	155	92	1894±156	90
1.0mM quanosine (9)	95	101	139	82	1463±196	78
EXPERIMENT 3 - 6.0 x 10^6 conidia, added at 17 hours						
CONTROL (8)	92	100	106	100	1155±203	100
1.0mM cAMP (9)	80	87	145	137	1808±278	156
1.0mM 5'-AMP (9)	106	115	130	123	1229±169	106
1.0mM adenosine (9)	93	101	163	154	1756±244	152

*Standard deviation of the mean.

The test agents were added at 17-19 hours, and the flasks harvested after 48-50 hours. (6-12 x 10^6 conidia).

occasions increased growth as well. However, the general effect was an increase in specific activities. The dose response to adenosine was tested in another experiment, and concentrations of the order of 10^{-4}M increased specific activities (Fig. 18). It is assumed that the adenosine was converted to cyclic AMP by the mycelia (Suttin and Rall, 1970), but the possibility exists that it may also have been transformed into compounds of the cytokinin class (see LeJohn et al., 1974), although there is no evidence for cytokinins in A. niger.

ADP had an important effect on the cultures, which was opposite to that of cyclic AMP. This agent enhanced growth and inhibited both citrate and YP synthesis (Table 11). ADP also promoted aggregation (Section IV). This result is not understood, but is interesting because the growth pattern in response to ADP was very similar to the SV-type of growth described in Section II.

Guanosine 3',5'-cyclic monophosphate (cyclic GMP) is the only other cyclic nucleotide known to be present in biological tissue. At 10^{-3}M this compound reduced growth and increased specific activities as did cyclic AMP, but was not as effective as cyclic AMP at lower concentrations (Table 11, Experiment 2). In another series of studies, cyclic GMP (0.3mM) caused a variable pattern of responses (Table 12). In experiment 1, citrate production was increased, but there was no effect on growth or YP (14% was considered to be significant under these conditions). In experiment 2, both growth and citrate accumulation were reduced a little; here YP synthesis was also retarded, and the cyclic GMP-treated pellets were larger. It is apparent that the response to cyclic GMP was complicated, and may have been related to the conidia

Figure 18: Dose response to adenose: stimulation of the specific activity of the mycelia. Adenosine was added at 17 hours and the flasks harvested at 50 hours. The control (n = 14) specific activity was 666. (7.0×10^6 conidia; n = 2).

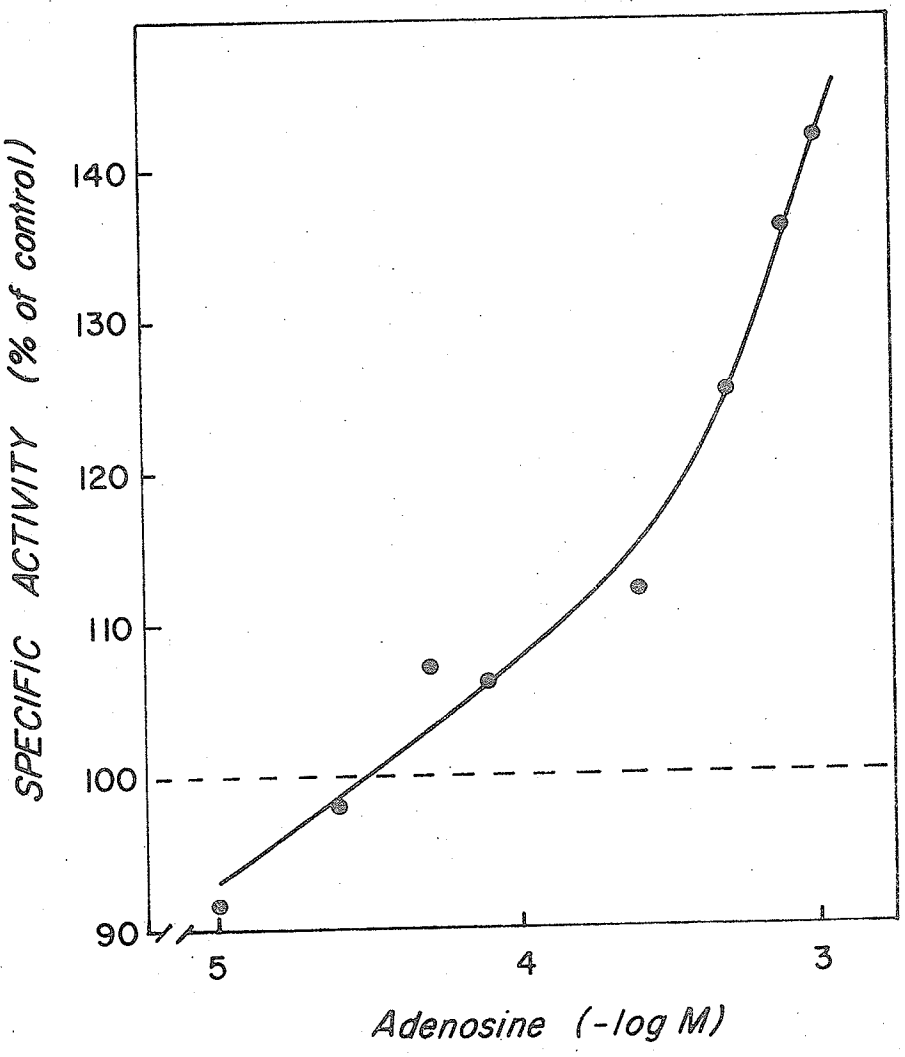

SPECIFIC ACTIVITY (% of control)
140
130
120
110
100
90
5
4
3
Adenosine (-log M)

Table 12

Influence of cyclic GMP on growth and citric acid accumulation.

	Dry Weight (mg)	Citrate µmoles	Specific Activity	% of Control
EXPERIMENT 1 - 1.1 x 10^7 conidia; n = 8				
CONTROL	71	200	2881	100
0.3mM cGMP	73	228	3294	114
0.1mM cGMP	73	208	2925	102
EXPERIMENT 2 - 6.9 x 10^6 conidia; n = 9				
CONTROL	62	124	2039	100
0.3mM cGMP	66	119	1823	89
0.1mM cGMP	69	138	2118	104
EXPERIMENT 3 - 8.9 x 10^6 conidia; n = 10				
CONTROL	65	106	1631	100
0.3mM cGMP	78	126	1626	100
0.1mM cGMP	64	108	1697	104

Cyclic GMP was added at 18-19 hours, and the flasks harvested after 48-50 hours.

density. Since the conidia density seemed to roughly define the physiological state at a particular time, further investigation of the results might prove interesting.

At high conidia densities, cyclic AMP always increased the specific activity of the mycelia. It is therefore assumed that the effect of cyclic GMP was of a "secondary" nature. Cyclic GMP has been observed to duplicate cyclic AMP effects in other systems, but its significance is not understoon (Hardman et al., 1971).

The response to N^6-2'-O-dibutyryl adenosine monophosphate (DB-cAMP) was variable and ambiguous. In some tests it behaved like cyclic AMP, in others it was without effect, and in still others it caused large increases in growth, with only small increases in citrate production (Table 13). This cyclic AMP analogue has been commonly used in studies on cyclic AMP physiology in cultured cells; it is normally more potent than cyclic AMP, presumably because of the enhanced membrane permeability associated with the lipophyllic side group. If such was the case here, it is possible that the increased growth sometimes observed may have been caused by the elevation of the "effective" intracellular nucleotide level, as growth oriented zinc replete mycelia contain high levels of cyclic AMP (Section VII). The mycelia do not take up large amounts of natural cyclic AMP (Section VII). On the other hand, the increased specific activity sometimes observed (experiment 1, flasks 2 and 3) may have resulted from nucleotide effects in another compartment of the cell, perhaps on the outside membrane. There is some circumstantial evidence that citrate accumulation is associated with the cell surfaces (Sections VI, VII).

Table 13

Influence of DB-cAMP and Na Butyrate on growth and citric acid accumulation.

Flask	Dry Weight (mg)	Citrate µmoles	Specific Activity
EXPERIMENT 1 - 6.6×10^6 conidia			
6-13; CONTROL	92	111	1203
1; 1.0mM DB-cAMP	111	117	1055
2; 0.7mM DB-cAMP	74	203	2743
3; 0.7mM DB-cAMP	63	151	2389
4; 2.0mM Na butyrate	82	65	798
5; 2.0mM Na butyrate	108	62	572
EXPERIMENT 2 - 8.3×10^6 conidia			
7-14; CONTROL	90	101	1067
1,2; 0.7mM DB-cAMP	78	86	1095
3.4; 0.5mM DB-cAMP	88	99	1189
5,6; 0.3mM DB-cAMP	86	95	1095
EXPERIMENT 3 - 4.9×10^6 conidia			
3-6; CONTROL	62	89	1446
1; 0.5mM DB-cAMP	73	100	1370
2; 0.3mM DB-cAMP	155	108	699

The agents were added at 21-23 hours, and the flasks harvested after 48-50 hours.

Sodium butyrate inhibited citrate accumulation, and also caused the mycelia to grow in the form of large white pellets (Table 13, experiment 1). This substance is known to mimick some of the effects of DB-cAMP in chinese hamster ovary cells (Wright, 1973). That author suggested that perhaps the activity of membrane bound adenyl cyclase might have been altered, or that conformational changes in the cell membrane may have occurred. Both possibilities would also seem to apply here.

Theophylline exhibited a complex effect when added to high density cultures, which was difficult to interpret without further experimentation. Theophylline at 2-4 mM increased citrate production with no effect on growth and morphology (Fig. 19). For some reason, however, very little citrate was produced in this particular experiment. Higher concentrations (7-17 mM) added at zero hours increased the pellet number and inhibited growth somewhat (Fig. 31, Section IV). When added at 16 hours, 5 mM theophylline increased growth a little, but 25 mM levels inhibited growth (Fig. 20). Theophylline alone inhibited citrate production, but theophylline in combination with cyclic AMP enhanced the cyclic AMP effect on both citrate accumulation (Fig. 20) and pellet number (Table 18, Section IV). In other experiments, high concentrations of theophylline added after 23 hours increased growth but decreased citrate production (Table 14). Finally, theophylline (15 mM) added after 40 hours growth increased citrate synthesis with no effect on growth (Fig. 10).

Some of these results may be interpreted to mean that theophylline

Figure 19: Dose response to theophylline added alone: stimulation of the specific activity of the mycelia. Theophylline was added after 15 hours, and the mycelia harvested after 48 hours. There was no theophylline effect on total dry weight; control = 83 mg. (2.1×10^7 conidia; n = 10).

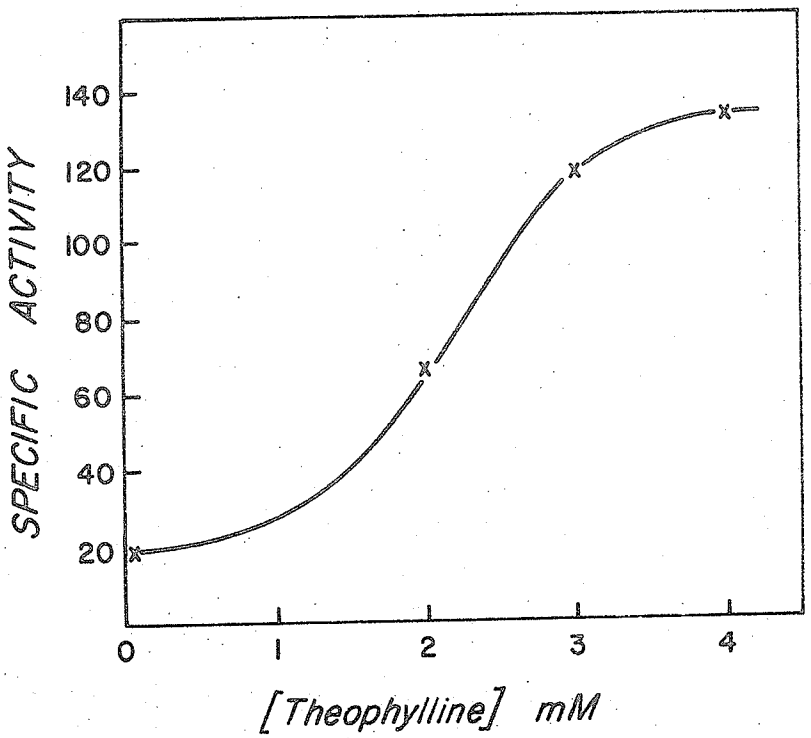

SPECIFIC ACTIVITY
140
120
100
80
60
40
20
0
1
2
3
4
[Theophylline] mM

Figure 20: Effect of theophylline alone and theophylline and cyclic AMP in combination on citrate accumulation and cell weight. Effectors were added after 15 hours growth, and the flasks harvested after 50 hours growth. (1.8×10^7 conidia; n = 9).

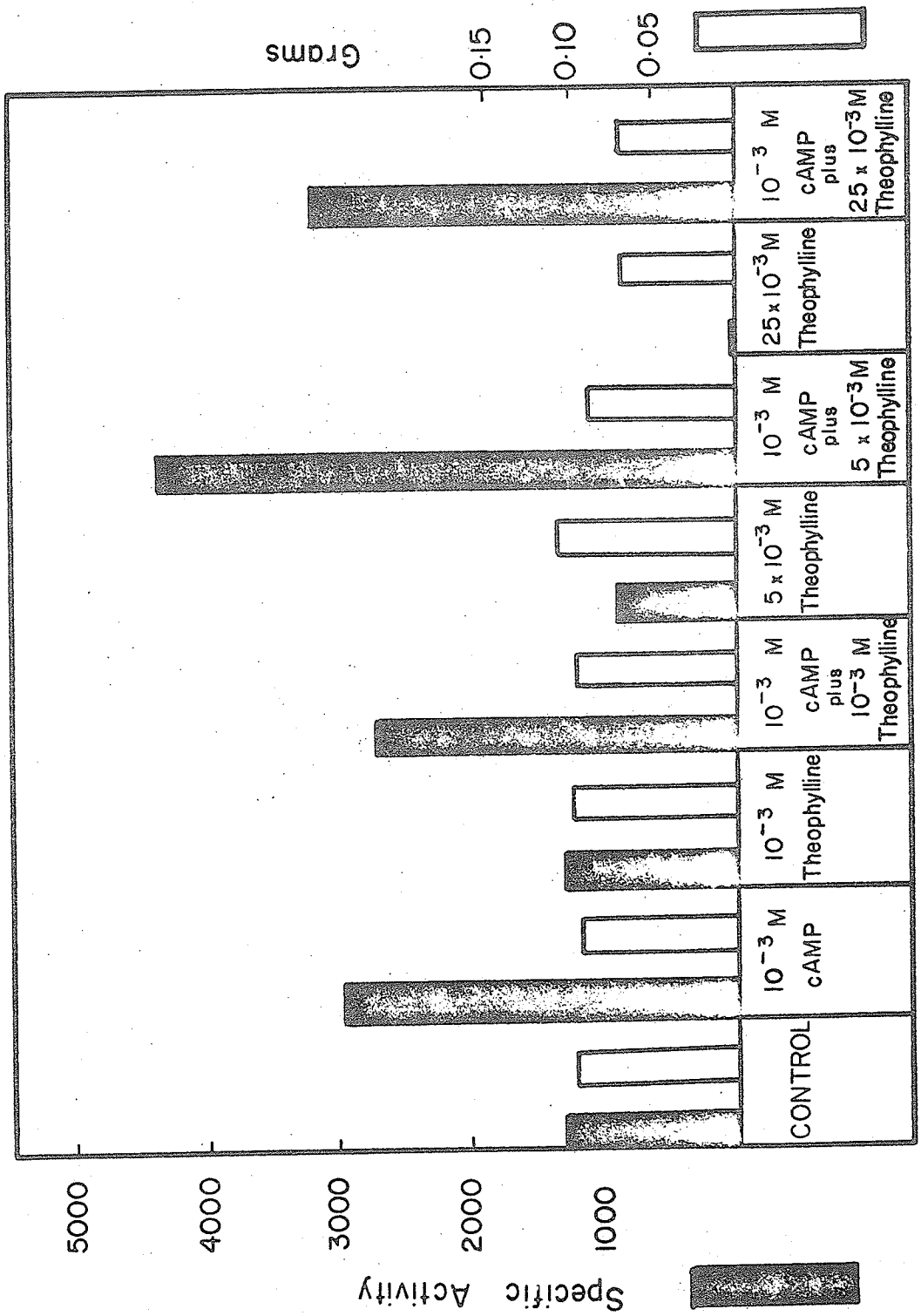

Specific Activity
5000
4000
3000
2000
1000
Grams
0·15
0·10
0·05
CONTROL
10⁻³ M cAMP
10⁻³ M Theophylline
10⁻³ M cAMP plus 10⁻³ M Theophylline
5 x 10⁻³ M Theophylline
10⁻³ M cAMP plus 5 x 10⁻³ M Theophylline
25 x 10⁻³ M Theophylline
10⁻³ M cAMP plus 25 x 10⁻³ M Theophylline

Table 14

Influence of theophylline and caffeine on growth and citrate accumulation.

	Dry Weight (mg)	Citrate µmoles	Specific Activity
CONTROL (n = 3)	62	89	1436
35 mM THEOPHYLLINE	-	13	-
30	96	42	438
25	76	50	658
20	62	55	887
15	-	46	-
10	-	75	-
6 mM CAFFEINE	45	16	356
5	-	16	-
4	-	25	-
3	-	21	-
2	-	44	-
1	-	43	-

The agents were added after 23 hours growth, and the flasks harvested after 50 hours. (5.0×10^6 conidia; n = 1).

elevated cyclic AMP levels by preventing the hydrolysis of the nucleotide, but that the effect observed was related to the physiological state of the mold. Thus, under citrate accumulating conditions (high added cAMP or after 40 hours growth) theophylline increased citrate production (Fig. 10). However, earlier in the fermentation (23 hours), when the mycelia were growth oriented, theophylline increased growth (Table 14), perhaps by increasing the intracellular nucleotide levels. Mycelia younger than 23 hours were somehow sensitive to the adverse effects of this agent and growth was inhibited at high concentrations of theophyllin.

The observation that theophylline inhibited citrate accumulation under most conditions presents an interesting possibility that there is a cyclic AMP "site" for citrate accumulation. Theophylline seems to behave like a competitive inhibitor for this site. For example, high concentrations of theophylline added alone may successfully compete with the endogenous cyclic AMP, and thereby cause inhibition of citrate production. However, this is reversed by added cyclic AMP (Fig. 20). The potent inhibition of citrate accumulation by relatively low concentrations of caffeine supports this idea (Table 14). Caffeine also inhibited growth at 6 mM, and at 15 mM caused almost complete inhibition when added at 23 hours. Obviously, a great deal more work is required to seriously test any of these suggestions.

Theophylline had other effects on the mold, independent of its relationship to cyclic AMP. Most noticable was an inhibition of YP synthesis, even in the presence of cyclic AMP. High concentrations of

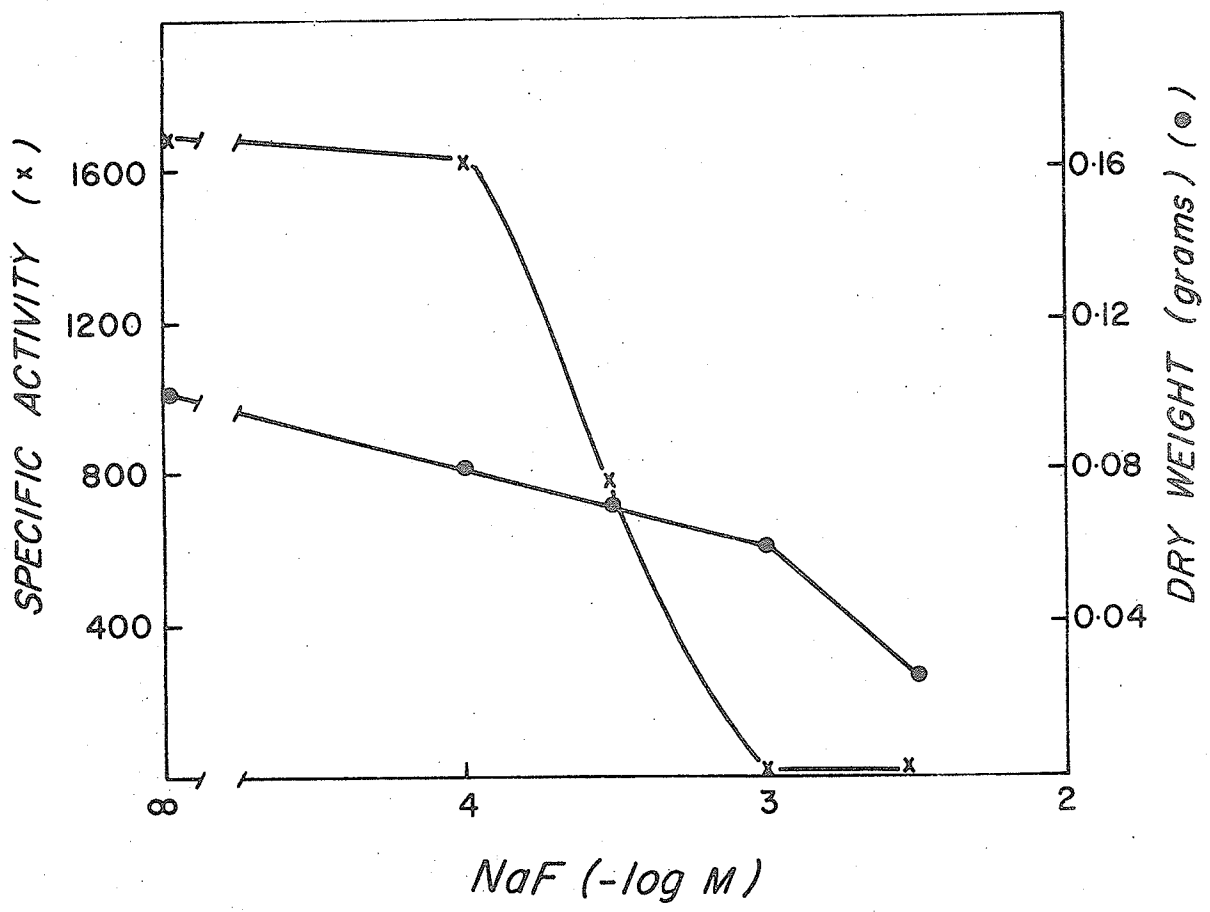
theophylline also seemed to prevent the formation of the regular pellet structure, causing the pellets to become "stringy" instead of smooth and compact.

The fluoride ion is known to be an activator of adenyl cyclase in many eucaryotic tissues, and it has also been reported to stimulate citrate accumulation by A. niger (Takami, 1967; 1968). This suggested a connection between the two phenomena. However, in these studies fluoride caused a dose dependent inhibition of growth, and a pronounced inhibition of citrate production (Fig. 21).

DISCUSSION

These results indicate that the citrate fermentation displays the typical trophophase-idiophase dynamics common to most microbial fermentations (Historical) in agreement with the data of Shu and Johnson (1948a). The other secondary metabolite, YP, was also synthesized in a similar manner. It has not been proved that cell proliferation had completely ceased before the idiophase was initiated (Weinberg, 1970), and in fact, the dry weight continued to increase to some extent as the citrate was being produced. However, it is clear that citrate accumulation was associated with at least a much reduced rate of growth, and there are several reasons to suppose that cell proliferation probably stopped before citrate accumulation. For example, in Section VII it is shown that the protein content of the idiophase mycelia was constant, but addition of zinc caused an increase in protein synthesis and termination of citrate production. Also, it is well known that large amounts of citrate are produced in replacement

Figure 21: Effect of NaF on growth and specific activity. NaF was added at zero hours, and the flasks harvested after 50 hours growth. (1.5×10^7 conidia; n = 3).


SPECIFIC ACTIVITY (x)
1600
1200
800
400
0·16
0·12
0·08
0·04
DRY WEIGHT (grams) (●)
∞
4
3
2
NaF (-log M)

cultures lacking a nitrogen or phosphate source so as to preclude growth (Szücs, 1944; Karrow and Waksman, 1947; Perquin, 1938). Further, the fact that the rate of citrate synthesis was linear argues that the acid was being produced by a non-expanding cell population. The initial lag (Fig. 11) presumably resulted because some of the mycelia stopped growing and began producing citrate before others. These citrate production kinetics are identical to those of other SM (Weinberg, 1970).

Added cyclic AMP either promoted or inhibited the increase in dry weight, depending upon the cultural conditions. This implies that cyclic AMP is a regulator of growth, and measurement of endogenous nucleotide levels supported this view (Section VII). Although dry weight is an experimentally convenient assay of growth, under some conditions it is not a very good one, for large increases in dry weight, which are caused by lipid and carbohydrate synthesis (Burrow et al., 1964), can occur after cell proliferation has ceased. Cyclic AMP is known to stimulate the breakdown of glycogen, as well as inhibit its synthesis, and also to stimulate lipolysis (Robison et al., 1968). It is conceivable then, that the nucleotide effects on dry weight may have been associated with the synthesis and degradation of these macromolecules. This seems unlikely though, for cyclic AMP influenced the increase in dry weight in very young cultures; these conditions are not normally associated with lipid or glycogen anabolism (Burrow, 1964). Further, cyclic AMP did not have an important influence upon the final dry weight obtained, only on the rate of increase.

The data are in accord with the idea that the mycelia are initially growth oriented, but under zinc deficient conditions, they eventually "sense" zinc deficiency, decrease their growth rate, and then differentiate into citrate accumulating cells. Although these studies clearly show that cyclic AMP inhibited growth and stimulated citrate synthesis under citrate accumulating conditions, they do not answer the important question as to whether the nucleotide "induced" the citrate accumulating state. That is, did cyclic AMP cause differentiation? In some experiments not reported, cyclic AMP inhibited the onset of citrate production in cultures grown at low conidia density, while in other experiments cyclic AMP had no influence on the onset, only on the rate of synthesis. On the other hand, cyclic AMP had a clear effect on the onset of synthesis of YP: at high conidia densities the onset was usually, but not always, increased, while at low densities the onset was always decreased. However, this relationship between cyclic AMP and YP was complex, and other unknown factors were involved (Section II). As there is no information available as to the nature of this pigment nor to the mechanism of its synthesis, its connection with cyclic AMP is not clear.

These results suggest that cyclic AMP is a regulator of growth in A. niger, but provide little information pertaining to its role in differentiation (as defined as the idiophase). However, concepts developed in other organisms indicate that this nucleotide may play a universal role as a regulator of growth and development in biological systems. In E. coli cyclic AMP is required for the synthesis of

catabolite-repressed enzymes (Pastan and Perlman, 1970), and high concentrations inhibit growth as well (Judewicz et al., 1973). In Haemophilus influenzae, cyclic AMP increases the frequency of transformation, a phenomenon which had been likened to secondary metabolism (Weinberg, 1970), and high concentrations also inhibited growth (Wise et al., 1973). Cyclic AMP is an integral part of the life cycle of D. discoidium. Nutrient depletion in this system is attended by growth termination, aggregation, migration, and differentiation (Bonner, 1971; Newell, 1971; Garrod and Ashworth, 1973). Cyclic AMP effects aggregation of the amoebae (Konijin et al., 1967), increases adhesiveness of individual cells in the aggregate (Konijin et al., 1968), and stimulates differentiation into stalk cells (Bonner, 1970).

Cyclic AMP also has profound effects on the cells of higher organisms. In animals, cyclic AMP stimulates the proliferation of lymphoidal and marrow cells (Whitfield, 1973), but inhibits cell division in some other types of cultured cells (Burk, 1968; Ryan and Henrick, 1968; Sheppard, 1971; Hsie and Puck, 1971; Johnson et al., 1971; Schultz and Gratzner, 1973); in the latter case the nucleotide may also induce irreversible differentiation (Prasad, 1973). In plants, there is some evidence that cyclic AMP may mediate the actions of some of the plant hormones, including indole acetic acid (Kamisaka and Masuda, 1970; Salomon and Mascarenhas, 1972), gibberellic acid (Galsky and Lippincott, 1969; Gilbert and Galsky, 1972; Kamisaka et al., 1972), and the cytokinisins (Wood et al., 1972; Wood and Braun, 1973). These are of course, well known regulators of growth and differentiation.

Very little is known about physiological aspects of cyclic AMP in filamentous fungi, but it has been reported that cyclic AMP induced the formation of fruiting bodies in Coprinus macrorhizus (Uno and Ishikawa, 1973a, b), and there is some evidence relating the nucleotide to phenomena associated with catabolite repression in yeasts (Sy and Richter, 1972a, b; Tsuboi et al., 1972). In the present studies, cyclic AMP inhibited growth for a considerable period of time before any other manifestation of the idiophase was observed, and when citrate accumulation finally began, the degree of response was intensified. This seemed to indicate a physiological "plan", and, along with the concepts developed in other systems, suggests that serious consideration should be given to the possibility that the nucleotide is a regulator of differentiation in A. niger. That cyclic AMP influenced "adhesiveness" also suggests this possibility (Section IV), for aggregation of the conidia and hyphae is probably important in developmental control.

With the exception of the "transformed" type of growth described in Section II, the trophophase-idiophase decision was controlled primarily by the concentration of the zinc ion, and the cultures responded to added cyclic AMP in a different manner according to the phase they were in. However, in all cases it seemed as if the nucleotide served to promote the activity characteristic of that particular phase. This suggests that cyclic AMP does not initiate or direct the flow of information, but simply amplifies it; control is at another level, and the initial signal is the concentration of the zinc ion. This is in accord with the well known role of cyclic AMP as a second messenger in mammalian systems (Robison et al., 1968).

It was postulated in Section II that zinc deficiency leads to the synthesis of a growth inhibitor. This hypothetical substance could possibly be connected in some way not only to the control of development but also to the metabolism of cyclic AMP. Extracellular growth regulators are known in mammalian systems, and one class of effectors, called chalones, has been reported to be related to cyclic AMP (Marks and Grimm, 1972).

There are other interpretations to the relationship between cyclic AMP and citrate accumulation shown here. Cyclic AMP is known to be a regulator of glycolysis in some mammalian tissues, the effect being mediated through phospholylase (Robison et al., 1968) and phosphofructokinase (PFK) (Monsour and Setlow, 1972). Phospholylase is activated in Neurospora (Tellez-Inon and Torres, 1970), and in yeast there is an increase in NADH oscillations and a shift in the steady state oxidation-reduction level towards oxidation (Chance and Schoener, 1964). Added cyclic AMP might then stimulate glycolysis, and the excess citrate produced under the restricted growth conditions might then spill over into the medium.

Another possibility is that cyclic AMP activates the actual secretion of citrate from the cell (Section VI). Cyclic AMP involvement in secretion and membrane function is well known (see Robison et al., 1971). This last idea would be particularily interesting if it could be shown that associated with the differentiation into the idiophase there was the induction of a mechanism concerned with the specific secretion of citrate from the cells.

SECTION IV

REGULATION OF THE AGGREGATION OF CONIDIA AND HYPHAE BY CYCLIC AMP

Filamentous fungi cultivated in agitated, submerged, liquid cultures may assume either a filamentous (Foster, 1949) or a pellet (Kluyver and Perquinn, 1933a; Burkholder and Sinnot, 1945) mode of growth. Although this is a very common observation, it is not understood what physiological ramifications ensue from either morphological type. Similarly, very little is known as to the precise nature of the process which effects the aggregation process (Smith and Anderson, 1973).

The pellets arise from the initial clumping of individual conidia and germlings (Burkholder and Sinnott, 1945; Takahashi et al., 1959a; 1959b; 1960; Galbraith and Smith, 1969), and the ability to form pellets is believed to be under genetic control (reviewed by Srb, 1972). Among the external factors known to influence the formation of the pellets include the composition (Burkholder and Sinnott, 1945; Foster, 1949) and pH (Pirt and Callow, 1959; Galbraith and Smith, 1969) of the medium, and the size of the inoculum (Camici et al., 1952; Steel et al., 1955; Trinci, 1970). In addition, pellet morphogenesis may be modified by such agents as L-sorbose (Tatum et al., 1949), ferrocyanide (Martin and Waters, 1952; Steel et al., 1955; Clark, 1962; Section VI), EDTA (Choudhary and Pirt, 1965; Section VI), deoxycholate (Dorn and Rivera, 1966), certain lipids (Millis et al., 1963), methanol (Section VI), snake venom, and con A (Section II).

Cytochemical examinations of the pellets have revealed that

they are very heterogeneous in nature (Clark, 1962; Yanagita and Kogane, 1963). The pellet consists of a thick cortex of branched hyphae; the center is hollow, a result of the autolysis of the older sections of the hyphae, and cell proliferation apparently occurs only on the outer surface (discussed by Trinci, 1970). Hyphae in different parts of the pellet appear to be both cytologically and physiologically differentiated (Yanagita and Kogane, 1963), as are fungus colonies on solid surfaces (Park and Robinson, 1966), and it has been suggested that a pellet may be regarded as a surface colony in three dimensions (Yanagita and Kogane, 1963).

It is believed that for fungi growing on solid media, cell-cell interactions are important in the regulation of the growth and differentiation (Park and Robinson, 1966). This is also considered to be true in the development of higher organisms (Stoker, 1967; Rutter et al., 1973). Therefore, it is possible that the contiguity of the hyphae in the pellets may similarly influence growth and differentiation of fungi in this morphological form. In support of this is the observation that filamentous fungi proliferate exponentially when the growth mode is filamentous (Zalokar, 1959; Pirt and Callow, 1960; Borrow et al., 1964; Choudhary and Pirt, 1965; Pirt, 1966; Carter and Bull, 1969; Trinci, 1969; 1970), but display cube root kinetics when the growth is in the pellet form (Emerson, 1950; Marshall and Alexander, 1960; Pirt, 1966; Trinci, 1970).

Cyclic AMP has been shown to affect the proliferation of cells from various types of organisms (Historical, Section III). Cyclic AMP has also been implicated as an agent which influences the adhesion of

several diverse types of cells. It increases the adhesion of cultured cells to the substratum (Johnson and Pastan, 1972; Grinnel et al., 1973) decreases the agglutinability of transformed cells by plant lectins (Sheppard, 1971; Hsie et al., 1971), regulates contact inhibition of cultured cells (Sheppard, 1971; Hsie et al., 1971; Hsie and Puck, 1971; Johnson et al., 1971), increases the adhesiveness of the amoebae of D. discoidium during the aggregation phase (Konijin et al., 1968), and inhibits blood platelet aggregation (reviewed by Salzman and Weizenberger, 1972).

In this section it is demonstrated that cyclic AMP influences the aggregation of both conidia and germlings of A. niger growing in medium M-3. Its effects on cell adhesiveness seem to correlate in an approximate way with its effects on growth (Section III); in the trophophase aggregation was enhanced, but following the CTP the nucleotide promoted de-aggregation instead. The significance of these findings is not clear from these preliminary studies, but the implication is that cyclic AMP may regulate development not only by affecting growth and secondary metabolism (Section III), but also by influencing the formation of the fungal thallus.

A. THE DEVELOPMENT OF THE PELLET

The biology of pellet formation by A. niger has been described by Galbraith and Smith (1969), and the present author's observations were essentially the same except that the kinetics of clumping seemed to be a little more rapid and vigorous under the present conditions. However, the qualitative conclusions offered here were derived from

repeated observations and occasional microscopic examination, rather than a rigorous empirical study. Conidia grown on medium M-3 agar slants seemed to have a greater tendency to clump than conidia grown on M-1 agar slants; they also seemed more sensitive to cyclic AMP.

Single conidia gradually aggregated both while floating on the surface of the medium, and after sinking beneath the surface. It usually took from five to nine hours for the clumps to sink into the medium. Before germination the conidia were very hydrophobic, and this undoubtedly contributed to their initial tendency to clump. Aggregation continued, accompanied by swelling and germination, until, after 15 hours, large, irregular clumps had formed. These then seemed to "break-up" to form the eventual pellet structure (Martin and Waters, 1952; Galbraith and Smith, 1969c). The process required a nutrient medium, and was poisoned by cyanide (Galbraith and Smith, 1969c; confirmed here). The tendency to aggregate seemed to decrease with age, which is a common property of cells in multicellular organisms (Curtis, 1967), and clumping was essentially complete by about 24 hours. However, it is not known whether this reflected an age-dependent decrease in adhesiveness of the hyphae, or rather the physical mechanics of the pellet-fluid system, such as the momentum of the pellet movement in a shaking flask.

B. INFLUENCE OF PELLET SIZE ON GROWTH AND ACIDOGENESIS

It is undoubtedly true that under standardized cultural conditions, the degree of aggregation observed represents an equilibrium situation between the adhesiveness of the mold, and the sheer forces in the fluid operating on the aggregate. It is also likely that the degree

of aggregation has an influence upon the growth and metabolism of the thallus. This was shown by the following experiment (Table 15). Medium M-3 was inoculated with conidia at high cell densities: one set of flasks was agitated at 220 rpm on a rotary shaker (A), another set was agitated at 160 rpm (B), and a third set was agitated at 160 rpm and then transferred to the 220 rpm shaker after 16 hours (C). Treatment A mycelia grew as many small pellets, while both B and C flasks formed only about one-fifth the number of pellets and these were considerably larger in diameter. Thus the diminished degree of turbulence allowed more clumping to occur, and in this case it had been completed by 16 hours.

The degree of agitation also had some influence upon the amount of citrate produced, and in particular the specific activity of treatment C mycelia decreased by 35%. It seems likely that this was related to the morphology of the pellets, as oxygenation of the cultures was probably not a factor under these conditions. Shu and Johnson (1948a) had earlier shown that a rapid shaker speed was more beneficial to citrate accumulation than a slow one, a result which they reasonably ascribed to the degree of oxygenation of the cultures. They did not comment upon the pellet morphology but these results in Table 15 suggest that this may have been important as well. This author has grown A. niger in sparged carboys, or in other vessels such that the amount of agitation was low, although the degree of oxygenation of the cultures was probably high. Invariably, the mold grew in the form of one or a few, large, white clumps, and produced no citrate. These observations imply that the kind and degree of aggregation may

Table 15

Influence of shaker agitation rate on growth, citrate accumulation, and aggregation.

Treatment	Dry Weight (mg)	Citrate	Specific Activity	Pellet Number
A. 220 rpm (5)	101	3.4	1680	2754
B. 160 rpm (6)	84	2.0	1200	524
C. 160-220 rpm (3)	94	2.1	1095	551

Flasks in treatment C were agitated at 160 rpm for 15 hours, and then transferred to a 220 rpm shaker. All the flasks were harvested after 50 hours growth (1.5×10^7 conidia).

play a role in the citrate accumulating process. This concept is discussed in more detail below.

C. ASSAY OF ADHESION

The adhesion between conidia and between hyphae was conveniently studied by first ensuring good separation of the inoculating conidia, and then allowing growth to proceed under standardized conditions. The fluid volume, the vessel size and shape, and the rpm of the rotary shaker were some of the important parameters kept constant. Aggregation, a reflection of adhesion, was then determined by visual observation of conidia clumping patterns, or by a quantitative radioactive assay (see below and Methods). Since the eventual number of vegetative pellets formed was related to the adhesiveness of the conidia, germlings, and young hyphae, this quantity was also assumed to be a convenient assay of adhesion. However, the pellet number yielded no information as to the events occurring early in the fermentation, and from these preliminary studies it was apparent that the aggregation of "young" mycelia was complex, and was not always reflected by the final pellet number (see below).

Aggregation of cells from other organisms has been routinely studies by a technique similar in concept to the one employed here. Individual cells are allowed to aggregate in spinner flasks under standardized conditions, and the number of clumps are determined (see Curtis, 1967).

D. INFLUENCE OF CYCLIC AMP ON AGGREGATION

Cyclic AMP had an important influence on the adhesiveness of

cells under these experimental conditions. Cyclic AMP caused a large increase in the aggregation of conidia (Fig. 22), which was observed at all conidia concentrations tested (Fig. 23), and either before or after germination had occurred. These qualitative results were later confirmed by the development of a quantitative assay. Conidia were pre-labelled with ^{14}C, and then subjected to "differential filtration", first through a 61μ pore size nylon mesh, followed by a 0.45μ Millipore filter (see Methods). Data obtained by this technique confirmed in all cases the qualitative results. Fig. 24 shows the effect of cyclic AMP on conidia aggregation using both the quantitative and qualitative techniques. Note that the control 0.45μ Millipore filter contained less conidia following pre-filtration through the nylon mesh.

A close view of a conidia aggregation is presented in the micrograph in Fig. 25. The conidia in the center of the clump (INSERT) are small, unswollen, and devoid of germination tubes, while the individual conidia surrounding the clump are large, swollen, and have emitted or are about to emit germination tubes. Apparently, the conidia in the centers of the aggregates do not germinate, a result which has been suggested before (Foster, 1949).

E. THE CTP OF ADHESION

Cyclic AMP stimulated the clumping of conidia at high conidia densities, and under these conditions promoted growth as well (Section III). However, after the T-CTP had passed, growth was inhibited. It was therefore of interest to see whether cyclic AMP had a similar

Figure 22: Cyclic AMP (2.5 mM) stimulation of conidia aggregation as compared to controls (nothing added). The three replicate samples illustrate the degree of variability commonly obtained in a set of five flasks. The controls (left column) appear as a dense film of relatively unaggregated conidia, while the cyclic AMP treated conidia (right column) have clumped so that the white Millipore filter can be seen in the background. 8.2×10^6 conidia per flask were used in this experiment.

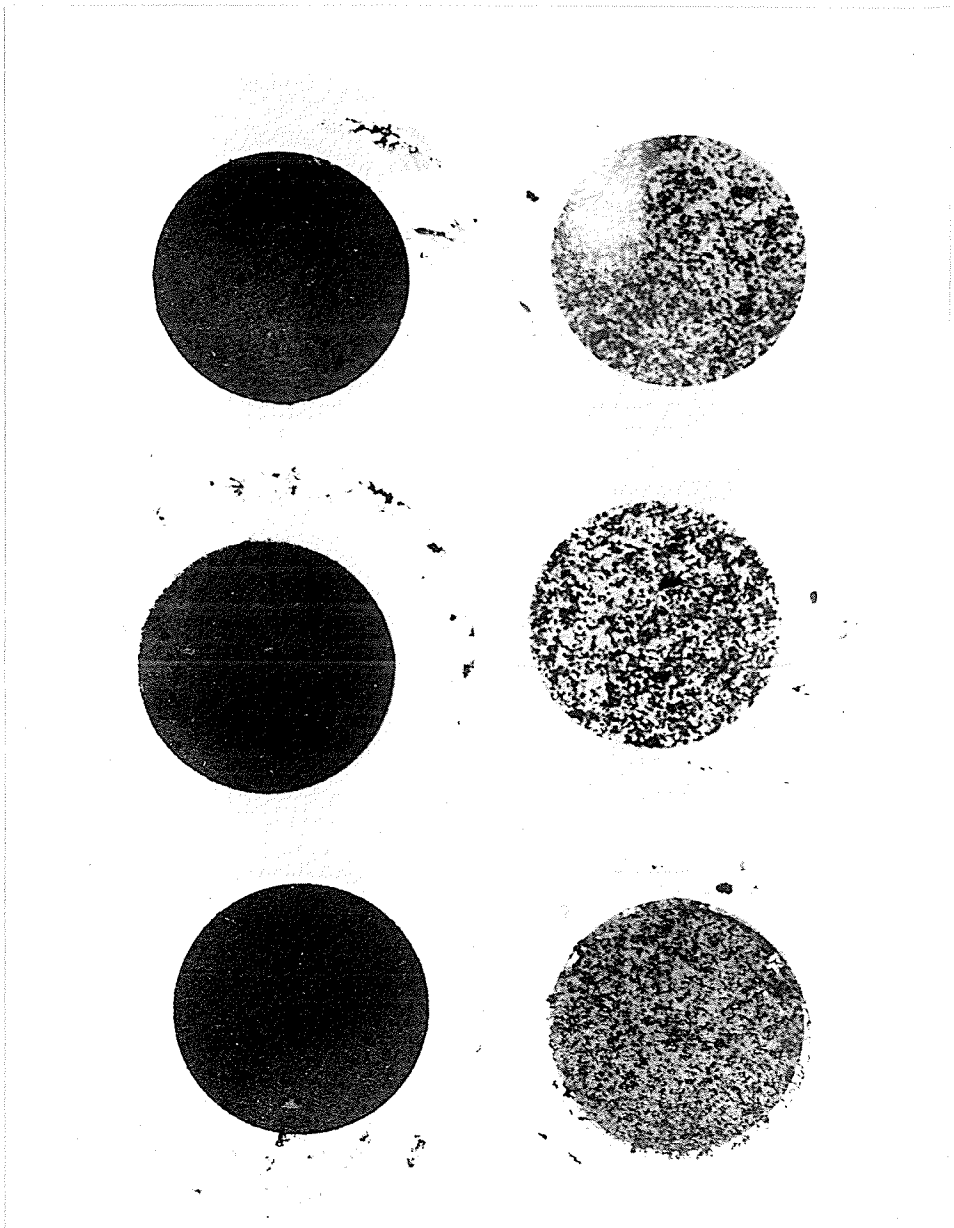


Figure 23: Cyclic AMP (1.0 mM) stimulation of conidia aggregation at different conidia concentrations. Top, 2.4×10^5 conidia; middle, 9.5×10^5 conidia; bottom, 2.4×10^6 conidia.

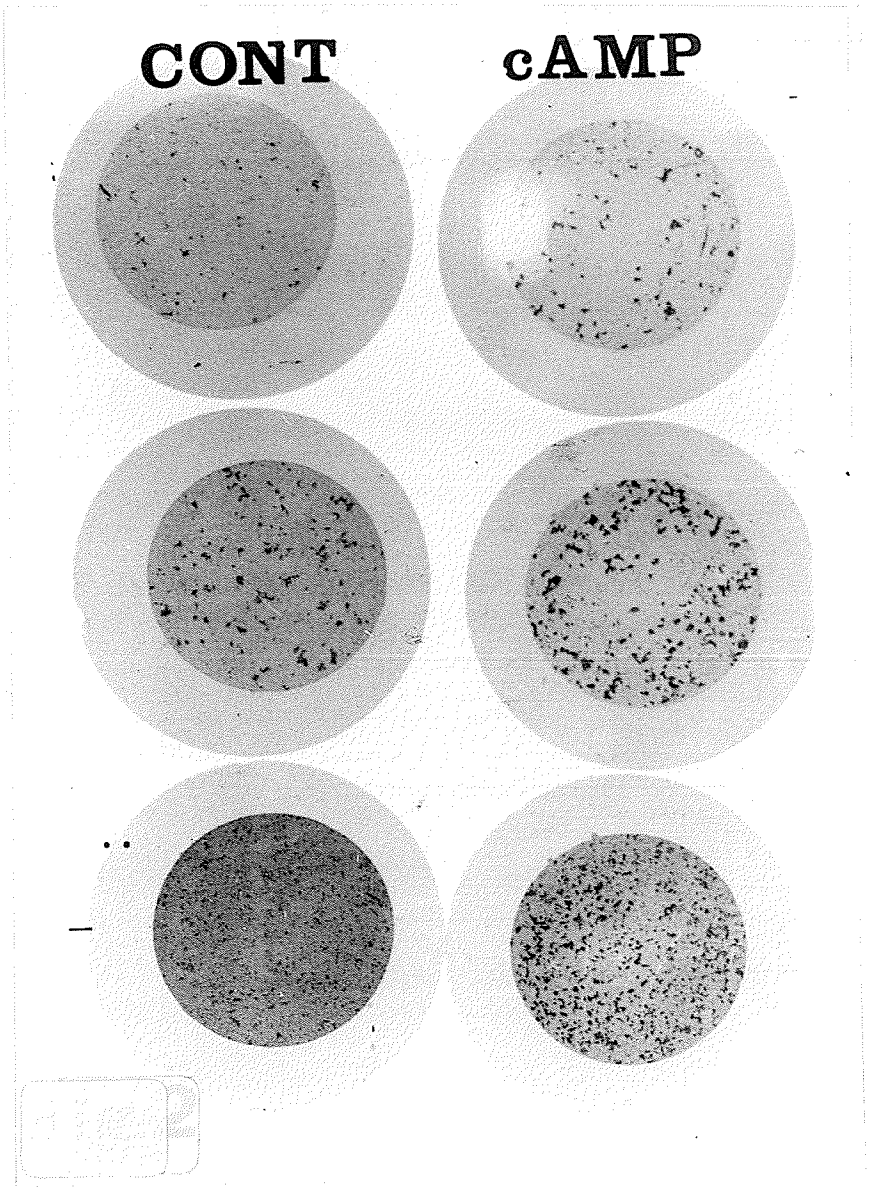

CONT
cAMP

Figure 24: Promotion of conidia aggregation by cAMP (1.8 mM) after six hours growth: comparison of qualitative and quantitative assay (left: control, right: cAMP). Qualitative: all the conidia were collected on a 0.45 μ Millipore filter, and aggregation usually scored (top). Quantitative: ^{14}C labelled conidia were first filtered through a 61 μ pore size nylon mesh (bottom), which retained the large clumps, and then through a 0.45 μ Millipore filter (middle) which collected the remaining unaggregated conidia. Radioactivity in the nylon mesh: cAMP (left), 216 cpm; control (right), 97 cpm. (5.1×10^6 conidia; n = 5).

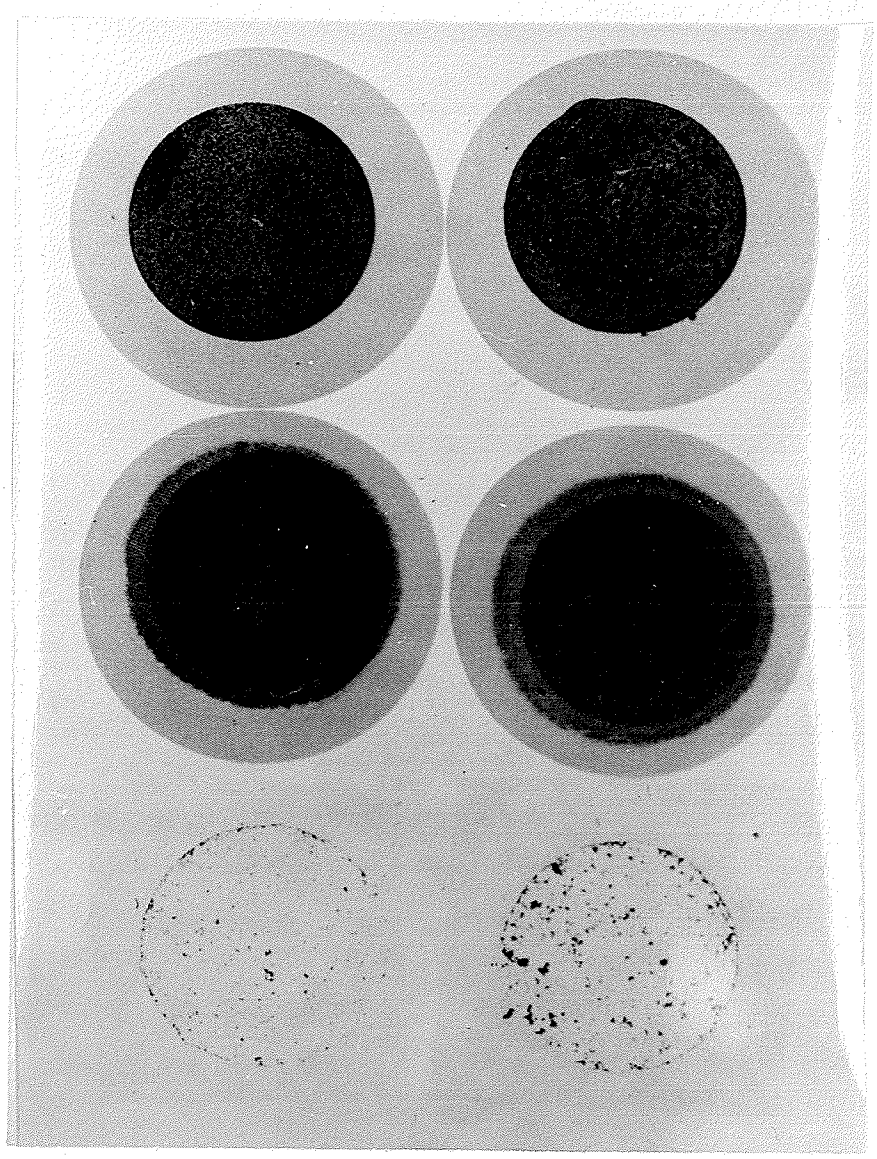
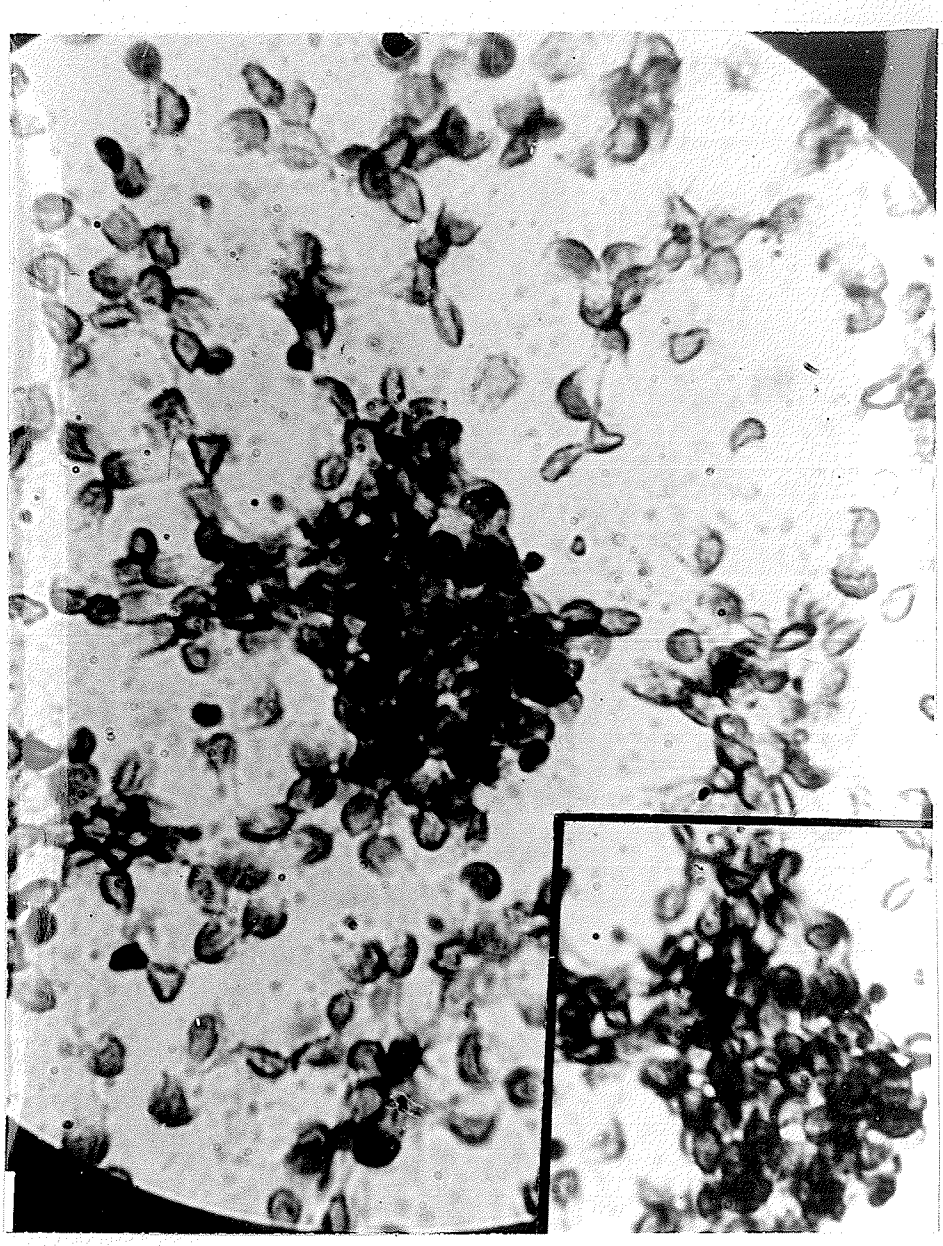


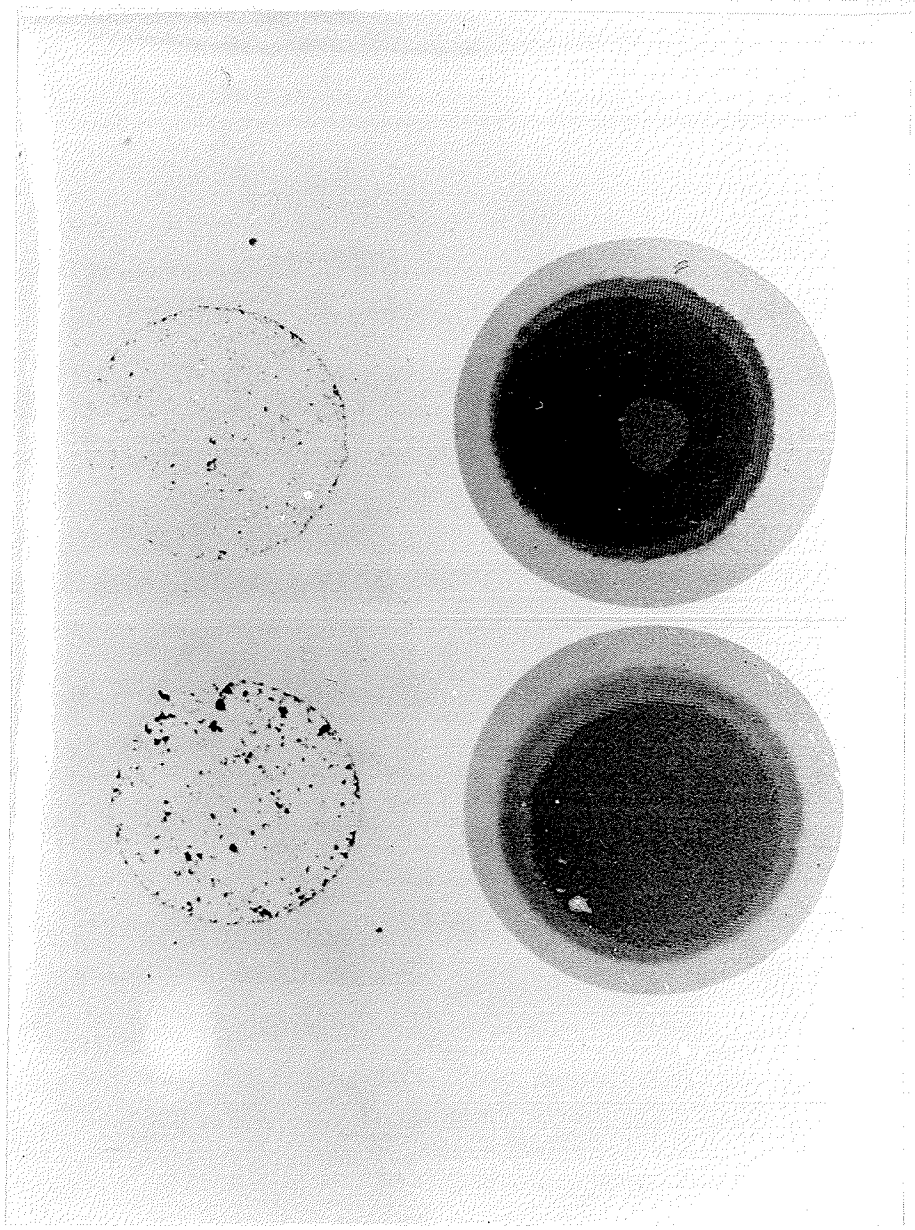
Figure 25: Micrograph illustrating that conidia aggregation inhibits germination. INSERT: the conidia clump at a slightly different focus. The conidia in the center of the clump are small, unswollen, and devoid of germination tubes. The conidia outside the clump are much larger, some show germination tubes, and many of the others have translucent sections of the conidia wall. (x 1000).



adverse effect on aggregation. ^{14}C conidia were inoculated into medium M-3 containing only 0.2 µmole sucrose instead of the regular 1.17 millimoles, and the flasks were harvested after 24 hours. Very little growth occurred, and the germlings were only a few septa long; thus aggregation was tested in the relative absence of growth. Cyclic AMP caused a decrease in the degree of aggregation of germlings (Fig. 26), whereas at Ca. 6 hours in the regular medium M-3 the nucleotide had enhanced aggregation (Fig. 24). Therefore, it is apparent that as with the growth response, the adhesion response to cyclic AMP also underwent a CTP such that adhesion was inhibited rather than augmented. Furthermore, the result in Fig. 26 indicates that this alteration could occur in the comparative absence of growth. This suggests that the conidia and germlings may secrete some substance which brings about the physiological change.

An experiment was conducted in order to test whether the cyclic AMP effect in Fig. 26 was a result of the inhibition of aggregation, or the promotion of de-aggregation. Flasks were inoculated at high conidia densities in the low sucrose medium as in Fig. 26, and after 26 hours incubation, ten flasks were made 5 mM cyclic AMP, with another ten as controls. The cultures were then harvested 19 hours later. Microscopic examination revealed germlings five to ten septa long. The control cultures contained loosely formed hyphal clumps, with the occasional unaggregated hyphae. On the other hand, three of of the cAMP cultures were totally filamentous, and another three were almost the same, with only very small and loose clumps and many individual hyphae. The other four cAMP flasks were similar to the controls. Since all the germlings had already clumped before the

Figure 26: Inhibition of conidia and germling aggregation by cAMP in medium M-3 containing 0.2 µmole sucrose. Cyclic AMP (1.8 mM) was added at zero hours, and the flasks were harvested after 24 hours. Radioactivity in the nylon mesh: cAMP (top); 146 cpm; control (bottom), 352 cpm; (5.5×10^6 ^{14}C labelled conidia; n = 6).



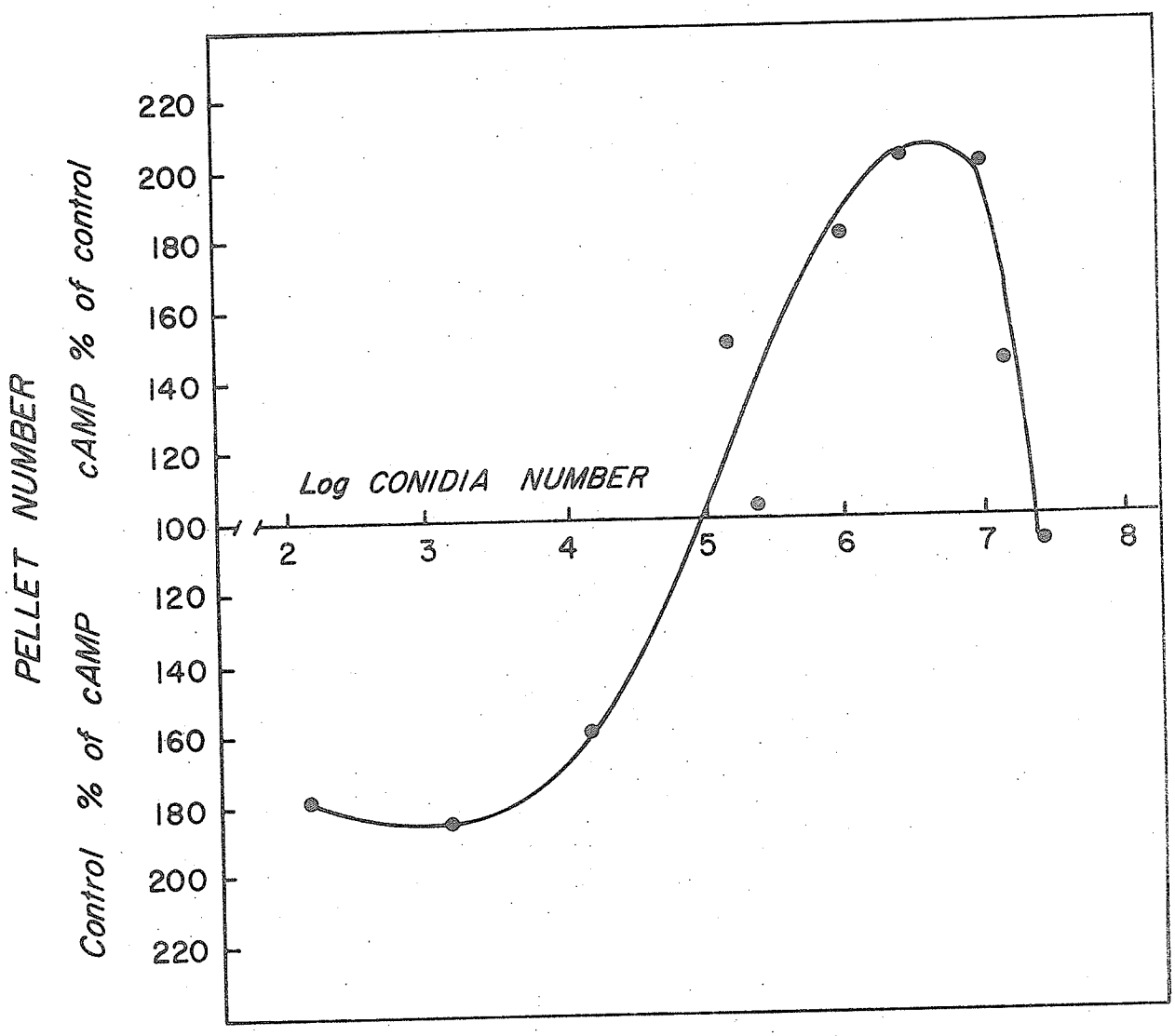
cyclic AMP was added, it was concluded that the nucleotide had promoted de-aggregation rather than inhibited further aggregation. The four cAMP-insensitive cultures may have passed the phase in which cyclic AMP was able to effect de-aggregation, for the nucleotide had no influence on the final pellet number when added to regular growth cultures after about 24 hours.

F. DOES THE CTP OF ADHESION REFLECT THE SAME PHYSIOLOGICAL EVENT AS THE CTP OF GROWTH?

At high conidia densities, cyclic AMP initially promoted growth and aggregation, but after approximately 24 hours (after the T-CTP), cyclic AMP both inhibited growth and promoted de-aggregation. This seemed to suggest a relationship between the two phenomena. That is, whereas there was a CTP with respect to growth, there was also a CTP with respect to adhesiveness, and it was of interest to investigate whether the two transition points occurred at a similar time. Conidia were inoculated at different densities into the regular medium M-3, with and without cyclic AMP, and the final number of pellets counted in order to determine the C-CTP. The T-CTP and the C-CTP are believed to be manifestations of the same physiological event (Section III). Cyclic AMP increased the number of pellets in flasks inoculated at conidia densities greater than about 10^5, and decreased the number of pellets at lower conidia densities (Fig. 27). Note that the C-CTP (24) determined for growth was of the same order of magnitude (Fig. 13, Section III).

Therefore, it seemed that if the T-CTP occurred early in the fermentation (high conidia density), cyclic AMP effected de-aggregation,

Figure 27: Influence of cyclic AMP on the number of pellets formed in flasks inoculated at different conidia densities. The cyclic AMP (1.8 mM) was added at zero hours and the flasks harvested after 70 hours (n = 4-7).

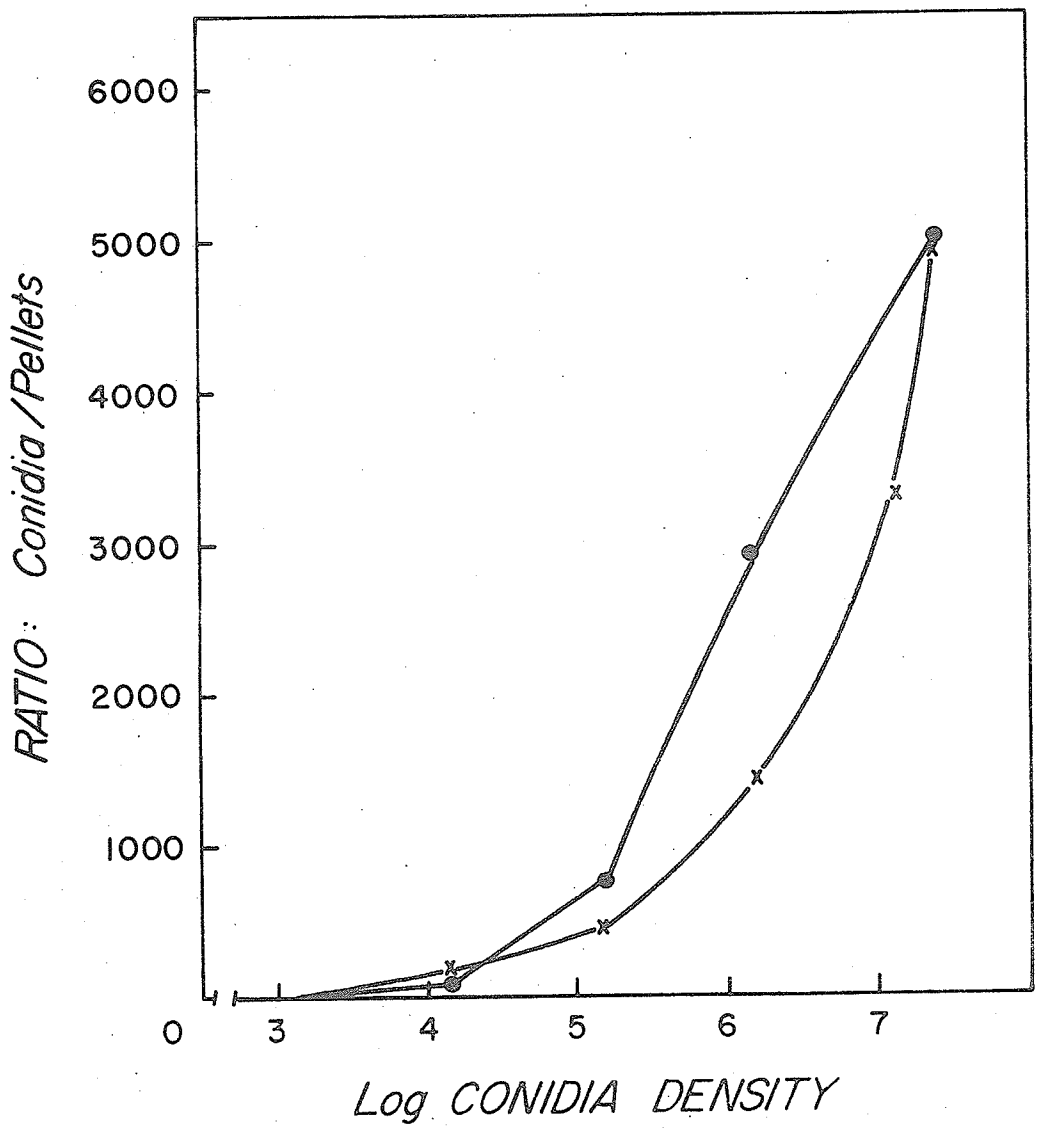

PELLET NUMBER
Control % of cAMP
cAMP % of control
220
200
180
160
140
120
100
120
140
160
180
200
220
Log CONIDIA NUMBER
2
3
4
5
6
7
8

and this was translated into an increase in the number of pellets formed. However, in normal cultures most of the pellets had been formed by 24 hours and consequently if the T-CTP occurred after the de-aggregation response had become refractive to cyclic AMP (low conidia density), only the aggregation-promotion effect was manifested, and the number of pellets formed was decreased. For example, the growth-associated T-CTP (640 conidia) was 46 hours; therefore, cyclic AMP decreased the pellet number (108 vs 200)(Fig. 12, Section III). It is therefore assumed that the CTP with respect to growth and aggregation relate to the same physiological response to zinc deficiency (Section III), and are associated with the differentiation to the citrate accumulating state.

Some circumstantial evidence which supports this view is derived from the fact that the conidia concentration is one of the most important parameters in the formation of pellets (Camici et al., 1952). In this investigation it was determined that the conidia/pellet ratio increased slowly at low conidia levels, but rose sharply once the conidia concentration of about 10^5 conidia/flask was reached (Fig. 28). It is probably significant that this was also the same conidia concentration at which the physiological responses of the mycelia to cyclic AMP changed. That is, the C-CTP (24 hours) of growth (Fig. 13, Section III) and the C-CTP of adhesion (Fig. 27) were both about 10^5 conidia. Similarly, in the experiment illustrated in Fig. 28 cyclic AMP reduced the conidia/pellet ratio at conidia densities greater than 10^5 conidia/flask, but increased it at lower conidia numbers.

It was pointed out in Section III that the CTP of the mycelia was probably controlled by the zinc ion, and in medium M-3 this was

Figure 28: Relationship between the conidia density and the number of pellets formed in the presence (✕) and absence (●) of 1.8 mM cAMP. Cyclic AMP was added at zero hours, and the flasks harvested at 70 hours. The ratio of conidia/pellets approaches one at low conidia densities. Ratio at 640 conidia (2.5 mM cAMP): cAMP, 5.9; control, 3.2.

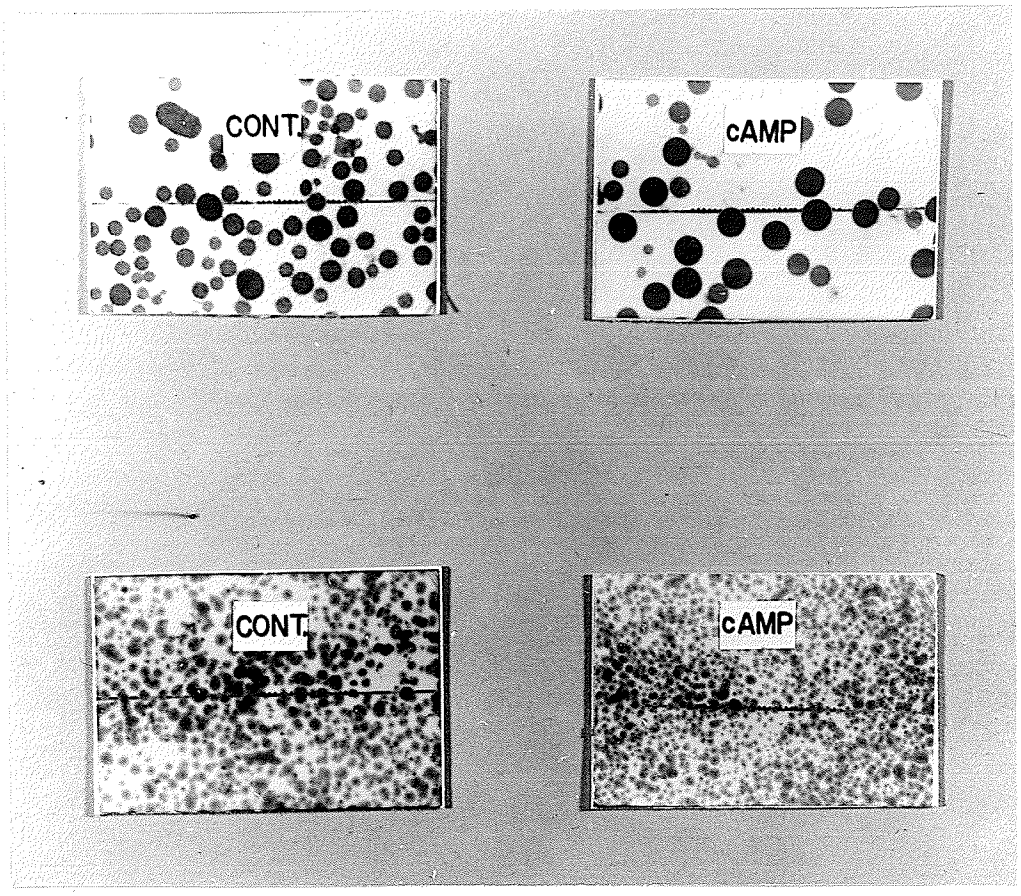

6000
5000
4000
3000
2000
1000
0
3
4
5
6
7
RATIO: Conidia / Pellets
Log CONIDIA DENSITY

related to the mass of the mycelia in the culture flask (Figs. and 13). In Section II it was postulated that zinc deficiency occasioned the synthesis of a substance which inhibits growth, and possibly has other physiological properties. Therefore, the results in Fig. 28 are explained by assuming that at a conidia concentration of about 10^5 conidia/flask, sufficient quantities of this or some other substance have accumulated in the flask to exert a pronounced effect on the physiological state of the culture. That is, the hyphae change in their response to cyclic AMP, they become less adhesive, growth-inhibited, and they differentiate into the idiophase.

At very high conidia densities, cyclic AMP did not have any influence upon the final number of pellets attained (Figs. 27 and 28). These pellets were generally not well defined and tended to be quite irregular in shape. Apparently only some of the original conidia aggregates actually grew, for many clumps of ungerminated conidia could be seen within the pellet structure. Perhaps at very high conidia densities the concentration of the putative growth inhibitor was sufficiently high so that the response of the cultures to exogenous cyclic AMP was modified a second time, but in such a way that they lost their ability to respond to the nucleotide.

The appearance of the pellets formed at high and low conidia densities, and in the presence and absence of cyclic AMP is shown in Fig. 29. At high conidia densities, only very small pellets were formed, and cyclic AMP increased the number and decreased the size. On the other hand, the opposite result was observed at low conidia numbers. The difference in pellet size caused by cyclic AMP at the two

Figure 29: Illustration of the effect of cyclic AMP on the number and size of the pellets formed at high and low conidia densities. Cyclic AMP (10^{-3}M) was added ten hours after inoculation. Top: 1.4×10^{4} conidia; harvested after 56 hours. Bottom: 1.4×10^{7} conidia; harvested after 118 hours. The distance between the marks on the scale is 1 mm.


CONT.
cAMP
CONT.
cAMP

conidia densities was probably related to both the degree of aggregation and the effect of the nucleotide on growth. Cyclic AMP inhibited growth at high conidia levels, but enhanced it for a considerable period in low conidia cultures (Section III).

Although it is not shown clearly in the photograph, cyclic AMP also caused the pellet surface to become hard and smooth. This was observed at all conidia densities. As is discussed below, this may be a reflection of the eventual inhibitory influence of the nucleotide on growth, and perhaps also an effect on the branching of the hyphae.

G. SPECIFICITY AND SENSITIVITY TO CYCLIC AMP

The ability to promote the aggregation of conidia was specific to cyclic AMP, and the following nucleotides were ineffective at 1.5 mM: adenosine, AMP, ADP (Fig. 30); cyclic GMP, guanosine, GDP, GTP (Fig. 31); and dibutyryl cyclic AMP (Fig. 32 , experiment 4). ATP (Fig. 30) and 2'3'-cyclic AMP (Fig. 32 , experiment 5) both evoked a weak response. The ATP effect may be the result of its conversion to cyclic AMP via adenyl cyclase; ATP had similarly mimicked the cyclic AMP stimulation of citrate accumulation (Section III). The response to 2',3'-cyclic AMP may be due to the structural similarity. 5'-GMP had a profound influence, larger than cyclic AMP (Fig. 31); this will be discussed below.

The aggregation of conidia was also influenced by the animal hormones glucagon and L-epinephrine, promoting and inhibiting respectively (Table 16). These hormones regulate cyclic AMP levels in cells of many mammalian tissues (Robison et al., 1968), and accordingly may be exerting a similar effect here. Glucagon is known to activate the

Figure 30: Influence of adenine nucleotides and cyclic AMP (all 1.5 mM) on conidia aggregation. (5.0×10^6 conidia).

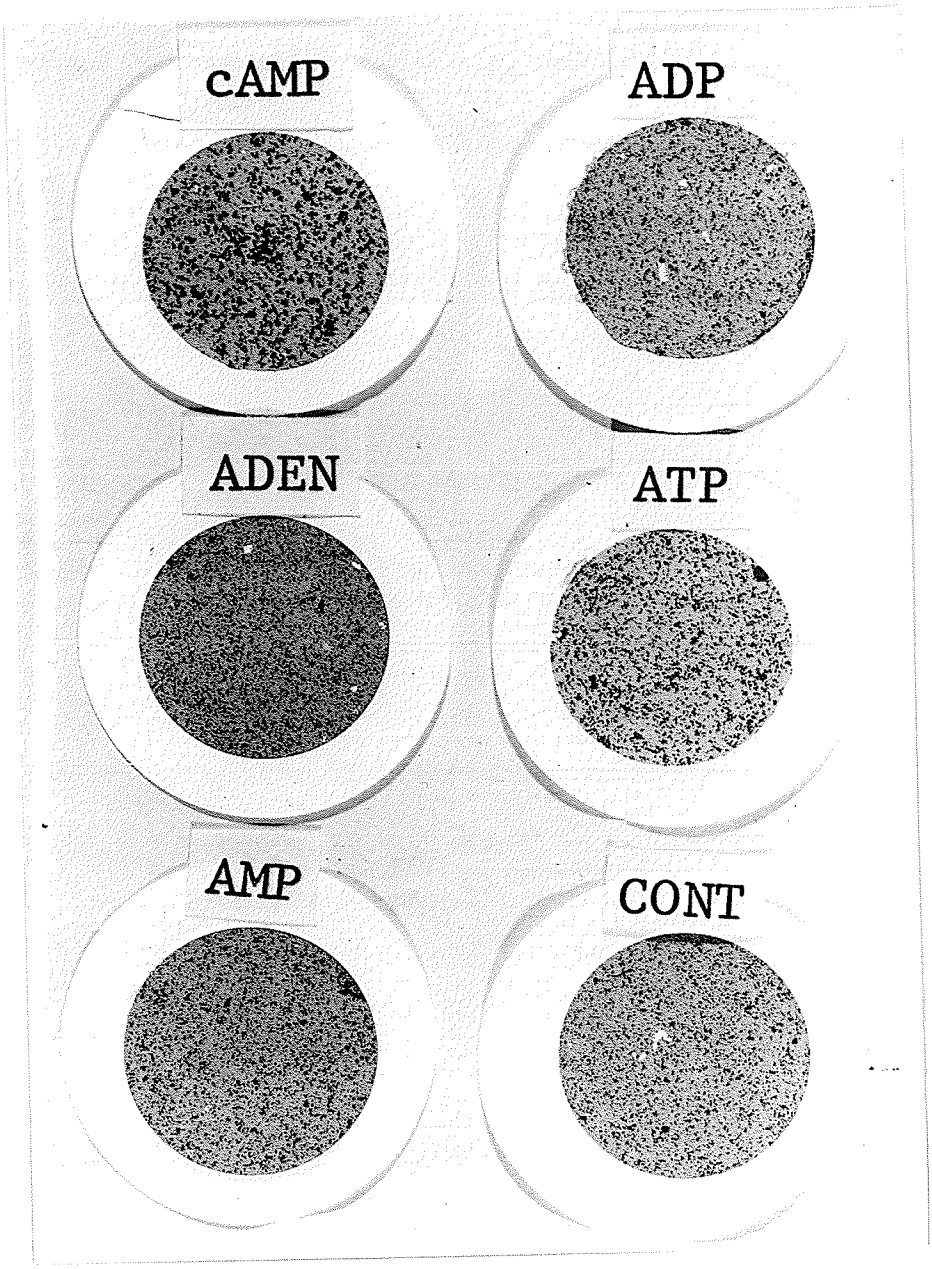

cAMP
ADP
ADEN
ATP
AMP
CONT

Figure 31: Influence of guanine nucleotides and cyclic AMP (all 1.5 mM) on conidia aggregation. GDP (not shown) was the same as the control. (4.0×10^6 conidia).

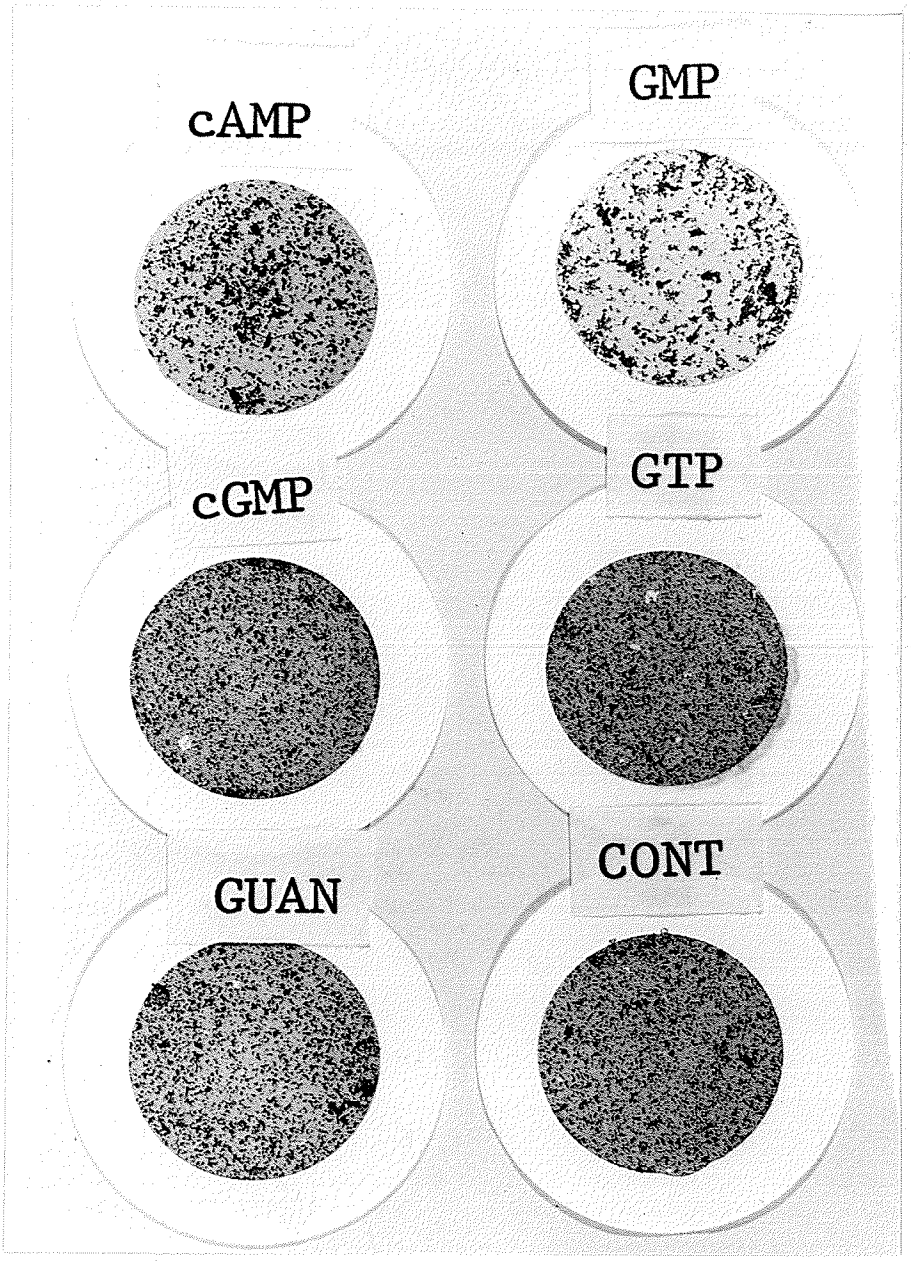

cAMP
GMP
cGMP
GTP
GUAN
CONT

Figure 32: Influence of cyclic AMP analogues on conidia aggregation. (1) 1.5 mM cyclic AMP (2) 3 mM theophylline (3) 1.5 mM cyclic AMP plus 3 mM theophylline (4) 1.5 mM N^6-2'-O-dibutyryl adenosine monophosphate (5) 1.5 mM 2', 3'-cyclic AMP (4.5×10^6 conidia).

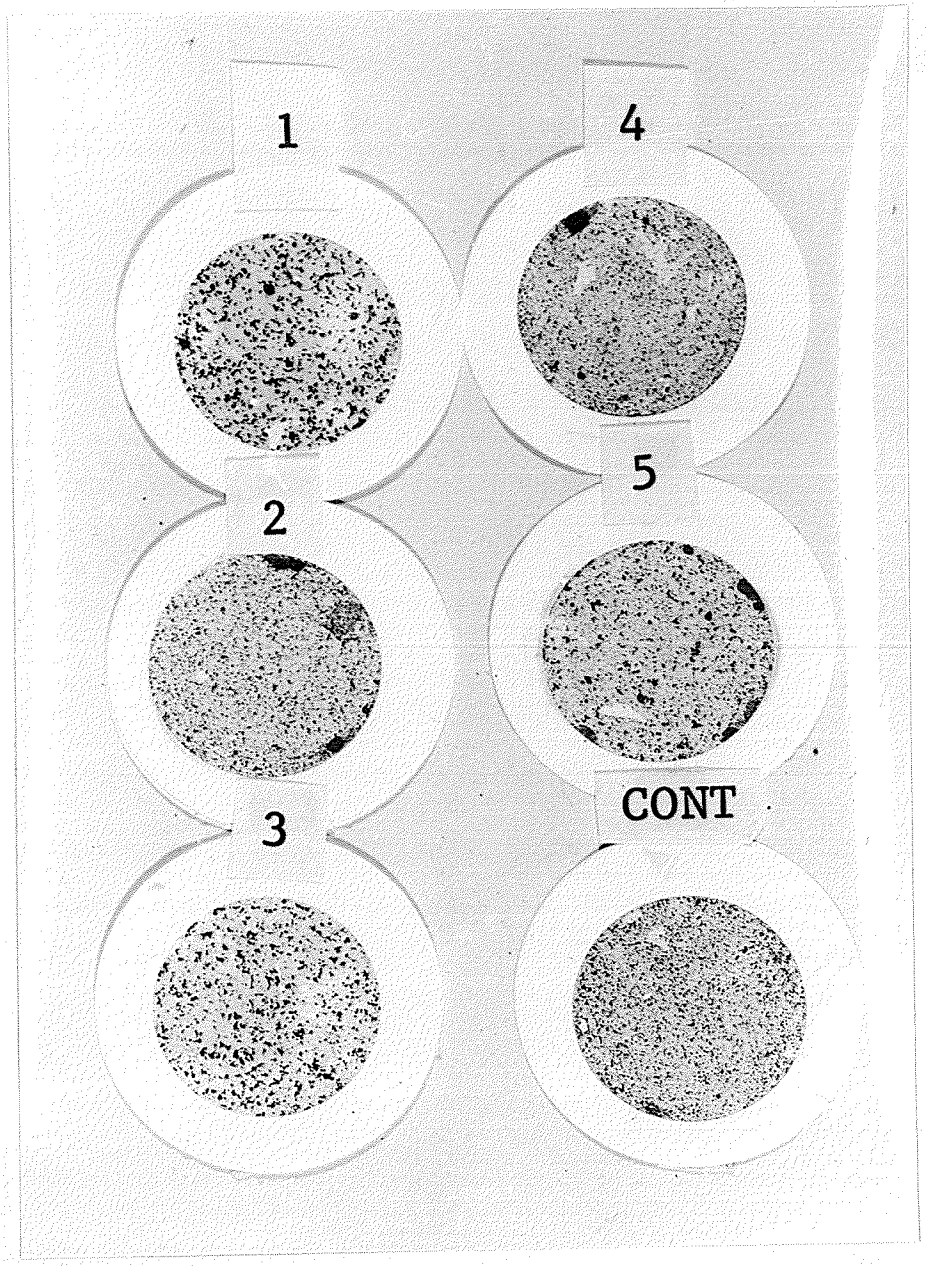

1
4
2
5
3
CONT

Table 16

Influence of glucagon and epinephrine on conidia aggregation after six hours.

	cpm	% of Control
Control	183	100
1 mg glucagon	234	128
10^{-3}M L-epinephrine	140	77

5.3×10^6 conidia; n = 5. The quantitative radioactive assay was used (see Methods).

adenyl cyclase of N. crassa, and insulin to inhibit (Flawia and Torres, 1973), a finding which supports this possibility.

The increase in pellet number observed at high conidia densities was specific for cyclic AMP and the mycelia responded to the other nucleotides generally in the same way as they had with respect to citrate accumulation (Section III). However, these results were not quantitized. ATP tended to increase the pellet number but was much less potent than cyclic AMP. In two trials, adenosine both increased (Table 11, experiment 3) and then decreased (Table 11, experiment 1) the pellet number; this response correlated with the effect of adenosine in citrate production. AMP was without influence.

ADP concentrations of the order of 10^{-5}M caused a 50% decrease in the pellet number (Table 17) although it had little effect on the initial aggregation of conidia (Fig. 30). ADP at 10^{-3}M also increased growth and inhibited citrate and YP production (Table 17 and Section III). It is interesting that cyclic AMP and ADP exert similar effects on both high conidia density cultures of A. niger and blood platelets. Cyclic AMP inhibits aggregation of blood platelets, while ADP promotes it (see Greengard and Robison, 1972; Robison et al., 1971) possibly by lowering endogenous cyclic AMP levels. ADP also increases adhesiveness of cells of D. discoidium and some animal tissues (cited in Born, 1967). As pointed out previously, ADP induced a growth form similar to that of SV (Section II), so it is possible that, along with cyclic AMP, this nucleotide may also play a role in the development of this organism.

Theophylline (1 mM) increased the number of pellets formed in high conidia density cultures either alone, or in combination with cyclic AMP (Table 18). A. niger cultures sometimes formed a "ring" of

Table 17

Effect of ADP on growth, YP formation, and pellet number.

	Dry Weight (mg)	Pellet Number	Yellow Pigment
CONTROL	33	2990	5+
10^{-3}M ADP	44	1334	2+
10^{-4}M ADP	34	1435	5+
10^{-5}M ADP	35	1534	5+

ADP was added at zero hours, and the flasks harvested after 51 hours. (4.0×10^6 conidia; n = 5).

Table 18

Effect of theophylline alone or in combination with cyclic AMP on growth, specific activity and pellet number.

	Dry Weight (mg)	Specific Activity	Pellet Number
CONTROL	98	125	$2114^{\pm}311$
10^{-3}M cAMP	96	281	$5629^{\pm}259$
10^{-3}M THEOPH.	100	130	$3087^{\pm}571$
10^{-3}M cAMP and 10^{-3}M THEOPH.	98	261	$5966^{\pm}208$

The test agents were added after 15 hours growth, and the flasks harvested after 50 hours. (1.7×10^7 conidia; n = 9).

mycelia on the side of the flask at the air-liquid interface. Conidia or germlings were deposited on the glass by the swirling motion of the liquid, and as growth proceded more cells adhered. The tendency to form this ring was probably related to the adhesiveness of the hyphae to the glass and to each other on the glass. Theophylline added to the cultures considerably reduced the formation of this ring of mycelia (Fig. 33). Growth was also inhibited, and the number of pellets formed increased. These results, along with those in Fig. 34, support the belief that cyclic AMP decreases the adhesiveness of hyphae growing at high conidia densities, because theophylline is believed to elevate cyclic AMP levels by inhibiting the nucleotide phosphodiesterase.

No attempt was made to determine the sensitivity of all the aggregation responses to various concentrations of exogenous cyclic AMP. Conidia were comparatively insensitive to the nucleotide, and a concentration of about 0.5 mM was required to promote clumping. However, the post-CTP hyphae seemed to be more sensitive. For example, cyclic AMP concentrations of the order of 10^{-5}M inhibited the formation of the ring of mycelia on the culture flask (Fig. 34).

DISCUSSION

Pellets are formed as a result of the aggregation of conidia, germlings, and hyphae, and in the liquid shake flask cultures employed here, the number and size of the pellets attained were dependent upon the amount of turbulence in the culture vessel. However, pellet formation was also a reflection of avidity of the mycelia, and this was

Figure 33: Influence of theophylline added alone at zero hours on growth and pellet formation. The flasks were harvested after 47 hours. The control values were: dry weights (●), 55 mg; pellet number (O), 1235; mycelia ring (x), 8.6 mg. (4.0×10^6 conidia; n = 7). The mycelia ring was collected and weighed.

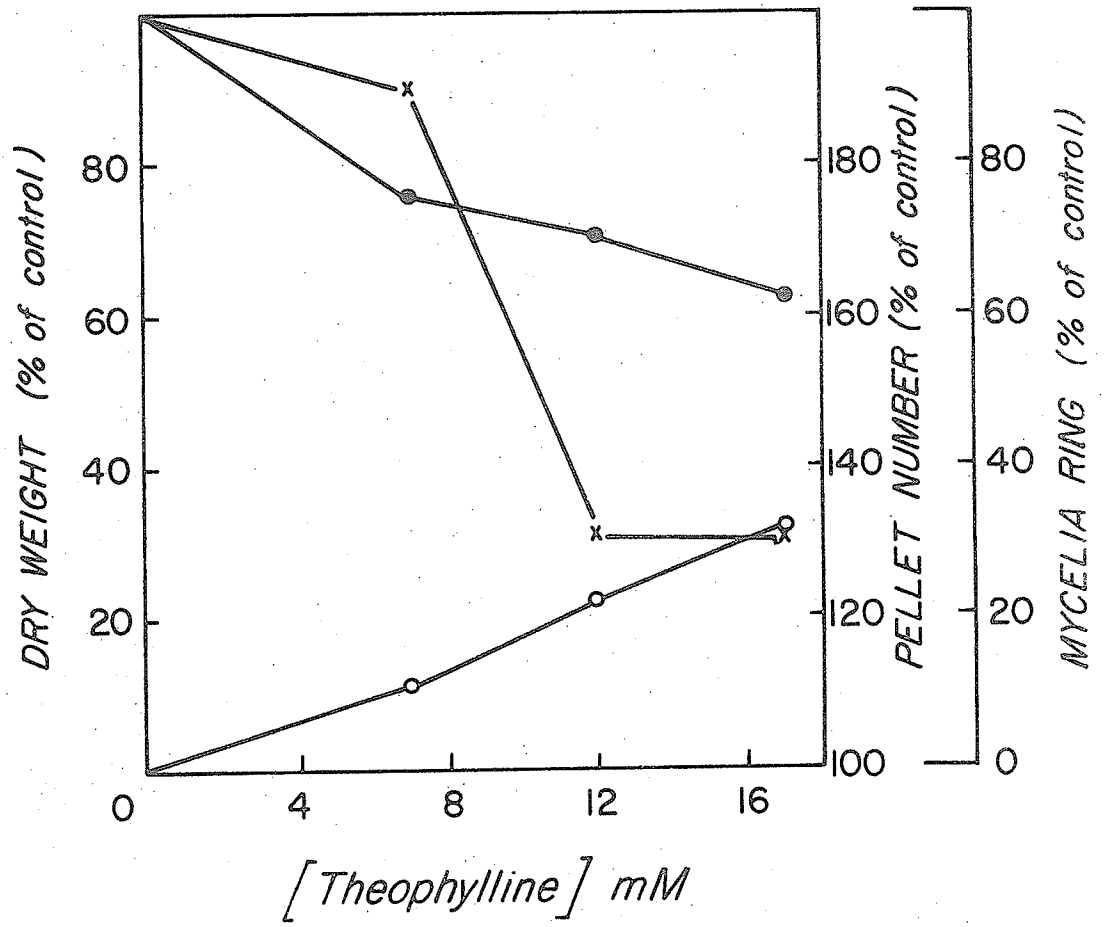
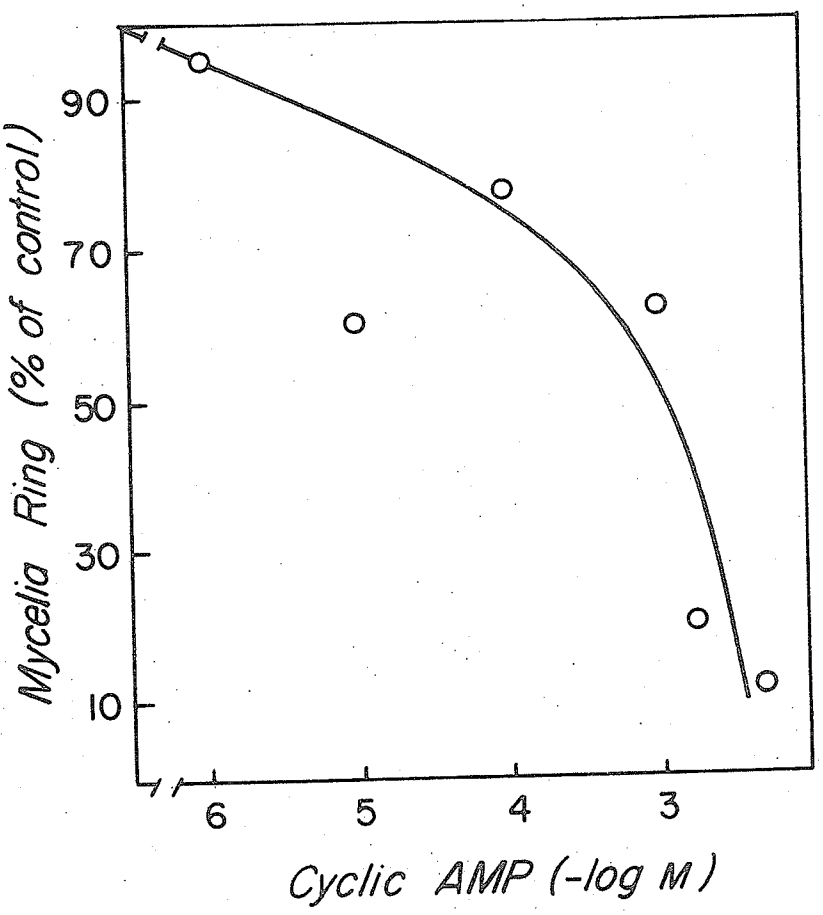

DRY WEIGHT (% of control)
PELLET NUMBER (% of control)
MYCELIA RING (% of control)
[Theophylline] mM
0
4
8
12
16
20
40
60
80
100
120
140
160
180

Figure 34: Inhibition by cyclic AMP of the formation of a "ring" of mycelia in the culture flasks at the air-liquid interface. The graph implies a dose dependent, cyclic AMP-mediated decrease in the adhesiveness of the mycelia to the culture flask. Cyclic AMP was added after 11 hours growth, and the flasks harvested after 31 hours. Each point was compiled from the sum of the ring weights of 15 flasks. The control weighed 1.4 mg per flask. (8.0×10^6 conidia).


Mycelia Ring (% of control)
90
70
50
30
10
6
5
4
3
Cyclic AMP (-log M)

related to the physiological state of the organism, which was in turn governed by the concentration of zinc in the medium and by the conidia concentration. In the trophophase the cells were highly adhesive and cyclic AMP augmented acretion, but after the transition to the idiophase (after the CTP) the avidity decreased, and cyclic AMP promoted de-aggregation. Therefore, the evidence is consistant with the idea that cyclic AMP is not only a regulator of growth and secondary metabolism (Section III), but also of cellular avidity. As was the case with growth and secondary metabolism, cyclic AMP did not appear to influence the trophophase-idiophase "decision". This is in accord with the view that cyclic AMP operates in A. niger as a second messenger, and that the primary signal is the zinc ion (Section III).

Very little is understood concerning either the causes or the effects of pellet formation, so it is of interest to speculate on the biological ramifications of these results. It was pointed out earlier that 5'-GMP had a profound influence on conidia clumping, larger than cyclic AMP (Fig. 31). Interesting in this respect is the recent report of the isolation of N,N-dimethyl-guanosine from D. discoidium, a structural analogue of GMP, and a putative germination inhibitor (Bacon and Sussman, 1973). Autoinhibition of spores and conidia, especially under crowded conditions, has been reported previously in fungi and Actinomycetes (Cochrane, 1958).

These observations suggest a possible physiological role for conidia aggregation: that cyclic AMP and GMP (or a metabolite) may be acting alone or synergistically to effect a population control mechanism; the number of conidia which germinate is regulated by clumping. Since

the vegetative pellets arise from the conidia aggregates, it follows that any agent which promotes clumping would reduce the number of pellets formed. This would ensure that under crowded conditions, such as within a fructification, there would be an adequate nutrient supply to maintain a viable population with full reproductive capacity. Unfavorable nutrient conditions are believed to favor the pellet type of growth, rather than the more luxurious filamentous type (Foster, 1949). Further evidence for this view is that the conidia/pellet ratio rises sharply once a certain conidia concentration is reached (Fig. 28), and that the conidia in the center of the clumps do not appear to germinate (Fig. 25).

In addition to the possible regulation of germination, cyclic AMP enhanced the acretion of the trophophase mycelia. Thus the nucleotide may contribute to the rapid and orderly development of the fungal thallus when the organism is in the trophophase. However, following the CTP cyclic AMP inhibited growth, and promoted de-aggregation and eventually acidogenesis. The significance of this finding is not clear, but since the response was specific to cyclic AMP, it is likely that it is of some consequence to development of the organism.

It has been repeatedly observed that citric acid accumulation by submerged cultures is invariably correlated with a particular pellet morphology (Snell and Schweiger, 1949; reviewed by Perlman and Sih, 1960). Growth is reduced, and the pellets are always very numerous, small, smooth, and hard. Acidogenic agents and treatments which produce this morphology include ferrocyanide (Martin and Waters, 1952; Clark, 1962; Section VI), EDTA (Choudhary and Pirt, 1965; Section VI), removal

of trace metals from the medium (Sanchez-Marroquin, 1970), methanol (Section VI), certain lipids (Millis et al., 1963), con A (Section II), and mutations (Gardiner et al., 1956; Trumpy and Millis, 1963).

On the other hand, agents and treatments which increase the size of the pellet often reduce citrate production. These include a low degree of turbulence in the medium (Table 15), ADP (Table 17), low conidia number (Section II), boiled snake venom, and pellet handling (Section II). In addition, citric acid is never produced in high yields by cultures which grow in the filamentous form (Steel et al., 1955; Millis et al., 1963; Clark et al., 1966; Choudhary and Pirt, 1966) which is probably a mode associated with growth.

Is is therefore apparent that citrate accumulation is usually associated with a specific type of growth pattern, characterized by reduced growth, and an increased number of small, smooth pellets. The observation that cyclic AMP also caused this morphology raises the possibility that the growth inhibition and de-aggregation responses may be morphogenesis associated with the citrate accumulating idiophase. The smooth and hard surface of the pellet caused by cyclic AMP and the other agents may be a manifestation of growth inhibition and concomitant increases in sub-apical branching. Large pellets are probably poorly acidogenic because of the reduced surface/volume ratio and consequent decreased mass transfer efficiency (Aiba et al., 1965; Blakebrough, 1967). However, it is also possible that the acidogenic ability of the mycelia is subject to cell-cell interactions in the pellet.

As indicated above, removal of trace metals from the medium influenced the pellet formation, as did addition of EDTA and ferrocyanide.

This indicates that metal ions are involved in the mechanism of adhesion. Filamentous growth of A. niger growing in high sucrose media was favored by iron (Snell and Schweiger, 1949; Choudhary and Pirt, 1965) and manganese (Clark et al., 1966). These ions did not exert a large effect in the present investigation, which was carried out at low sucrose levels. Zinc reduced the pellet number somewhat, but it was difficult to study this because there was sufficient zinc present in medium M-3 to allow a large degree of aggregation to occur in the absence of added zinc. Nevertheless, zinc (but not iron, manganese, or calcium) reversed the EDTA-induced clumping of conidia, which suggests that zinc is in fact important in the aggregation process (Section VI).

Calcium is known to be associated with the avidity of other types of eucaryotic cells (Curtis, 1967), including D. discoidium (Mason et al., 1967) and yeasts (Morris, 1966). Cyclic AMP is believed to be linked to calcium metabolism in many systems (Rasmussen, 1970; Whitfield, 1973), and also has been shown in some instances to affect cellular adhesion (see introduction this section). This analogous situation provides further circumstantial evidence that the metabolism of zinc and cyclic AMP may be connected in this organism and that the effect of cyclic AMP on adhesion may be related to the "mobilization of zinc" (Section VII).

One further observation on the mechanism of pellet formation is that it seems to be related to the glucose concentration. In the present studies, high levels of sucrose (5%) caused either completely filamentous growth, or at least very loose and filamentous pellets (not shown). However, filamentous growth was never observed in low sucrose

media (0.8%). High sucrose also inhibits flocculation in yeasts (Morris, 1966), which suggests that the aggregation process in fungi may be governed by catabolite repression. Cyclic AMP is known to be involved in catabolite repression in some bacteria (Pastan and Perlman, 1970), and may play a similar role in yeasts (Sy and Richter, 1972a, b; Tsuboi et al., 1972). This observation may have some bearing on the results presented here pertaining to the mechanism of action of cyclic AMP in pellet acretion.

SECTION V

DEMONSTRATION OF ADENYL CYCLASE AND CYCLIC AMP BINDING PROTEIN AND SOME PRELIMINARY STUDIES ON TWO PHOSPHODIESTERASES, INTRACELLULAR AND EXTRACELLULAR

Adenyl cyclase was first described by Sutherland et al. (1962) and cyclic AMP specific phosphodiesterase by Butcher and Sutherland (1962). Since that time, these enzymes have been extensively studied in many mammalian tissues, but to a much lesser degree in plants and lower organisms. In the case of fungi, adenyl cyclase has been demonstrated in N. crassa (Flawia and Torres, 1972), Saccharomyces fragilis (Sy and Richter, 1972b), and C. macrorhizus (Uno and Ishikawa, 1973b). Phosphodiesterase has been shown in Saccharomyces carlsbergensis (Spezialli and Van Wijk, 1971), C. macrorhizus (Uno and Ishikawa, 1973b), and N. crassa (Scott and Solomon, 1973). This section proves the presence in A. niger of adenyl cyclase, a protein(s) capable of binding cyclic AMP, and two apparently distinct phosphodiesterases, one intracellular and the other extracellular.

The enzymes were routinely prepared from 48 hour lyophilized mycelia grown in medium M-2 (0.8% sucrose) as described in Part VI of the Methods. Fresh mycelia gave similar results. Adenyl cyclase and the cyclic AMP binding protein were extracted with the following buffer: 0.1 M Tris-Cl, pH 8.0; 10^{-4}M $MgCl_2$; 10^{-4}M DTT; and 20% glycerol at 4°C. The intracellular phosphodiesterase was extracted with 0.1 M Tris-Cl, pH 8.0, containing 10^{-4}M DTT, at 4°C (TD buffer). The term "crude extract" refers to the semi-particulate supernatant obtained when the homogenized mycelia were centrifuged at 48,000 x g for 30 minutes. All

the enzymes studied were completely inactivated by heating at 95°C for 30 seconds. In all experiments heat denatured enzymes were used as control blanks, and the values subtracted from the test results.

A. Adenyl Cyclase

Extensive investigations were carried out in order to purify adenyl cyclase from crude extracts of this organism. Although some degree of success was achieved, these results were inconclusive mainly because of the presence of other enzymes which interfered with the reaction under study. The following experiment is presented which nevertheless clearly demonstrates the presence of an activity capable of forming cAMP from ATP, and also provides a preliminary examination of its distribution in various cell fractions obtained by differential centrifugation.

Lyophilized cells (1.0 g) were suspended in 13 ml of the extraction buffer (EB) and homogenized on ice with a Bellco ground glass tissue homogenizer (Methods, Part VI). All the following steps were carried out at 1 to 4°C. The crushed cell suspension was centrifuged at 2,500 x g for ten minutes, and the supernatant decanted. The pellet was washed in 10 ml of the EB, centrifuged again at 2,500 x g, and the two supernatants were combined. The pellet was resuspended in 8 ml of EB and called the "800 x g pellet"; this was presumed to represent a fraction containing membranes and cell walls. The 800 x g supernatant was further centrifuged at 17,000 x g for ten minutes, and the supernatant decanted. The small pellet was washed with 5 ml of EB, centrifuged again for ten minutes at 17,000 x g, and the supernatants combined. The pellet was resuspended in 3 ml of EB, and called the "17,000 x g pellet";

this was presumed to represent the mitochondria fraction. The 17,000 x g supernatant was still further centrifuged for one hour at 200,000 x g in a Beckman Ultracentrifuge, the supernatant decanted, and the pellet resuspended in 3 ml of EB.

The various fractions obtained were assayed for adenyl cyclase activity as described in Part XIII C 2 of the Methods, and the results are shown in Table 19. Most of the activity appeared to be located in the 200,000 x g supernatant, with a lesser amount in the 800 x g pellet, representing the crude membrane fraction. Thus the enzyme appears to be soluble in this organism, or perhaps more likely easily solubilized. The adenyl cyclase is considered to be membrane-bound in all mammalian tissues (Robison et al., 1971a), N. crassa (Flawia and Torres, 1972), and S. fragilis (Sy and Richter, 1972b). The enzyme from C. macrorhizus was studied in the "soluble fraction", as was that of Brevibacterium liquificans (Hirata and Hayaiski, 1967; Ide et al., 1967). In E. coli adenyl cyclase has been reported to be particulate (Ide, 1969), but easily solubilized (Tao and Lipmann, 1969; Tao and Huberman, 1970).

B. Cyclic AMP Binding Protein

An activity capable of complexing cyclic AMP and binding to cellulose acetate membrane filters was discovered in crude extracts. This technique has been utilized to study similar proteins in other organisms (Gilman, 1970; Walton and Garron, 1970; Sy and Richter, 1972a). The activity remained in the supernatant after protamine sulfate treatment (see Part C of this section) but was completely precipitated by 60% ammonium sulfate. Chromatography of a dialysed 50% ammonium sulfate

Table 19

Demonstration and distribution of adenyl cyclase in A. niger cell fractions.

	Volume of Cell Fraction (ml)	Prior to TLC cpm x 10^3	cAMP Spot cpm x 10^3	Adenosine Spot cpm x 10^3
800 x g Pellet	12	278	310	6
17,000 x g Pellet	5	69	41	2
200,000 x g Pellet	4	50	8	1
200,000 x g Supernatant	25	1804	1788	1

The data calculated to represent the total activity in the full volume of the cell fraction. See text for further details.

precipitate on Schleicher and Schuell DEAE-cellulose revealed two peaks of activity (Fig. 35).

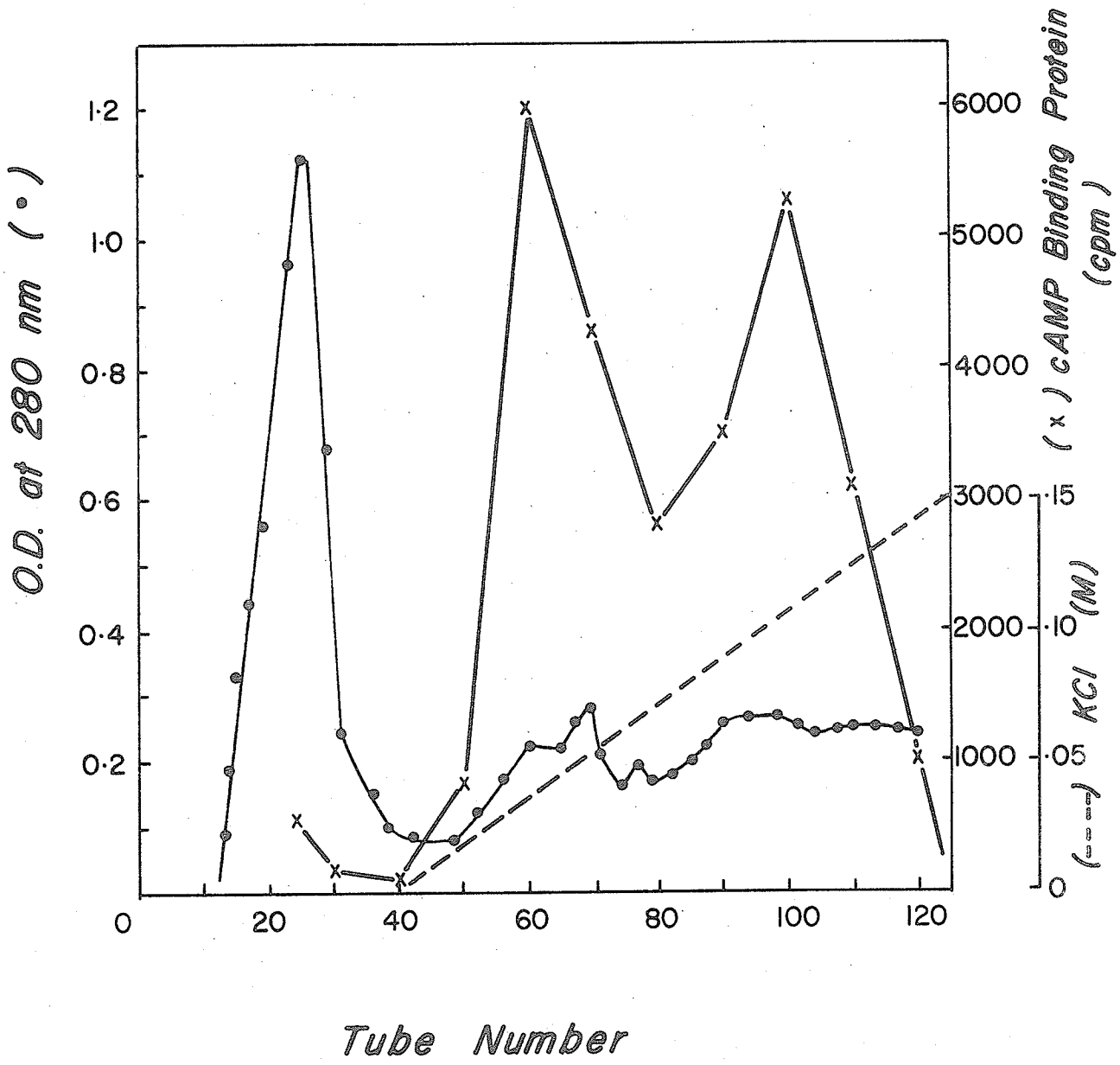
It is not known whether this cyclic AMP binding activity represents a cyclic AMP dependent protein kinase, such as has been shown in N. crassa (Tellez-Inon and Torres, 1970) and many mammalian tissues (see Greengard and Robison, 1972). Other proteins which are not protein kinases are known to bind cyclic AMP. One is the CAP protein found in E. coli, which is required for the transcription of β-galactosidase (Pastan and Perlman, 1970). A cyclic AMP binding protein which is not a protein kinase has been found in yeast, but its function is not known (Sy and Richter, 1972a).

C. Intracellular Cyclic AMP Phosphodiesterase

Crude extracts contained a phosphodiesterase which hydrolysed cyclic AMP to AMP. Since there was also a strong phosphomonoesterase present, snake venom was added after the termination of the reaction to convert all the AMP into adenosine. The rate of formation of adenosine from cyclic AMP, as determined by either the Dowex or paper chromatography techniques (Methods), then yielded the reaction rate.

The intracellular phosphodiesterase (I-PDE) was partially purified according to the following procedure. Unless otherwise indicated all the steps were carried out at 4°C. Lyophilized mycelia were homogenized in 13 volumes (v/w) of TD buffer, and a crude extract prepared. A protamine sulfate suspension was added to the crude extract in the ratio of 3.5 mg protamine sulfate to 1 ml of crude extract. The slurry was stirred for 30 minutes, and then centrifuged at 48,000 x g for 15 minutes. Solid enzyme grade ammonium sulfate was slowly added to the stirring

Figure 35: Demonstration of two peaks of cAMP-binding protein activity by DEAE-cellulose chromatography. The column was equilibrated in TD buffer, and eluted with a linear KCl gradient, 800 ml TD buffer to 800 ml 0.15 M KCl in TD buffer.


O.D. at 280 nm (•)
(x) cAMP Binding Protein (cpm)
(---) KCl (M)
Tube Number
1·2
1·0
0·8
0·6
0·4
0·2
0
20
40
60
80
100
120
6000
5000
4000
3000
2000
1000
·15
·10
·05
0

protamine sulfate supernatant until 50% saturation was reached. Stirring was continued for 30 minutes, and then the suspension centrifuged at 10,000 x g for 20 minutes. The pellet, which contained the majority of the enzyme activity, was redissolved in one-third the original volume of 40 mM TD buffer, and dialysed in a Zeinth dialyser against the same buffer. Ethanol (95%, -20°C) was slowly added to the stirring enzyme solution until the concentration was 35% and then the stirring continued at -20°C for 30 minutes. The suspension was centrifuged at 20,000 x g for 15 minutes, and the supernatant discarded. The pellet, which contained the enzyme activity, was suspended in 50 ml of 50 mM TD buffer containing 0.14 M KCl, and stirred at 2°C for two days. The insoluble portion was removed by centrifugation, and the soluble fraction saved for further purification. A 2.5 x 45 cm glass column was packed with Schleicher and Schuell DEAE-cellulose and equilibrated in 50 mM TD buffer containing 0.14 M KCl. The soluble portion from the ethanol step was applied and the column was washed with 300 ml of equilibrating buffer. The enzyme was then eluted with a linear gradient of KCl, 500 ml of 0.14 M KCl to 500 ml of 0.31 M KCl, all in 50 mM TD buffer. The peak of enzyme activity released by 0.2 M KCl was collected, dialysed for 18 hours against two changes of one liter 10 mM TD buffer, concentrated ca.25 times by Aquacide, and stored at 2°C. Under these conditions the enzyme was stable for several months.

The concentrated enzyme obtained from the DEAE column was utilized in all the studies to be reported here. The specific activity of this fraction was approximately 30 fold higher than that of crude

extracts. Disc electrophoresis of the fraction in polyacrylamide gels revealed about eight protein bands. As there was no phosphomonoesterase activity present, the radioactivity in the 5'-AMP spot on the paper chromatogram yielded the reaction rate, and this assay was used to obtain the data. In all studies the rate of reaction was proportional to the enzyme concentration (Fig. 36), and the time of reaction (Fig. 37). The protein concentration of the enzyme sample was 1.7 mg/ml, and 10 µl (17 µg) of enzyme were in the kinetic experiments.

The optimum hydrogen ion concentration of the enzyme was determined to be pH 7.5 (Fig. 38). The enzyme was most active in bicine buffer so this was used for additional studies.

A preliminary investigation of the metal requirement yielded ambiguous results. Assay of the enzyme in the presence of 10^{-3}M EDTA caused a 60% inhibition of activity which suggested the influence of a metal. In an attempt to remove the metal, 0.5 ml of the enzyme preparation was dialysed at 4°C for five days against 500 ml of 5 mM EDTA in 10 mM bicine buffer, pH 7.5. The enzyme was then further dialysed for 24 hours against two liters of 10 mM bicine buffer, pH 7.5, to remove the EDTA. This procedure caused only a 25% decrease in activity, suggesting that the putative metal was tightly bound.

The enzyme did not appear to require magnesium for its activity, although concentrations higher than 10^{-3}M caused higher reaction rates (Figs. 39, 40). The effect of magnesium seemed to be to increase the Vmax rather than to change the affinity of the enzyme for cyclic AMP (Fig. 41). Preliminary studies indicated that this activation may be a common property of divalent metals, including zinc

Figure 36: Cyclic AMP hydrolysis as a function of the I-PDE concentration. The reaction was carried out for 20 minutes in the presence of 2.5 mM cAMP and 10 mM $MgCl_2$. Protein concentration is 1.7 mg/ml.

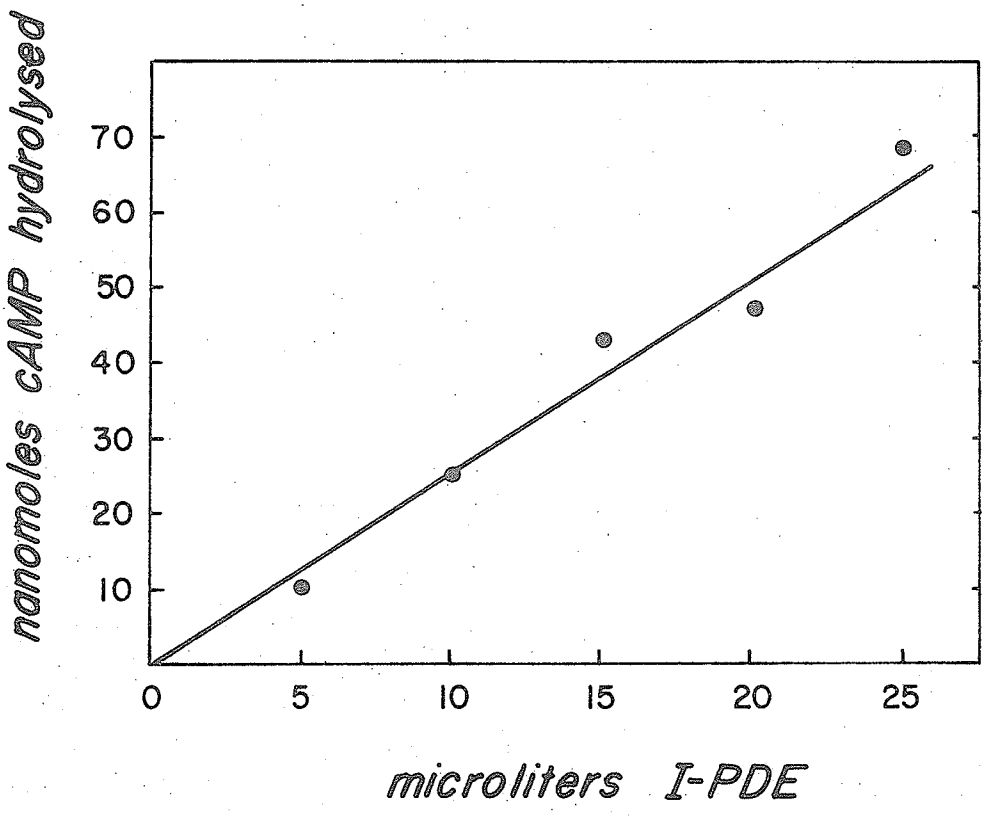

nanomoles cAMP hydrolysed
70
60
50
40
30
20
10
0
5
10
15
20
25
microliters I-PDE

Figure 37: Cyclic AMP hydrolysis by I-PDE as a function of the time of reaction. The reaction was carried out in the presence of 7.5 mM $MgCl_2$, using 10 µl (17 µg) of enzyme protein.

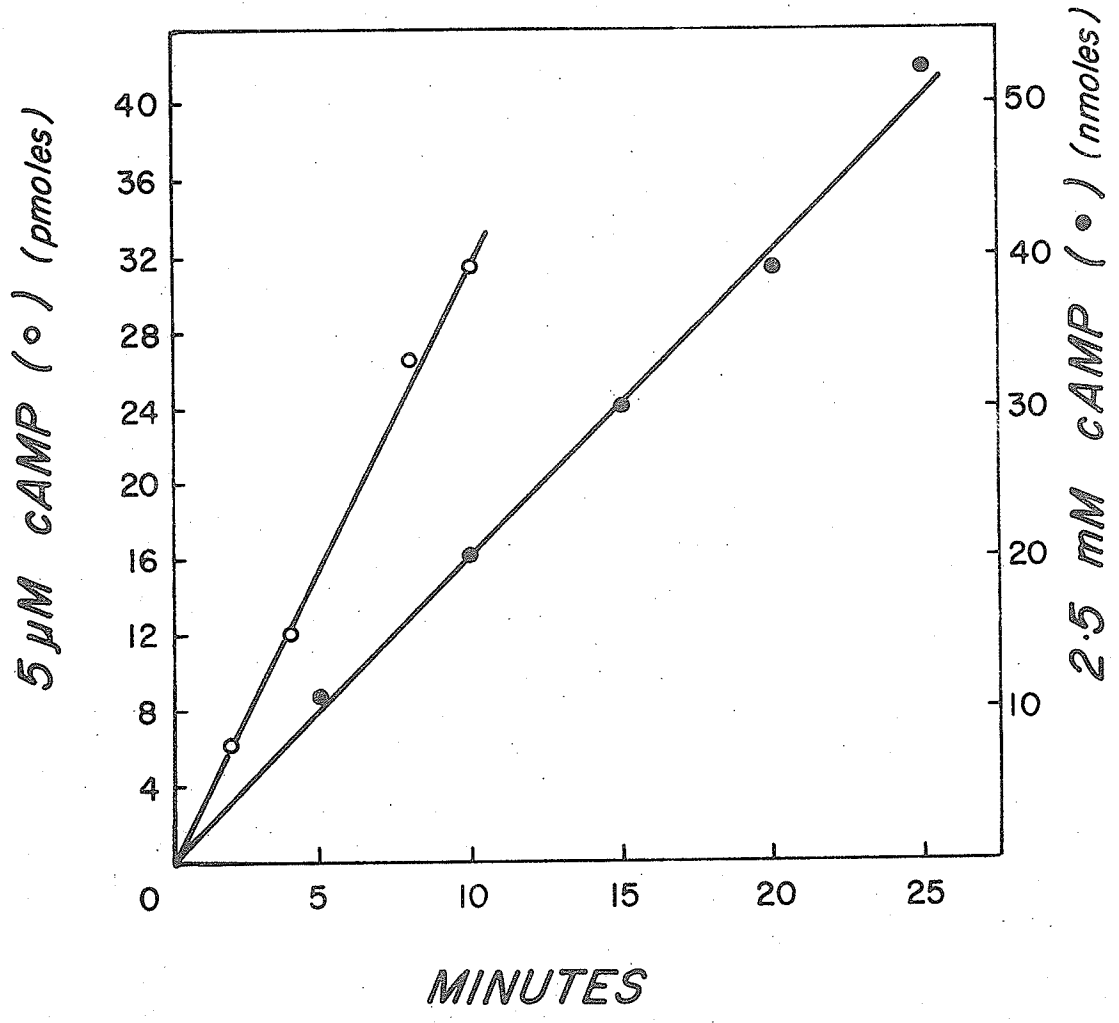

5 μM cAMP (○) (pmoles)
2.5 mM cAMP (●) (nmoles)
MINUTES
0
4
8
12
16
20
24
28
32
36
40
10
20
30
40
50
5
10
15
20
25

Figure 38: Influence of pH on the activity of I-PDE. The assay conditions were 2.5 mM cAMP, 7.5 mM $MgCl_2$, and 34 µg enzyme protein. Buffers used (all 65 mM): Tris-Cl (●), Tris-Acetate (○), Hepes-KOH (x), Bicine-KOH (△), Mops-KOH (⊙), Tes-KOH (□), Mes-KOH (+), Pices-KOH (◍).

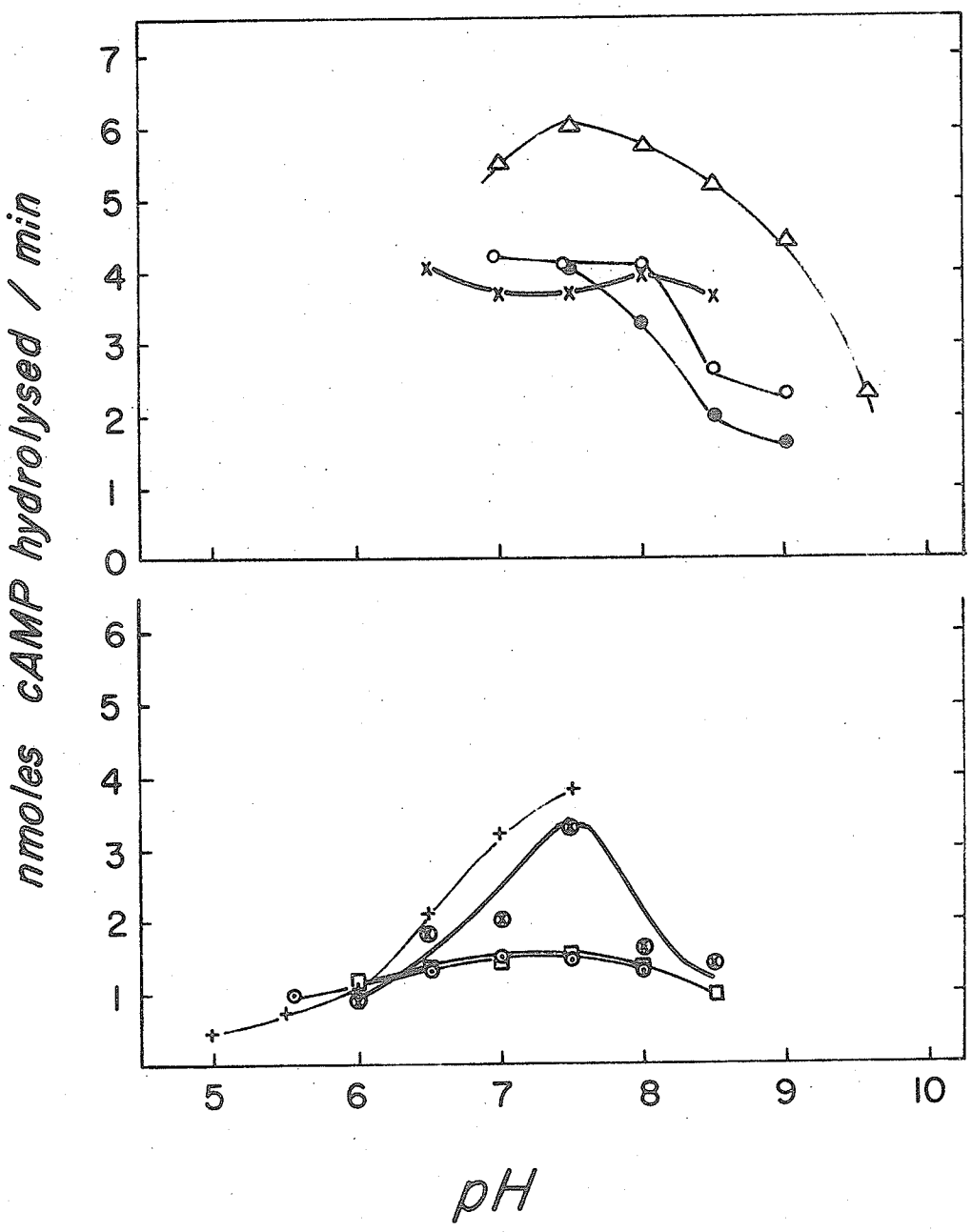

nmoles cAMP hydrolysed / min
pH
0
1
2
3
4
5
6
7
8
9
10

Figure 39: Double reciprocal plot of the velocity of I-PDE versus the $MgCl_2$ concentration at various (high) levels of cyclic AMP. Concentration of cAMP: 10 mM (●), 5 mM (O), 2.5 mM (X), 1.0 mM (△), 0.5 mM (□).

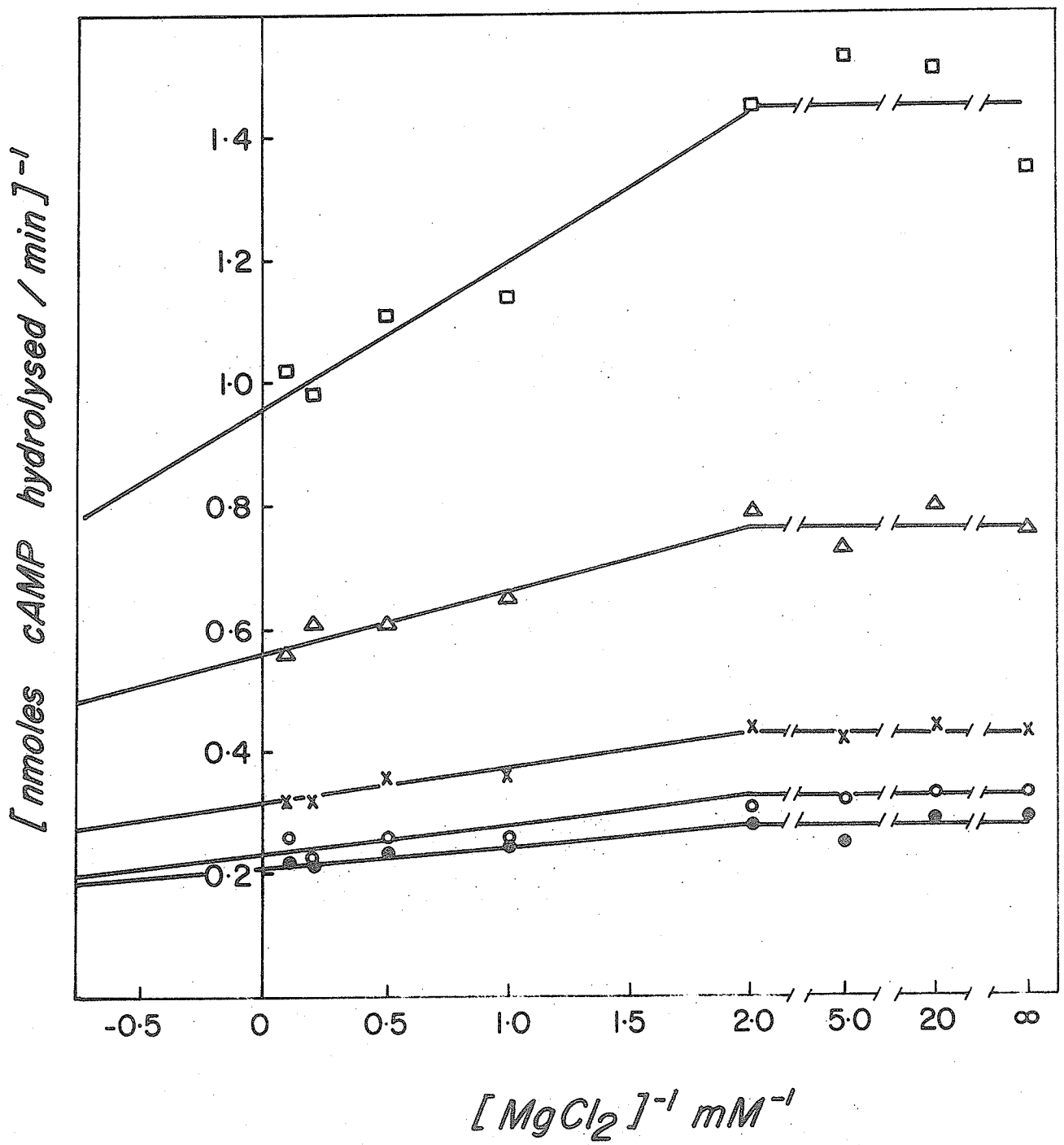

[nmoles cAMP hydrolysed / min]⁻¹
1·4
1·2
1·0
0·8
0·6
0·4
0·2
-0·5
0
0·5
1·0
1·5
2·0
5·0
20
∞
[MgCl2]⁻¹ mM⁻¹

Figure 40: Double reciprocal plot of the velocity of I-PDE versus the $MgCl_2$ concentration at various (low) levels of cyclic AMP. Concentration of cAMP: 0.25 mM (●), 0.10 mM (○), 0.05 mM (x), 0.025 mM (△), 0.01 mM (□).

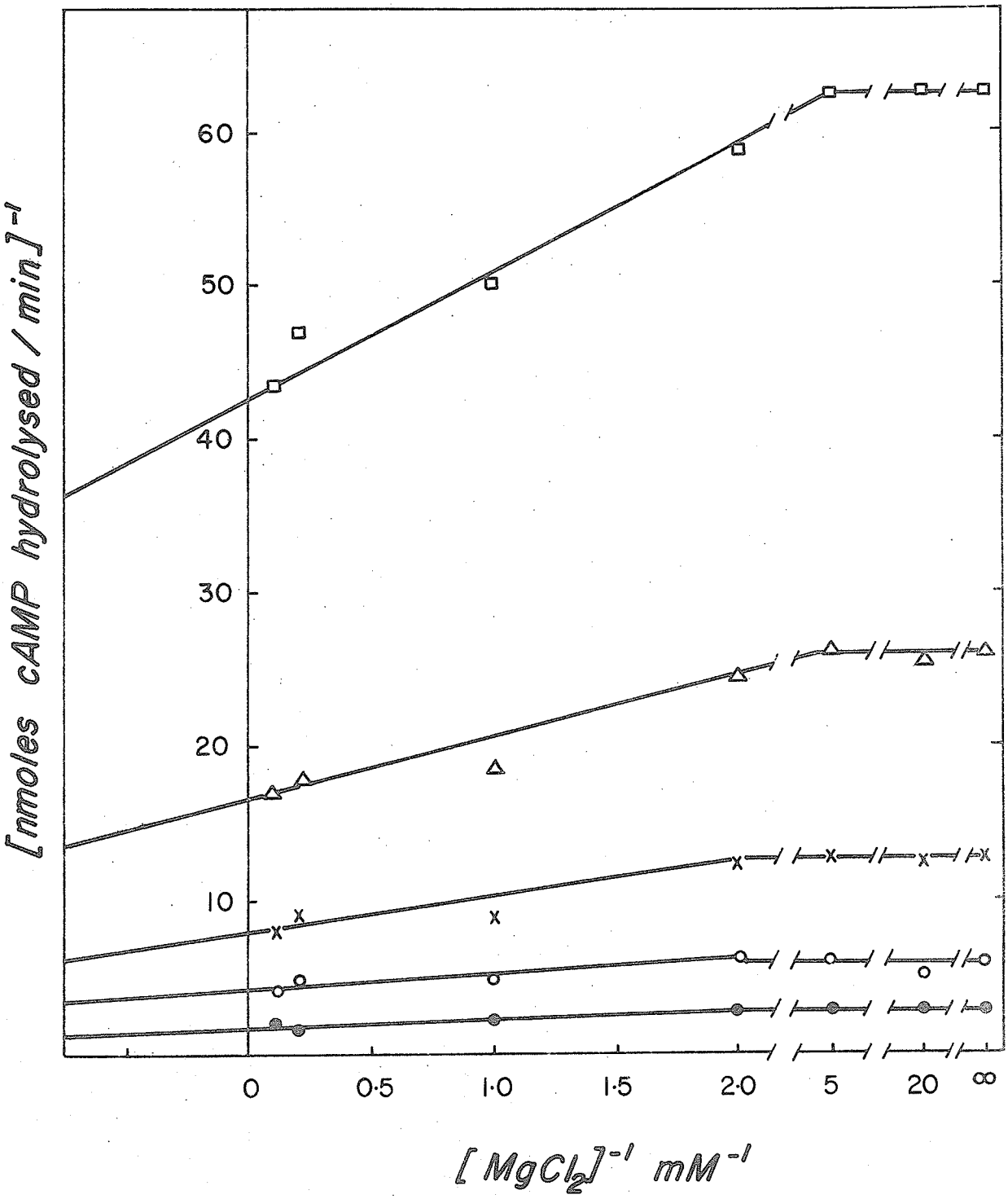

[nmoles cAMP hydrolysed / min]⁻¹
60
50
40
30
20
10
0
0·5
1·0
1·5
2·0
5
20
∞
[MgCl₂]⁻¹ mM⁻¹

Figure 41: Double reciprocal plot of the I-PDE velocity versus the cAMP concentration at various levels of $MgCl_2$. Concentration of $MgCl_2$: 10 mM (●), 5 mM (O), 1 mM (X), 0.5 mM (Δ), zero $MgCl_2$ (■).

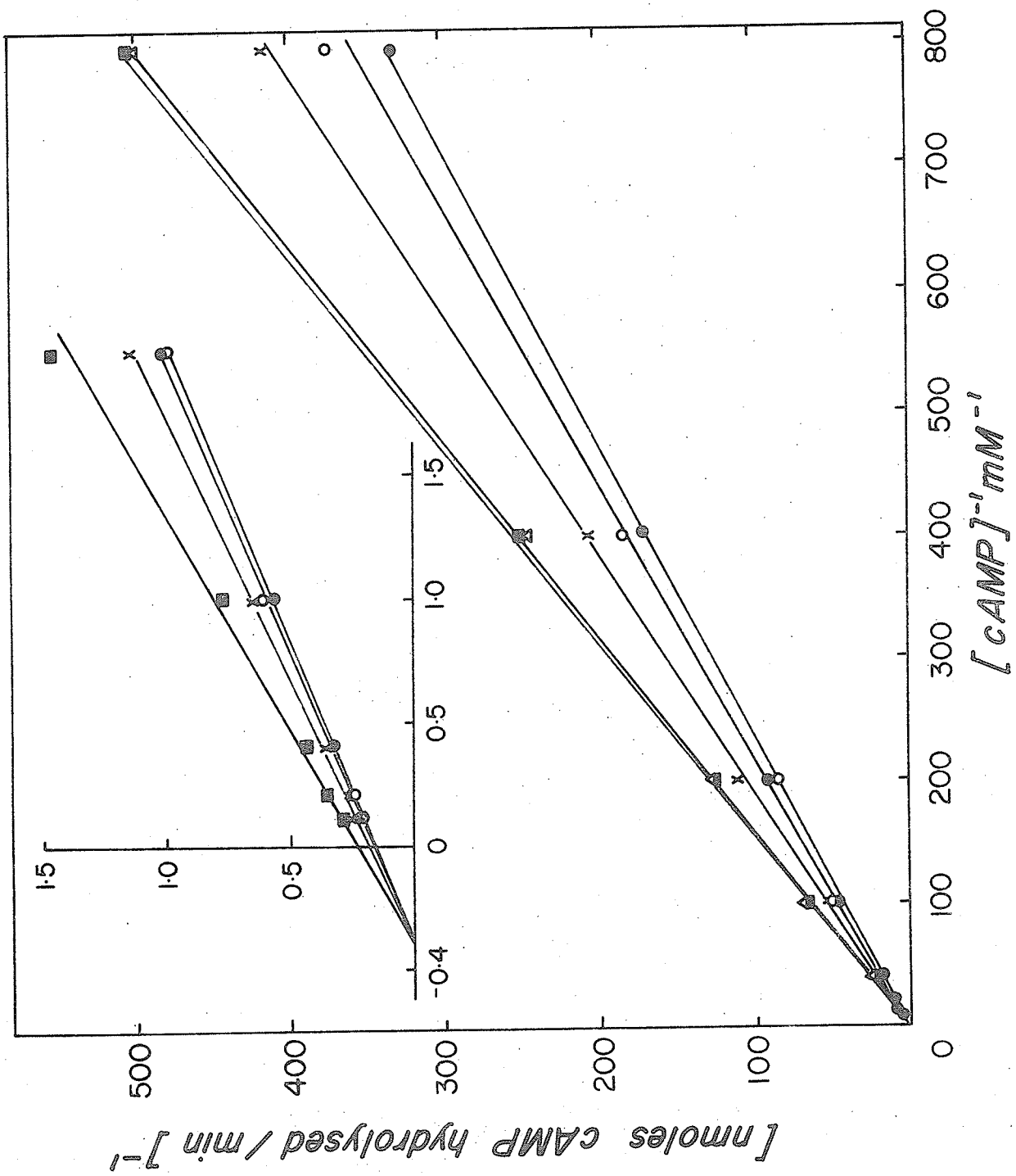
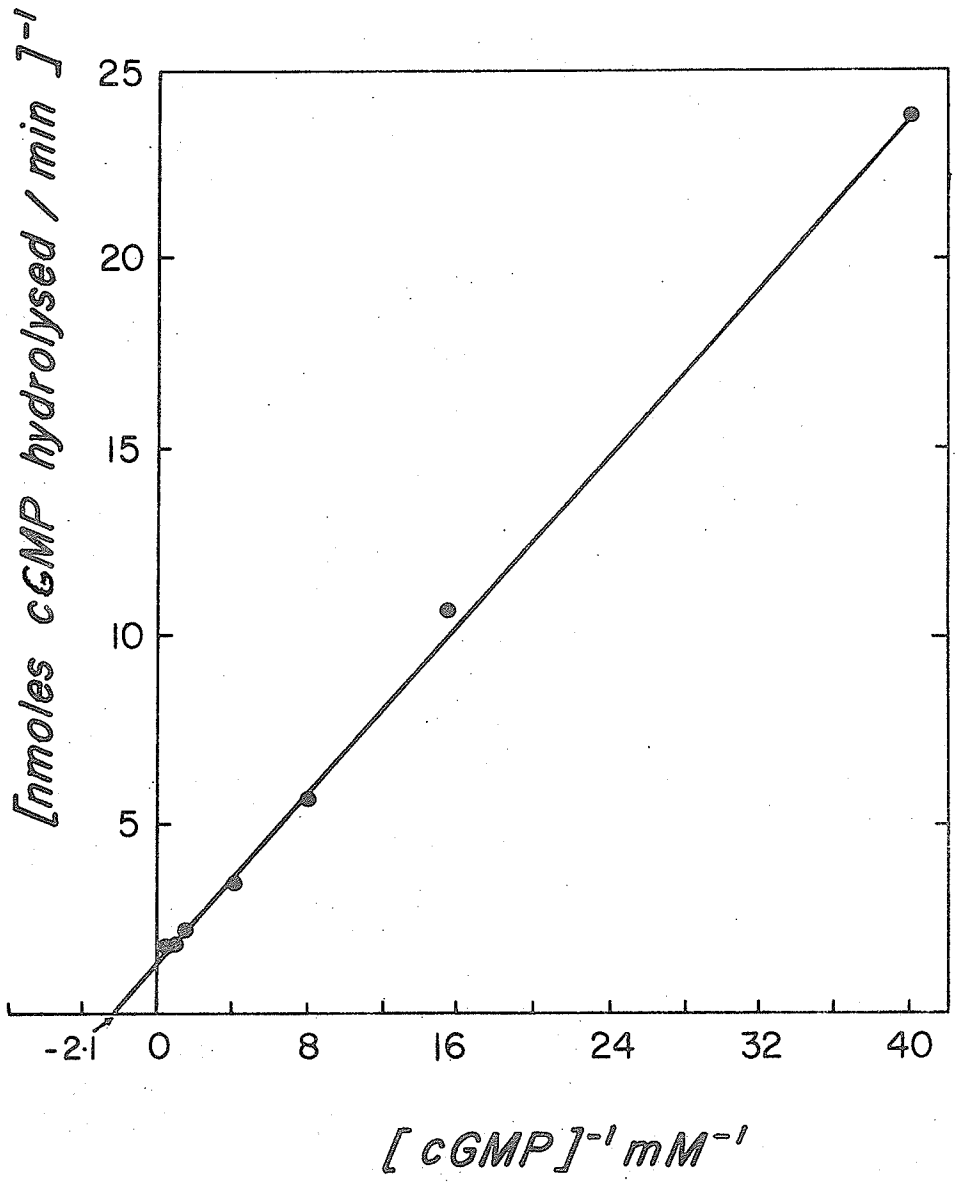

[nmoles cAMP hydrolysed / min]⁻¹
[cAMP]⁻¹ mM⁻¹
0
100
200
300
400
500
600
700
800
-0·4
0
0·5
1·0
1·5

Figure 42: Double reciprocal plot of the velocity of I-PDE versus the cyclic GMP concentration in the presence of 10 mM $MgCl_2$. (U-^{3}H-cGMP, 2.3 Ci/mmole; 108,000 dpm added).


[nmoles cGMP hydrolysed / min]-1
25
20
15
10
5
-2·1
0
8
16
24
32
40
[cGMP]-1 mM-1

and calcium. In view of the significance of the two metals in cyclic AMP metabolism, further investigation on this point is warranted. The phosphodiesterase from N. crassa was similarly inhibited by EDTA, but magnesium, manganese, and calcium, up to 10 mM, did not affect the activity.

The Km of the enzyme for cyclic AMP was estimated to be 2.5 mM (Fig. 41). A similar value was obtained when crude extracts were tested. This enzyme was also capable of hydrolysing the phosphodiester bond of cyclic GMP, with a Km of 0.48 mM (Fig. 42).

D. Extracellular Phosphodiesterase

The medium from 48 hour cultures was found to contain activities which were capable of hydrolysing cyclic AMP to AMP, and also AMP to adenosine. For the studies of this extracellular phosphodiesterase (E-PDE) the medium was concentrated ten times at 4°C using Aquacide. Since the medium contained a strong phosphomonoesterase, snake venom was added after the reaction was terminated to convert all the AMP to adenosine. The radioactivity in the adenosine spot on the paper chromatogram then yielded the reaction rate.

The E-PDE showed a broad pH optimum of from pH 2.5 to pH 4.5, and there was no activity at a neutral pH (Fig. 43). The activity was identical when assayed in either K-citrate or K-acetate. All subsequent studies were carried out in K-citrate buffer at pH 3.5.

The Km for cyclic AMP was estimated to be 18 μM (Fig. 44), which is two orders of magnitude lower than that of the I-PDE. This result is to be considered preliminary, however, because there appeared to be other factors present in this crude enzyme preparation which interfered

Figure 43: Influence of pH on the activity of E-PDE. Assay conditions: 60 µM cAMP (17,000 cpm/nmole), 20 mM cAMP, 34 mM buffer, one hour at 30°C. K-citrate (●), Tris-acetate (O).

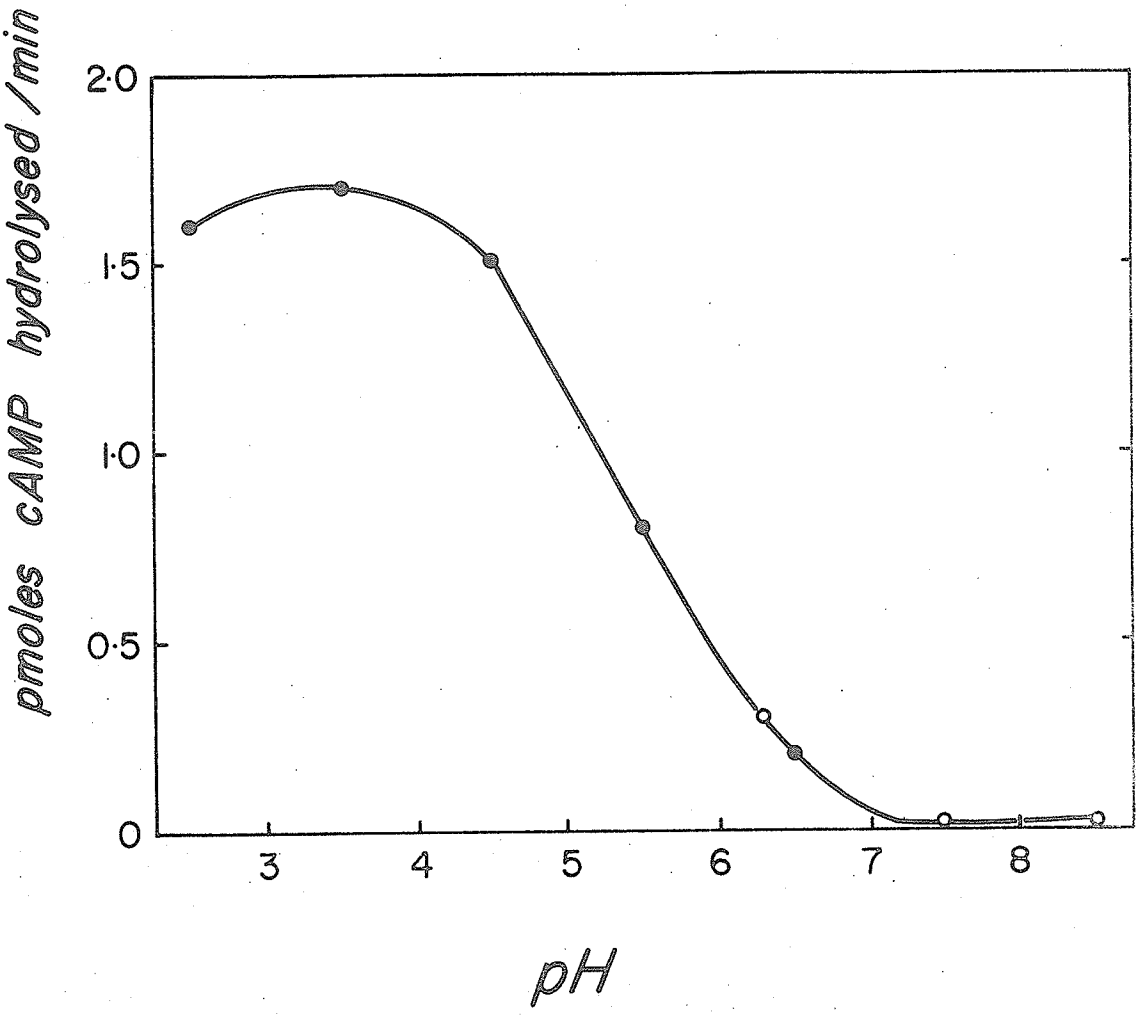
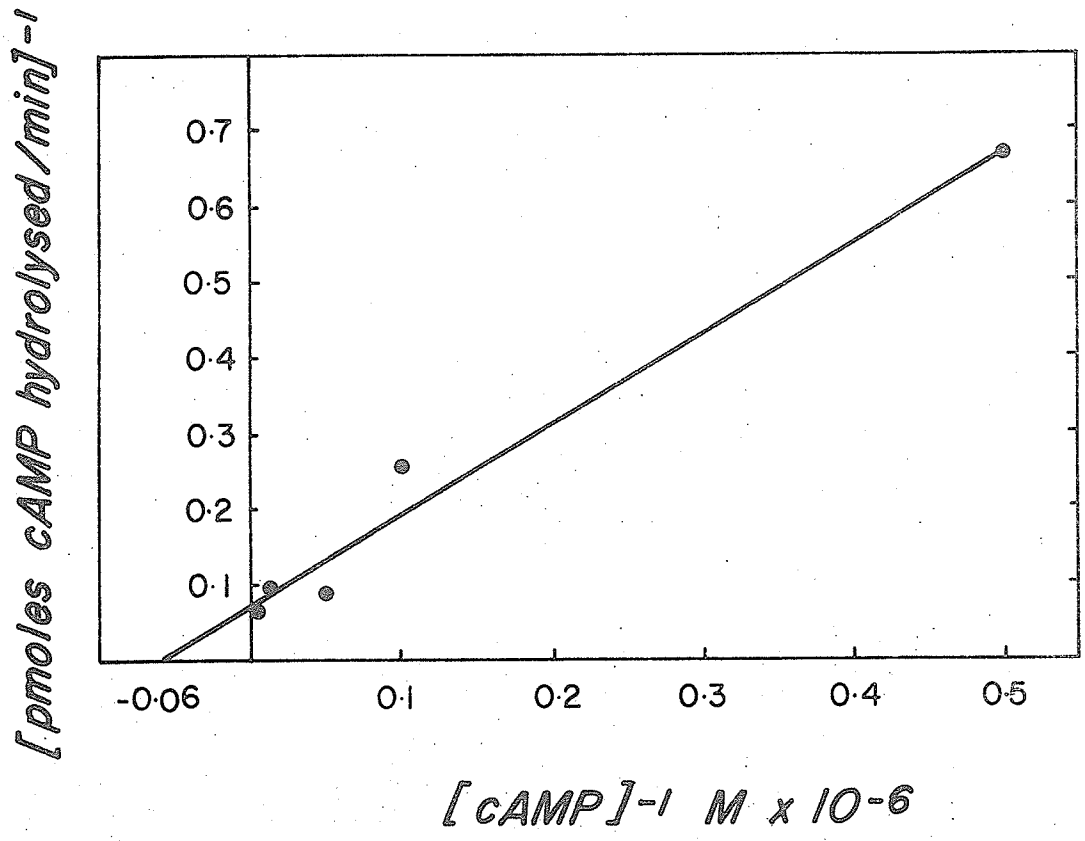

pmoles cAMP hydrolysed /min
2·0
1·5
1·0
0·5
0
3
4
5
6
7
8
pH

Figure 44: Double reciprocal plot of the E-PDE velocity versus the cyclic AMP concentration. Assay conditions: 38,000 dpm, 10 mM $MgCl_2$, 50 minutes at 30°C.


[pmoles cAMP hydrolysed/min]-1
0·7
0·6
0·5
0·4
0·3
0·2
0·1
-0·06
0·1
0·2
0·3
0·4
0·5
[cAMP]-1 M x 10-6

with the reaction. In some experiments, the reaction rate was found to be linear with time for at least three hours, but in others this was not the case (Fig. 45). Under these circumstances the rate of the reaction would eventually become zero, even though only 10% of the available cyclic AMP had been hydrolysed. This is a curious result, for the enzyme was stable for at least several days at 24°C, and for months at 4°C. In addition, there is some evidence for the presence of an "inhibitor" in the preparation, which may be dialysable (Table 20). It is to be expected that the reaction rates from the various enzyme preparations would be additive when combined, but this was not the case. Studies on this enzyme and the factors which regulate its activity should prove highly interesting.

DISCUSSION

It has been shown that in cells of D. discoidium which respond chemotactically to cyclic AMP there are at least two or possibly more phosphodiesterases (Malchow et al., 1972). One enzyme is membrane-bound, and its activity increases in the developmental stage prior to aggregation; no increase is found in non-aggregating mutants. This enzyme has a low Km (10-15 μM) and appears to have similar properties to an enzyme secreted by the cells (Pannbacker and Bravard, 1972). Another phosphodiesterase is apparently found both intracellularly and extracellularly (Malchow et al., 1972). The extracellular form has a Km of 4 μM (Riedel et al., 1972), and its activity is regulated by a low molecular weight proteinacious inhibitor which is released into the culture medium when growth is terminated by nutrient exhaustion (Riedel and Giersch, 1971). The activity of the membrane-bound enzyme was not

Figure 45: Hydrolysis of cAMP by E-PDE as a function of time. Assay conditions: 60 µM cAMP, 20 mM $MgCl_2$, 20 mM K-citrate, pH 3.5, 30°C.

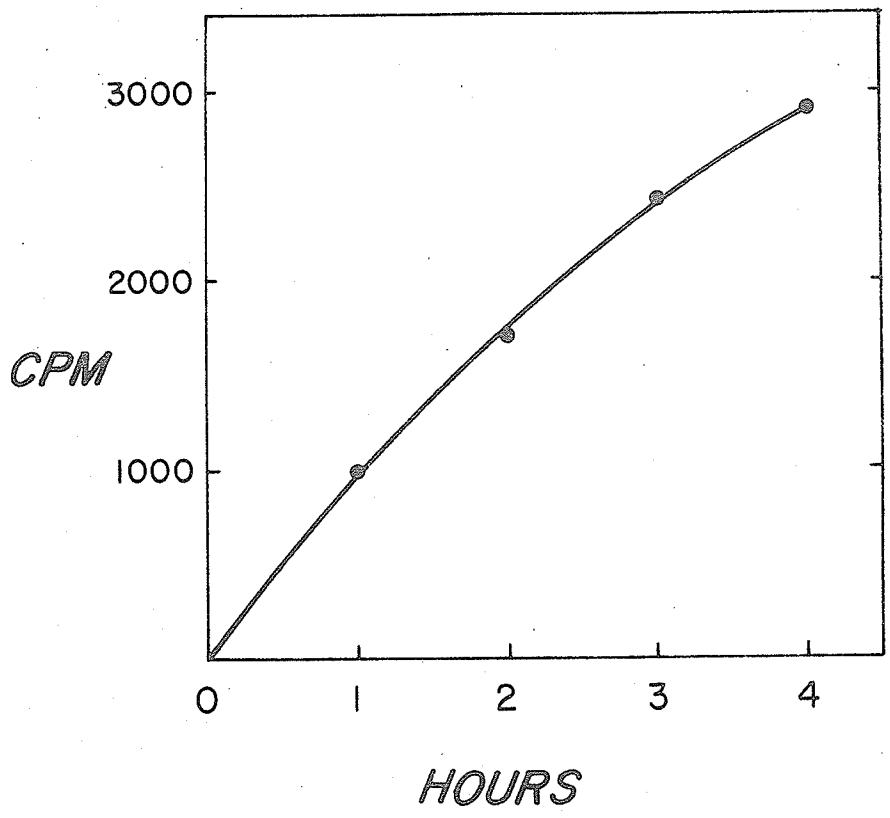

3000
2000
1000
CPM
0
1
2
3
4
HOURS

Table 20

Evidence for the presence of an inhibitor for E-PDE.

Test	cpm Expected	cpm Obtained	Per Cent of Expected Result
40 µl Unconcentrated Media (1)		2979	
25 µl Media, 10 x Concentrated (2)		4946	
25 µl Media, 10 x Concentrated (3) Dialysed		8133	
40 µl (1) Plus 25 µl (2)	7925	5308	67
40 µl (1) Plus 12.5 µl (2)	5452	4487	82
40 µl (1) Plus 25 µl (3)	11112	5408	49
40 µl (1) Plus 12.5 µl (3)	7046	4904	70

Assay conditions: 1.0 mM cAMP (1300 cpm/µmole), 20 mM $MgCl_2$, 20 mM K-citrate, pH 3.5, one hour at 30°C.

as potently affected by this inhibitor (Malchow et al., 1972). Evidence is available which indicates that an operative enzyme-inhibitor system is required for the proper chemotactic response to cyclic AMP (Giersch et al., 1972). Possibly a third distinct extracellular phosphodiesterase is also present, which has a Km of 2 mM (Chang, 1968; Chassy et al., 1969). However, there is evidence that the high and the low Km activities represent two kinetically different, interconvertible forms of the same enzyme (Chassy, 1972). All these enzymes have slightly alkaline pH optima. There is also a soluble intracellular phosphodiesterase with a pH optimum of about 4.5, but this enzyme is not specific for cyclic AMP (Reidel et al., 1972).

Cells from other organisms also contain kinetically different phosphodiesterases. Physarum polycephalum possesses both particulate and extracellular enzymes (Murray et al., 1971). The N. crassa enzyme reported is both soluble and particulate, and has both high and low affinity forms for cyclic AMP (Scott and Solomon, 1973). This is well known for many tissues of the rat (Thompson and Appleman, 1971), including cultured 3T3 cells (D'Armiento et al., 1972). Cultured chicken embryo fibroblasts have been reported to contain two dissimilar phosphodiesterases, one particulate and one soluble (Russell and Pastan, 1973). The particulate enzyme can also be found in the soluble fraction. It may be a general feature of cells to contain both high and low affinity forms of cyclic AMP phosphodiesterases, and A. niger seems to be one further example.

These preliminary results demonstrate that not only does A. niger contain cyclic AMP both inside and outside the mycelia (Section VII),

but also many of the enzymes known to be associated with the metabolism of the nucleotide. This argues that the observed responses to cyclic AMP (Sections III, IV) represent natural physiological phenomena. In addition, it seems that the cyclic AMP degrading machinery in this organism may be similar in nature to that of D. discoidium. Since the extracellular cyclic AMP is of crucial importance in the regulation of chemotaxis (Konijin et al., 1967), adhesion (Konijin et al., 1968) and differentiation of stalk cells in D. discoidium (Bonner, 1970), by analogy, this may also be true for A. niger.

SECTION VI

INFLUENCE OF EDTA, FERROCYANIDE, AND METHANOL ON THE CITRIC ACID FERMENTATION

It is well established that a surfeit of trace metals in the medium has an adverse influence on the accumulation of citric acid by A. niger, generally by favoring vegetation at the expense of acidogenesis (Historical). From an historical point of view, this was one of the greatest obstacles that had to be overcome in order to achieve a successful commercial fermentation from cheap sugar sources. A practical solution to this problem was offered by Mezzadroli (1938) and Perlman et al. (1946a), who suggested that interfering metals might be removed by the formation of complexes with ferrocyanide ions, and this method has been employed by many workers (reviewed by Perlman and Sih, 1960). Another technique has been to desensitize the mold to the trace metals by treatment with methanol and other low molecular weight alcohols and esters (Moyer, 1953). A third procedure has been the use of mutagenic agents to produce trace metal-insensitive strains (Historical).

Although the success of these methods is well established, their mechanism of action is not understood. Ferrocyanide is not the only metal complexing agent which is acidogenic, and others (Perlman and Sih, 1960), including EDTA (Choudhary and Pirt, 1966; Qadar and Abdullah, 1971) have also been used. It is therefore likely that the effects of these agents relate to their ability to interfere in some way with the normal trace metal metabolism of the cultures. Nevertheless, it is not clear whether they act directly on the fungal cells, or

whether they merely precipitate or complex the metals in the medium, and thereby prevent their utilization (Choudhary and Pirt, 1966).

Martin (1955) suggested that ferrocyanide acted directly on the mold by reducing the rate of cell growth during the fermentation (sucrose in synthetic medium by surface culture), and this interpretation was supported by the finding that ferrocyanide inhibited isocitric dehydrogenase (Ramakrishnan et al., 1955) and aconitase (Suzuki et al., 1966). On the other hand, Horitsu and Clark (1966) determined that concentrations of ferrocyanide which markedly enhanced acidogenesis in resting cells had no significant influence on either isocitric dehydrogenase activity, glucose breakdown, or terminal oxidation. High concentrations of ferrocyanide inhibited the growth of their submerged cultures (fermenting beet molasses), but had little influence upon the efficiency (specific activity of the mycelia) of acid production. Since ferrocyanide was known to precipitate many metals from the molasses mash (Clark et al., 1965) and addition of manganese antagonized the effect of ferrocyanide on growth, morphology, and acidogenesis (Clark et al., 1966), Horitsu and Clark (1966) concluded that "ferrocyanide benefits the fermentation only indirectly by precipitating or making biologically inactive interfering metals, particularly manganese".

This section described some preliminary experiments on the effects of EDTA, ferrocyanide, and methanol on the citrate fermentation as carried out in the present investigation. It is concluded that the chelating agents influence the fermentation through a direct action on the mold, probably by binding to metals at specific sites on the cell surface, and thereby rendering the metals "biologically unavailable". Methanol may also act by affecting the ability of the mycelia

to utilize the metals. As the response of the cultures to these agents was similar in many respects to that to cyclic AMP (Sections III, IV), it is considered that the results in this section may shed light not only on the role of the trace metals in the fermentation, but also on the mechanism of action of cyclic AMP.

Experimental Conditions

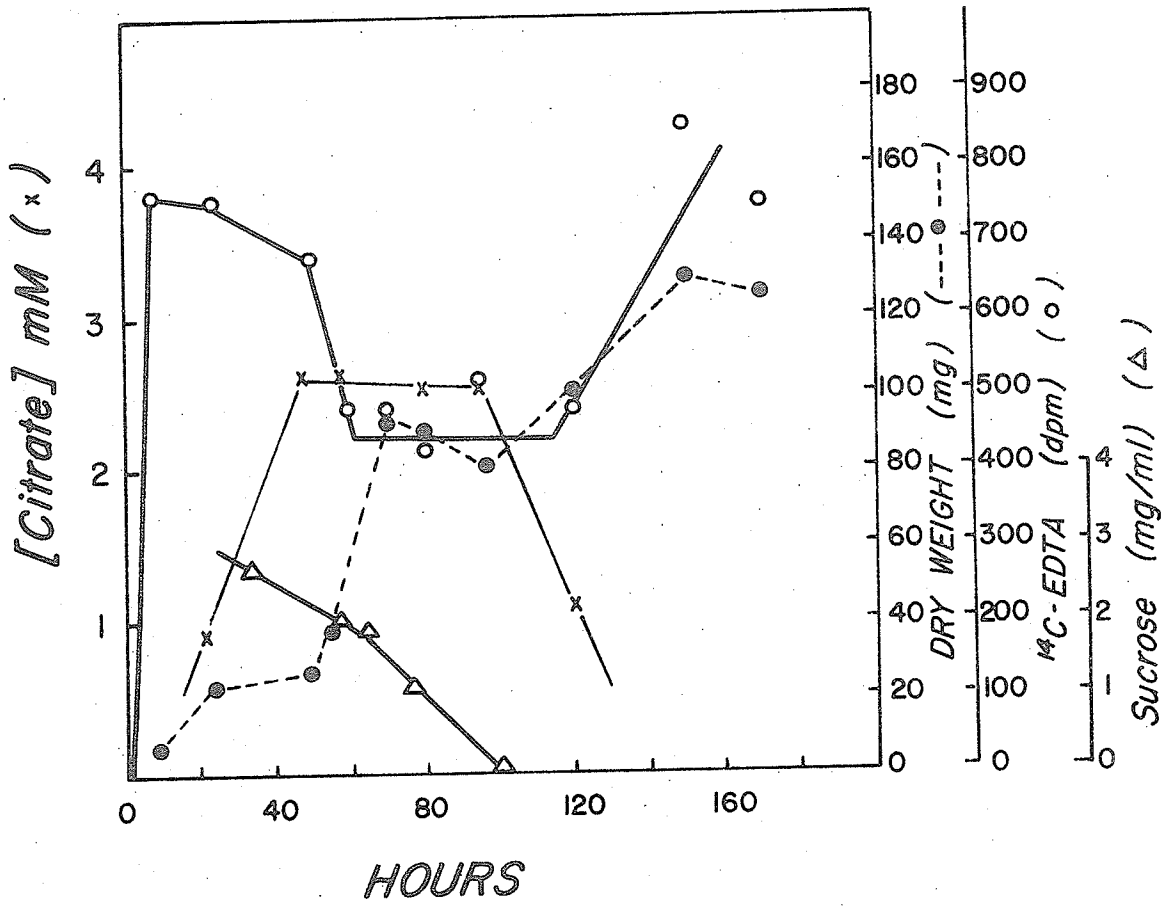
Unless otherwise indicated, all the experiments with EDTA were carried out in 100 ml of medium M-2 (0.4% sucrose) in 250 ml flasks agitated on a rotary shaker at 200 rpm and 28°C. The EDTA (7 mM) was added before the medium was sterilized. The experiments with ferrocyanide and methanol were carried out as described in the text and in Part IV of the Methods.

A. EDTA

Under the experimental conditions of this investigation, citric acid did not accumulate in medium M-2 because it contained too high a concentration of zinc (Section I). This medium favored abundant growth, and the production of relatively large, white pellets with a filamentous periphery. However, addition of a sufficient quantity of EDTA to the medium early in the trophophase caused inhibition of growth, a modification of the pellet morphology, and stimulation of citrate synthesis.

The dynamics of the fermentation with EDTA were complex, and growth appeared to occur in a step-wise manner. In the presence of 7 mM EDTA growth became arrested early in the fermentation (ca. 24 hours), and this was accompanied by the start of secondary metabolism (Fig. 46). Citrate continued to be synthesized at a linear rate until 50 hours, when acidogenesis was suddenly terminated and growth re-initiated. The

Figure 46: Effect of EDTA (7 mM, 3200 cpm per µmole) on growth and citric acid accumulation. The uptake of EDTA by the mycelia is also shown.


[Citrate] mM (×)
DRY WEIGHT (mg) (---●---)
^{14}C-EDTA (dpm) (○)
Sucrose (mg/ml) (△)
HOURS
0
40
80
120
160
1
2
3
4
20
60
100
140
180
200
300
400
500
600
700
800
900

citrate in the medium then remained constant for the remainder of this second growth period, and when the sucrose was finally exhausted the acid was re-utilized. The rate of disappearance of sucrose from the medium seemed to increase in association with the stop in the acidogenesis and increase in growth.

It is highly probable that the effect of EDTA on the fermentation was related to its metal chelating properties. Therefore, a likely explanation for the behaviour of the cultures with respect to EDTA was that the drop in pH caused by the citric acid in the medium resulted in dissociation of the EDTA-metal complexes (Pribil, 1972), and consequently the metals then became available to the mold, which allowed the switch back into the trophophase. In support of this interpretation is the absorption characteristics of the EDTA to the mycelia (Fig. 46). During the idiophase the mycelial content of EDTA was high, but coincident with the termination of acidogenesis the content dropped by ca. 45%. Thus the decrease in pH apparently prevented continued binding of the ligand to the pellets, and it was released into the medium. The increased uptake of EDTA at 150 hours in Fig. 46 may be associated with a rise in pH resulting from the re-utilization of citrate. It is unlikely that the EDTA was metabolized by the cultures, as no microorganism has ever been demonstrated to possess the ability (Hutner, 1972).

As a more direct test of the influence of pH, the fermentation was carried out in the presence of EDTA, but with an initial pH value varying from 2.2 to 3.5, the latter being the initial pH of medium M-2. Only the mycelia grown at a pH of 3.0 or 3.5 displayed the typical EDTA-type of growth pattern, while cultures at pH 2.6 were indistinguishable from those grown in the complete absence of the agent (Table 21).

Table 21

Effect of the initial pH on EDTA-induced acidogenesis.

Initial pH	54 Hours		79 Hours		96 Hours	
	Citrate (mM)	Medium pH	Citrate (mM)	Medium pH	Citrate (mM)	Medium pH
3.5	3.3	2.9	6.0	2.7	0	2.7
3.0	2.5	-	6.5	2.7	5.0	2.6
2.6	0	-	0	-	0	2.7
2.2	0	-	0	-	0	2.3

The growth at pH 2.2 was similar to that at pH 2.6, but was somewhat retarded. It is therefore concluded that the data of Fig. 46 can be explained on the basis of the relationship between the sequestering properties of EDTA and the pH of the culture fluid.

It is not known whether the EDTA had a direct action on the mold tissue, or whether it functioned by merely depriving the cultures of metals in the medium, and thereby preventing their uptake. It is difficult to answer this question unequivocally, but since the EDTA was assimilated in large amounts by the mycelia (Fig. 46), and since EDTA produced pellet morphologies (see below) which were considerably different from that formed in medium M-3 (lacking trace metals), it is the author's opinion that the effect was directly on the mold.

Some oblique support for this interpretation is obtained from the characteristics of the EDTA uptake. It is possible that the EDTA may have been assimilated along with the metals, in the form of an EDTA-metal complex, and in this way could interfere with the transport of the metals. There are, however, reasons to believe that the EDTA uptake was not directly related to the metal transport, but rather occurred by a process of absorption or adsorption to the mycelia. For example, the assimilation of the ligand did not correlate with the growth of the mycelia, because the highest counts were attained when the cultures were still in the conidia phase (Fig. 46). In fact, growth and uptake studies in the early stages of the fermentation showed that the EDTA uptake had been completed before growth even commenced (Fig. 47). Furthermore, the amount of EDTA taken up was proportional to its concentration in the range tested, and this is not in accord with an active transport mechanism, by which metals are likely to be assimilated (Fig. 48).

Figure 47: Early growth and uptake of EDTA (7 mM, 3200 dpm per μmole).

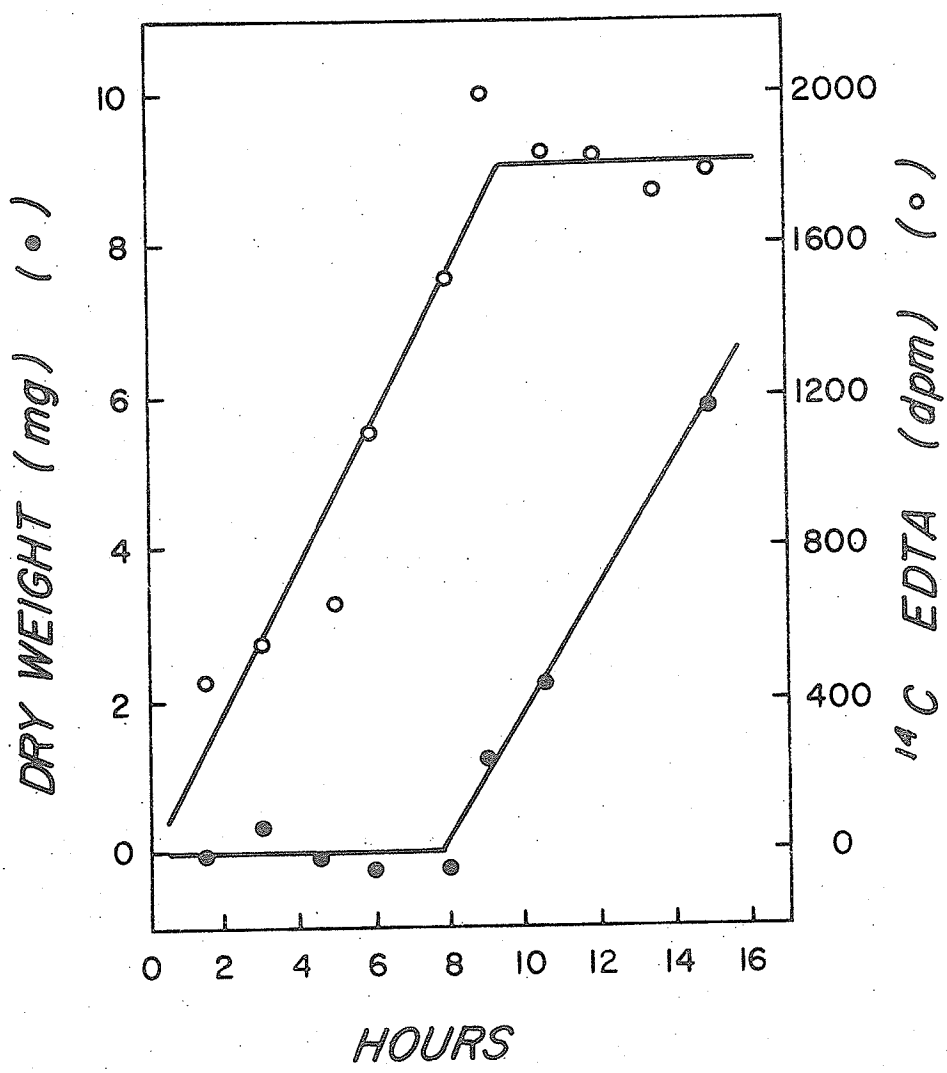
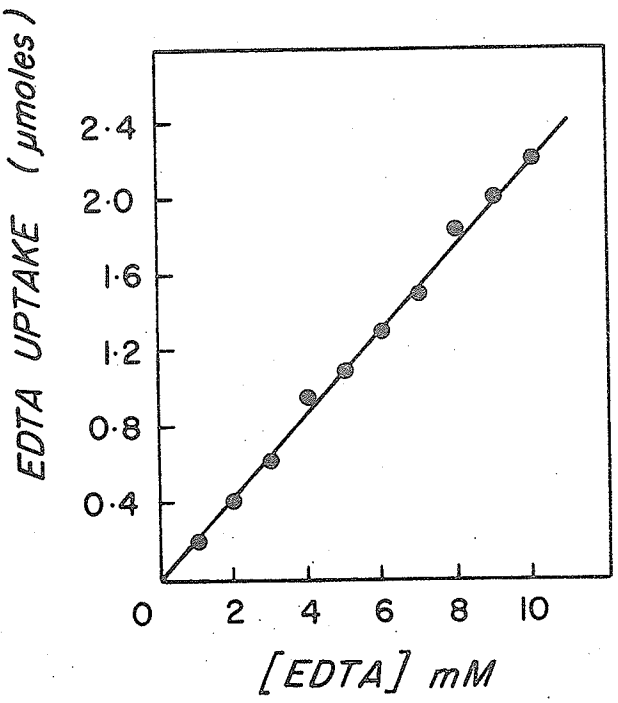

DRY WEIGHT (mg) (●)
^{14}C EDTA (dpm) (○)
HOURS
0
2
4
6
8
10
12
14
16
0
400
800
1200
1600
2000

Figure 48: The relationship between the EDTA concentration and its uptake by conidia. Conidia were inoculated into medium M-2 containing varying amounts of EDTA (10^6 cpm), and the flasks harvested after eight hours growth.

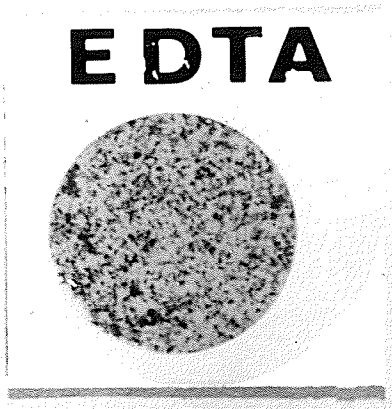

EDTA UPTAKE (μmoles)
2·4
2·0
1·6
1·2
0·8
0·4
0
2
4
6
8
10
[EDTA] mM

Therefore, the EDTA probably influenced the fermentation through a direct action on the mold. The results suggest that the agent was assimilated through a process of absorption and adsorption rather than by active transport. Since the most important property of EDTA is its ability to chelate metals, the most likely mechanism of uptake was by the binding of the ligand to metals on or in the mycelia. The finding that the pH of the medium influenced the mycelial content of EDTA indicates that some or all (in this case 45%) of the EDTA-binding sites were located on the cell surface. Moreover, that the acidogenic ability of EDTA was also related to the pH of the medium leads to the important conclusion that the critical control sites with respect to the trophophase-idiophase decision in the citrate fermentation reside in metals located on the surface of the cells. This will be discussed in more detail below.

In addition to its effects on growth and secondary metabolism, EDTA also had a profound influence on the aggregation of conidia, the number of pellets finally formed, and their morphology. Addition of EDTA to medium M-3 resulted in a large increase in the clumping of conidia (Fig. 49). This effect was observed at 10^{-4}M EDTA, but not at 10^{-5}M EDTA (not shown), and was reversed by zinc, but not manganese or calcium (Fig. 50). In fact, 10^{-6}M zinc had a more potent EDTA-sparing effect than did the other metals at 10^{-5}M (Fig. 51). As the binding constants for EDTA and the other metals are of the same order of magnitude at this pH (Pribil, 1972), this indicates a unique role for zinc in the aggregation of conidia.

In contrast to zinc, addition of iron enhanced conidia aggregation, both in the presence and absence of EDTA (not shown). This result

Figure 49: Stimulation of conidia aggregation by EDTA (10^{-4}M) as compared to a control with nothing added (5.2 x 10^{6} conidia; n = 5).


EDTA

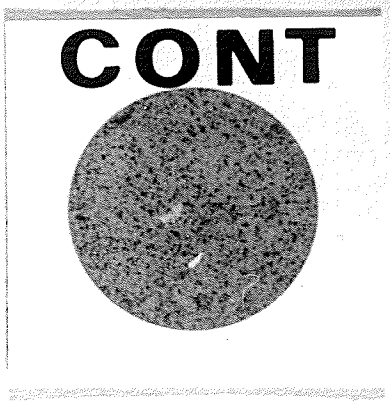

CONT

Figure 50: Reversal of the EDTA-stimulation of conidia aggregation by zinc, but not calcium or manganese. The concentration of EDTA was 1.5×10^{-4}M and that of the metals was 10^{-4}M (5.3×10^{6} conidia; n = 5).

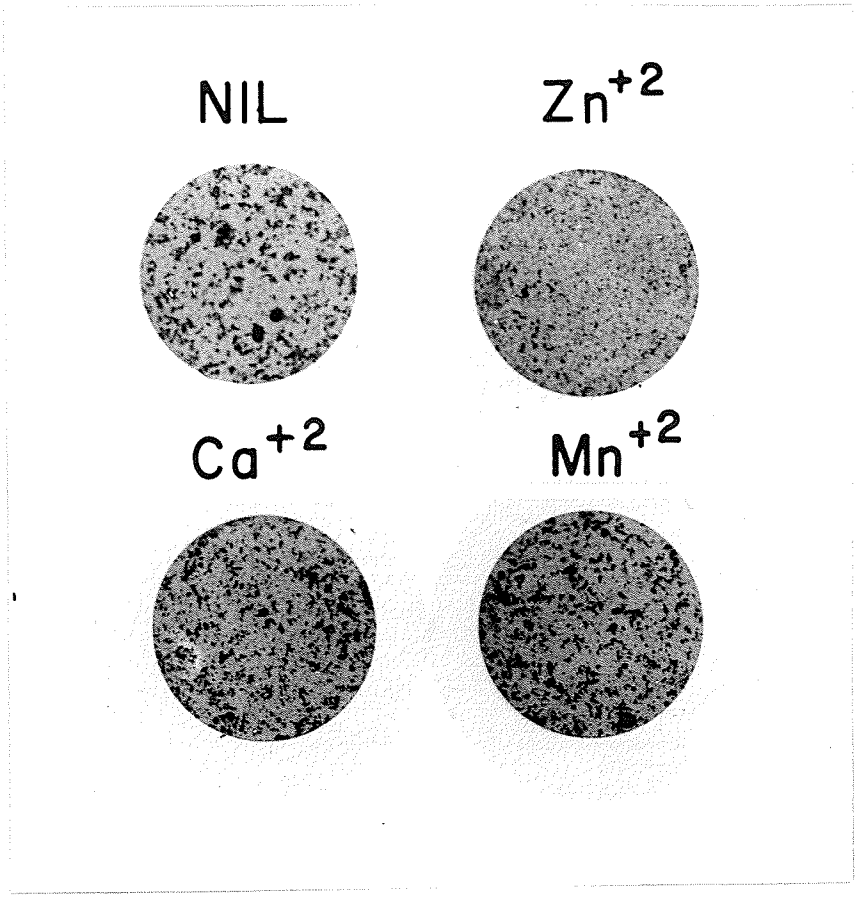
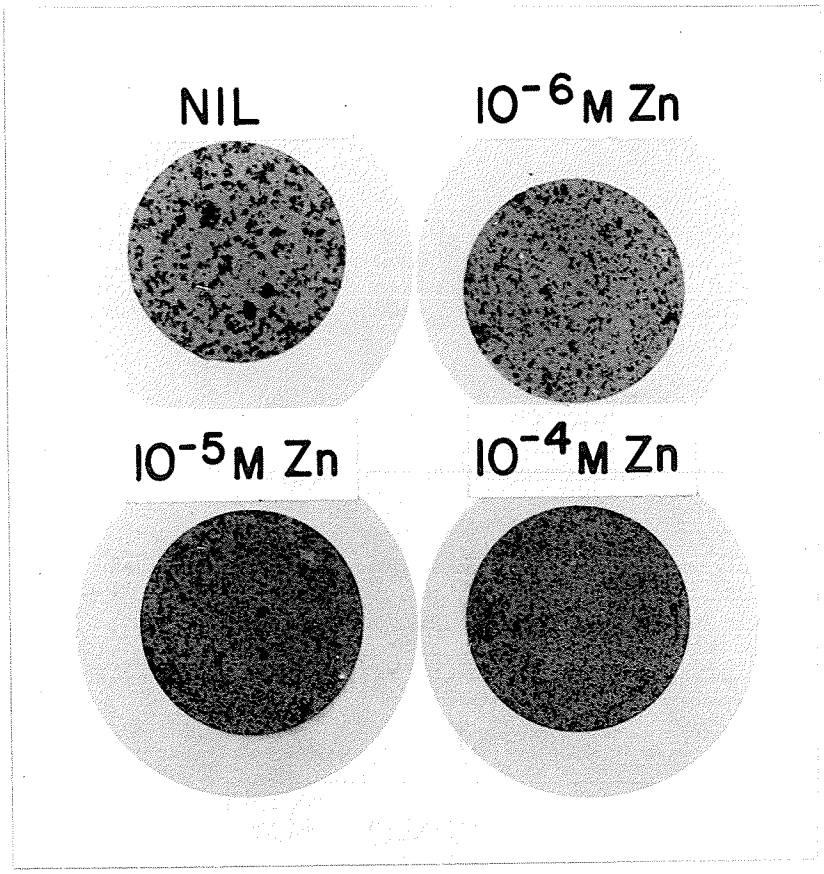

NIL
Zn^{+2}
Ca^{+2}
Mn^{+2}

Figure 51: Reversal of the EDTA-stimulation of conidia aggregation by 10^{-6}M, 10^{-5}M, and 10^{-4}M zinc. The EDTA concentration was 1.5×10^{-6}M (5.3×10^{5} conidia; n = 5).


NIL
10^{-6}M Zn
10^{-5}M Zn
10^{-4}M Zn

is not understood, and was not investigated further. However, iron did not have a significant effect on the final pellet morphology while zinc did (Section VII).

Although EDTA promoted the aggregation of conidia, it also increased the final number of pellets attained (Table 22). Conidia aggregation normally leads to a reduction in the number of pellets, so apparently the EDTA caused de-aggregation of the clumps at a later stage in the fermentation. This was precisely the same effect that cyclic AMP had on pellet acretion (Section IV).

Associated with the increase in pellet number, EDTA also caused the mold to grow in the form of small (<1.0 mm), hard, and smooth pellets, with a "gravel-like" consistency. This result has been reported before (Choudhary and Pirt, 1965; Qadar and Abdullah, 1971).

B. Potassium Ferrocyanide

Potassium ferrocyanide caused a response very similar in nature to that described for EDTA: inhibition of growth, increased citrate synthesis, increased pellet numbers, and changed pellet morphology. The dose response to ferrocyanide is shown in Fig. 52. The agent was significantly more potent in medium M-3 than in medium M-2, indicating that trace metals were involved (Fig. 52). Ferrocyanide at 0.5 mM caused a 40% reduction of growth in M-2, but higher concentrations did not reduce the growth further; instead the amount of citrate synthesis was increased. The agent had little influence on growth in medium M-3, but increasing concentrations caused a very large increase in acidogenesis.

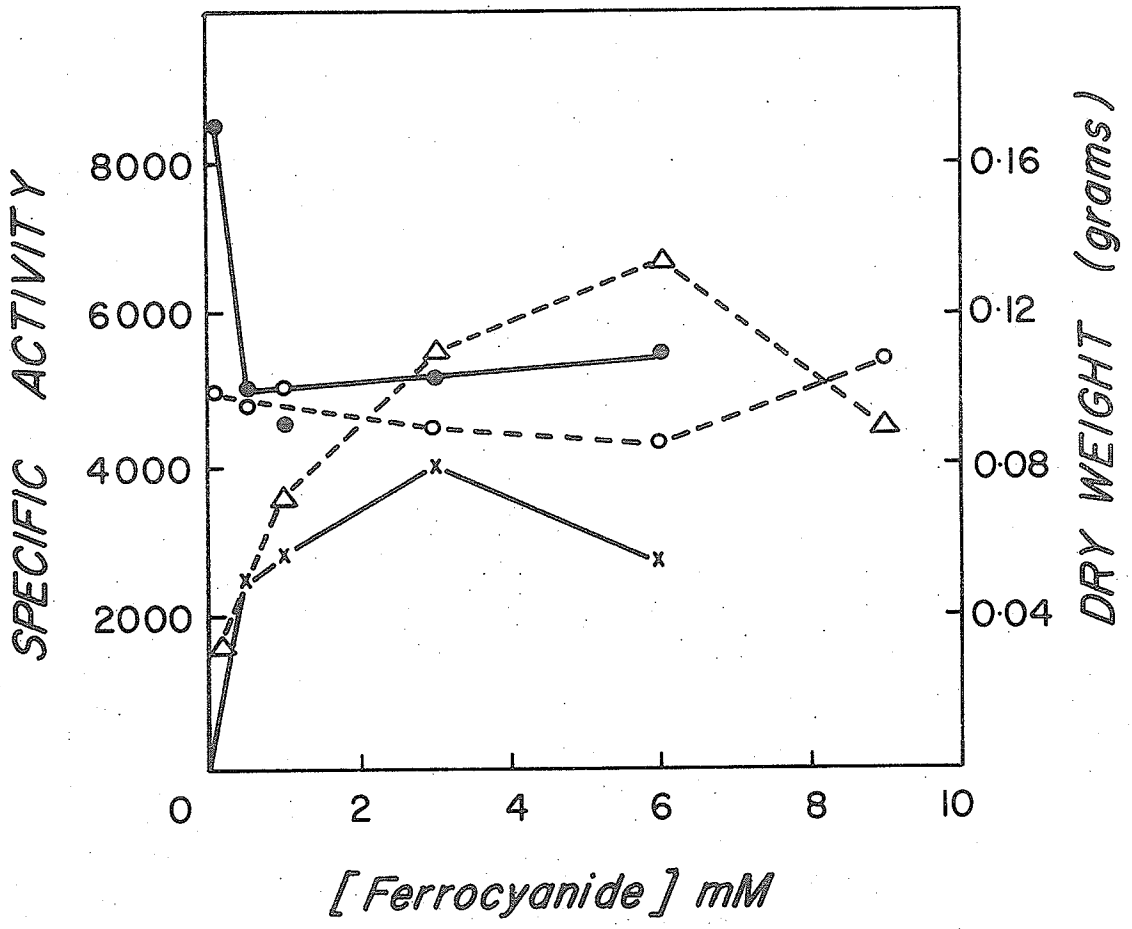
Table 22

Effect of EDTA added at zero hours to medium M-3 (0.8% sucrose) on the final pellet number.

Effector	Pellet Number
0.2 mM EDTA	4877
Control	2502

(10^7 conidia; n = 7).

Figure 52: Effect of potassium ferrocyanide on growth and citric acid accumulation in media M-2 and M-3. The ferrocyanide was added after 15 hours growth, and the cultures harvested after 50 hours. Medium M-2 (full line): specific activity (x), dry weight (●). Medium M-3 (broken line): specific activity (Δ), dry weight (o) (1.5×10^7 conidia).


SPECIFIC ACTIVITY
8000
6000
4000
2000
0·16
0·12
0·08
0·04
DRY WEIGHT (grams)
0
2
4
6
8
10
[Ferrocyanide] mM

It is not known whether the ferrocyanide was effective by acting directly on the mold, or whether it simply deprived the cultures of metals by complexing them in the medium and preventing their utilization. However, in agreement with the observations of Choudhary and Pirt (1965) the pellets became blue-green in color (ferrocyanide-metal complexes) indicating that the ligand had been taken up by the mycelia. A similar conclusion can be derived from the work of Clark (1962), who reported that the particles of precipitate which were formed in the medium after ferrocyanide treatment became attached to the surface of the growing hyphae and were eventually assimilated by them. It is therefore considered that, as was the case with EDTA, the primary effect of ferrocyanide was to act directly on the mold tissue.

It was not determined whether ferrocyanide exerted an influence on the aggregation of conidia, because this agent inhibited conidia germination at this pH (Martin, 1955; Choudhary and Pirt, 1965). However, as with EDTA, addition of ferrocyanide to medium M-2 after 15 hours growth resulted in an increase in the number of pellets formed (Table 23). A similar effect was observed in medium M-3.

Ferrocyanide also had an influence on the pellet morphology similar to that described for EDTA, the pellets becoming small, hard, and smooth. Similar observations on pellet morphology have been made by any authors (Martin and Waters, 1952; Steel et al., 1955; Clark, 1962; Choudhary and Pirt, 1965).

C. Methanol

Methanol added to medium M-3 at zero hours resulted in large

Table 23

Effect of ferrocyanide added at 15 hours to medium M-2 (0.8% sucrose) on growth, citric acid production, and the final pellet number after 50 hours growth.

Effector	Dry Weight (mg)	Citrate (μmoles)	Pellet Number
1.0 mM potassium ferrocyanide	90	245	3842
Control	1715	0	2035

(1.5×10^7 conidia; n = 2).

increases in citric acid production (Table 24). In the experiment shown, methanol had no influence on growth, but in other experiments growth was retarded to some extent. Unlike ferrocyanide and EDTA, this agent did not cause citric acid accumulation in medium M-2, although it did inhibit growth (Table 24). Moyer (1953) reported that 3% methanol caused decreased growth and increased acidogenesis in media containing trace metal levels too high for efficient citric acid accumulation to occur. Perhaps the trace metal levels in medium M-2 used here were far too high, or the low sucrose (0.8% here versus ca. 15% for Moyer) was a factor.

Methanol inhibited the aggregation of conidia (not shown) and this was translated into an increase in the number of pellets formed (Table 24). This effect was observed in both M-2 and M-3 media. Increasing concentration of methanol tended to cause a similar pellet morphology as described for EDTA and ferrocyanide. At 6% methanol in medium M-2, growth was severely inhibited and the pellets were very small, smooth, and hard, with a "gravel-like" consistency.

DISCUSSION

EDTA, ferrocyanide, and methanol had a profound influence on the growth, secondary metabolism, and morphology of the pellets under these experimental conditions. It is significant that the responses of the cultures to these agents was similar in many respects to the responses to cyclic AMP described in Sections III and IV. For example, both cyclic AMP and EDTA: (1) stimulated conidia aggregation, (2) increased the pellet number at high conidia densities, (3) inhibited growth, (4) stimulated citrate accumulation, and (5) caused a small,

Table 24

Influence of methanol added at zero hours to media M-2 and M-3 (0.8% sucrose) on growth, citric acid accumulation, and pellet number.

	Dry Weight (mg)	Citrate (μmoles)	Specific Activity	Pellet Number
Medium M-3				
2 1/2% Methanol	85	560	5688	3017
Control	83	175	2108	2754
Medium M-2				
3% Methanol	134	Trace	--	2495
6% Methanol	26	0	--	>50,000
Control	177	0	--	2057

The flasks were harvested after 50 hours growth (9.9×10^6 conidia; n = 4).

smooth, hard type of pellet morphology. Both ferrocyanide and methanol similarly enhanced acidogenesis, increased the pellet number, produced the same type of pellet morphology, and in some cases reduced growth. Therefore, these observations raise the possibility that the mechanism of action of cyclic AMP and these agents may have similar aspects.

Citric acid did not accumulate in medium M-2 because of the presence of zinc (Section I), but addition of EDTA or ferrocyanide to this medium induced citrate production, and produced the other changes described above. Based on these results it is assumed that these agents act by interfering with the normal zinc metabolism. As both EDTA and ferrocyanide were taken up by the cells, it is considered that the effect was probably directly on the mold. The characteristics of the EDTA uptake argue that it was assimilated by binding to metals on the mycelia. The relationship between the pH of the medium and the binding of, and the biological response to EDTA indicates that the critical metal binding sites are located on the exterior of the cells, in communication with the pH changes in the medium. There is also other evidence which places the locus critical for control of acidogenesis on the cell surface (Section VII).

Accordingly, these observations are consistent with the concept that the chelators complex zinc ions at certain specific sites on the cell surface, render them "biologically unavailable", and thereby effect secondary metabolism. Methanol may act by interfering with the operation of proteins which are located on the cell exterior, and which are involved with zinc metabolism. Therefore, in the presence of these agents, the cultures "perceive" a zinc deficiency, although the medium

is actually replete with zinc. This model could explain why, in the work of Choudhary and Pirt (1966), EDTA and ferrocyanide did not reduce the quantity of trace metals present in the mycelia, although removal of the metals from the medium did. That is, these effectors need not interfere with the mycelial content of the metals in order to influence the course of the fermentation.

As pointed out above, there may be a similarity in the mechanism of action of these agents and cyclic AMP. Accordingly then, cyclic AMP may also function by reducing the quantity of zinc on the cell surface which is "effectively" available to the organism. One way this could be accomplished might be through the phosphorylation, by cAMP-dependent protein kinases, of certain proteins located on the cell surface which are concerned with zinc metabolism. The important consequence of this scheme is that when the concentration of zinc ion is low, that is in the critical range with respect to secondary metabolism, high (exogenous) levels of cyclic AMP would cause a relatively large effective decrease in the biological availability of zinc. Thus cyclic AMP would be a potent amplifier of the information that "zinc is deficient", and secondary metabolism would be initiated or augmented.

This model could also partly explain why zinc replete mycelia were refractive to added cyclic AMP (Fig. 14, Section III). If it is assumed that an on-off equilibrium exists between zinc and the critical cell surface loci, then high concentrations of zinc in the medium would shift the equilibrium towards saturation of the zinc sites. This could prevent the translocation by cyclic AMP of zinc away from the loci.

It is the author's opinion that in this organism the metabolisms

of zinc and cyclic AMP are closely connected (Section VII), and the results presented in this section add support to this idea. Particularly strong circumstantial evidence is that both cyclic AMP and EDTA promoted conidia aggregation, and that this effect was antagonized by zinc with EDTA (Figs. 50, 51) and also with cAMP (Section VII).

The manner in which this link between zinc and cyclic AMP is accomplished is, of course, highly significant. It is known that zinc regulates the levels of cyclic AMP: high zinc leads to high intracellular but low extracellular cyclic AMP; while low zinc leads to comparatively low intracellular but comparatively high extracellular cyclic AMP (Section VII). The model presented above provides for a mechanism by which, under certain circumstances of zinc deficiency, cyclic AMP may regulate the (biological) levels of zinc. It is believed that extracellular cyclic AMP is more important in this respect than is intracellular cyclic AMP (Section VII). Therefore, it is possible that not only may zinc regulate the level of cyclic AMP, but also cyclic AMP may regulate the "level" of zinc.

It is contended that the idiophase in the citrate fermentation is a differentiated state (Historical, Section III), and that under the conditions of this investigation the onset of the idiophase was controlled by the concentration of zinc. EDTA and ferrocyanide were apparently able to influence zinc metabolism in such a way as to cause differentiation to the idiophase even in the zinc replete medium M-2. On the other hand, methanol and cyclic AMP (Section III) were ineffective in M-2, undoubtedly because the zinc level was too high. However, once the idiophase was realized all the effectors were highly acidogenic. Therefore, these agents had a stimulatory influence on the accumulation

of citric acid in addition to their influence on the differentiation process. It is of interest then, to speculate on the acidogenic mechanism of these agents, especially in terms of the model which has been proposed above.

Firstly, these agents could affect the regulatory properties of phosphofructokinase in such a way that glucose catabolism is stimulated (Section I). Cyclic AMP could accomplish this by activating phosphofructokinase (Section III), but it is not clear how this could be achieved by methanol and chelating agents. Secondly, these agents could interfere with the normal operation of the TCA cycle (Historical), although it is again not clear whether or not the TCA cycle is operative during the period of citrate accumulation (Ahmed et al., 1973).

A third possibility is that associated with the idiophase is a mechanism concerned with the specific secretion of citrate from the cells. There are no systems in which the mechanism of secretion is completely understood, but it is known that in animals the calcium ion is involved in some way in this phenomenon (Rubin, 1970). Therefore, as a continuation of the theme that in A. niger zinc may functionally replace calcium (Section VII), cyclic AMP, methanol, and the chelating agents may be acidogenic because they influence the availability of zinc at the critical sites on the cell surface, and thereby stimulate the secretion of the citrate from the cells. It is well known that cyclic AMP is involved in secretion (Robison et al., 1971b). This idea could explain why Horitsu and Clark (1966) observed a very large stimulation by ferrocyanide of citrate synthesis in resting cell suspensions, even though the ligand had no influence upon terminal respiration.

SECTION VII

A POSSIBLE LINK BETWEEN ZINC AND CYCLIC AMP METABOLISM: THE TELEOLOGY OF CITRIC ACID ACCUMULATION

Under the conditions of this investigation, the dynamics of citrate fermentation were controlled by the concentration of zinc ion. High zinc favored vegetation at the expense of secondary metabolism, while low zinc favored secondary metabolism at the expense of vegetation (Section I). In medium M-3, which contained zinc only as a contaminant, the fermentation was biphasic: following conidia germenation, mycelia accrued in the trophophase, but when zinc eventually became deficient, the mycelia differentiated into the idiophase (Section III). Associated with the transition to the idiophase was an alteration in the avidity of the hyphae, with the trophophase hyphae being the more adhesive (Section IV). The role of zinc in this process of differentiation seemed to be regulatory, rather than permissive, because under certain circumstances in medium M-3 abundant growth could in fact be achieved, and with a paucity of acidogenesis (Section II).

Cyclic AMP was also important in the regulation of growth, citric acid accumulation, and adhesion (Sections III, IV). Cyclic AMP stimulated growth and aggregation in the trophophase, acidogenesis and de-aggregation in the idiophase, and inhibited growth in the period of phase transition. Since these parameters were controlled by zinc, an obvious possibility is that the metabolism of zinc and cyclic AMP may be physiologically connected. The finding that EDTA can simulate many of the effects of cyclic AMP is evidence which lends good support to this

concept (Section VI).

The idea that cations may be linked to the metabolism of cyclic AMP is not a novel one. Rasmussen (1970) has pointed out a remarkable correlation between calcium and cyclic AMP metabolism in animal cells, and on the basis of this has suggested that the physiological responses to cyclic AMP may be mediated through calcium ions. Similarly, Whitfield et al. (1973) have suggested that calcium and cyclic AMP may be partners in the regulation of the proliferation of cells from the bone marrow and thymus gland, and that cyclic AMP may even be the ion's intracellular agent in this process.

If these hypotheses are true for animal systems, they may also apply in a general way to the mode of action of cyclic AMP throughout the plant and animal kingdoms. However, calcium is not an important metal to some filamentous fungi, and in the case of A. niger a calcium requirement has actually never been demonstrated, despite diligent attempts (Lilly, 1965). On the other hand, zinc is of great significance in many aspects of the physiology of A. niger (reviewed in Section I). Therefore, it is possible that in A. niger zinc may functionally replace calcium, and that the metabolism of cyclic AMP may be connected to zinc rather than to calcium.

In Section VI it was suggested that cyclic AMP may cause "mobilization" of zinc away from critical control sites on the cell surface, and thereby stimulate acidogenesis (citrate secretion) by reducing the "effective" zinc concentration on the cell surface. In Section III it was proposed that cyclic AMP was a second messenger, and that the primary signal was zinc. This section provides evidence in support of these two

possibilities, and based on the validity of the assumptions, advances a teleological reason for citrate accumulation.

Association of Zinc and Cyclic AMP with Conidia Aggregation

One of the properties of cyclic AMP was its ability to stimulate the clumping of conidia (Section IV), and this same result was produced by EDTA (Section VI). Since the EDTA effect was antagonized by zinc (but not calcium or manganese), a likely possibility is that EDTA affects conidia aggregation by complexing zinc ions located on the surface of the conidia. It follows then that cyclic AMP may sililarly act by "mobilizing" zinc ions on the surface of the conidia. It was difficult to obtain unequivocal data on this point because added zinc had an influence on growth, and aggregation was to some extent a function of growth. The general result obtained was that added zinc repressed the cyclic AMP stimulation of clumping in the very early stages of growth, but not later when the zinc effect on growth became manifest (Fig. 53). This result is then, in accord with the idea that cyclic AMP effects conidia aggregation by mobilizing zinc away from critical sites on the cell surface, and that these sites are re-saturated by added zinc.

Association of Zinc and Cyclic AMP with Growth and Acidogenesis

Mycelia grown in high zinc media were characterized by abundant growth and a lack of citrate production, and added cyclic AMP had little influence of either of these two parameters (Section III). Table 25 summarizes the results obtained when the fermentation was carried out with calcium, manganese, iron, and zinc (all 10^{-5}M), and in the presence

Figure 53: Reversal by zinc of the cAMP-stimulation of conidia aggregation using the differential filtration technique. The cyclic AMP (1.5 mM) and zinc (10^{-4}M) were added at zero hours. Cyclic AMP (✕); Cyclic AMP plus zinc (○); Control (●) (5 x 10^6 ^{14}C conidia; n = 5).

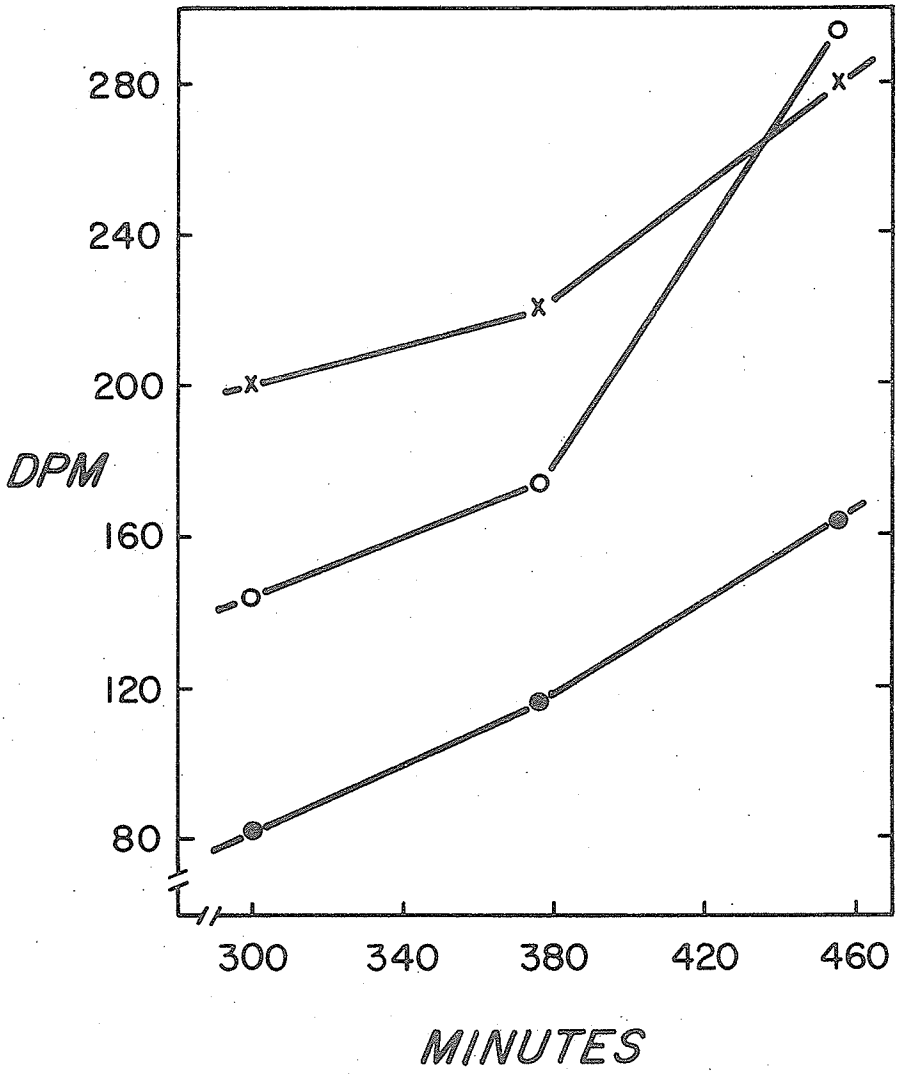

280
240
200
160
120
80
DPM
300
340
380
420
460
MINUTES

Table 25

Effect of 10^{-5}M metals on growth and citrate accumulation in the presence and absence of 10^{-3}M cyclic AMP as compared to controls with nothing added.

	Dry Weight (mg)	Specific Activity
Control	50	2415
cAMP	45	4136
$CaCl_2$	52	3131
cAMP plus $CaCl_2$	60	4308
$MnCl_2$	65	2177
cAMP plus $MnCl_2$	60	2736
$FeCl_3$	55	2593
cAMP plus $FeCl_3$	45	2597
$ZnSO_4$	163	338
cAMP plus $ZnSO_4$	157	475

The effectors were added at zero hours and the flasks harvested after 51 hours (1.2×10^7 conidia; n = 7).

and absence of cyclic AMP. Cyclic AMP enhanced citrate synthesis both in the presence and absence of any added metals and in the presence of calcium. Calcium was without influence on the cAMP-stimulation of acidogenesis at any ion concentration ranging from 10^{-7}M to 10^{-4}M (Table 26). These data support the belief that calcium is not involved in either the citrate fermentation by A. niger, or with the metabolism of cyclic AMP.

A result from the experiment in Table 25 which may be of particular importance was the finding that iron, and to a lesser extent manganese, while having no influence by themselves on the citrate fermentation, significantly antagonized the acidogenic effect of cyclic AMP. This result was not confirmed, but if true it suggests that cyclic AMP metabolism may be connected with not only zinc, but also with iron, and perhaps manganese. It is noteworthy that in other investigations carried out in high sucrose media by many different workers, zinc, iron, and manganese have all been implicated as critical elements in the citrate fermentation (Historical).

The Intracellular Levels of Cyclic AMP are High in Proliferating Cells

It was of interest to determine how the endogenous levels of cyclic AMP varied under conditions of growth and citric acid accumulation, particularly with respect to the zinc concentration. The cultures were grown in 500 ml lots of media M-2 and M-3 in two liter flasks on a rotary shaker at 160 rpm. At various time intervals the mycelia were harvested and analysed for cyclic AMP, protein, and citrate as described in the Methods. The growth medium was analysed for cyclic AMP, citrate, sucrose and protein. After 42 hours growth, zinc (1.55 μM) was added to

Table 26

Effect of calcium on cyclic AMP (10^{-3}M) stimulation of citric acid accumulation over the control (nothing added).

	Dry Weight (mg)	Specific Activity
Control	95	369
cAMP	76	1378
cAMP plus 10^{-7}M $CaCl_2$	85	1232
cAMP plus 10^{-6}M $CaCl_2$	81	1355
cAMP plus 10^{-5}M $CaCl_2$	92	1304
cAMP plus 10^{-4}M $CaCl_2$	86	1285

The effectors were added at zero hours and the flasks were harvested after 69 hours (10^7 conidia; n = 7).

two M-3 cultures, and the flasks harvested 6 and 12 hours later and tested for changes in the levels of these same compounds.

In cultures grown in medium M-2, growth (increase in total protein) was terminated by sucrose exhaustion after 50 to 60 hours, and this was accompanied by a small pulse of citrate appearing in the medium (Fig. 54). Thus the typical trophophse-idiophase dynamics were displayed, with the secondary metabolite appearing after growth termination. During the trophophase, the intracellular cyclic AMP levels were high, ca. 23 pmoles/mg dry weight mycelia, but when growth ceased the levels fell dramatically to ca. 4 pmoles/ mg.

A somewhat different picture was observed with the citrate accumulating mycelia grown in medium M-3. As illustrated in Fig. 55A, total protein did not increase over the investigated time span of 42 to 58 hours, so it is clear that the cultures were not growing. This is the result expected from the mycelia in the idiophase. The intracellular cyclic AMP concentration was ca. 8 pmoles/mg dry weight mycelia (Fig. 55A) which is only one-third of the concentration in the trophophase mycelia in medium M-2 (Fig. 54A). Addition of zinc to the acidogenic cultures in medium M-3 resulted in cessation of citrate synthesis (Fig. 55B); and by 12 hours in an increase in the total protein (growth) and a six fold rise in the intracellular cyclic AMP, from 8 to 52 pmoles/mg dry weight mycelia (Fig. 55A).

Zinc also had a significant effect on the protein content of the mycelia. In medium M-2 protein accounted for 35% of the dry weight of the mycelia at the end of the trophophase, while in medium M-3 protein accounted for only 21% of the dry weight, or 60% of that of medium M-2

Figure 54: Correlation of intracellular and extracellular cAMP levels with growth, sucrose utilization, and acidogenesis in a fermentation carried out in medium M-2. A: Intracellular (except sucrose). B: Extracellular. The extracellular cAMP concentration was 4.7 nM at 24 hours, and 0.8 nM at 49 hours (7.7×10^7 conidia/500 ml cultures).

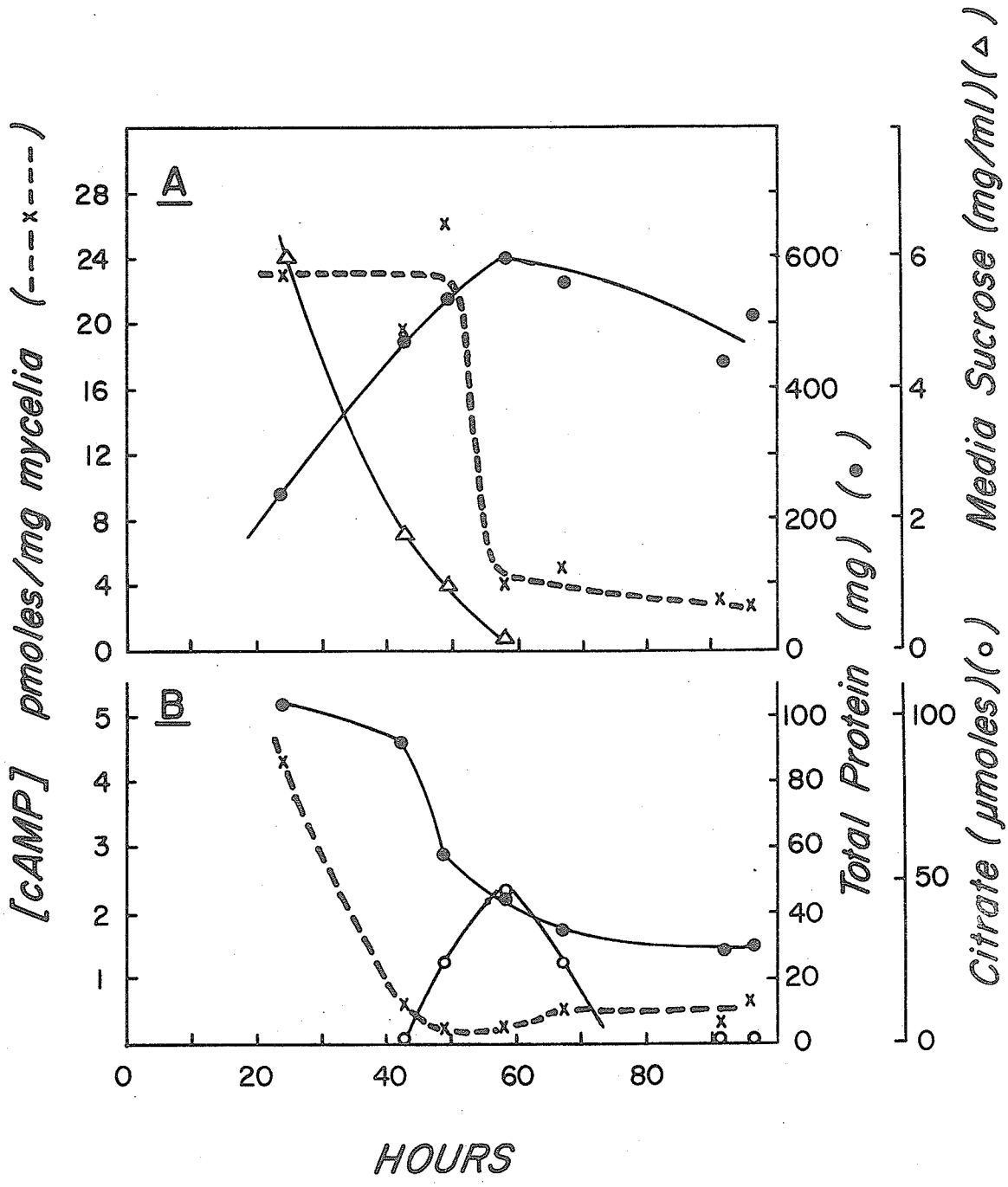
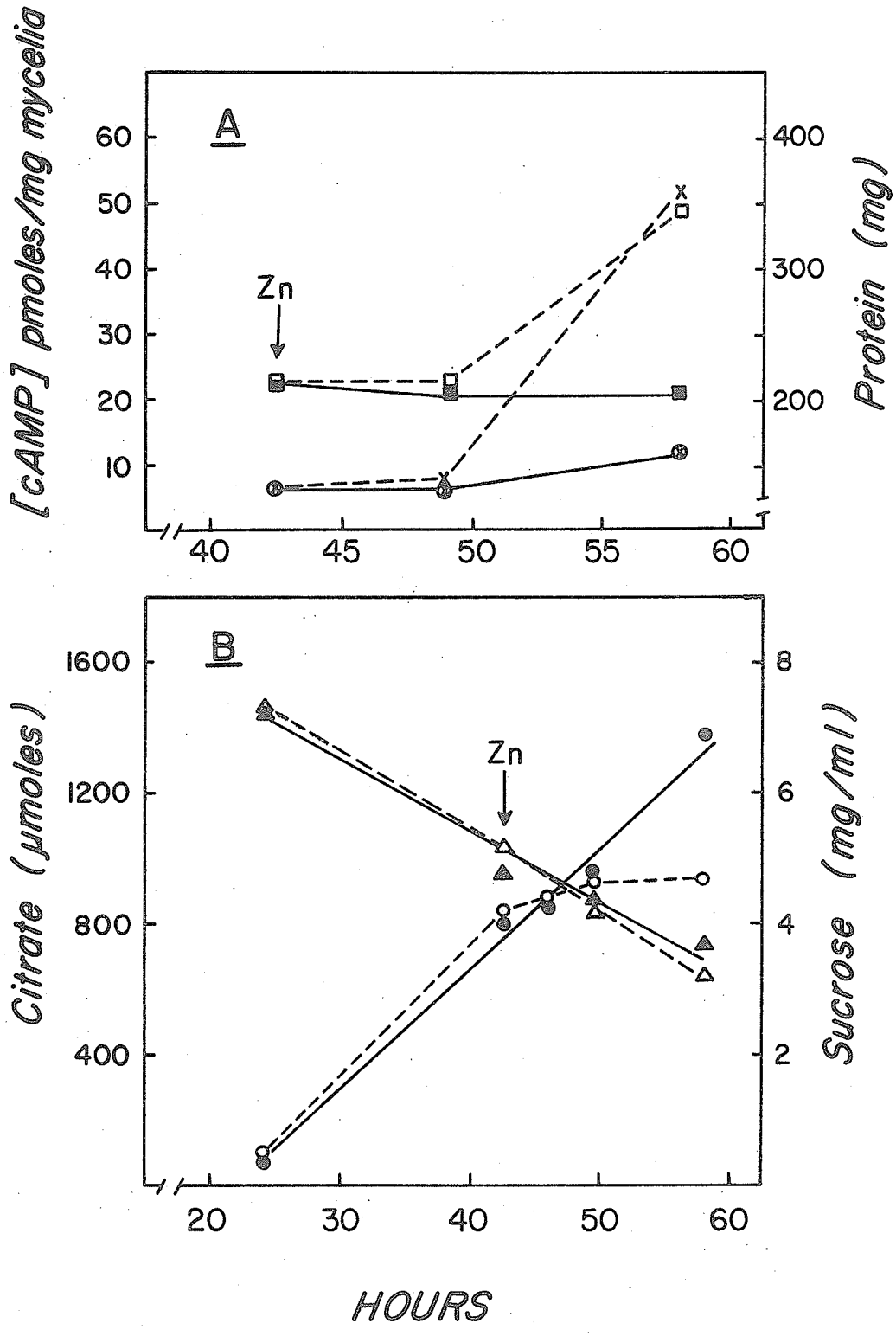

A
B
[cAMP] pmoles/mg mycelia (---x---)
Total Protein (mg) (•)
Media Sucrose (mg/ml)(△)
Citrate (μmoles)(○)
HOURS
0
20
40
60
80
28
24
20
16
12
8
4
0
600
400
200
6
4
2
5
3
1
100
50

Figure 55: Influence of zinc addition (1.55 µM, arrow) on the endogenous cAMP and protein levels of mycelia accumulating citrate in medium M-3. A: Intracellular zinc added (broken line): cAMP (⊗), Protein (□). Control (full line): cAMP (⊗), Protein (■). B: Extracellular zinc added (broken line): citrate (○), sucrose (△). Control (full line): citrate (●), sucrose (▲). The extracellular cAMP concentration was 3.5 pmoles/mg mycelia (7.9 nM). There were 77 mg protein. Zinc addition did not change the extracellular cAMP or protein concentrations (7.7×10^7 conidia/500 ml cultures).


[cAMP] pmoles/mg mycelia
Protein (mg)
A
Zn
60
50
40
30
20
10
400
300
200
40
45
50
55
60
B
Zn
Citrate (μmoles)
Sucrose (mg/ml)
1600
1200
800
400
8
6
4
2
20
30
40
50
60
HOURS

mycelia. This, along with the fact that zinc addition resulted in protein synthesis (Fig. 55A), is consistent with previous observations that zinc is required for growth and for the synthesis of nucleic acids and proteins (Section I).

It therefore seems that under growth oriented conditions, such as are produced by high zinc, the intracellular cyclic AMP levels are high, but when growth is absent, as in the case of sucrose exhaustion or zinc deficiency, the intracellular cyclic AMP levels are low. This, coupled with the observation that in the trophophase added cyclic AMP stimulates growth (Section III), strongly implies that high intracellular cyclic AMP levels are required for cell proliferation to occur.

Whitfield et al., (1973) suggested that cyclic AMP might be the intracellular partner of calcium in the regulation of cell proliferation, because the calcium concentration influenced the intracellular levels of cyclic AMP. Zinc similarly affected the intracellular cyclic AMP concentrations in this investigation, so an analogous partnership may exist between zinc and cyclic AMP in A. niger. However, it is obvious that other factors also controlled the intracellular levels of cyclic AMP. For example, sucrose exhaustion was attended by growth termination and a dramatic fall in the levels of the nucleotide; this was in the zinc replete medium M-2. Apparently then, high zinc was necessary but not sufficient to maintain high intracellular levels of cyclic AMP.

The Extracellular Levels of Cyclic AMP are High in Acidogenic Cells

When the mycelia were grown in medium M-2 the extracellular levels of both cyclic AMP and protein were high in the early part of the

fermentation, but then fell to low levels at later stages, both before and after growth had terminated (Fig. 54B). The concentration of cyclic AMP stabilized at about 0.5 pmoles/mg dry weight of mycelia (0.8 nM), and that of protein at ca. 30 mg/flask. On the other hand, when the growth was carried out in medium M-3 the protein and especially the cyclic AMP levels were much higher than that observed in medium M-2. The protein concentration was 77 mg/glask, over twice that in M-2, and the cyclic AMP concentration was 3.5 pmoles/mg dry weight of mycelia, 15 times that in M-2, or 7.9 nM, nine times more concentrated. The significance of the difference in extracellular protein is not clear, but it is considered that the difference in cyclic AMP concentrations may have relevance with respect to the acidogenic mechanism of cyclic AMP (see below).

Extracellular Cyclic AMP May Regulate Physiology in the Idiophase

Addition of cyclic AMP to trophophase mycelia elicited enhanced growth and adhesion, but addition to cells which had passed the CTP resulted in inhibition of growth and adhesion, and eventually in augmented acidogenesis (Sections III, IV). Therefore, it is apparent that in the idiophase the cells had differentiated in such a way that their response to cyclic AMP was altered. As discussed above, added cyclic AMP enhanced growth in the trophophase, and trophophase mycelia contained high intracellular cyclic AMP, and on this basis it was concluded that high intracellular cyclic AMP was a requirement for growth. Using a similar line of reasoning, since added cyclic AMP reduced growth and adhesion, and stimulated acidogenesis in the idiophase, it could be expected that idiophase mycelia might also contain

high intracellular cyclic AMP, but that in this case the altered response which was observed upon addition of the nucleotide was merely a reflection of the differentiation which had occurred in the cultures. However, idiophase cells were actually low in cyclic AMP, 8 pmoles/mg dry weight of mycelia (Fig. 55A), only one-third that of the trophophase cells (Fig. 54A), a result which seems to be inconsistent with the supposition that the acidogenic site of added cyclic AMP is intracellular.

One explanation for this finding is that the acidogenic site of cyclic AMP is in fact intracellular, but that the cyclic AMP is compartmentalized, not only within individual septated cells, but also from cell to cell throughout the hyphal filament; this cannot be discerned by the measurement of nucleotide levels in pellet cultures. That is, in the trophophase the majority of the cyclic AMP may be in the meristematic apex, while in the idiophase all the cyclic AMP could be located only in the acidogenic sections of the hyphae. In this case, 8 pmoles/mg mycelia could actually be a high concentration, and indeed, it was twice that observed in the M-2 mycelia once growth had terminated (Fig. 54A).

Another possibility is that while intracellular cyclic AMP may be critical for cell proliferation in the trophophase, extracellular cyclic AMP may be critical for the parameters which it affects in the idiophase. Under these circumstances, the comparatively high concentrations of extracellular cyclic AMP found in the idiophase cultures assume significance.

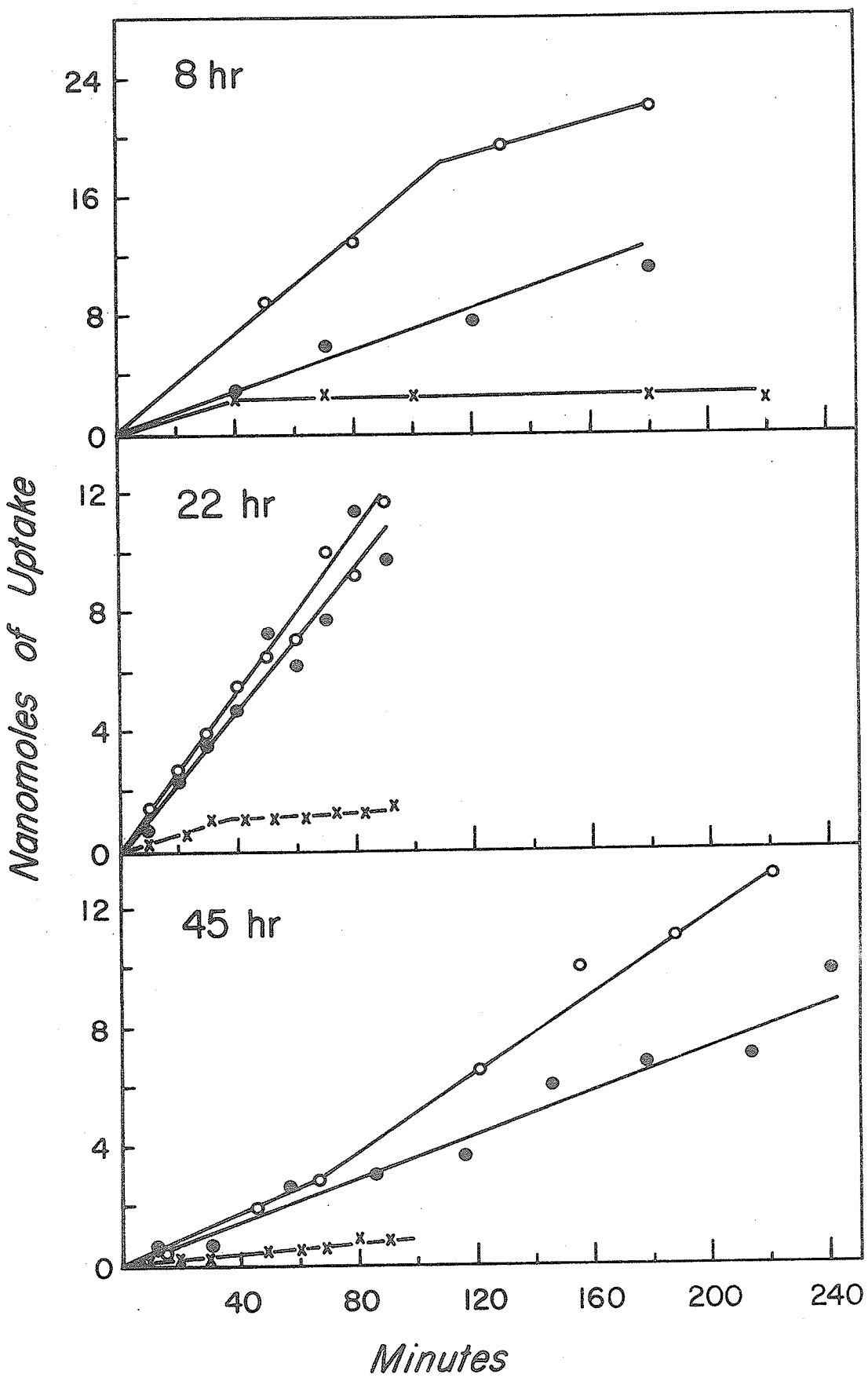
It has been pointed out previously that the critical control sites with respect to acidogenesis seem to be located on the surface of the

cells (Section VI). There is a considerable amount of evidence in favor of this assumption, as many agents which are likely to act (specifically) on the cell surface exert a profound influence on the patterns of growth, citric acid accumulation, and aggregation. These include SV, con A, PHA, pellet "handling" (Section II), butyrate, theophylline, caffeine (Section III), hyphal contiguity (Section IV), EDTA, ferrocyanide, methanol (Section VI), and certain lipids (Millis et al., 1963). Other evidence is that citric acid accumulation is favored by a low pH (<3.0), as oxalate and gluconate are produced under more alkaline conditions (Historical); it is unlikely that the hydrogen ion concentration in the cytosol varies significantly. The curious effect of temperature on the fermentation (Historical) is also explicable in these terms, for the "fluidity" of the cell membrane, and perhaps the activity of certain enzymes concerned with acidogenesis, both of which are likely to be involved in citrate production, are likely to be a function of temperature.

Therefore, if the control loci with respect to acidogenesis are situated on the cell surface, it is reasonable to assume that these loci may be affected by extracellular cyclic AMP. Accordingly, cyclic AMP added in the idiophase may operate directly on the cell surface, and may not have any intracellular function. In fact, added cyclic AMP was not taken up in large amounts by the mycelia, although this was not the situation with adenosine and AMP (Fig. 56).

Furthermore, if exogenous cyclic AMP regulates growth, adhesion, and acidogenesis in the idiophase, then it is imperative that there be some mechanism by which the extracellular concentration of the nucleotide be regulated. It was demonstrated in Section V that this organism

Figure 56: Uptake of 10^{-5}M cAMP (x), 5'-AMP (O), and adenosine (●) by mycelia in medium M-3 after 8, 22, and 45 hours growth. Top: 7.0×10^6 conidia under standard growth conditions. Middle and Bottom: 0.7×10^6 conidia in 5 ml of medium in 20 ml glass vials agitated on a rotary shaker at 28°C and 220 rpm.


8 hr
22 hr
45 hr
24
16
8
0
12
8
4
0
12
8
4
0
40
80
120
160
200
240
Nanomoles of Uptake
Minutes

possesses an extracellular phosphodiesterase (E-PDE), and preliminary data were presented which indicated that there may be an endogenous inhibitor for this enzyme, analogous to the D. discoidium system. Table 27 illustrates that the activity of the E-PDE was considerably lower in cultures grown in the acidogenic medium M-3, than in medium M-2. This result could account for the high levels of cyclic AMP found in M-3 cultures, and for the low levels of the nucleotide found in M-2 cultures.

DISCUSSION

These results are consistent with the belief that in the citrate fermentation by A. niger there is a connection between the metabolism of zinc and cyclic AMP. However, the nature of this connection seems to depend upon the physiological state of the organism. Under conditions which allowed growth (sufficient sucrose) the zinc ion determined the differentiated state; high zinc favored the trophophase, while low zinc favored the idiophase. In the trophophase the intracellular concentration of cyclic AMP was high, but in the idiophase the concentration was low. This suggests that cyclic AMP may be the intracellular agent of zinc in the thallogenic process in the trophophase. On the other hand, in the acidogenic idiophase the extracellular cyclic AMP concentration was high (Fig. 55, legend), and there are reasons to believe that the exogenous nucleotide may be significant in regulating growth, adhesion, and acidogenesis in the idiophase. There are also reasons to believe that the mechanism of action of cyclic AMP in the idiophase is associated with zinc. That is,

Table 27

Extracellular phosphodiesterase activity in media M-2 and M-3 after 75 hours growth.

	E-PDE Activity	Citrate (mM)
M-2 Mycelia	7.2	0.1
M-3 Mycelia	1.6	4.2

The activity represents the nmoles cAMP hydrolysed per gram dry weight of mycelia per minute (6.3×10^6 conidia; n = 2).

EDTA simulated and zinc antagonized the response of the cultures to cyclic AMP. The mode of action of cyclic AMP is not known, but as was suggested in Section VI the nucleotide may act on the idiophase mycelia by some mechanism involving the "mobilization" of zinc ions away from critical control sites located on the surface of the cells. Thus, while in the trophophase cyclic AMP may be the agent of zinc, in the idiophase, zinc may be the agent of cyclic AMP. According to this scheme then, in the trophophase zinc controls the biological concentration of cyclic AMP, and in the idiophase cyclic AMP controls the biological concentration of zinc.

There are precedents for the belief that extracellular cyclic AMP is significant in the regulation of physiology in the idiophase. In fact it has been suggested previously that it may be a property of microorganisms that they respond to exogenous cyclic AMP (Konijin et al., 1968). The most extensively studied organism in this respect is D. discoidium, which responds chemotactically to exogenous cyclic AMP, and which regulates the extracellular levels of the nucleotide by means of an E-PDE and an E-PDE inhibitor (Bonner, 1971; Gerisch et al., 1972). Since A. niger also possesses an E-PDE and possibly and E-PDE inhibitor (Section V), the biology of these two organisms with respect to exogenous cyclic AMP may be analogous.

These observations lead to a biological model which seems to summarize the available information and which explains how citric acid accumulation could give positive evolutionary advantages to the fungus. Consider the fate of conidia from citrate accumulating strain of A. niger which have fallen into a region of comparative zinc deficiency. The

conidia germinate, and in this process secrete cyclic AMP and the putative GMP metabolite discussed in Section IV. Both these agents increase the adhesiveness of the conidia and hyphae. If the conidia density is high, then the localized concentration of these agents would be high; this leads to conidia aggregation and consequent inhibition of germination (Section IV).

Once germination has been completed the hyphae begin to proliferate in the trophophase. The intracellular cyclic AMP concentration is high, and the nucleotide serves to promote the growth and adhesion of the hyphae. Growth continues until an essential nutrient such as sucrose is exhausted; then the intracellular nucleotide concentration declines, and no further growth takes place. The fungus may now ensure its survival by conidiating.

Exogenous cyclic AMP may also be significant in regulating adhesion in the early stages of growth, a possibility supported by the finding that trophophase cultures contained high levels of extracellular cyclic AMP early in the fermentation (Fig. 54B). In the latter part of the trophophase the extracellular cyclic AMP was low, suggesting that exogenous cyclic AMP is physiologically insignificant at this stage. The extracellular levels of the nucleotide are presumably regulated by the E-PDE and its putative inhibitor.

If the essential nutrient which is depleted is not sucrose, but rather zinc, a different situation applies. Growth is terminated as with sucrose, but now the organism differentiates into the acidogenic idiophase. It is not known what the nature of this differentiation process is, but it could involve a modification in the regulation of

glucolysis, the operation of the TCA cycle, and perhaps the induction of a mechanism concerned with the specific secretion of citrate from the cells (Section VI).

Also intrinsic in the process of differentiation is a complete alteration in physiology with respect to cyclic AMP. The intracellular cyclic AMP declines (precluding growth) and the extracellular cyclic AMP rises. In the case of the exogenous nucleotide there is preliminary evidence that this occurs by inhibiting the activity of the E-PDE (Section V; Table 27). The high concentrations of extracellular cyclic AMP then inhibit growth and adhesiveness, and stimulate acidogenesis. This could be accomplished by "mobilizing" zinc ions away from critical control sites on the cell surface (Sections VI, VII), and thereby reducing the effective biological concentration. It is possible that cyclic AMP may also play a role in the actual process of differentiation (at the gene level), although there is no evidence for this.

The accumulation of the citric acid now has important consequences to the organism. As acidogenesis continues there is a large rise in the local concentration of the acid. This has the effect of solubilizing zinc and other minerals from previously insoluble complexes in the soil, and thereby rendering them biologically available. The fresh supply of zinc then re-induces the trophophase. By using this scheme acidogenic soil fungi would be able to control an important aspect of their existence, that is, the availability of zinc and other inorganic nutrients. Accordingly, as is the case with conidiation, the acidogenic idiophase also has survival value, and in fact may be an alternate differentiated state to that of conidiation.

Thus, cyclic AMP is an important tool to the organism. In the trophophase the nucleotide contributes to the overall development of the fungal thallus by first regulating germination, and then by promoting growth and adhesion. However, under zinc deficient conditions the nucleotide enhances the survival potential of the mold by stimulating physiological phenomena which bring about the increased availability of essential mineral nutrients. Central in this argument is that the three conditions of growth, conidiation, or acidogenesis never occur simultaneously (Historical). This is true for all secondary metabolites (Weinberg, 1970).

In these studies zinc controlled growth and citrate accumulation, so it is assumed that this ion is used to monitor the environment for inorganic nutrients. However, as iron, manganese, copper, and possibly phosphate are known to be important in the fermentation, a similar pattern may exist for these ions as well with other strains of citrate-producing soil fungi, or perhaps under different experimental conditions from those used here.

There are other highly significant observations which lend support to this model. Trace metals are known to increase or decrease secondary metabolism in many other microorganisms in a manner independent of growth, which suggests regulation (Historical; Weinberg, 1970). There are certain "key" metals involved: in Bacillus sp. the ion is manganese; in other bacteria and Actinomycetes the metal is iron; and in filamentous fungi and some Actinomycetes it is zinc. In all tests magnesium and calcium were without effect. In addition to the various organic acids produced by fungi, many other unrelated secondary metabolites form strong

complexes with metals (Weinberg, 1970; Perlman, 1965). In the case of iron the metal or the metal-ligand complex often regulates the synthesis of the product (Nielands, 1957; Weinberg, 1970; Hutner, 1972). The pedogenic and edaphic action of microorganisms, especially fungi, is well known, and considerable attention has been given to the role of organic acids as solubilizers of mineral matter in soils (for reviews see Stevenson, 1967; Zajic, 1969; Silverman and Munoz, 1970; Alexander, 1971).

Other hypotheses have been advanced to explain why secondary metabolites are produced. Among many are: 1) waste products of cellular metabolism; 2) reserve food storage materials; 3) breakdown products derived from cellular macromolecules; 4) in the case of antibiotics, protection against competing microorganisms; and 5) "safety valve" shunts of very low molecular weight precursors into innocuous products (Foster, 1949). The last is most popular (Woodruff, 1966; Weinberg, 1970) because it considers the process of secondary metabolism to be of much more importance to the organism than the specific products, and therefore applies to all the diverse products produced by microorganisms. The idea is that when growth is terminated there is a large build up of toxic primary intermediates because feedback regulatory mechanisms are not adequate; the intermediates then induce or activate enzyme systems (Bu'Lock, 1967) and are thereby detoxified by conversion into innocuous secondary metabolites. Secondary metabolism also enables the organism to remain viable for extended periods of time by keeping metabolism operative in the absence of growth (Bu'Lock, 1961).

This does not appear to be an entirely satisfactory explanation for the citrate fermentation, however, because of the complicated controls which appear to regulate it. If citrate is excreted only to be removed from the mycelia, why then does cyclic AMP amplify all the conditions which favor acidogenesis? These include inhibition of growth, and promotion of citrate production, sucrose utilization (Section III), and hyphal de-aggregation (Section IV). If the "purpose" of citrate accumulation is merely to shunt away harmful primary metabolites or to maintain a viable metabolism, then it does not seem likely that this "purpose" would require stimulation by cyclic AMP. In addition, although citrate accumulates only under the limiting growth conditions of zinc deficient media (Section I), this seems to be a regulatory property for there is sufficient zinc present to allow extensive growth (Section II). These observations imply a teleology to this phenomenon of a more profound nature than to "maintain mechanisms essential to cell multiplication in operative order when that cell multiplication is no longer possible" (Bu'Lock, 1961) or to provide "safety valve" shunts (Woodruff, 1966; Weinberg, 1970).

CONCLUSIONS AND PROSPECTS

CONCLUSIONS AND PROPECTS

It is concluded that, in common with other mycological fermentations, the citric acid fermentation is biphasic, consisting of a trophophase and an idiophase. The two phases represent alternate differentiated states, as does hyphal growth and conidiation. The idiophase may have evolved because of its survival value to the organism, for the accumulation of citric acid could liberate the mold from zinc and other mineral deficiencies.

Under the experimental conditions employed in this investigation, the growth and development of the fungus were controlled primarily by the concentration of the zinc ion. Cyclic AMP appears to be the tool by which at least some aspects of this regulation are brought about. Under conditions which allowed growth (sufficient sucrose) zinc increases the intracellular concentration of cyclic AMP, and the nucleotide in turn enhances growth and adhesion. Thus cyclic AMP is the second messenger with respect to zinc in the development of the fungal thallus.

When zinc is present in deficient quantities, the organism apparently differentiates from the trophophase into the idiophase. Intrinsic in this process is a complete alteration in the physiological responses to cyclic AMP. In the idiophase cyclic AMP decreases rather than increases the cell adhesiveness, and when the acidogenic mechanism is operative, the nucleotide stimulates citric acid production. During the period of transition between the trophophase and the idiophase cyclic AMP inhibits growth. In addition, there is circumstantial evidence which suggests that whereas intracellular cyclic AMP is

physiologically significant in the trophophase, it is the extracellular cyclic AMP which is significant in the idiophase.

There is no clear evidence indicating that cyclic AMP has an influence on the process of differentiation, although this could be a possibility, particularly in view of the fact that cyclic AMP reduces the growth rate in the period of phase transition. Actually, the change in the growth response of the mycelia to cyclic AMP, defined as the CTP, is the first overt manifestation that differentiation has commenced. Obviously though, cyclic AMP does not initiate differentiation; the signal for this is zinc deficiency.

It is postulated that the phase transition is also accompanied by the production of a fungus "hormone" which limits growth. It would be of interest to determine whether this putative "hormone" has an influence in regulating the concentrations of cyclic AMP through an effect on the adenyl cyclase or either of the two known phosphodiesterases.

Various lines of evidence indicate that the critical sites which control growth and development in both the trophophase and the idiophase are located on the surface of the cells. Apparently, modification of the surface architecture by "handling" the pellets or by treatment with SV induces a form of "neoplastic transformation" such that abundant growth may take place at the expense of acidogenesis. Con A and PHA were both thallogenic and acidogenic, so sugar moities seem to be involved in both growth and citric acid accumulation.

Studies with EDTA indicate that cations, presumably zinc, are also associated with the critical control sites on the cell surface. The similarities between the responses of the cultures to both EDTA and

cyclic AMP suggest that the two agents may have a similar mechanism of action. Therefore, it is postulated that in the idiophase exogenous cyclic AMP exerts its physiological effects by modifying the cells so that it interferes with the metabolism of zinc at the loci on the cell exterior. According to this model not only can zinc control the concentration of cyclic AMP, but also in the idiophase at low levels of zinc cyclic AMP can control the biological concentration of zinc (Section VI).

Cyclic AMP can also control the concentration of zinc in another way, by stimulating citrate accumulation and thereby enhancing the consequent solubilization of the ion. Therefore, the organism can use zinc to regulate its growth and development in such a way that, in effect, the concentration of the ion will determine its own biological availability. If this is so, then cyclic AMP is one of the tools by which this regulation is carried out.

This thesis has presented data from a series of investigations into the biology of the citric acid fermentation by A. niger. This type of study had never been performed previously, and as such was necessary, for it is pointless to investigate in depth only one aspect of such a topic without an understanding as to what relationship that particular aspect may have in the context of the overall phenomenon. As a result of this approach, however, the data are mainly descriptive in nature, and perhaps some of the conclusions which have been drawn may be somewhat speculative. Nevertheless, in the absence of further evidence, the interpretations of the data seem to be consistent with the information available not only from the citrate fermentation, but

also from other more extensively studied biological systems. The stage is now set for a detailed investigation into the biology and biochemistry of the various aspects of this topic which have been described here.

A further justification for the conclusions which have been drawn lies in their potential general significance. For example, very little is known concerning the regulation of development in mycological fermentations and the results of this study indicate so far that the citrate fermentation is a typical mycological fermentation. That is, the dynamics of the trophophase and the idiophase are the same as with other fermentations, and the synthesis of citrate and the many other diverse secondary metabolites is regulated by zinc (Weinberg, 1970). Thus the results presented in this thesis may be of general significance in the field of secondary metabolism.

The idea that growth and development is controlled by zinc and its partner cyclic AMP may also contribute to the general understanding of how, not only fungal cells, but also other types of eucaryotic cells regulate their growth and development. That is, it provides analogous support to the concepts developed by Rasmussen (1970) and Whitfield et al. (1973) concerning the physiological connection between calcium and cyclic AMP.

Another interesting finding was that cyclic AMP affected the adhesiveness of the cells, in a manner which depended upon the physiological state, which in turn was defined by zinc. To the author's knowledge an influence of cyclic AMP on cell adhesiveness is not generally accepted, although it has been shown in D. discoidium

(Konijin et al., 1968), blood platelets (reviewed by Salzman and Weizenberger, 1972), and the detachment of cultured cells from the substratum (Johnson and Pastan, 1972; Grinnel et al., 1973). In accord with the argument that in A. niger zinc functionally replaces calcium, this finding raises the possibility that a similar connection may apply with respect to calcium, cyclic AMP, and cell adhesiveness in other systems.

Finally, in Section II evidence was presented which indicates that A. niger is capable of undergoing "neoplastic transformation". It would be of interest to investigate this phenomenon, particularly with regard to the metabolism of zinc and cyclic AMP, as calcium and cyclic AMP are known to be involved in some way in the mechanism of carcinogenesis (Schultz and Gratzner, 1973; Whitfield, 1973). It is not inconceivable that such a novel study could provide additional insight into the general mechanism of the cancer problem.

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