A PHYSIOLOGICAL STUDY OF *ASPERGILLUS NIGER* WITH RESPECT TO CITRATE ACCUMULATION

by

JACQUELINE MAIDA MARIE DZUBA

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TO MY PARENTS

A B S T R A C T

ABSTRACT

The physiology and controls involved in citrate-accumulation by cultures of A. niger were studied.

Citric acid was accumulated by a selected strain of A. niger only if zinc was carefully excluded. Cultures were grown in shake flasks on a rotary shaker at 28°C.

The addition of Zn to the medium at any time up to 35 hours at least temporarily inhibited citric acid production and reduced the final yield. The addition of Zn increased the dry weight and sucrose utilization of cultures as compared to controls. The effects were greater with increased concentrations of Zn and were progressively less as Zn was added later.

Distinct patterns of internal metabolite levels were found in citrate-accumulating cultures. High levels of internal citrate were established early and late in the growth period in citrate-accumulating cultures but not in those that contained Zn. Internal sucrose levels were similar in both cultures until after 40 hours, when sucrose levels remained relatively high in control cells, but were depleted from Zn-grown cells. Protein and RNA levels of citrate-accumulating cultures were lower than those of Zn cultures, except for the first 20 hours of growth; cell volumes and dry weights were also lower.

On the other hand, DNA content of control cultures was maintained at a higher level than in Zn cultures for the first 30 hours of growth.

Branching of A. niger mycelia on a solid medium could be induced with staled medium from citrate-accumulating cultures, but not from Zn-cultures, or by citric acid at a concentration as low as 10^{-16}M. Seven other organic acids caused some branching when tested at 10^{-15}M. Eight others, including iso-citric acid could not cause branching, nor could EDTA or ferrocyanide. Citric acid at 10^{-5}M, undiluted staled growth medium from accumulating cultures, or 10^{-15}M citric acid plus 2×10^{-4}M cAMP caused inhibition of growth before regrowth as subapical branches.

Exogenous citric acid added to accumulating cultures at levels normally found in staled medium induced earlier and higher accumulation of the acid in the medium and made sucrose utilization curves more distinctly biphasic, with the lag at 35 hours enhanced. Re-utilization of citric acid was also enhanced. Citric acid could not induce extra accumulation by Zn cultures, but did promote re-utilization. Exogenous citric acid removed the lag at 35 hours from sucrose utilization curves of Zn cultures.

Levels of exogenous citric acid as high as 23.4 mM did not repress citrate accumulation, but did decrease the rate of sucrose utilization of accumulating cultures.

Citric acid production was repressed and growth promoted when DNA synthesis was inhibited with mitomycin C or when mitochondria protein synthesis was inhibited with chloramphenicol. Inhibition of RNA synthesis with actinomycin D repressed citric acid production, but did not cause any change in growth as compared to control cultures. Inhibition of cytoplasmic protein synthesis with cycloheximide repressed both citric acid accumulation and growth.

Control of citric acid accumulation by Zn and citric acid, possibly mediated by cAMP was postulated. The absence of Zn permitted accumulation of citric acid. Citric acid leaked into the medium early in growth triggered differentiation into the idiophase, characterized by a highly branched pellet structure and citric acid accumulation.

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TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	vi
LIST OF FIGURES	ix
LIST OF TABLES	xii
ABBREVIATIONS	xiii
INTRODUCTION	1
HISTORICAL	5
GROWTH PARAMETERS OF FUNGI	6
MORPHOGENS	8
1. Biological morphogens	8
2. Chemical morphogens	13
CHARACTERISTICS OF SECONDARY METABOLITE ASSOCIATED GROWTH	18
CONTROL OF SECONDARY METABOLITE PRODUCTION	20
CONTROL OF CITRIC ACID PRODUCTION	23
MATERIALS AND METHODS	28
CULTURE	29
MEDIA	30
1. Brain-heart Infusion Agar (BHI)	30
2. Malt-yeast Extract Medium (MYE)	30
3. M-1	31
4. M-3	31
5. Assay Medium for Branching	31

Table of Contents Continued

	Page
STOCK SOLUTIONS	32
GROWTH CONDITIONS	33
SAMPLING AND PREPARATION FOR ANALYSES	34
CHEMICAL ANALYSES	37
1. Citric acid	37
2. Sucrose	38
3. Protein	39
4. RNA	39
5. DNA	39
BRANCHING FACTOR	40
1. Plate assays	40
2. Extraction of branching factor	41
SOURCES OF CHEMICALS	43
RESULTS	46
GENERAL METABOLISM	47
ZINC EFFECTS	55
1. Cultural response to zinc	55
2. Cellular response to zinc	71
BRANCHING RESPONSE	101
1. Endogenous branching factor	101
2. Branching response to organic acids	106
EFFECT OF ADDED CITRIC ACID	110
ANTIBIOTIC EFFECTS	123

Table of Contents Continued

	Page
DISCUSSION	135
METABOLISM ASSOCIATED WITH THE PRODUCTION OF CITRIC ACID AND THE EFFECTS OF ZINC	137
EFFECTS OF CITRIC ACID ON THE MORPHOLOGY ASSOCIATED WITH CITRIC ACID ACCUMULATION	141
CELLULAR PROCESSES REQUIRED FOR CITRIC ACID ACCUMULATION	144
CONTROL OF CITRIC ACID ACCUMULATION	146
REFERENCES	149

LIST OF FIGURES

Page

Figure 1. Citric acid production by A. niger cultures 49

Figure 2. Atypical citric acid accumulation by cultures insensitive to Zn deficiency 52

Figure 3. Effects of the addition of varying concentrations of Zn on citric acid production and dry weight 57

Figure 4. Effect of increased concentrations of zinc on citric acid production 60

Figure 5. Effect of increased concentrations of zinc on average dry weight 62

Figure 6. Effect of increased zinc concentrations on sucrose utilization 64

Figure 7. Logarithmic decrease in citric acid production and specific activity due to increasing zinc concentrations 66

Figure 8. Effect on citric acid production of the addition of Zn at 0 or 20 hours 68

Figure 9. Effect on citric acid production of the addition of Zn at 0, 25, 30 and 35 hours 70

Figure 10. Effect on dry weight of the addition of Zn at 0, 25, 30 and 35 hours 73

List of Figures Continued

		Page
Figure 11.	Effect on sucrose utilization of the addition of Zn at 0, 25, 30 and 35 hours	75
Figure 12.	Sucrose concentrations in the mycelia and media of control and Zn cultures	77
Figure 13.	Citric acid concentrations in the mycelia and media of control and Zn cultures	80
Figure 14.	Cell volume of control and Zn cultures	83
Figure 15.	Semi-log plot of citric acid accumulation by control and Zn cultures	85
Figure 16.	Semi-log plot of sucrose utilization by control and Zn cultures	88
Figure 17.	Citric acid concentrations in the mycelia and media of extensively washed samples....	90
Figure 18.	Sucrose concentrations in the mycelia and media of extensively washed samples....	93
Figure 19.	Protein content of control and Zn cultures.	95
Figure 20.	RNA content of control and Zn cultures.....	98
Figure 21.	DNA content of control and Zn cultures.....	100
Figure 22.	Patterns of growth of A. niger hyphae on Park's medium plates.....	103
Figure 23.	Effect of exogenous citric acid on total medium concentration of citric acid of control cultures	112

List of Figures Continued

Page

Figure 24. Net production of citric acid by control cultures given exogenous citric acid 114

Figure 25. Effect of exogenous citric acid on sucrose utilization rates of control cultures 116

Figure 26. Effect of exogenous citric acid on total and net medium levels of citric acid in Zn cultures 118

Figure 27. Effect of exogenous citric acid on sucrose utilization of Zn cultures 122

Figure 28. Effect of exogenous citric acid on total medium concentration of citric acid of cultures insensitive to Zn deficiency..... 125

Figure 29. Net production of citric acid by Zn-deficiency insensitive cultures in the presence of exogenous citric acid 127

Figure 30. Medium citric acid levels of cultures grown in M-3, M-3 with citric acid added and M-3 with citric acid replacing sucrose as carbon source 129

Figure 31. Sucrose utilization by cultures grown in M-3 or M-3 with citric acid added..... 131

LIST OF TABLES

Page

TABLE I. Correlation between citric acid concentration and branching response..... 108

TABLE II. Activity of organic acids and other substances as branching agents..... 109

TABLE III. Effects of metabolic inhibitors on citric acid production and dry weight.... 133

TABLE IV. Effects of metabolic inhibitors on citric acid production and dry weight when added at different times..... 134

ABBREVIATIONS

BHI	Brain-heart infusion agar
c-AMP	Adenosine 3',5'-cyclic monophosphate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamenetetraacetic acid
MYE	Malt-yeast extract
NAD(H)	Nicotinamide adenine dinucleotide (reduced)
RNA	Ribonucleic acid
Tween-80	Polyoxyethylene (20) sorbitan monoleate
YP	Yellow pigment

INTRODUCTION

INTRODUCTION

The study of accumulation of citric acid by Aspergillus niger began and, at least initially, was accomplished largely through the need for improved industrial production. Interest in metabolic pathways, secondary metabolism and processes of differentiation has since induced non-corporate investigators into the field.

A great deal of effort has been aimed at establishing proper growth conditions and optimal substrate and salt concentrations for maximal citric acid production in both industrial and laboratory scale fermentations. It should be noted that despite this tremendous effort, conflicting data still exist, probably due to strain specificity and strain variability.

Because of the emphasis on high yields of citric acid and mechanisms of control based largely on industrial interests, the morphology and growth of the fungus itself was basically ignored except where it served as an indicator of good citrate-producing conditions - A. niger was merely the vehicle and not the target of study.

Some workers along the way have described the mode of growth. The characteristic growth forms in submerged and surface cultures became a criterion, although by no

means infallable, for detecting citrate-accumulating, as opposed to non-accumulating, cultures. Although as early as 1917 Currie commented on the accumulation of citrate to the exclusion of conidiation, and in 1949 Snell and Schweiger listed the lack of conidiation as a criterion for citric acid accumulation, not until 1956 (Gardiner et al) was the striking coincidence between growth form and citric acid production described. In 1962 Clark described the small, round, hollow-centered pellet with a dense periphery and no filamentous mycelia as the morphology of A. niger under citric acid-accumulating conditions. This morphology has also been noted by Perlman and Sih (1960), Martin and Waters (1952), Choudhary and Pirt (1965) and Sanchez-Marroquin et al (1970).

The surge of interest in secondary metabolites and in the differentiated states they represent has lead to descriptions of various systems and good reviews by Bu'Lock (1961), Demain (1973) and Weinberg (1970, 1971). Weinberg considered citric acid a primary metabolite, Bu'Lock called it a "shunt metabolite", while Demain (1968) considered it an important industrial metabolite and included it with other secondary metabolites, the control of production of which has yet to be determined.

Wold (1974) realized the significance of the transition from growth to citric acid-accumulating phase exhibited by A. niger, and carefully described the morphology and growth of the organism with respect to defining a differentiated state.

Most studies have concentrated on the nature of cultures before, or in the absence of, citric acid accumulation, or during actual accumulation. Differences observed between the two states have helped to define the differentiation involved but have not defined the controlling factors or the process of differentiation. Thus, for example, differences observed in enzyme levels might be in fact a cause of citric acid accumulation, but they are more likely an effect.

The present study was undertaken as an attempt to understand the mechanisms involved in the process of differentiation which leads to the "idiophase" generally designated as the period of secondary metabolite synthesis, and in particular the synthesis of citric acid by Aspergillus niger.

H I S T O R I C A L

HISTORICAL

GROWTH PARAMETERS OF FUNGI

The growth parameters of fungal colonies and pellets under "normal" conditions have been well described. "Normal" conditions imply growth which is not associated with specialization or strong environmental influence. A nearly exponential growth rate up to a designated length, followed by septation and a linear growth rate was described for Geotrichum candidum (Fiddy and Trinci, 1976,b). Branching occurred almost invariably behind septa after a fairly specific time period. Aspergillus nidulans was also shown to grow exponentially, with septa formed in groups, the cycles of which approximated the organism's doubling time; single branches were usually formed behind the septa, also at an exponential rate correlated with septation (Fiddy and Trinci, 1976 a). In neither case was subapical branching normally found. In 1970 Trinci rejected the exclusion of subapical and apical branching as normal forms of morphogenesis and published a thorough report of these branch types in A. nidulans and G. lactis without, however, having given any indication of their frequency of occurrance. The same author, six years later, specified that branching of A. nidulans and G. candidum hyphae was normally

lateral, and not subapical or apical (Fiddy and Trinci, 1976 a, b). Katz et al (1972) also described the growth of A. nidulans. They observed that extension at any growth rate was proportional to both growth rate and length of hyphae, and that branching occurred when hyphae had a capacity to extend greater than that which the growth rate allowed. Katz et al (1972) as well as Fiddy and Trinci (1976a), postulated a build up of some cytoplasmic factor which might initiate branch formation. Aspergillus oryzae growth was compared in static and shaken liquid cultures by Nishi et al (1968). They found that growth in static culture resulted in longer hyphae with more branches; DNA synthesis (nuclear division) was more rapid near the apex but RNA and protein synthesis occurred to a greater extent distally.

Saxena and Sinha (1973) described conidiation of A. nidulans and the pellet mode of growth in submerged culture. Martinelli (1976) also studied the same phenomenon, and reported conidiation in response to nitrogen and carbon source depletion unless citrate or acetate were used as carbon source, in which case no conidiation occurred.

Normal growth and morphogenesis of fungi, including possible participation of growth factors produced by the mycelia, were described in detail by Park and Robinson (1966). Bracken (1967) reviewed the ultra-structure of fungi and its relationship to growth and morphogenesis. In 1970 Grove and Bracken described specifically the structure and morphogenesis of hyphal tips.

MORPHOGENS

In addition to reports on "normal" growth patterns of fungi, there have been many reports on morphogenesis resulting from environmental manipulation. These profound effects ranged from changes due to strictly physical parameters such as osmotic pressure and temperature to complex effects caused by chemicals both endogenous and exogenous to the mycelia.

1. Biological morphogens

Park and Robinson (1966) have written a good theoretical account of the roles played by fungal hormones or growth factors on the morphogenesis of hyphae and the development of the adult mycelium. Because of its complex life cycle, the slime mold Dictyostelium discoidium has received a lot of attention as a model for morphogenesis in eukaryotes. In 1968 Gerisch described in detail the process of aggregation and differentiation which occurred in Dictyostelium species in response to a chemotactic signal. The focus of attention on the "aggregation factor" which served as the chemotactic signal resulted in publications from several workers who studied the effects of the signal and its identity. The signal was found to be 3', 5' cyclic AMP (Konijn et al, 1968; Bonner et al, 1969), modulated by an extracellular phosphodiesterase (Bonner et al, 1969).

In 1973 Malkinson et al isolated a c-AMP binding protein from D. discoidium with a Ka of 3×10^{-8} to 14×10^{-8} M and Gregg and Nesom 1973 showed evidence of a direct response of the plasma membrane in the formation of particles visible under the electron microscope due to the combined action of Ca^{++} and c-AMP. Differentiation in non-aggregating mutants could be induced by the diffusion of "acrasin", or c-AMP, from wild strains separated by cellulose (Darman et al, 1975). Mato and Konijn (1975) showed that aggregation could be induced 3-8 hours earlier than normal by activating c-AMP receptors with ATP in the medium, but not with ADP or AMP. A threshold value of c-AMP of 3.6×10^{-9} M/mm would induce aggregation of 50% of D. discoidium amoebae (Mato et al, 1975). Since the amoebae responded to a concentration gradient, by assuming the amoebae length to be 10 μ, a concentration difference over the length of the amoebae was calculated as 3.6×10^{-11} M; if the c-AMP source was 4.3×10^{-9} M at a distance of 5.9 mm the sensitivity of detection or concentration difference between the two ends of the amoebae would be 0.9% of the total concentration, and a difference of 12 occupied receptors between the ends of the amoebae was calculated. Moreover, taking into account the fact that movement was by pseudopod formation, a concentration gradient over the length of a one micron pseudopod of 3.6×10^{-12} M c-AMP, or a difference of 0.01 occupied receptors was calculated.

Klein and Darmon (1976) found evidence for an extra cellular macromolecule differentiation stimulating factor (DSF), which might be a glycoprotein the appearance of which determined the time at which amoebae became responsive to c-AMP pulses. DSF acted in cooperation with c-AMP and could cause amoebae to respond earlier than normal but only after a certain time of development; the site of action was proposed as the synthesis or activation of the c-AMP binding protein.

A growth inhibitor (Yarger et al, 1974) and a transcription inhibitor (Yarger and Soll, 1975) produced by D. discoidium and found in the media of stationary phase cultures have also been reported.

D. discoidium also produces a self-inhibitor of spore germination (Russell and Bonner, 1960) which has been identified as 2-diethylamino 6-oxypurine riboside; 50 µg/ml of the compound inhibited 10 x 10^2 spores/ml by 100% (Bacon et al, 1973) by preventing synthesis of proteins necessary for development (Bacon and Sussman, 1973).

Slime excreted by Physarum flavicomum inhibited cell division and was normally found only in cells that were not dividing; it was capable of producing changes in respiration and macromolecular synthesis in P. flavicomum haploids, and could affect morphology, respiration and cell division of Bacillus subtilis (Henney and Asgari, 1975). Polysphondilium violaceum also produced an acrasin, or

aggregation factor of 1500 daltons molecular weight which might be a peptide (Wurster et al, 1976).

A number of reports have ascribed to fungi the production of growth factors which controlled sporulation and/or outgrowth of spores as normal regulatory mechanisms. Some evidence suggested that diffusible factors formed by Geatrichum candidum controlled sporulation (Park and Robinson, 1969) and germination (Park and Robinson, 1970). The self-inhibitor of bean rust uredospores was identified as cis or trans methyl 3,4,-dimethoxycinnamate which inhibited germination by 50% at a concentration of 5×10^{-3} µg/ml (Macko et al, 1970). Inhibitors of wheat stem rust uredospores were identified as cis and trans isomers of methyl 4-hydroxy-3- methoxycinnamate, which inhibited germination by 50% at a concentration of 8×10^{-9} M (Macko et al, 1971). The germination self-inhibitor of sunflower, corn and snapdragon rust was identified as cis methyl 3,4-dimethoxycinnamate which inhibited the fungi tested at a concentration range from 0.01 to 3.68 ng/ml (Macko et al, 1972). Aspergillus nidulans also produced a germination inhibitor (Scott et al, 1972) which, like the methylferulates active in rust uredospores, could be washed out with Tween 80 or diethyl ether (Scott and Alderson, 1974).

Aspergillus oryzae produced both stimulatory and inhibitory substances during growth which could effect immature neighbouring hyphae (Meyrath and McIntosh, 1964). Two proteins with molecular weights of 28,000 and 11,000 daltons were partially purified from young A. oryzae

mycelia, and were found to inhibit growth (Meyrath and Roth, 1973).

Park suggested (1963) that all fungi produced growth regulators - which might be identical - that affected the morphology of producing species and of various other fungi. Fusarium oxysporum, through the release of succinic acid into the medium, was found to induce branching in the producing organism and in A. niger and Geotrichum candidum (Robinson, 1972). A fungal hormone from Fusarium oxysporum induced vacuolation and inhibited growth in its own hyphae and those of other fungi including A. niger (Park and Robinson, 1964, 1967; Robinson and Park, 1965). This substance was described as a red crystalline material (C_{20} $H_{14}O_8$) which induced vacuolation of 30 species of fungi, at a concentration of 0.01 ng/l (Cornforth et al, 1971).

Volatile metabolites released from nine bacteria and one ascomycete caused abnormal shortening of conidiophores of A. giganteus Wehmer, thin-walled vesicle-like swelling in Fusarium oxysporum f. conglutinans (Wollenw.) Snyder and Hanson, Penicillium irridicatum Westling, Trichoderme viride Pers et Fr and Zygohnchus viullemini Nanynslowski; increased septation in T. viride and Z. viullemini; and increased branching and hyphal distortion of Z. viullemini. The morphological effects and their presence or absence correlated with the species and the amount of growth of the producing organism (Moore-Landecker and Stotzky, 1973).

The diterpenes sclarol and 13-epi-sclarol isolated from leaves of Nicotiana glutinosa were found to inhibit growth of colonies of fungi by causing increased hyphal branching. The nineteen fungi studied, including A. niger and F. oxysporum, were normally found as plant pathogens (Bailey, Vincent and Burden, 1974).

Penicillium janczewskii zal. produced a "curling factor" which markedly affected the morphology of Botrytis Allii at concentrations of 1 µg/ml of the partially purified compound (Brian et al, 1945).

A. niger produced a cyclic peptide called malformin which caused malformations to bean and corn roots at concentrations of 10^{-4} µg per plant (Curtis, 1961).

2. Chemical morphogens

The variety of effects, composition and source of morphogens active in biological systems is vast. Chemical induction of "colonial paramorphs" of Neurospora and Syncephalastrum has been described (Tatum, Barrett and Cutter, 1949) whereby exogenously added sodium desoxycholate at 0.01%, Tergat 7 at 0.0015%, gammahexane at 0.025% or ℓ-sorbose at 0.05% induced colony morphologies resembling those of genetic mutants. Inositol would not reverse the effect of gammahexane, but various other carbon sources could reverse the effect of ℓ-sorbose and other surfactants tested had varying degrees of activity to that of Tergat.

In the words of Bartnicki-Garcia and Lippman (1972) "apical growth is a delicate balance between wall synthesis and wall lysis". Any mechanism which tends to disrupt this balance, either specifically or non-specifically, tends to cause morphological change, or in the extreme case, lysis. Hyphae of Mucor rouxii could be induced to lyse by treatment with hypertonic or hypotonic solutions, depending upon culture conditions; occasionally by acids or bases, or treatment with salts, or by a rapid change in temperature. Colonies of Fusarium oxysporum challenged with a concentration increase or decrease from 0.76 M of medium constituents in solution, ceased growth and then branched; any treatment which resulted in the cessation of growth for more than sixty seconds resulted in branching (Robertson, 1958). The internal pressure of hyphal tips of A. niger v. Leigh was measured and the osmotic equivalent was found to be slightly in excess of that of the growth medium; hyphae could rapidly re-equilibrate in response to imposed variations (Park and Robinson 1966). Application of hypertonic solutions lead to the build-up of cell wall material in the absence of sufficient internal pressure to maintain extension growth, so that subsequent morphogenesis of the apex depended upon the degree of cell wall completion before a pressure differential was again achieved. Subsequent morphogenesis occurred as simple regrowth, as dichotomous branching, subapical branching or lateral branching, depending upon the time involved.

Hyphal tips of Dendryphiella salina burst when challenged with low concentrations of non-metabolizable sugars such as 3-0-methyl glucose and ℓ-sorbose, but at higher concentrations bursting was repressed (Thornton, Galping and Jennings, 1976). Similar effects were observed with sea water and hydrochloric acid, but metabolizable sugars such as glucose or fructose were not active. The effects were explained as the result of transport into the hyphae to such an extent that the osmotic potential was increased enough to cause bursting; high concentrations maintained a high external osmotic pressure and thus protected the tips. Similar Rizvi and Robertson (1965) found that hyphal tips of N. crassa would disintegrate in response to non-metabolizable sugars such as ℓ-sorbose but not in response to fructose or dextrose, although arrestment of growth, some plasmolysis, recovery and regrowth occurred. N. crassa hyphae treated with snail enzyme in the medium grew with a colonial type of morphology with a high rate of apical branching, but in bathing solutions, snail enzyme resulted in plasmolysis before regrowth. Addition of both ℓ-sorbose and snail enzyme to the medium resulted in a reduction of sorbose-induced disintegration, possibly through the release of metabolizable sugars from the medium by snail enzyme. A high percentage of Mucor rouxii hyphae flooded with 0.01 M acetic acid (pH 3.4 to 5.5 adjusted with NaOH), 0.01 M EDTA (pH 7.0), or 0.01 M sodium hexametaphosphate

(pH 5.7 to 6.8) would burst, but those flooded with 0.01 to 100 mM NaOH (pH 9 to 13) or distilled water did not burst; polyethyleneglycol could partially protect hyphae as could Ca^{++} to a lesser extent (Dow and Rubery, 1975). These results were explained as resulting from the antagonistic effects of H^{+} and Ca^{++} on the physical properties of the wall polymers whereby cell extension was inhibited by Ca^{++} and thus hardening occurred, while H^{+} had the opposite effect. N. crassa hyphae damaged by flooding with water or by cutting rapidly plugged the septal pores with hexoganol crystals identified as ergosterol, and then resumed growth by the production of intra-hyphal branches from the plugged septa, side branches just below them, or multiple branches from the lateral walls (Trinci and Collinge, 1974). This mechanism was also assumed to be responsible for progressive ageing and lateral branching in normally maturing mycelia.

Other physical parameters reported to result in morphogenesis of fungi included cell density (Aldridge and Pye, 1976; Hashimoto et al, 1975) and γ-irradiation Kuzin et al, 1976).

Metabolizable sugars such as maltose, but not non-metabolizable sugars such as xylose or α-methyl glucoside, blocked subsequent development and morphogenesis of Dictyostelium discoideum but this inhibition could be partially overcome by the addition of nanomolar concentration of c-AMP only if development had been allowed to

occur beyond the formation of c-AMP binding sites (Ramsdorf et al, 1976). Three yeasts, Schizosaccharomyces pombe, Pichia farinosa and Saccharomyces cerevisiae could be lysed by 2-deoxyglucose or its derivatives, probably by interference with the resynthesis, by the addition of glucose, of the glucan layer which was broken to allow cellular growth (Johnson, 1968).

Different amino acids had the ability to shorten or lengthen the internodes of Mucor hiemalis and to increase or decrease the degree of lateral branching, with an increase in lateral branching also resulting in branches being initiated closer to the hyphal tips, opposing actions of these amino acids were also detected (Plunkett, 1966).

Distortion of A. niger hyphae, including stunting, branching, and curling, was induced by Vincent (1947) with methyl ρ-hydroxybenzoate, anisic, salicylic and benzoic acids and phenol at concentrations of 3×10^{-4} molar, at which concentrations these effects were readily detected; ρ-hydroxybenzoic acid and m-hydroxybenzoic acid had very little morphogenic ability.

Inhibition of protein synthesis with cycloheximide and osmotic shock have both been shown to cause increased septation and branching in hyphae of A. nidulans (Katz and Rosenberger, 1971).

CHARACTERISTICS OF SECONDARY METABOLITE ASSOCIATED GROWTH

Weinberg (1970, 1971) defined secondary metabolite production as a differentiated process resulting in the accumulation of apparently useless materials once growth has stopped, and also (1970) described the differentiation process as specific enzyme induction required for secondary metabolite production. Demain (1968) discussed the roles of feedback induction and catabolite repression in controlling production of industrial metabolites, and thereby assumed a differentiated process was involved.

Demain (1973) described the technique, now nearly out-moded, due to a rapid increase in the number of mutants sought, of detecting better producers of secondary metabolites through the use of morphological mutants. A morphological mutant of Neurospora crassa, co$_{2}$ -2 accumulated glucose-6-phosphate because of a point mutation involving the structure of G-6-P dehydrogenase; the strain had a "dense compact and highly branched colony structure as compared to the spreading filamentous form of the wild type strain" (Brody and Tatum, 1966). Similar mutations resulting in glucose-1-phosphate accumulation and a high branching frequency were described for three other N. crassa morphological mutants with low phosphoglucomutase levels (Brody and Tatum, 1967). Other morphological mutants with G-6-P dehydrogenase defects have also been described (Scott and Tatum, 1976).

The morphology of organisms during the accumulation of secondary metabolites, i.e. in the idiophase, as opposed to the growth or trophophase is rather characteristic. Currie (1917), and later Snell and Schweiger (1949), commented on the lack of conidiation as a characteristic of citric acid-accumulating Aspergillus niger. The exclusion of conidiation to secondary metabolite production has been termed a general feature by Weinberg (1970). Pellets of A. niger accumulating citrate were small, round and smooth, with none of the filamentous mycelial growth which was found under non-accumulating conditions (Clark, 1962). The production of gibberelic acid by Gibberella fujikoroii was accompanied by cessation of growth and vacuolation of the hyphae (Barrow et al, 1964). Duckworth and Harris (1949) described in detail the morphological variations which occurred in Penicillium chrysogenum upon the initiation of penicillin production, the most notable being increased branching, which was attributed to the shake-flask method of culture, but which has also been found since then in secondary metabolite producing cultures.

Production of alkaloids by Penicillium cyclopium Westling was preceded and accompanied by a definite differentiation process during which penicilli and conidia were produced and new specific enzymes were synthesized (Nover and Luckner, 1974). Aspergillus nidulans synthesized

low molecular weight phenols during the exponential phase of growth which were further converted to melanin concommitantly with conidiophase formation as the growth phase declined (Carter and Bull, 1969). Bu'Lock's review on intermediary metabolism (1961) mentioned morphological changes associated with penicillin production by P. chrysogenum and tetracycline production by Streptomyces species.

CONTROL OF SECONDARY METABOLITE PRODUCTION

A definite change in morphology occurs in fungi with the onset of secondary metabolite production. As there are a variety of substances which can influence the morphology of fungi, a variety of factors affect or control secondary metabolite synthesis. Secondary metabolism is, in fact, extremely sensitive to any variation from the conditions optimum for accumulation.

As evidenced by the N. crassa morphological mutants which accumulate G-6-P and G-1-P (Brody and Tatum, 1966, 1967) production of secondary metabolites was affected, if not controlled, by mutation. Lysine-requiring mutants of yeast accumulated α-ketoglutarate (Bhattacharjee et al, 1967). Another yeast mutant, a glutamate auxotroph, accumulated citric acid, succinic acid and α-ketoglutaric acid (Crocker and Bhattacharjee, 1973). Variations in penicillin production among 'wild' strains of A. nidulans

were shown to be genetically controlled, so that the capability to produce penicillin could be associated with four linkage groups (Cole et al, 1976).

Metabolism and secondary metabolite production of Penicillium uriticae were found to be closely linked, so that the induction of the idiophase could be described as a differentiated process resulting from stepwise enzyme induction (Bu'Lock et al, 1974). Similarly Nover and Luckner (1974) described the differentiated process resulting in antibiotic production by P. cyclopium Westling. Production of alkaloids by P. cyclopium Westling could be inhibited with cycloheximide and was resumed when the inhibitor was removed, initially at a rate much higher than that found in uninhibited cultures (Nover and Muller, 1975).

The role of trace metals has proven to be of central importance to secondary metabolite production. Weinberg (1962, 1970) has reviewed trace metal involvement in the idiophase development and has listed trace metals specifically involved in enhancing or depressing accumulation of metabolites in different systems.

Substrate and carbon sources present in the fermentation medium are important. Flavin production by Candida species and Torulopsis fumate-0-3 was enhanced by the provision of hydrocarbons other than glucose and subject to inhibition by iron (Dikanskaya and Gorobtsova, 1975). The addition of 0.5% of one of the Tweens (40, 60 or 80), particularly

Tween 80, but not the respective free fatty acids, enhanced alkaloid synthesis by Aspergillus fumigatus, probably by mediating substrate transport across the cell membrane (Rao and Rao, 1975). Growth and aflatoxin production were not supported by the same carbon sources, although the best growth was also achieved with those carbon sources-glucose, ribose, xylose and glycerol-which supported aflotoxin production (Davis and Deiner, 1968).

Limitation of nitrogen, phosphorous, sulfur or magnesium induced the synthesis of citrate or isocitrate by Candida lipolytica; with glucose as the carbon source citrate was the product, whereas hexadecane as the carbon source resulted in a mixture of citrate and isocitrate. Moreover, if growth was limited by thiamine the products were ketoacids-on glucose the product was pyruvate, and on hexadecane the product was α-keloglutarate (Rozinov et al, 1973). In the presence of 0.1% butyrate Streptomyces kitasatoensis produced leucomycin A-1 to a greater extent, but in its absence leucomycin A-3 was the major product (Omura et al, 1976).

Other factors have also been found to be important in controlling secondary metabolite production. Phosphate levels over 1 mM inhibited candicidin production by Streptomyces griseus, without affecting the protein or RNA synthesis necessary for antibiotic production (Martin and Demain, 1976). Weinberg (1974) reviewed the roles of

phosphate and temperature in the control of secondary metabolism. Increased aeration enhanced growth of Penicillium expansum, but suppressed patulin accumulation (Woodhead and Walker, 1975).

CONTROL OF CITRIC ACID PRODUCTION

Many of the same or similar, factors that control secondary metabolite production in general have been found to regulate the citric acid fermentation.

Mutagenesis of Aspergillus niger with ultra-violet rays and a combined action of nitrosomethyl urea and u.v. rays resulted in a mutant that produced 22% more citric acid and half as much gluconic and oxalic acids as the parent strain (Shcherbakova et al, 1974). Higher-yielding mutants were also produced with continuous γ-irradiation (Kuzin et al, 1976). Chang and Terry (1973) established that citric acid producing ability was inherited through nuclear genes.

Growth conditions have been extensively studied, and have been found to be important parameters seriously affecting yields of citric acid. A low initial pH (Currie, 1917; Foster, 1949) improved the yield of citric acid and decreased the amount of oxalic acid which was formed concommitantly, especially at higher pH (Wehmer, 1891; Foster, 1949) due to induction of oxalacetase (Lenz et al, 1976). The temperature at which citric acid production was usually carried out was a compromise

between optimum growth temperature and optimum accumulation temperature which tended to be about 10°C apart (Kovats, 1946; Perlman, 1947; Yamada and Hidaka, 1964). Sufficient aeration has generally been acknowledged as necessary. A requirement for oxygen for citric acid production or to enhance citric acid production has been shown by Perquin (1938), Shu and Johnson (1948 a), Shu (1953), Martin and Waters (1952), Clark and Lentz (1961), Lockwood and Schweiger (1967) and Horitsu (1971). A requirement for sufficient aeration for growth to occur was also indicated by Martin and Waters (1952), Clark and Lentz (1961) and Lockwood and Schweiger (1967). However, the necessity of controlled, non-excessive aeration for growth has also been reported (Perquin, 1938; Horitsu, 1971).

Various carbon sources have been investigated as potential raw materials for industrial citrate production (Hang et al, 1975; Stell et al, 1954), but use of substances such as sugar beet molasses or brewery spent grain liquor was coupled with the difficulty of developing efficient techniques to overcome trace metal contamination (Noguchi and Johnson, 1961; Clark, 1962; Choudhary and Pirt, 1965). High concentrations (10-20%) of chemically pure sucrose have been shown to be optimal for citric acid production; higher levels of sucrose inhibited growth while lower levels decreased the yield of citric acid (Johnson, 1954).

Requirements for sources of N, Mg, S and P as well as carbon and oxygen have been disputed only as to the chemical forms and concentrations at which the citric acid

yield was optimal; this difficulty probably has arisen only because of strain variability.

Opinions on trace metal nutrition and control have been far more diverse. Optimal trace metal components and concentrations have differed for growth and for citric acid accumulation. Zinc at low concentrations (ie. trace amounts) is probably necessary for growth; for the production of citric acid different authors have indicated an enhancement of production by Zn (Perlman and Sih, 1960; Porges, 1932) or inhibition by excess Zn (Currie, 1917; Shu and Johnson, 1948 b; Tomlins et al, 1950, 1951; Trumpy and Millis, 1963; Sanchez-Marroquin et al, 1970; Wold and Suzuki, 1976 a; Banik, 1976). Sanchez-Marroquin et al (1970) reported optimum citric acid production of up to 98.7% yields from carbon sources in the presence of low concentrations of Fe, Zn and Cu. However the validity of the results was coloured by the fact that the carbon sources used had trace metals in them. Banik (1976) found 1.0 µg/ml of Fe, Mn and Zn to be optimal for citric acid production with sucrose as the carbon source, while Cu, Co and Mo inhibited production. Wold and Suzuki (1976 a) in a carefully controlled study found only Zn to be important as a control of acidogenesis. High Zn favored growth, while low Zn, below 1.0 µM, favored citric acid production. They also found (1972, 1976 b) that 3',5'-cAMP enhanced both growth and acidogenesis, i.e. worked in conjunction with the control effected

by Zn. Studies have also indicated some degree of control by iron (Shu and Johnson, 1948 b; Perlman and Sih, 1960; Das and Nandi, 1969; Currie, 1917; Tomlins et al, 1950, 1951; Trumpy and Millis, 1963; Snell and Schweiger, 1949; Banik, 1976) and by manganese (Clark et al, 1976; Perlman and Sih, 1960; Das and Nandi, 1969; Banik, 1976; Kubrith and Rohu, 1977).

Aided by the use of radioactively-labelled substrates, workers have established the metabolic pathways involved in both growing and citric acid-accumulating A.niger. Isolation of specific enzymes has confirmed these pathways as well as having shown that genetic defects in enzyme constitution was not to blame for the accumulating characteristic. More recently, kinetic mechanisms of postulated controlling enzymes have been determined in order to elucidate a difference between those of citrate-accumulating and non-accumulating cultures. Metabolic pathways, including the tricarboxylic acid cycle (Weinhouse and Lewis, 1949; Lewis and Weinhouse, 1951; Cleland and Johnson, 1954; Ramakrishnan 1954; Martin, 1954), the Embden-Meyerhof pathway (Cleland and Johnson, 1954; Shu et al, 1954), a glucose-repressible glyoxylate cycle (Collins and Kornberg, 1960) and at least an indication of the hexose monophosphate pathway (Smith et al, 1971) have been confirmed. The presence of TCA cycle enzymes under non-accumulating conditions (Martin; 1954; Ramakrishnan, 1954) and under accumulating conditions (Ramakrishnan et al, 1955; Ahmed et al, 1972)

has been shown. Bertrand and De Wolf as well as Smith et al (1971) have detected several EMP enzymes. Ahmed et al (1972) found a functional citrate synthase, succinic dehydrogenase, fumarase, NAD and NADP-linked isocitric dehydrogenase and malic dehydrogenase at all stages of the fermentation. No indication of control at the level of NADP-specific isocitric dehydrogenase (Chan et al, 1965; Horitsu and Clark, 1966), pyruvate carboxylase (Feir and Suzuki, 1969) or phosphofructokinase (Chung, 1975; Bilous, 1977) purified from A. niger has been found.

Besides the enhancement of citric acid production by cAMP (Wold and Suzuki, 1976 a) promotion of aggregation of the conidia in submerged liquid culture by cyclic AMP and 5'GMP has also been found (Wold and Suzuki, 1973).

MATERIALS AND METHODS

MATERIALS AND METHODS

CULTURE

The early part of this work was done with a strain of Aspergillus niger isolated from A. niger NRC A-1-233 (same as A. niger Wis 72-4, ATCC 11414, Perlman et al, 1946). Due to strain deterioration, use of this strain was abandoned. New cultures of A. niger NRC 401121 and ATCC 26550 (isolated from NRC A-1-233, Wold and Suzuki, 1976 a) were obtained. A strain isolated from ATCC 26550 was used for the majority of this work since it most closely resembled the original NRC strain with respect to growth on various media, sporulation, Zn-sensitivity and citric acid production.

Originally cultures were maintained on a synthetic medium (M-1 of Feir and Suzuki, 1969) and subcultured to brain-heart infusion agar (BHI). All work was done with cultures grown up on BHI. Because of rapid strain deterioration, a new means of maintaining stocks was sought. Subsequently, a stock of spores grown on agar slants of malt-yeast extract medium (MYE) was stored in dry sterile soil at $4^{\circ}C$. For each experiment, an aliquot was shaken out onto a slant of MYE and incubated for three days. Dry spores were then transferred with

a wire loop to a fresh slant of MYE. A dry inoculum was used to minimize carry-over of trace metals. After sporulation, 5 ml of 0.5% Tween 80 was added to the slant and the spores were gently suspended into it with a wire loop. This suspension was used to inoculate slants of BHI. This process of transfer to BHI after growth was then repeated twice, after which the spore suspension obtained was used to inoculate experimental flasks or petri plates as required.

MEDIA

1. Brain-heart Infusion Agar (BHI)

This medium was used to grow cultures for experimental use and was made up as recommended on the label (BBL). Screw cap test tubes (30 ml) containing 10 ml of the medium were sterilized by autoclaving at 250°C, 15 psi, for 10 minutes, then set at a 10° angle so that the agar hardened to form slants.

2. Malt-Yeast Extract Medium (MYE)

This medium was prepared in slants (as described above) with the following composition (w/v): malt extract, 3%; yeast extract 0.5%; dextrose, 0.5%; agar 1.5% in Millipore-deionized distilled water.

The medium was recommended by the National Research Council of Canada for maintenance of A. niger.

It proved superior to the synthetic M-1 (described below) and BHI for stock culture maintenance.

3. M-1

The medium was used by Feir and Suzuki (1969) for A. niger maintenance and was prepared in four parts. The composition was: Part A, sucrose, 8.8 g; $(NH_4)_2HPO_4$, 1.65 g; $FeSO_4.7H_2O$, 0.01 g; $MnSO_4.4H_2O$, 0.001 g; water 300ml: Part B, $MgSO_4.7H_2O$, 0.2 g in 100 ml water; Part C, $CaCl_2.H_2O$, 0.1 g in 100 ml water; Part D, K_2HPO_4, 0.1 g; $Na_2HPO_4.7H_2O$, 28.8 g; NaH_2PO_3, 13.9 g, water 500 ml (pH 6.6 to 6.8).

All water used was Millipore-deionized distilled. The four solutions A, B, C and D were autoclaved separately and then mixed asceptically. For the preparation of slants, 1.5% agar was added to solution A.

4. M-3

The medium was used by W.S.M. Wold (1974) for citrate accumulation studies. The composition was: sucrose 8.0 g; NH_4PO_3, 2.5 g; KH_2PO_4, 2.5 g; $MgSO_4$, 0.25 g; Millipore-deionized distilled water to one liter.

Salts were prepared as a 50 X concentrated solution, autoclaved and stored.

5. Assay Medium for Branching

The medium was used by David Park (1961) to study the effects of culture staling in Fusarium oxysporum and

to study the morphogenic effects of various fungi on one another (1963). This medium was ideal for the study of hyphal branching because it was dilute and clear so that microscopic observations could be made easily. The composition of the medium was: glucose, 0.7 g; $MgSO_4.7H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4NO_3, 0.1 g; agar, 15 g; Millipore-deionized distilled water to one liter.

Ten milliliters of medium were used per (15 x 100 mm) Petri plate. The salts for this medium were prepared as 100 X concentrated stock solution and autoclaved.

STOCK SOLUTIONS

Zinc sulfate was made up at 44.4 mg $ZnSO_4$ per liter of deionized distilled water and sterilized by autoclaving at $250^{o}F$ for 15 minutes. It was added to the medium at 0.4 mg $ZnSO_4$ per liter in most cases, or at other concentrations as indicated in the results section, to inhibit the accumulation of citric acid.

Tween 80 (0.5 ml) was dissolved in 100 ml of Millipore-deionized distilled water. The solution was autoclaved at $250^{o}F$ for 15 minutes.

Mitomycin C was dissolved (1.0 mg/ml) in M-3 or ethanol where indicated. Sterile medium was injected directly into the vial and the solution was stored frozen.

Chloramphenicol was dissolved (1.0 mg/ml) in M-3 or (sterile) or added asceptically to the culture as a powder because of its low solubility.

Cycloheximide and actinomycin D were dissolved (1.0 mg/ml) in sterile M-3 by injecting medium into the vials; the solutions were stored frozen.

Citric acid was prepared as a 1.257 M stock solution of citric acid monohydrate in Millipore-deionized distilled water. The solution was autoclaved and stored in an acid washed glass bottle.

GROWTH CONDITIONS

Cultures for stocks were grown on the media described. Sporulation was usually complete after four days growth, at which time a culture was either sub-cultured or used directly. Incubation was at 28 ± 1°C in screw cap test-tubes with the caps loosened to ensure adequate aeration; lack of sufficient aeration resulted in poor or no sporulation.

Park's medium plates were inoculated with spores suspended in 0.5% Tween 80 at about 10^7 spores/ml. The plates were incubated at 28°C for three days prior to use.

Liquid media were dispersed 55 ml per 250 ml flask or 20 ml per 125 ml flask into Erlenmeyer flasks

that had been washed in 6N HCl and rinsed twice in Millipore-deionized distilled water.

Liquid media were always inoculated with 0.5 ml of spore suspension per 55 ml of medium. The spore suspension contained 10^7 viable spores per ml of 0.5% Tween 80 and resulted in a final spore concentration of 1.8×10^5 spores per ml medium. A 1 in 5 dilution in 0.5% Tween 80 of the spore suspension containing 10^7 spores/ml gave a reading of 130 Klett units (red filter). Viable spore counts were obtained by growing diluted aliquots on MYE and/or M-1 and counting the colonies.

Flasks were incubated at 28 ± 1°C on a New Brunswick Scientific rotary shaker at 150 rpm for the required incubation time.

Zinc or citric acid were added before the cultures were inoculated, i.e. at "0" time and designated as "Zn" or "ca" culture, or at later times where indicated and designated both by the addition and the time of addition; for example Zn(20) designated a culture to which $ZnSO_4$ had been added after 20 hours of growth. Antibiotics were added from the stock solutions or as powders at 20 hours, or later, where indicated.

SAMPLING AND PREPARATION FOR ANALYSES

Samples of media for sucrose and citric acid determinations were withdrawn from flasks with sterile pipettes; care was taken to exclude mycelium from the samples. In cases where a dry weight determination was also

made, a sample of medium was taken after the mycelium had been filtered out. Samples were stored frozen at -16°C.

For the determination of dry weight, the contents of a flask were filtered by vacuum through a 61 µm nylon mesh on a Buchner funnel, then washed with Millipore-deionized distilled water. The mycelium was transferred to a pre-weighed glass vial and dried at 60°C for at least twelve hours. At least 10 hours were required for drying, and after 12 hours constant dry weight was established.

Before determination of internal sucrose, citrate, RNA, DNA and protein the mycelial volume was measured by displacement of water in conical centrifuge tubes. The samples were then either frozen or homogenized immediately in water before freezing.

Homogenization was done by hand using a Bellco 7 ml capacity ground glass homogenizer. Complete disruption of the mycelium was determined by observing samples under 160 power with a Zeiss Jena phase contrast microscope. If the homogenate were frozen, cell material tended to coalesce and had to be rehomogenized before analysis.

Homogenized samples was used directly for the protein determination.

For the determination of RNA, the homogenates were centrifuged for 15 minutes in a Sorvall bench top centrifuge. The supernatants were diluted 1:15 in Millipore water.

For the determination of DNA, centrifuged homogenates were diluted 1:2 in water.

Before the determinations of internal sucrose and citric acid, 0.5 ml of homogenate was combined with 0.5 ml of water and spun 30 minutes in a Sorvall bench-top centrifuge. The pellet was washed in 1.0 ml of water, and the combined supernatants were assayed.

A second preparation for the determination of internal sucrose and citric acid was also used. Mycelia were collected by centrifugation at 15,000 rpm in a Sorvall model RC-5 for 30 minutes. The supernatants were used for the determination of external sucrose and citric acid concentrations. The mycelia were washed twice in 5.0 ml Millipore water and pelleted by centrifugation. One ml of water was added to each pellet before freezing. The mycelia were thawed and homogenized. For the determination of internal sucrose, samples of homogenate were centrifuged for 30 minutes in a Sorvall bench top centrifuge and the supernatants were assayed. No citric acid was found in these samples. Samples of homogenate were made to 0.5 N HCl and centrifuged as above. Citric acid assays were done on these supernatants.

CHEMICAL ANALYSES

Citric acid

Citric acid concentration was determined by using the coupled enzyme assay:

$$\text{citric acid} \xrightarrow{\text{citratase}} \text{oxaloacetic acid} + \text{acetic acid}$$

$$\text{oxaloacetic acid} + \text{NADH} + \text{H}^+ \xrightarrow[\text{dehydrogenase}]{\text{malic}} \text{malic acid} + \text{NAD}^+$$

and following the decrea in absorbance at 340 nm due to NADH oxidation (Daron and Gunsalus, 1962).

The reaction was carried out in a 3 ml silica cuvette containing: 1.6 ml 0.1 M NaH_2PO_4, pH 7.5; 1.0 ml 1.0 mM NADH in NaH_2PO_4 (0.1 M); 0.1 ml 0.02 M $MgCl_2.6H_20$; 0.1 ml citratase; 0.1 ml malic dehydrogenase. Malic dehydrogenase solutions were 100 µl of Boenringer-Mannheim $(NH_4)_2SO_4$ suspension in 5 ml water or 50 µl of Sigma $(NH_4)_2SO_4$ suspension in 5 ml water.

The solution was mixed in the cuvette and the absorbance (A) was measured on a Beckman Acta III recording spectrophotometer before and after the addition of 0.1 ml citrate sample. A plot of ΔA vs citrate concentration was prepared.

Citratase was prepared from Aerobacter aerogenes according to Daron and Gunsalus (1962). A. aerogenes from trypticase soy agar slants was grown up in a 20 l carboy which contained (g/l): trisodium citrate, 9.0; KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4.7H_20$, 0.4; adjusted to pH 7.0

with NaOH. The culture was grown at 37°C for 36 hours then collected by Sharples centrifugation. The cells were washed twice in cold 0.1 M phosphate buffer, pH 7.5, and collected by centrifugation at 15,000 rpm for 20 minutes in a Sorvall RC-2B superspeed centrifuge. The cells were resuspended in 50 ml phosphate buffer and sonicated for one hour in a Raytheon Sonic Oscillator. The suspension was centrifuged at 40,000 rpm (Ti-50 rotor) for one hour in a Spinco Model L centrifuge. The supernatant was decanted and stored at -16°C in 5 ml aliquots. The enzyme was stored successfully up to 18 months. Repeated freezing and thawing, however, caused a rapid loss of activity.

Sucrose

Sucrose concentrations were determined by the anthrone method (Seifter et al, 1950). Anthrone reagent was prepared by adding 2.0 g anthrone powder to one liter of concentrated sulfuric acid. The reagent was kept on ice. Six ml was placed in each of a series of 20 ml screw cap test tubes on ice. A 3 ml sample (10-150 µg sucrose) was layered on the reagent, and did not mix as long as the solutions were kept cold. The reaction was begun by tilting the tubes, the caps were removed and the mixtures were boiled for three minutes in a water bath. The tubes were cooled by being plunged into ice then the relative absorbance of each sample was measured using the red filter on a Klett-Summerson colorimeter.

Protein

Protein was determined by the Biuret method (Ennis Layne, 1957). After color development the tubes were centrifuged for 10 minutes in a Sorvall bench top centrifuge to remove the readily pelleted cellular debris. The extra time did not affect colour development as indicated by the linear bovine serum albumin standard curve.

RNA

RNA was determined by using the orcinol reagent (Schneider, 1957). A. 0.1 ml sample of the mycelial homogenate was diluted to 1.5 ml in water and treated with 1.5 ml of orcinol reagent. The mixture was boiled for 20 minutes in a water bath. The green color was read at 600 nm on a Unicam SP500 spectrophotometer.

DNA

DNA was determined with diphenylamine reagent (Schneider, 1957). Two ml of reagent was added to one ml of sample and boiled for 10 minutes in a water bath. The absorbance was read at 600 nm on a Unicam SP 500 spectrophotometer.

BRANCHING FACTOR

Plate assays

A. niger was grown for three days on Park's assay medium (Park, 1961) at 28° as three discrete colonies per Petri plate. A 5 µl portion of a test solution was placed at the leading edge of the hyphae. After thirty to forty-five minutes the hyphae were observed at a magnification of 160 by phase contrast microscopy to see if branching was induced. Three solutions per colony, or nine per plate, could be tested at one time; the test substance at one location did not interfere with that at any other site. Sterile liquid Park's assay medium was used as a control. Test solutions were not placed on the side of the colony nearest to the edge of the plate because an indiscriminatory branching effect occurred.

Cultures less than three days old gave variable results and tended to branch indiscriminately upon the addition of a test or control solution. Cultures older than three days produced colonies which were too large and thus too close to one another; the effects of neighbouring colonies on the leading hyphae masked the effects of applied solutions. Older colonies could be used if only one or two were grown per plate and crowding was avoided. Three day old cultures were also considered to be mature, as complete sporulation normally occurred at this time, although four days was sometimes required for sporulation of extensively subcultured stock cultures.

Materials to be tested were diluted in liquid Park's assay medium so as not to disturb the metabolite and osmotic balance of the growth medium. The solutions were made 1 mM in Millipore-deionized distilled water then diluted in Park's medium.

Citric acid was assayed on each plate at 10^{-15}, 10^{-16} and 10^{-17} M as well as the test substance. The concentration of 10^{-16} M citric acid was the lowest which gave a positive branching effect and 10^{-17}M citric acid served as a second negative control.

Samples were diluted $1:10^3$ serially from 1 mM in Park's assay medium, then 1:10 and finally 1:2 to establish accurate limiting dilutions.

Extraction of branching factor

The "branching factor" was found in both the medium and the mycelium of citrate-accumulating A. niger cultures.

Mycelium was suspended in 10 ml ethanol per gram wet weight. The slurry was stirred 40 minutes at room temperature, during which time the bright yellow colour of the mycelium was transferred to the solvent and left the cell debris a dull brown-grey colour. The suspension was centrifuged 10 minutes at 10,000 rpm in a Sorval RC-2B superspeed refrigerated centrifuge. The pellet was discarded and the supernatant was dried under an air stream; the sides of the tube were washed down with ethanol. During evaporation a white precipitate formed. The yellow

pigment (YP) remained liquid and collected as a yellow film on the walls of the test-tube. The material was suspended in five ml of Millipore-deionized distilled water.

The solution was filtered through Whatman No. 1 filter paper. The white precipitate and some of the yellow pigment was retained. The filtrate was pale yellow and cloudy. The filter paper was washed with an additional 5 ml of water.

Both the YP and the branching factor from the water fraction could be extracted into ether. The ether solution had to be dried and redissolved in water before it could be tested by the plate assay since ether disrupted cell membranes and thus interfered with the assay.

Acidification of the water fraction or of the ether extract (in water) caused the formation of a white suspension but did not decrease the activities.

The branching factor was stable to boiling for 10 minutes and to storage at 4°, 22° or $-16^{\circ}C$. It could not be dialyzed out of a $24A^{\circ}$ dialysis membrane in water or in NaOH at pH 9.0.

The extract at pH 9.0 was not as active as the water extract, but at pH 1.25 (with HCl) the activity was higher. Neither NH_4OH, NaOH or HCl used to adjust pH interfered with the plate assay at the concentrations used.

SOURCES OF CHEMICALS AND MEDIA

The following chemicals and media were obtained from:

Analar:

tartaric acid

J.T. Baker Chemical Company:

zinc sulfate

malonic acid

oxalic acid

Bausch and Lomb Optical Company:

potassium ferrocyanide

BBL:

brain-heart infusion agar

Boehringer Mannheim:

malic dehydrogenase

Difco:

bacto-agar

yeast extract

Eastman-Kodak:

malic acid

maleic acid

Fisher:

dextrose

ferric sulfate ($FeSO_4.7H_2O$)

manganese sulfate ($MnSO_4.4H_2O$)

magnesium sulfate ($MgSO_4.7H_2O$)

calcium chloride ($CaCl_2.2H_2O$)

disodium phosphate ($Na_2HPO_4.7H_2O$)

sodium phosphate (NaH_2PO_4)

potassium phosphate

citric acid monohydrate

ethylene diamine tetraacetic acid (EDTA)

orcinol

Matheson Coleman and Bell:

Tween 80

ℓ glutamic acid

diphenylamine

succinic acid

Nutritional Biochemicals:

bovine serum albumin

succinic acid

glutamic acid

oxaloacetic acid

cis-aconitic acid

Shawinigan Chemical Co.:

ammonium phosphate

ammonium nitrate

Sigma:

3',5' cyclic adenosine monophosphate (cAMP)

chloramphenicol

actinomycin D

cycloheximide

nicotinamide adenine dinucleotide (reduced)

malic dehydrogenase

isocitric acid

α-ketoglutaric acid

mitomycin C

bovine serum albumin

ribonucleic acid (yeast)

deoxyribonucleic acid (calf thymus)

pyruvic acid

fumaric acid

R E S U L T S

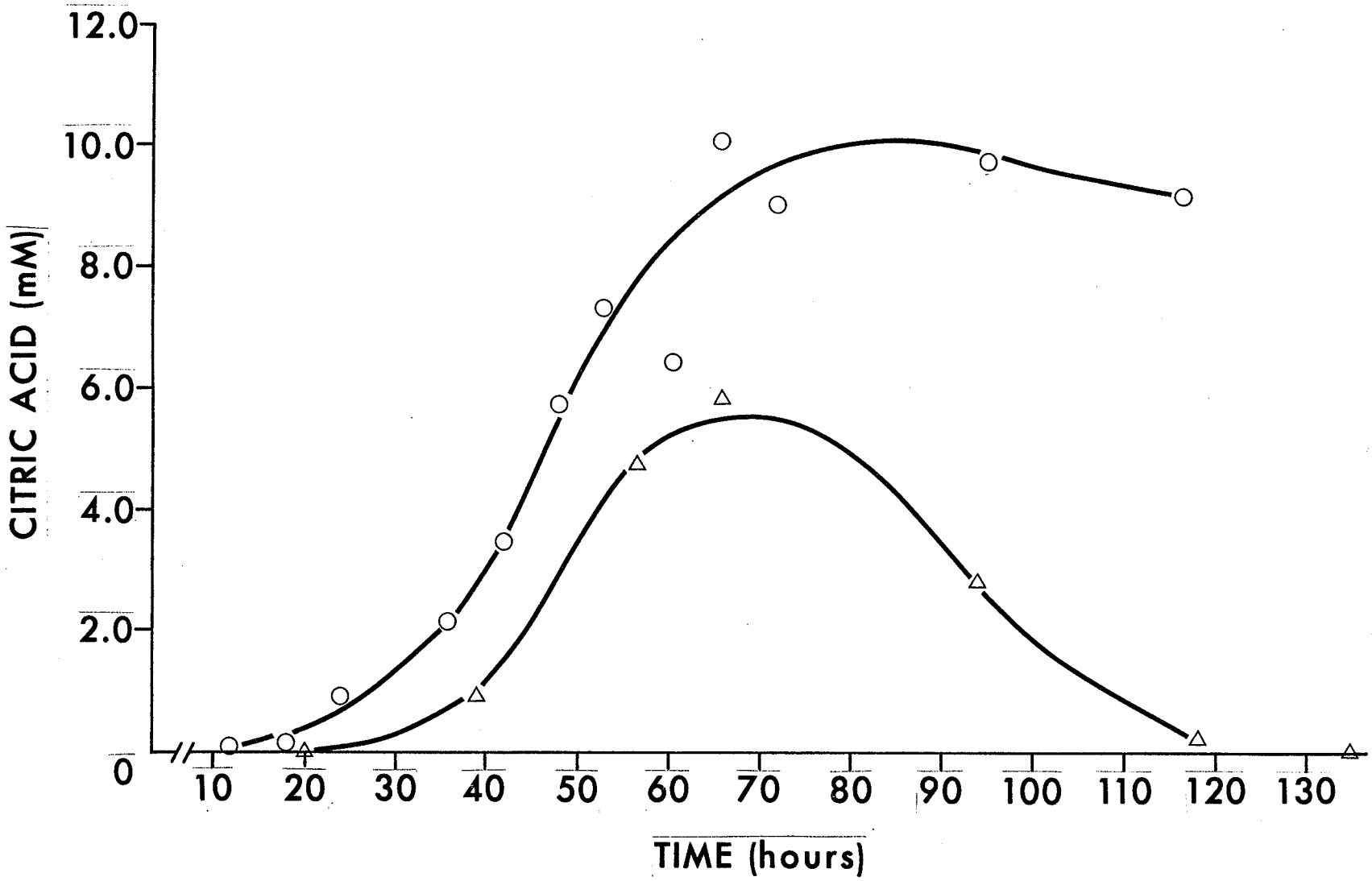
RESULTS

GENERAL METABOLISM

Aspergillus niger (NRC A-1-233 or ATCC 26550) was used to study the controls involved in the accumulation of citric acid. A "good" strain of A. niger possessed the following chara eristics of citrate-accumulation: a minimum of five millimolar citric acid accumulated in zinc-deficient medium, small (1-2 mm) compact yellow pellets formed in liquid Zn-deficient medium, increased growth and pellet size and decreased or no citric acid production were found in the presence of 0.4 mg/l $ZnSO_4$ (Fig. 1). Furthermore well defined sporulating colonies which produced large brownish-black conidia over the entire colony surface within three to four days formed on agar media, a low frequency of spontaneous branching of hyphal tips occurred on solid media, and branching of hyphal tips occurred in response to a challenge of 10^{-16} M citric acid.

A. niger deteriorated rapidly upon repeated subculturing. Citric acid production was reduced, time required for sporulation to occur increased, extent of sporulation decreased, effects of zinc upon metabolism became variable and gross colony morphology was altered.

Figure 1. Citric acid production by A. niger grown in citric acid accumulating conditions (0) and in the presence of Zn (Δ).


12.0
10.0
8.0
6.0
4.0
2.0
0
CITRIC ACID (mM)
10
20
30
40
50
60
70
80
90
100
110
120
130
TIME (hours)

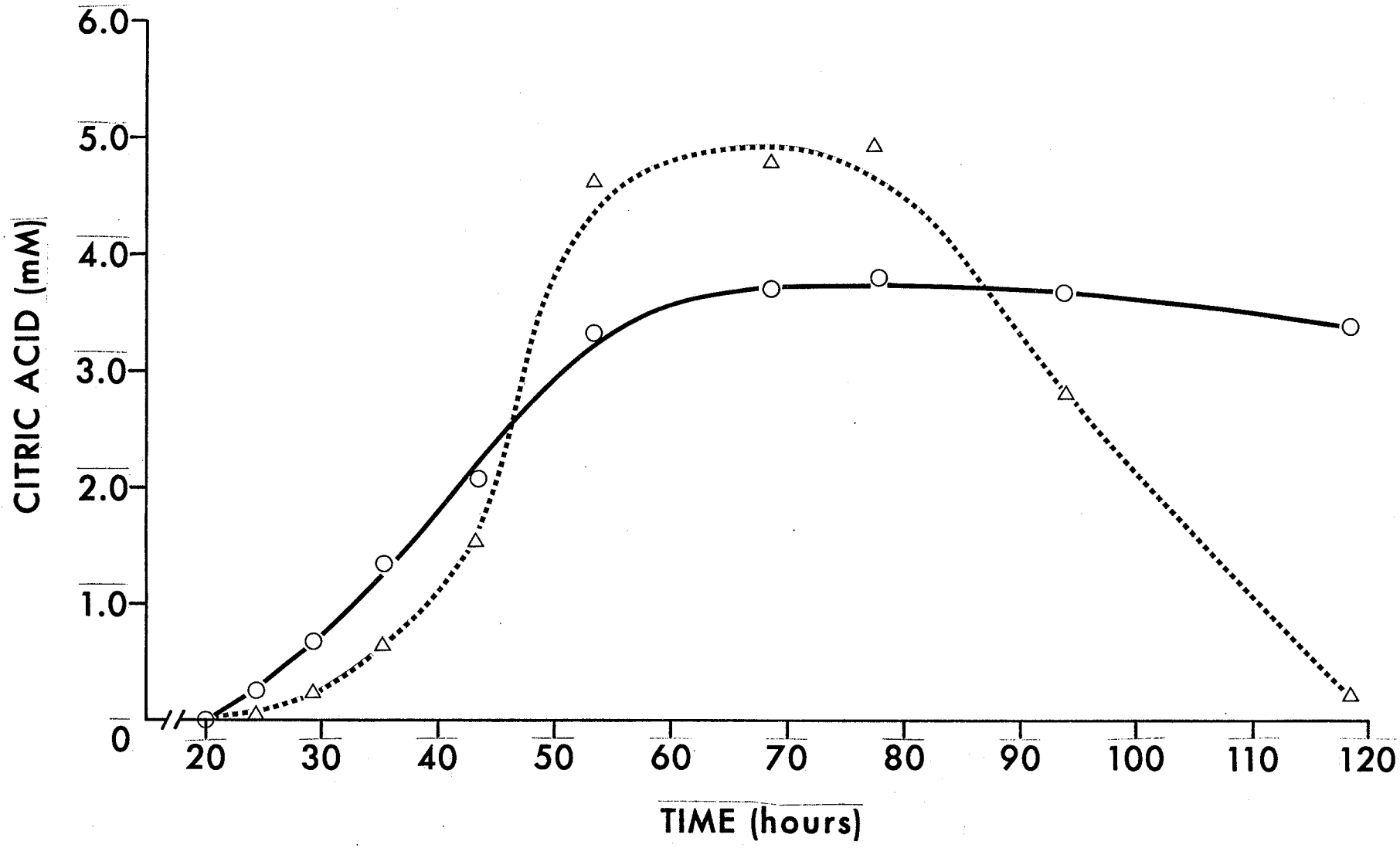
The final concentration of citrate produced on Zn-deficient medium was variable, however (Figs. 1, 3). This might reflect culture variability, trace Zn-contamination, or one of any other environmental fluctuationsto which the fermentation is susceptible.

The highest degree of stability was achieved by storing spores from malt-yeast extract (MYE) cultures in sterile soil at 4°C and culturing as required. Other methods of storage, including refrigeration on various media, always resulted in strain deterioration during the storage period.

All strains used were obtained by selecting for a characteristic morphology on solid media from single spore cultures. These colonies were then checked for the other desirable qualities. A good culture could often be selected from a degenerate culture by selecting single spore colonies in this way.

At times, a subculture from soil stocks was obtained which showed very poor citrate production under Zn-deficient conditions but normal characteristics in the presence of Zn (Fig. 2). These cultures usually produced as much, or more, citric acid in the presence of Zn than in its absence; reutilization in the presence of Zn occurred normally. The response to Zn-deficiency in increased citrate production was lacking, although the shape of the curve was normal. Further subculturing resulted in a normal culture.

Figure 2. Atypical citric acid production by A. niger strains having lost sensitivity to Zn-deficiency due to culture conditions. Cultures were grown in M-3 with (Δ) and without (0) the addition of 0.4 mg $ZnSO_4$/l. Each point is the average of three flasks.


CITRIC ACID (mM)
6.0
5.0
4.0
3.0
2.0
1.0
0
20
30
40
50
60
70
80
90
100
110
120
TIME (hours)

The ability to produce large amounts of citrate in response to Zn-deficiency was found to be of a cyclic nature in these cultures, and depended directly on the number of times the culture was grown on brain-heart infusion agar (BHI) before having been transferred to liquid culture and tested. Cultures grown an even number of times on BHI showed poor citric acid production, whereas cultures grown an odd number of times on BHI (first transfers from soil stocks were never tested) showed "normal" characteristics.

The adaptive response upon transfer to BHI only occurred from soil-stored stocks. Cultures that had been stored on MYE, BHI or the synthetic M-1 showed normal characteristics after any number of passages on BHI, subject to strain deterioration after extensive subculturing.

Growth on MYE, storage in soil and growth on MYE in the presence of soil from stocks subjected cultures to trace metal rich conditions. Upon transfer to BHI, cultures which had been acclimatized to Zn-rich conditions failed to accumulate Zn in their spores. At second and subsequent even-numbered passages a compensation effect might have occurred whereby spores stored trace metals. Thus in the absence of added Zn, enough of the metal might have been present in the spores to suppress citric acid accumulation. Presumably this effect would be lost after extensive subculturing.

Cultures of A. niger on solid media exhibited a normal branching response common to many fungi (Tatum et al, 1949; Robertson, 1958; Park, 1961, 1963; Plunkett, 1966; Park and Robinson, 1966 b; Trinci, 1970; Robinson, 1972). Hyphae branched when challenged with staled culture medium, dilute aqueous solutions of organic acids or when they grew within a few millimeters of the edge of a Petri plate or of another colony.

ZINC EFFECTS

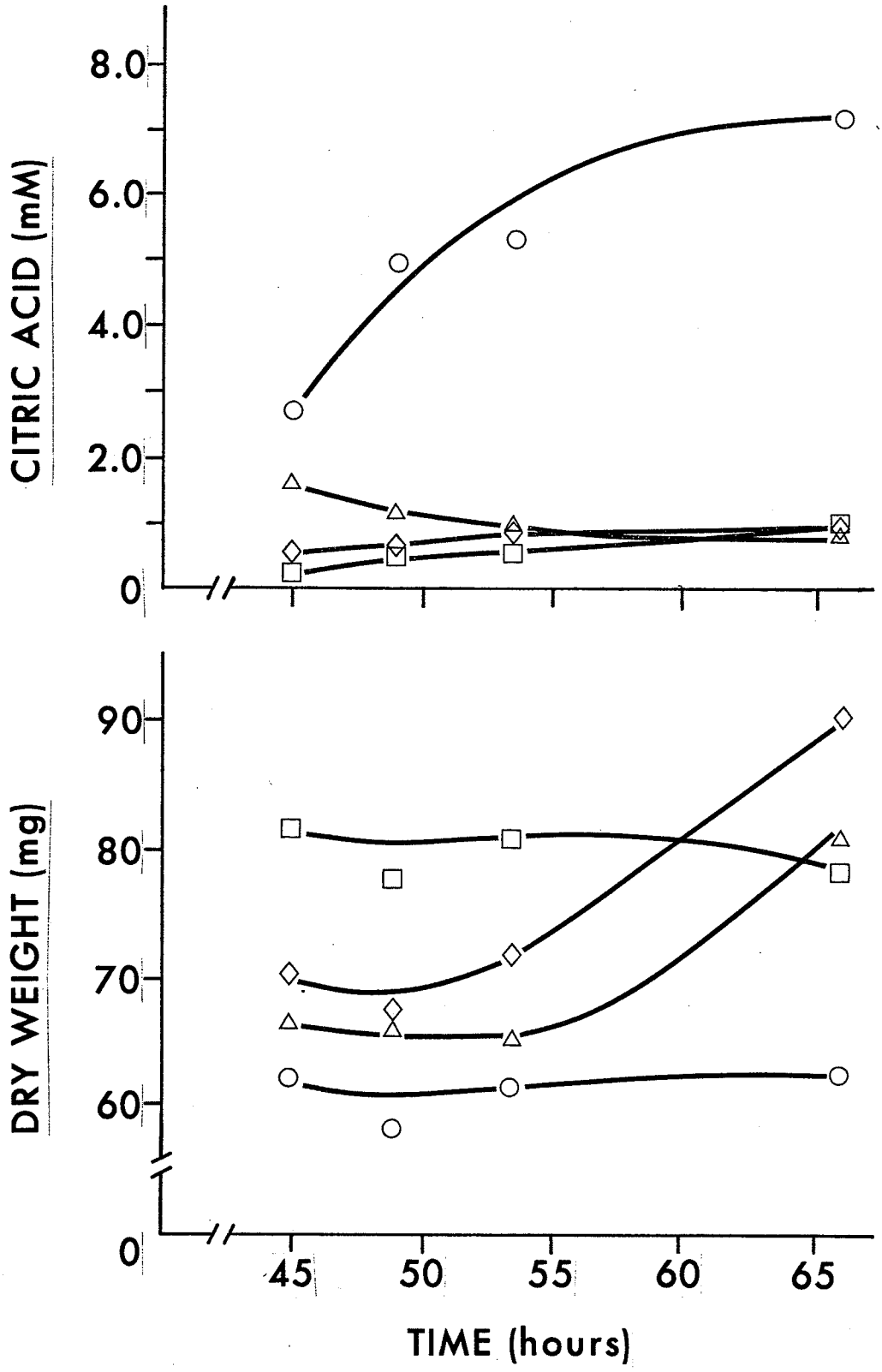
Cultural response to zinc

The addition of zinc as $ZnSO_4$ to the normal citrate-accumulation medium (M-3) inhibited the production of citric acid (Wold and Suzuki, 1976 a). A small amount of citric acid was usually produced even in the presence of Zn, but at very low levels (Fig. 1, Fig. 3a); this citric acid was reutilized to a greater extent than in non-Zn cultures (Fig. 1). In the presence of Zn, growth rather than citric acid production occurred, as indicated by increased dry weight (Fig. 3b).

The amount of citric acid produced decreased as the medium concentration of zinc increased, while the dry weight of the cultures increased (Fig. 3 a, b). In the presence of 0.8 mg/l of $ZnSO_4$ however (0.4 mg/l was normally used as an inhibitory concentration) dry weight did not continue to increase to the same extent as with lower Zn concentrations.

After 50 hours of growth, cultures began to enter the stationary phase of growth. Moreover, reutilization of citrate by Zn cultures commenced at a later time. Therefore the effects of Zn (added at zero time) on A. niger cultures were studied in comparison to non-Zn cultures at that time. Citric acid production at 50 hours decreased as the medium zinc concentration increased

Figure 3. Effects of the addition of Zn to medium M-3 on citric acid production (a) and on culture dry weight (b). Cultures were grown in medium to which 0.4 mg/l (Δ), 0.6 mg/l (◇) or 0.8 mg/l (□) $ZnSO_4$ were added before inoculation, or in the absence of Zn (0). Each point is the average of three flasks.


CITRIC ACID (mM)
8.0
6.0
4.0
2.0
0
DRY WEIGHT (mg)
90
80
70
60
0
45
50
55
60
65
TIME (hours)

(Fig. 4). Dry weight increased with increased levels of zinc, up to 0.4 mg/l of $ZnSO_4$ (Fig. 5). The amount of sucrose used by the growing mycelia was consistent with the dry weight curves. The amount of sucrose used increased with the increased concentration of zinc ion in the medium (Fig. 6).

Thus the amount of zinc in the medium affected the metabolism of sucrose. In the presence of Zn, the metabolism was increased in favour of protein production. The control by Zn can be indicated by a semi-logarithmic plot of log [citric acid] vs [Zn] or as the log of specific activity ($\frac{\text{umoles citric acid}}{\text{g dry weight}}$) vs [Zn] (Fig. 7).

To further test the control exerted by Zn, 0.4 mg/l of $ZnSO_4$ was added to the culture after citric acid accumulation had begun, that is at 20 hours after inoculation. A lag in citric acid accumulation followed by a small amount of renewed synthesis occurred (Fig. 8). However, much less citric acid was produced, and at an earlier time, in the Zn(20) cultures than in the Zn (0) culture.

Because dry weight and sucrose utilization of Zn(20) cultures approached those of Zn(0) cultures, these data have not been presented.

The effects of the addition of zinc to citrate-accumulating cultures at 25, 30 and 35 hours was also tested. The addition of Zn at each time showed the expected inhibition of production of citric acid, followed by re-initiation of production (Fig. 9). In each case, the

Figure 4. Effect of increased concentration of zinc on citrate production. Zinc was added as $ZnSO_4$ at zero time. Samples were taken at 50 hours. Each point is the average of three flasks.

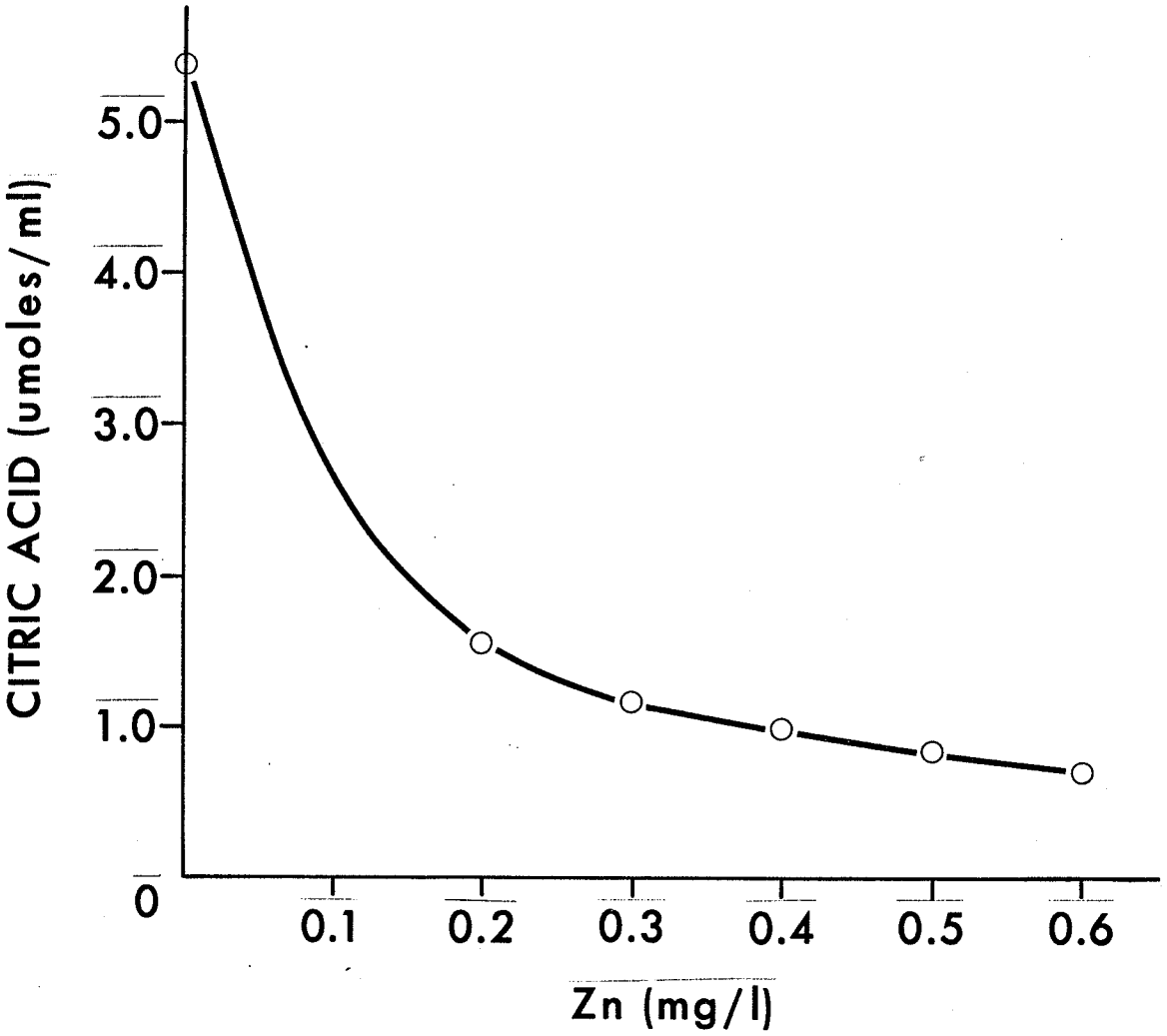

CITRIC ACID (umoles/ml)
5.0
4.0
3.0
2.0
1.0
0
0.1
0.2
0.3
0.4
0.5
0.6
Zn (mg/l)

Figure 5. Effect of increased zinc concentration on the average dry weight. Zinc was added as $ZnSO_4$ at zero time. Samples were taken at 50 hours. Each point is the average of three flasks.

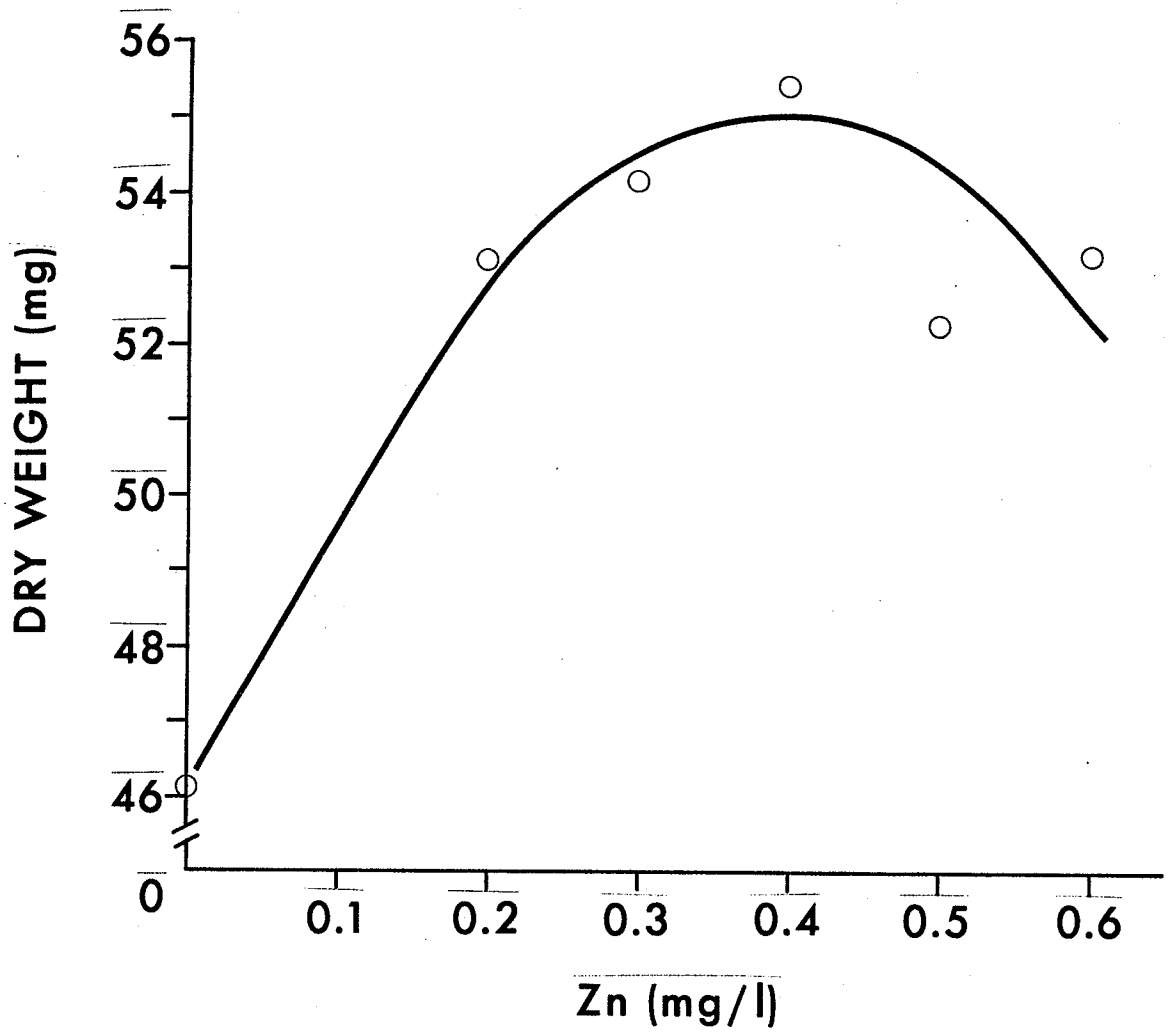

DRY WEIGHT (mg)
56
54
52
50
48
46
0
0.1
0.2
0.3
0.4
0.5
0.6
Zn (mg/l)

Figure 6. Effect of increased zinc concentration on sucrose utilization. Zinc was added as $ZnSO_4$ at 0 time. Samples were taken at 50 hours. Each point is the average of three flasks.

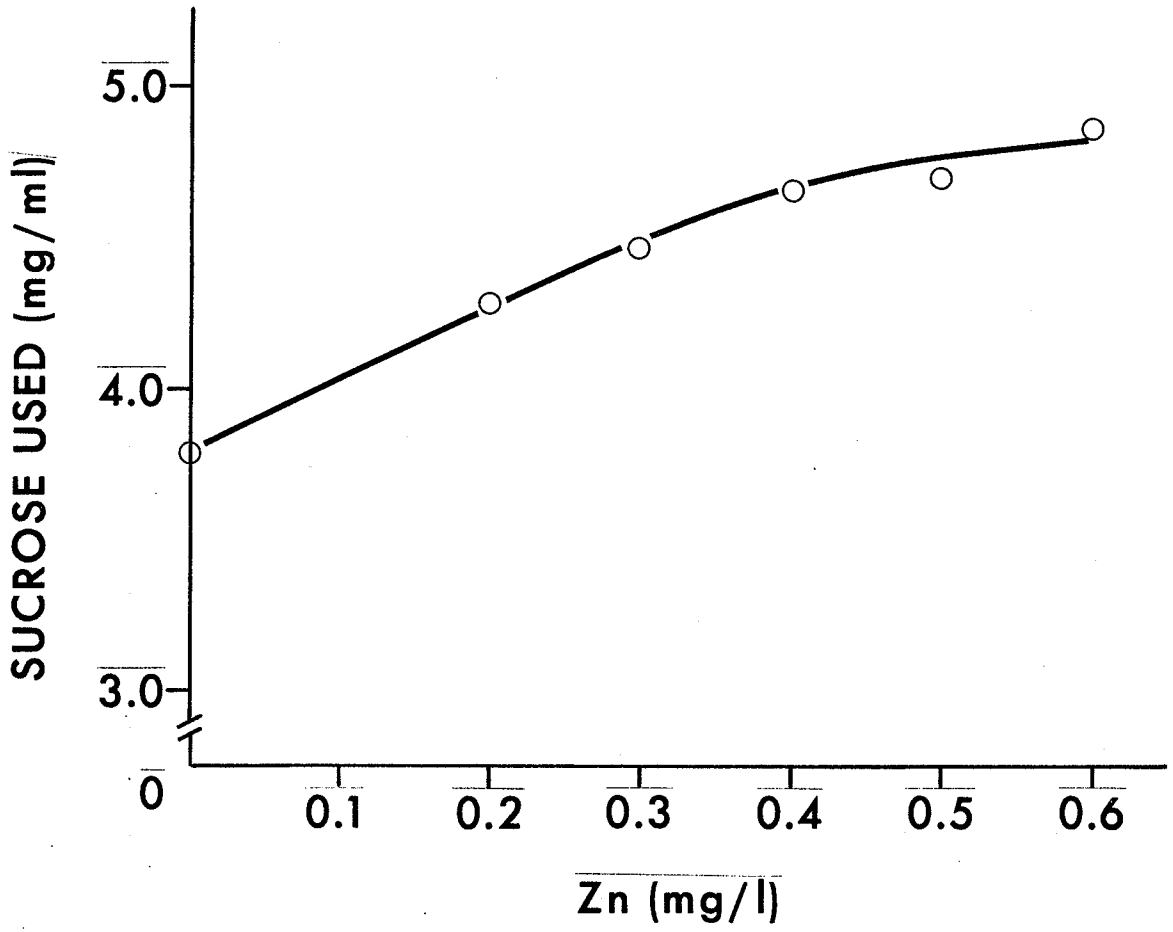

SUCROSE USED (mg/ml)
5.0
4.0
3.0
0
0.1
0.2
0.3
0.4
0.5
0.6
Zn (mg/l)

Figure 7. Control of A. niger metabolism by Zn. Zinc was added as $ZnSO_4$ at 0 time. Samples were taken at 50 hours. Each point is the average of three flasks.

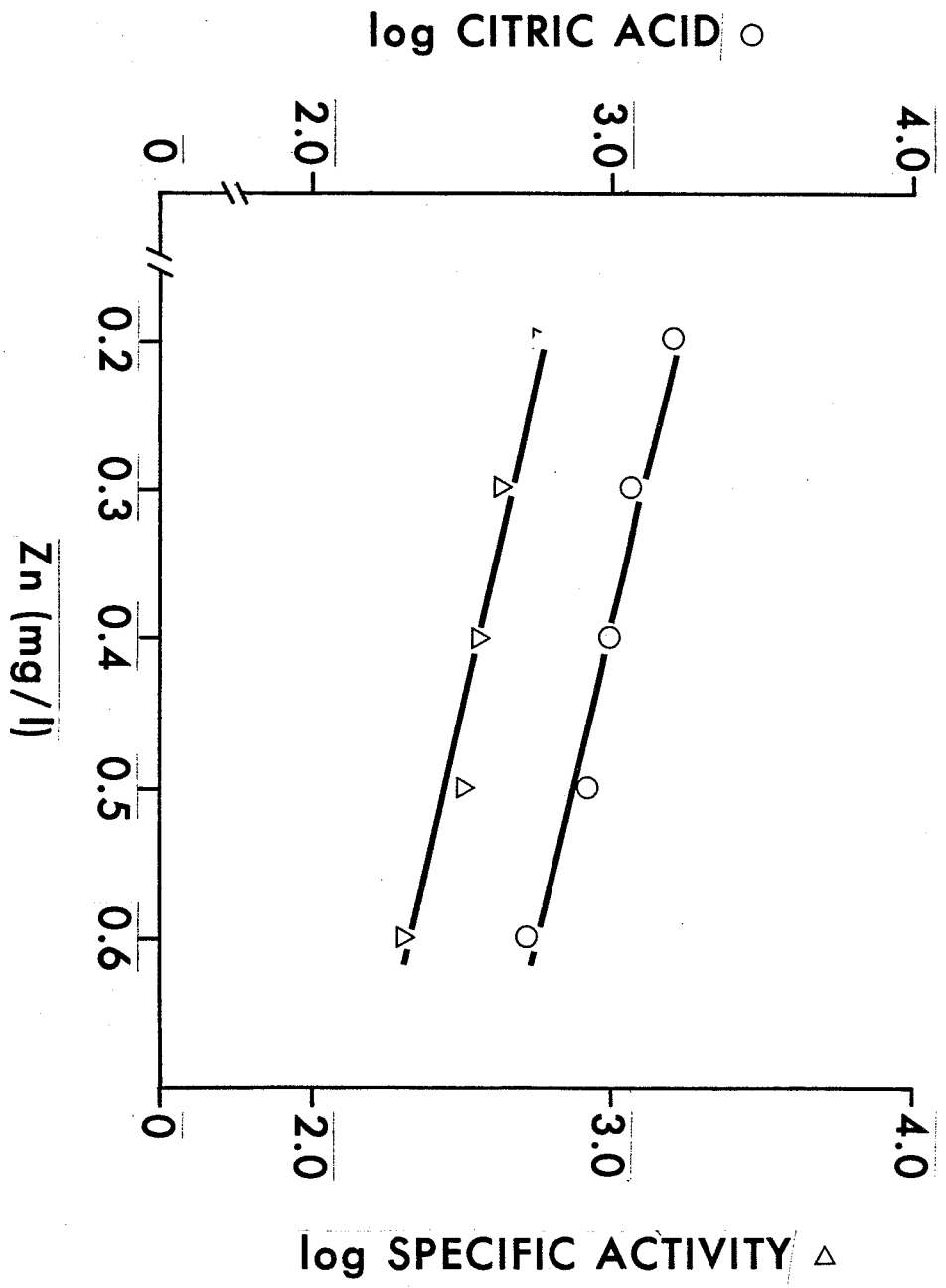

log CITRIC ACID ○
0
2.0
3.0
4.0
0.2
0.3
0.4
0.5
0.6
Zn (mg/l)
0
2.0
3.0
4.0
log SPECIFIC ACTIVITY △

Figure 8. Effects of the addition of zinc on citric acid production by A. niger grown in M-3. Zinc was added as $ZnSO_4$ at 0.4 mg/l at 0 time (Δ) or after 20 hours (◇). Control cultures had no zinc added (0). Each point is the average of three flasks.

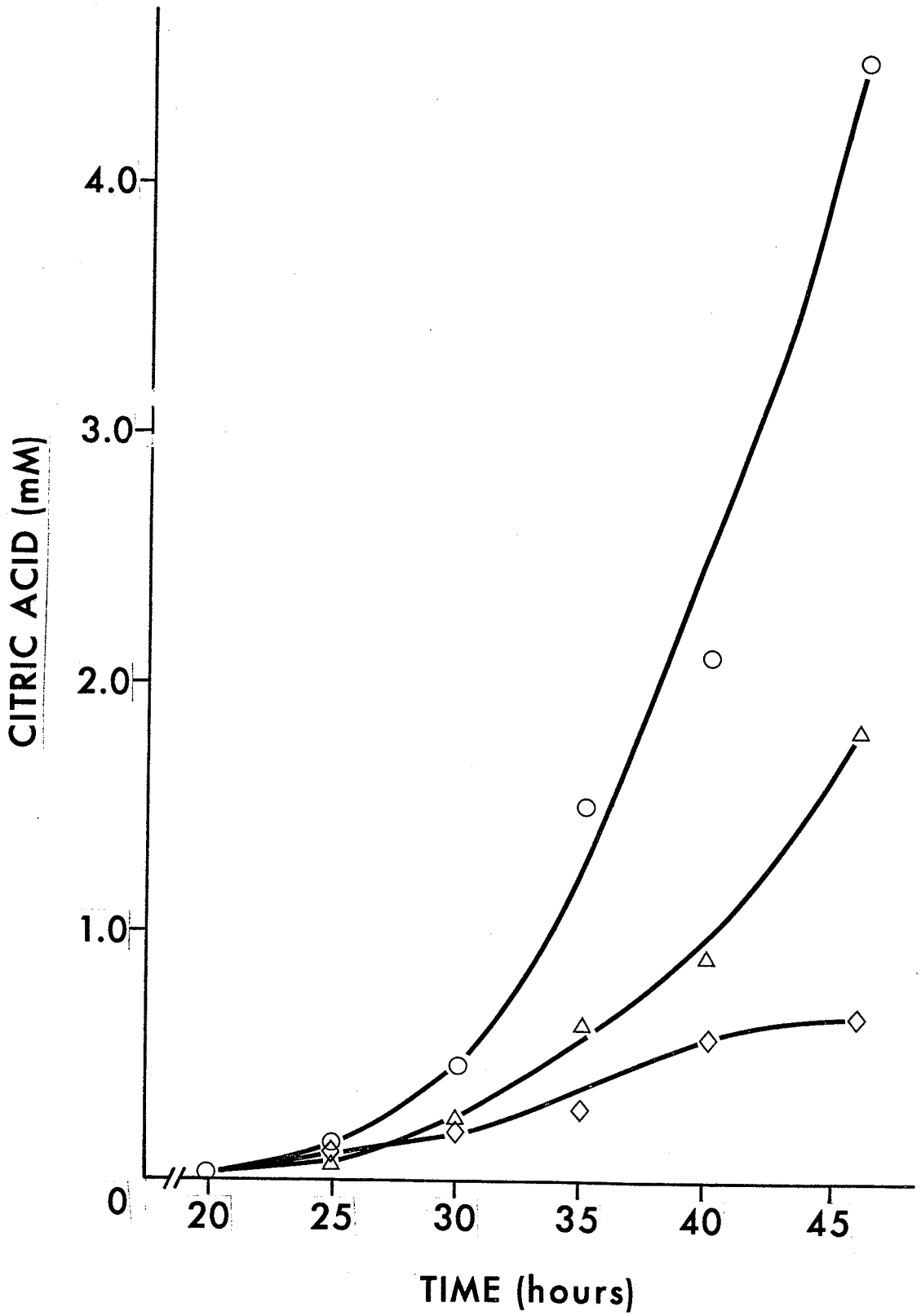
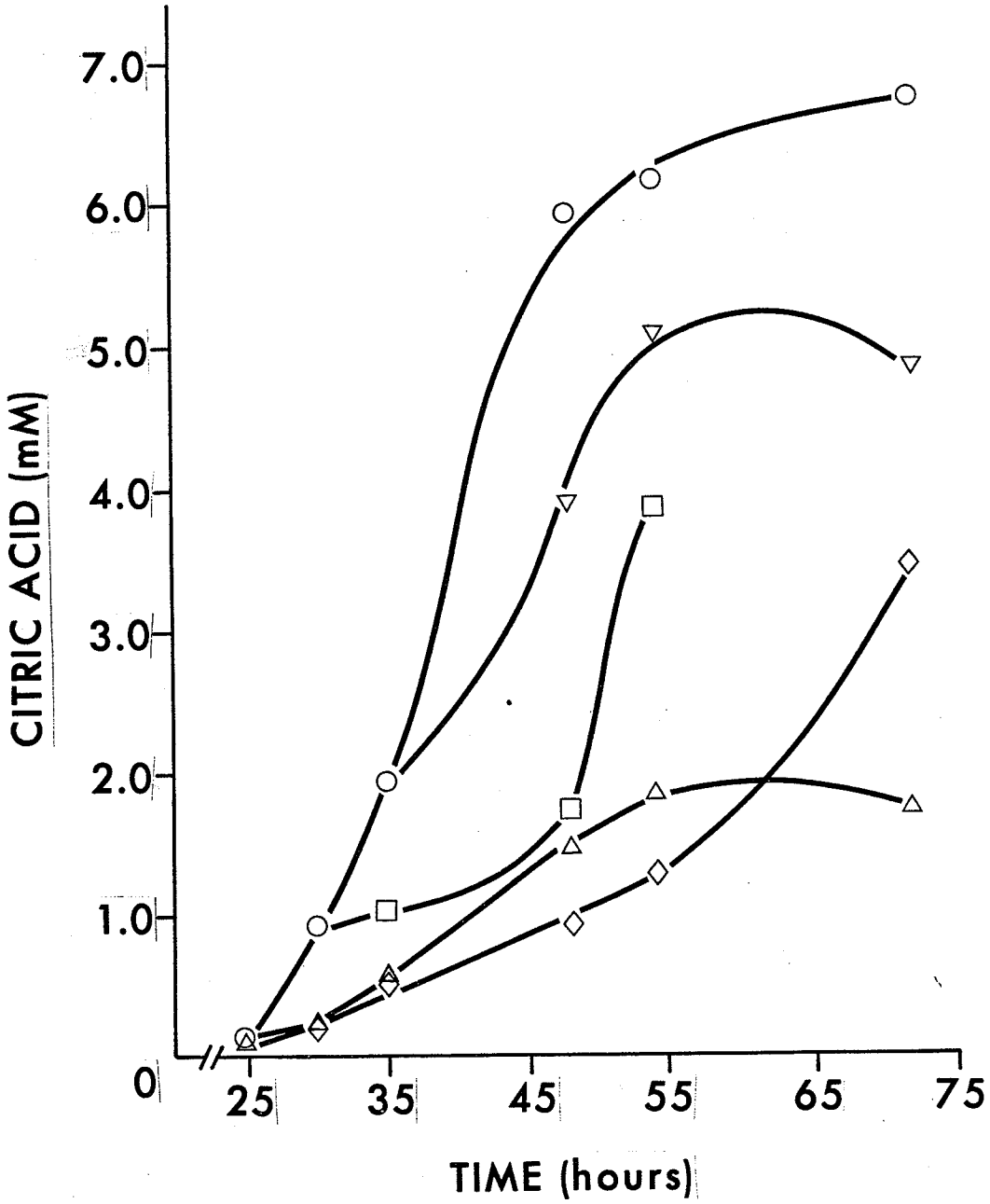

CITRIC ACID (mM)
4.0
3.0
2.0
1.0
0
20
25
30
35
40
45
TIME (hours)

Figure 9. Effect on citric acid production of the addition of Zn as $ZnSO_4$ to citrate-accumulating cultures at 0 (Δ), 25 (◊), 30 (□) and 35 hours (▽) after inoculation, compared to non-Zn (0) cultures. Each point is the average of three flasks.


7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
CITRIC ACID (mM)
25
35
45
55
65
75
TIME (hours)

concentration of citric acid finally achieved was much higher than that of Zn(0) cultures but lower than that of the non-Zn controls. The amount of citric acid produced was greater as the time of addition of Zn increased.

A difference was also evident in the increase in dry weight as Zn was added at later times (Fig. 10). An immediate, rapid increase in dry weight occurred, followed by a levelling off to a point much higher than the non-Zn controls, but lower than Zn(0) cultures.

After the addition of Zn, the rate of sucrose utilization also increased, after an initial lag (Fig. 11). The rate then increased to nearly that of Zn(0) cultures, with nearly as much sucrose having been consumed by the end of the experiment.

Cellular response to zinc

In addition to the more obvious differences in dry weight, sucrose utilization and citric acid production evident between control and Zn-grown cultures, more basic cellular differences could be studied. There were differences in the levels of DNA, RNA and protein in the cells of each group. Also, the concentrations of sucrose and citric acid within the cells of each group were significantly different.

The difference between Zn and control cultures with respect to the disappearance of sucrose from the medium has been dealt with. A more striking difference was seen in the levels of sucrose established inside the cells during the course of growth. As shown in Fig. 12, Zn- cells concentrated sucrose to about the level in the

Figure 10. Effect of the addition of Zn on dry weight of citrate-accumulating cultures. Zn was added as $ZnSO_4$ (0.4 mg/l) at 0 time (Δ), 25 (◇), 30 (□) or 35 (▽) hours after inoculation, or cultures were grown without Zn (0). Each point is the average dry weight of three flasks.

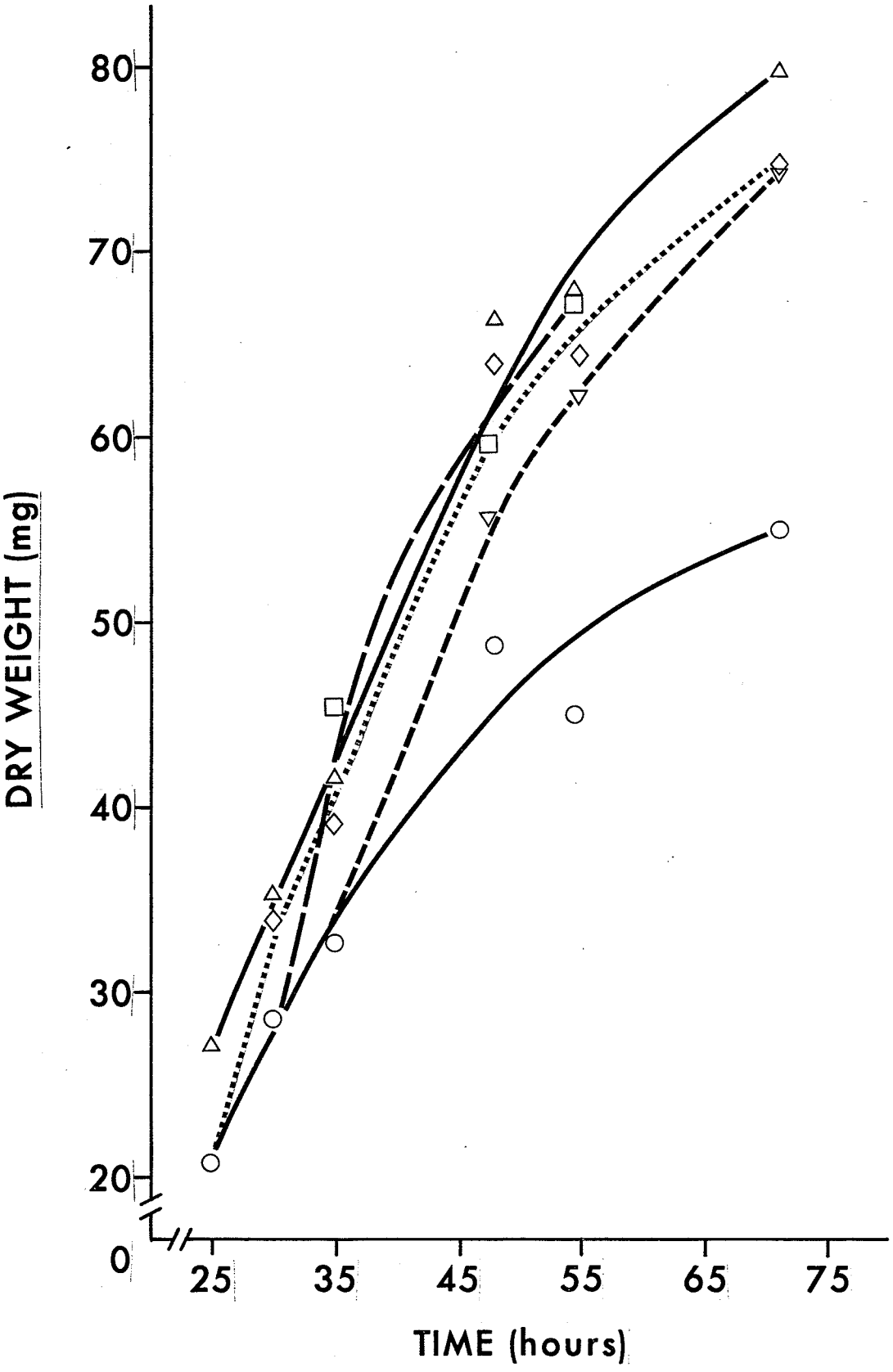

80
70
60
50
40
30
20
0
DRY WEIGHT (mg)
25
35
45
55
65
75
TIME (hours)

Figure 11. Effect of the addition of Zn on sucrose utilization by citrate-accumulating cultures. Zn was added as $ZnSO_4$ (0.4 mg/l) at 0 time (Δ), 25 (◇), 30 (□) or 35 (▽) hours after inoculation or cultures were grown without Zn (0). Each point is the average of three flasks.

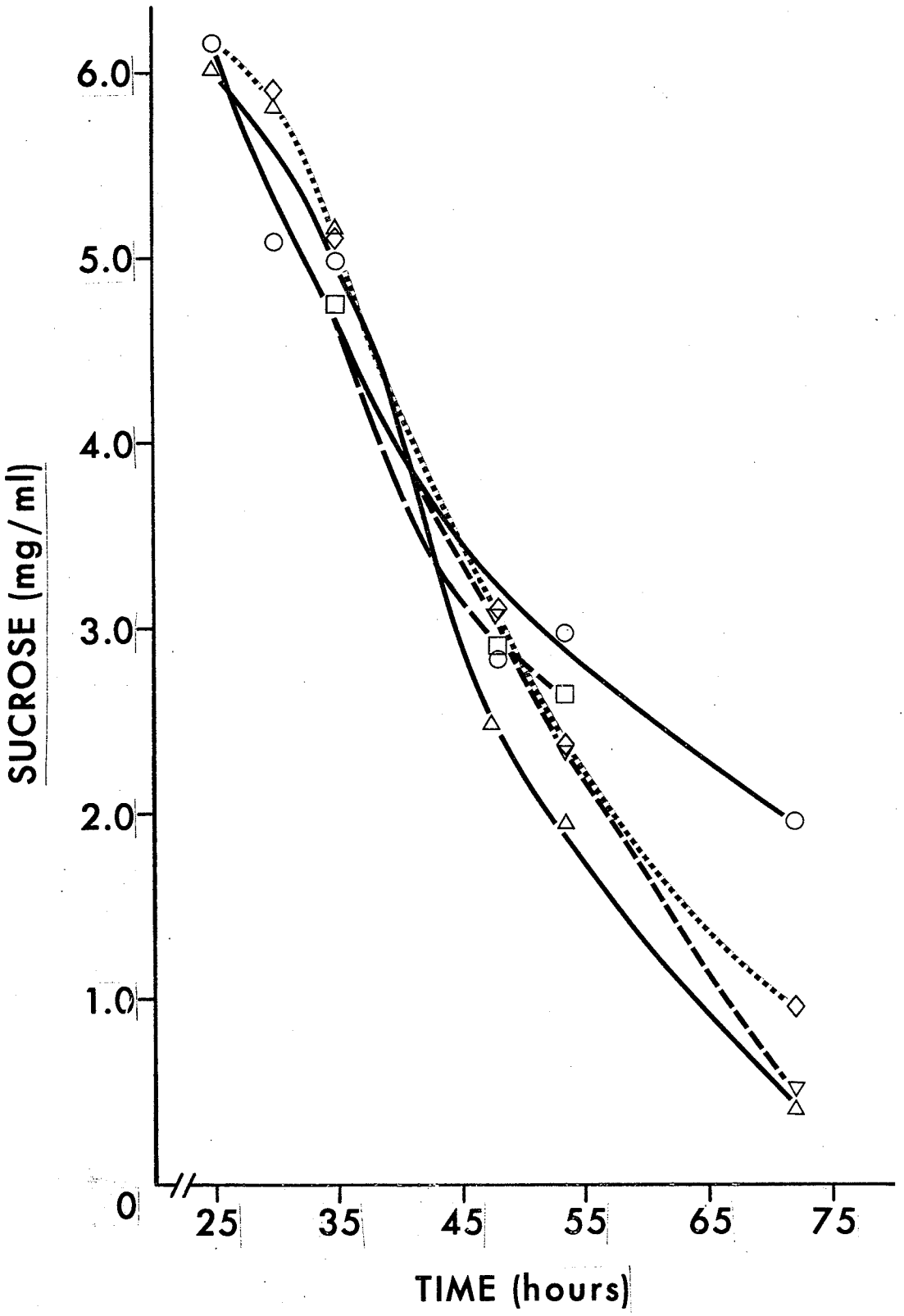
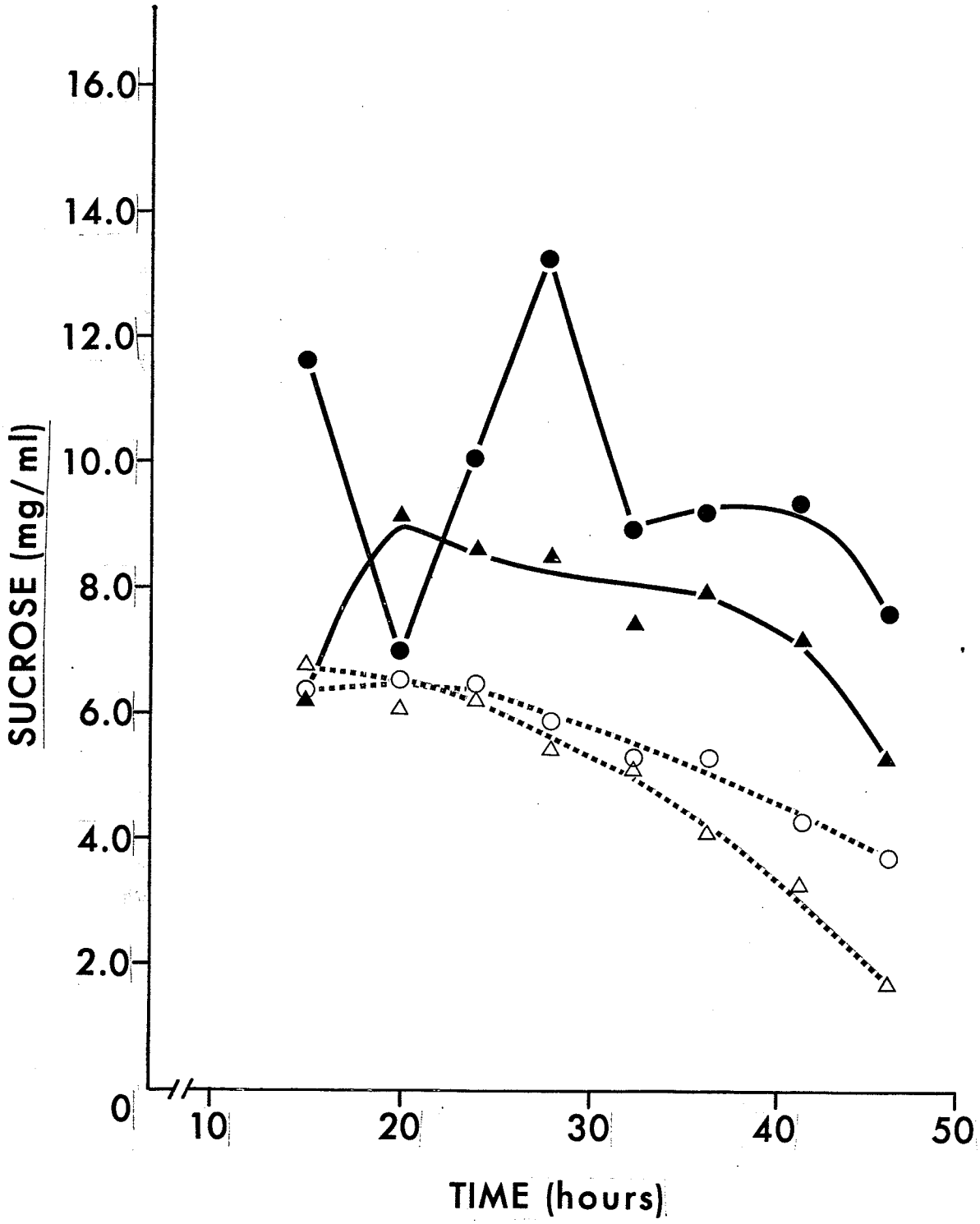

6.0
5.0
4.0
3.0
2.0
1.0
0
SUCROSE (mg/ml)
25
35
45
55
65
75
TIME (hours)

Figure 12. Sucrose concentrations in the medium (open symbols) and mycelium (closed symbols) of control (0) and Zn (Δ) cultures. Zn was added as $ZnSO_4$ before inoculation. Six flasks were sampled at each time point; six medium samples were assayed for external sucrose, three mycelial samples were homogenized and extracted as described in Materials and Methods for internal sucrose. Internal sucrose concentrations were based on cell volumes (Fig. 14) as determined in Materials and Methods.

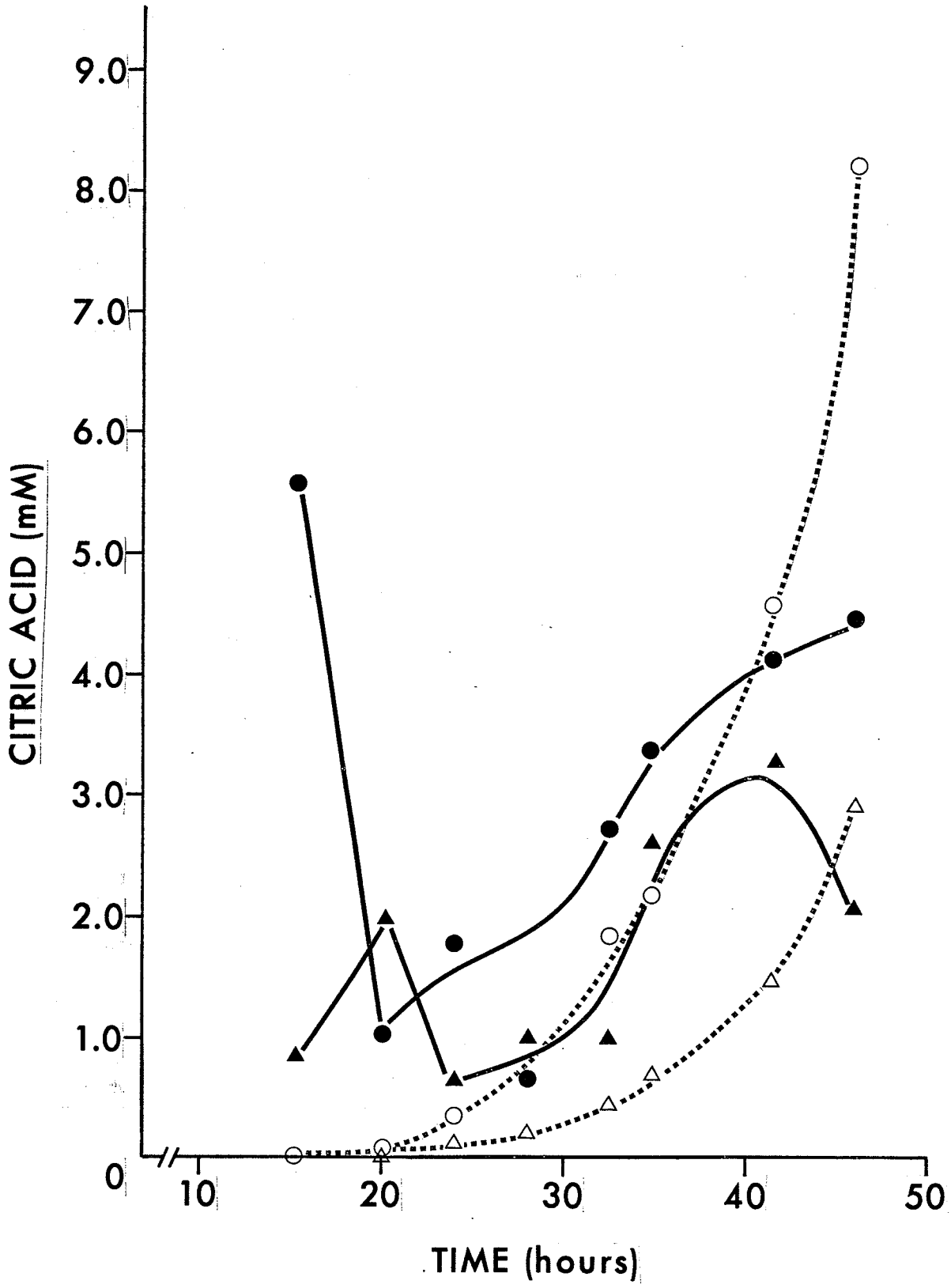

16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
SUCROSE (mg/ml)
10
20
30
40
50
TIME (hours)

medium by approximately 17 hours, accumulated a maximum amount by 19 hours, and then maintained nearly this level throughout growth. A slight depletion occurred progressively until about 35 hours, after which the internal concentration dropped. At no time, after the initial concentration, did the internal sucrose level drop below the medium level.

Control cultures accumulated sucrose internally to a much higher concentration during the first 15 hours; a concentration 1.5 times that of the external concentration was reached (Fig. 12). This was followed by a rapid drop to about 19 hours at which time citric acid was detectable in the medium (Fig. 12). Another increase in internal sucrose concentration then occurred concurrently with the logarithmic production of citric acid. This level then dropped again until about 32 hours and thereafter remained relatively constant; this was also the time period during which a significant shift in the rate of depletion of sucrose from the medium occurred (Fig's. 11, 12).

Some interesting differences were also seen in the internal concentrations of citric acid. The control, or citric acid-accumulating cultures, developed a high internal level of citric acid in the first 15 hours of growth (Fig. 13). This level then rapidly dropped until 20 hours, as did the internal sucrose level. At this time citric acid became detectable in the medium, although

Figure 13. Citric acid concentrations in the medium (open symbols) and mycelium (closed symbols) of control (0) and Zn (Δ) cultures. Zn was added as $ZnSO_4$ before inoculation. Six flasks were sampled at each time point; six medium samples were assayed for external citrate, three mycelial samples were homogenized and extracted as described in Materials and Methods. Internal citric acid concentrations were based on cell volumes (Fig. 14) as determined in Materials and Methods.


CITRIC ACID (mM)
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
TIME (hours)

small amounts were present earlier. From the sudden appearance of measurable citrate in the medium, it seems possible that the internal citrate was suddenly released. The internal citrate concentration at 15 hours was 5.5 mM; the cell volume was 0.15 ml and at 20 hours it was 0.26 ml. The dilution of citrate by cell size increase would make the internal citrate concentration 3.2 mM if no more citrate synthesis occurred. However the measured internal citrate concentration was 1.0 mM. This loss of citrate would account for one tenth of the medium citrate measured. However there was no reason to believe that continued citrate synthesis and export did not occur during this time. Thereafter high internal levels did not accumulate again until at about 25 hours, when rapid internal accumulation resumed, coupled with rapid external accumulation. Notuntil 40 hours, when the internal concentration began to level off - also where a break in the plot of log [citric acid] (external) vs time occurred (Fig. 15) - did the internal levels fall below the external level, which continued to increase.

Zn cells, on the other hand, did not accumulate such high levels of internal citric acid. Some concentration increase occurred up to 20 hours which was then depleted as in the control cultures, but at a later time. A nearly constant internal level was then maintained until about 32 hours, after which a rapid accumulation equal in rate but not in quantity to control cells occurred.

Figure 14. Total average cell volume (of three flasks) of mycelia per flask of control (0) and Zn(Δ) cultures, (sucrose and citrate data given in Fig's. 12 and 13).

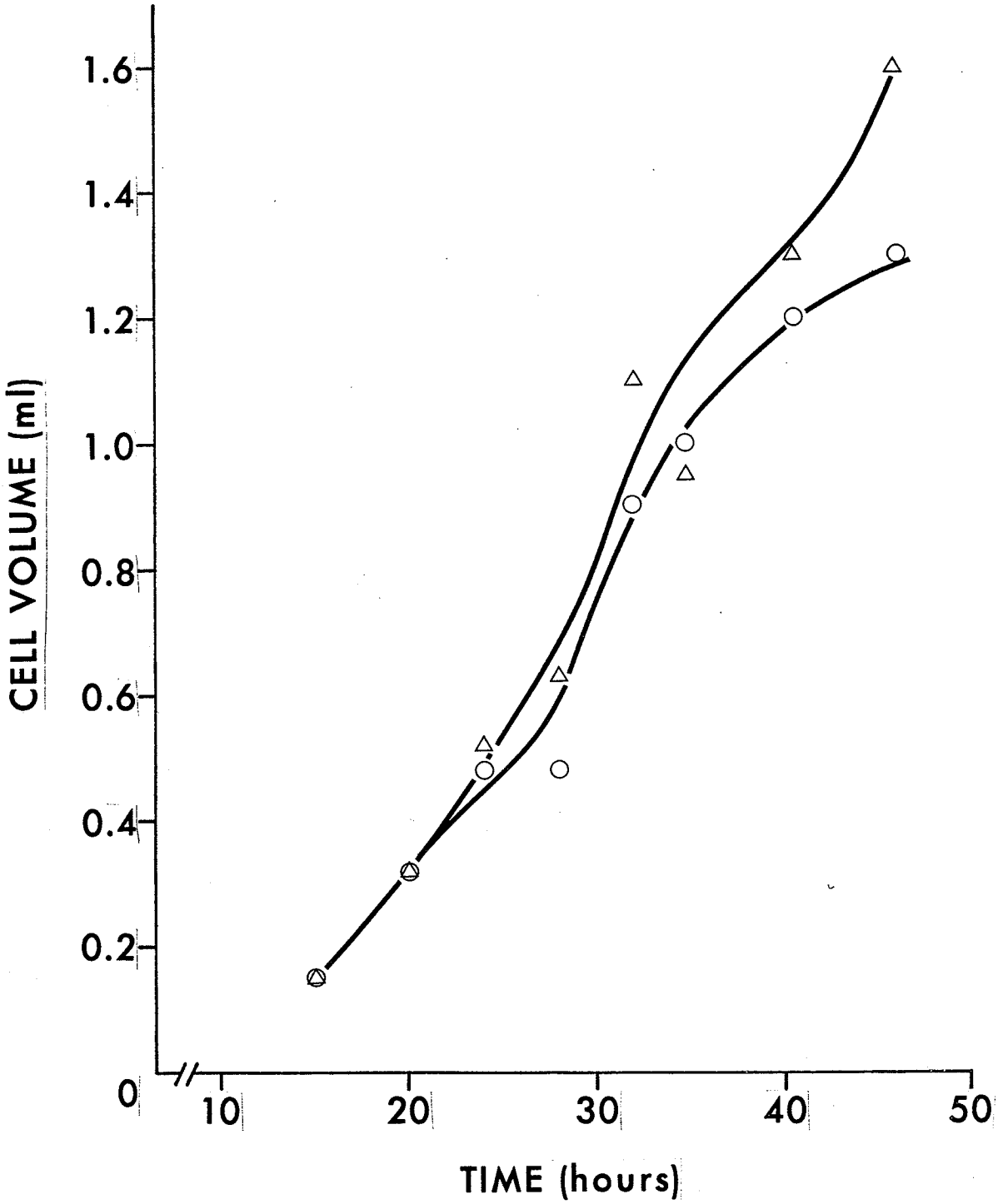
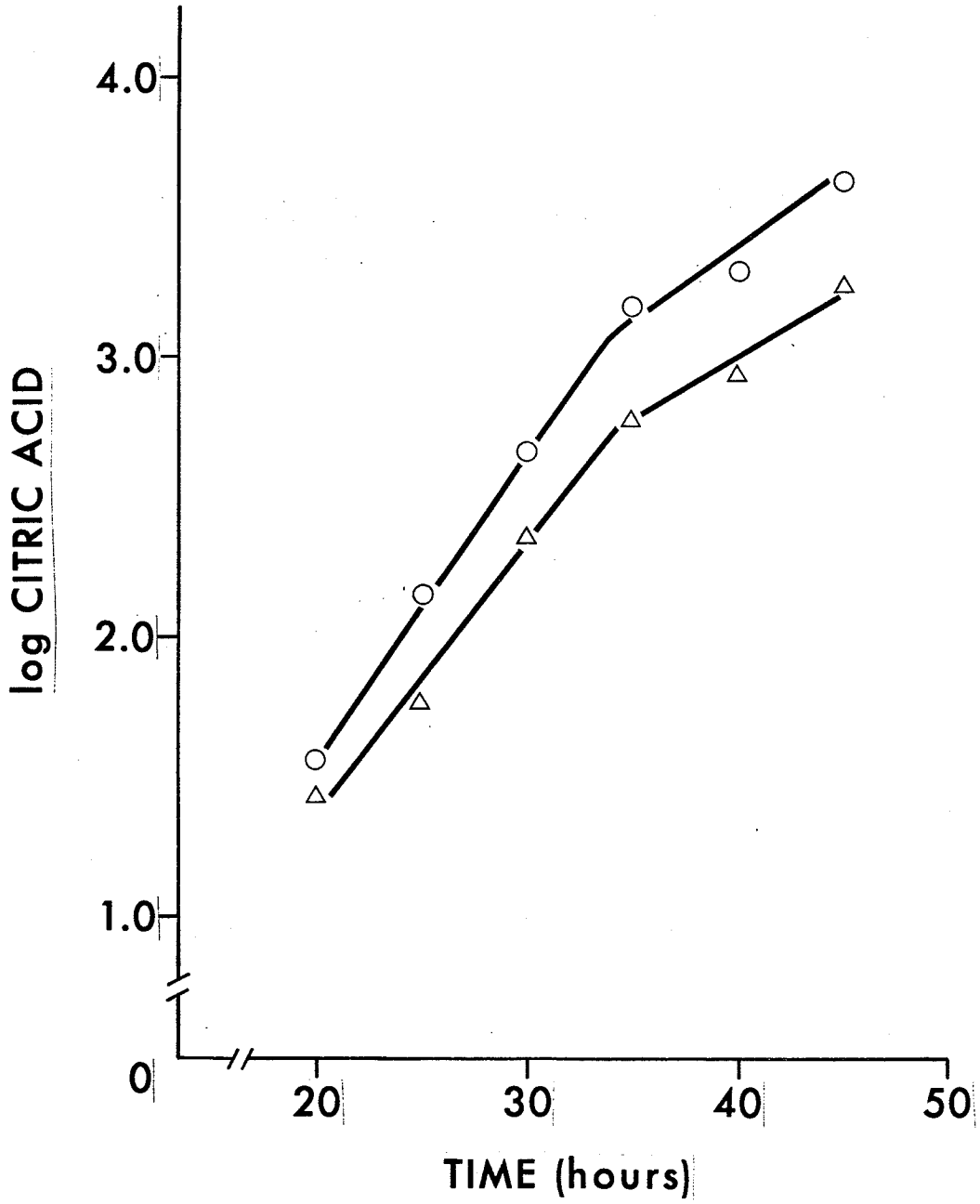

CELL VOLUME (ml)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
TIME (hours)

Figure 15. Semi-log plot of citric acid accumulation of control (0), citrate-accumulating, and Zn (Δ), non-accumulating, cultures. Culture conditions as described in Materials and Methods. Each point is the average of three flasks.


4.0
3.0
2.0
1.0
0
log CITRIC ACID
20
30
40
50
TIME (hours)

After 40 hours the internal citrate concentration again dropped quickly, accompanied by low levels of externally accumulating citrate.

In both Zn and control cultures, an internal build-up of citric acid occurred at 35 hours, where the shift in sucrose utilization also occurred (Figs. 11, 16).

The final depletion of citric acid from the Zn cultures probably resulted from complete metabolism for growth, as no more citrate usually accumulated after this time. Dry weight (Fig. 10) and protein content (Fig. 19) did continue to increase.

The aforementioned determinations were carried out with homogenates of unwashed mycelia (as described in Materials and Methods). If the mycelia were washed extensively in water before homogenization, very little or no citric acid, was detected in the homogenate. However if the homogenate was acidified with HCl to 0.5 N, citric acid was again released and a similar pattern to that of unwashed cells appeared (Fig. 16), although the concentrations obtained were lower. Citric acid was apparently bound, and the acidification with strong HCl released it from the insoluble cell components.

When Zn was added at 30 hours, the internal as well as external accumulation of citric acid stopped with a quite close correlation (Fig. 17).

Figure 16. Semi-logarithmic plot of sucrose utilization by A. niger under citrate-accumulating (0) and non-accumulating (Δ) conditions. Each point is the average of three flasks.

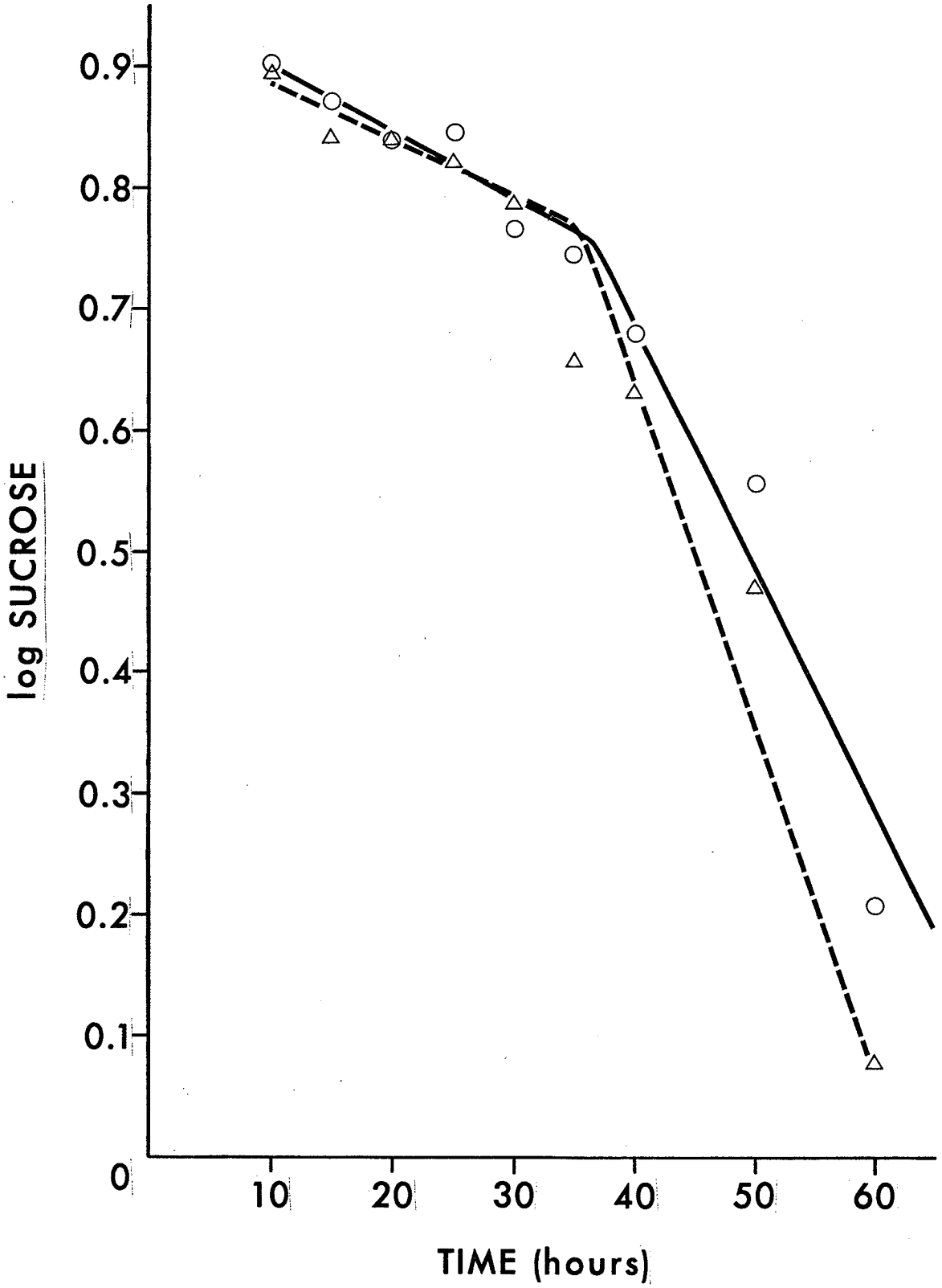
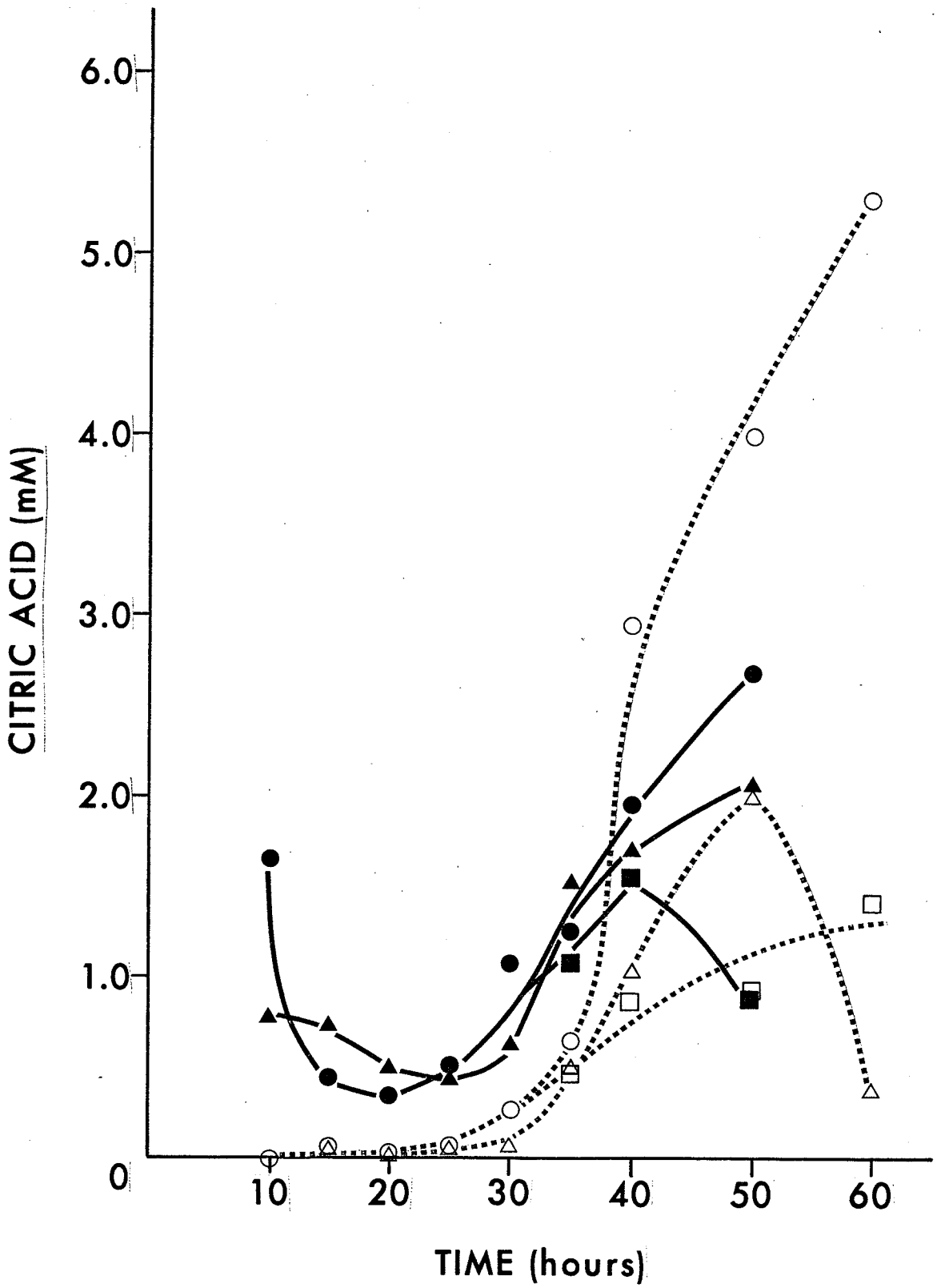

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
log SUCROSE
10
20
30
40
50
60
TIME (hours)

Figure 17. Levels of citric acid in the medium (open symbols) and in the mycelium (solid symbols)of extensively washed cultures. A. niger was grown in M-3 under citrate-accumulating conditions (0), with Zn added at inoculation (Δ), or with Zn added after 30 hours (□). Each point is the average of three flasks.


CITRIC ACID (mM)
6.0
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
TIME (hours)

The levels of internal sucrose were considerably different in extensively washed mycelia, although the pattern was similar (Fig. 18). A great deal of the sucrose was apparently reversibly attached to the cell surface especially at the 15 and 20 hour sampling times. At 10 hours, an initial high internal concentration of sucrose was still evident. This level dropped dramatically to 15 hours, and then internal concentration increased again. After 30 hours the internal sucrose levels of Zn-cells dropped; the sucrose levels in cultures with Zn added at 30 hours also dropped, but at a slower rate. The level remained relatively high and constant in control cultures. A drop did occur at 30 hours, however, concomittant with a drop in the rate of depletion of medium sucrose.

The protein content of the cultures under the two different growth conditions is shown in Fig. 19. In the presence of Zn, proteins synthesis was continuous from the beginning of the growth period. A decrease in protein production rate occurred between 30 and 40 hours followed by a rapid increase.

Non-Zn cultures had a higher protein content than Zn cultures until about 20 hours. A lag in protein production between 20 and 30 hours was followed by increased synthesis from 30 to 40 hours, after which no net increase was found.

Figure 18. Internal concentrations of sucrose in A. niger cultures, as described in legend to Figure 17 (closed symbols). Sucrose in the medium (open symbols). Cultures were grown under citrate-accumulating conditions (0), with Zn added at inoculation (Δ), or with Zn added after 30 hours (□). Each point is the average of three flasks.

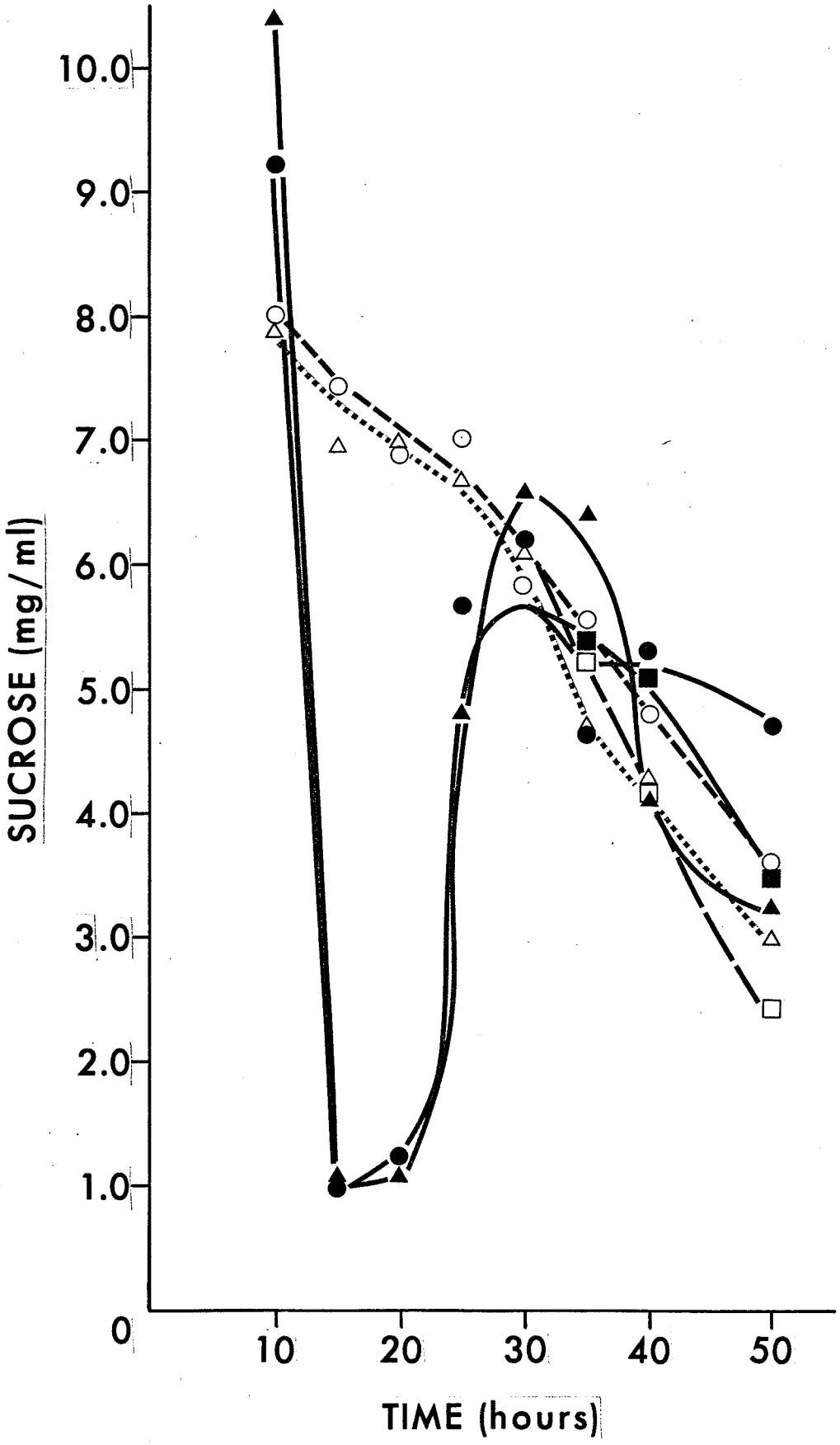
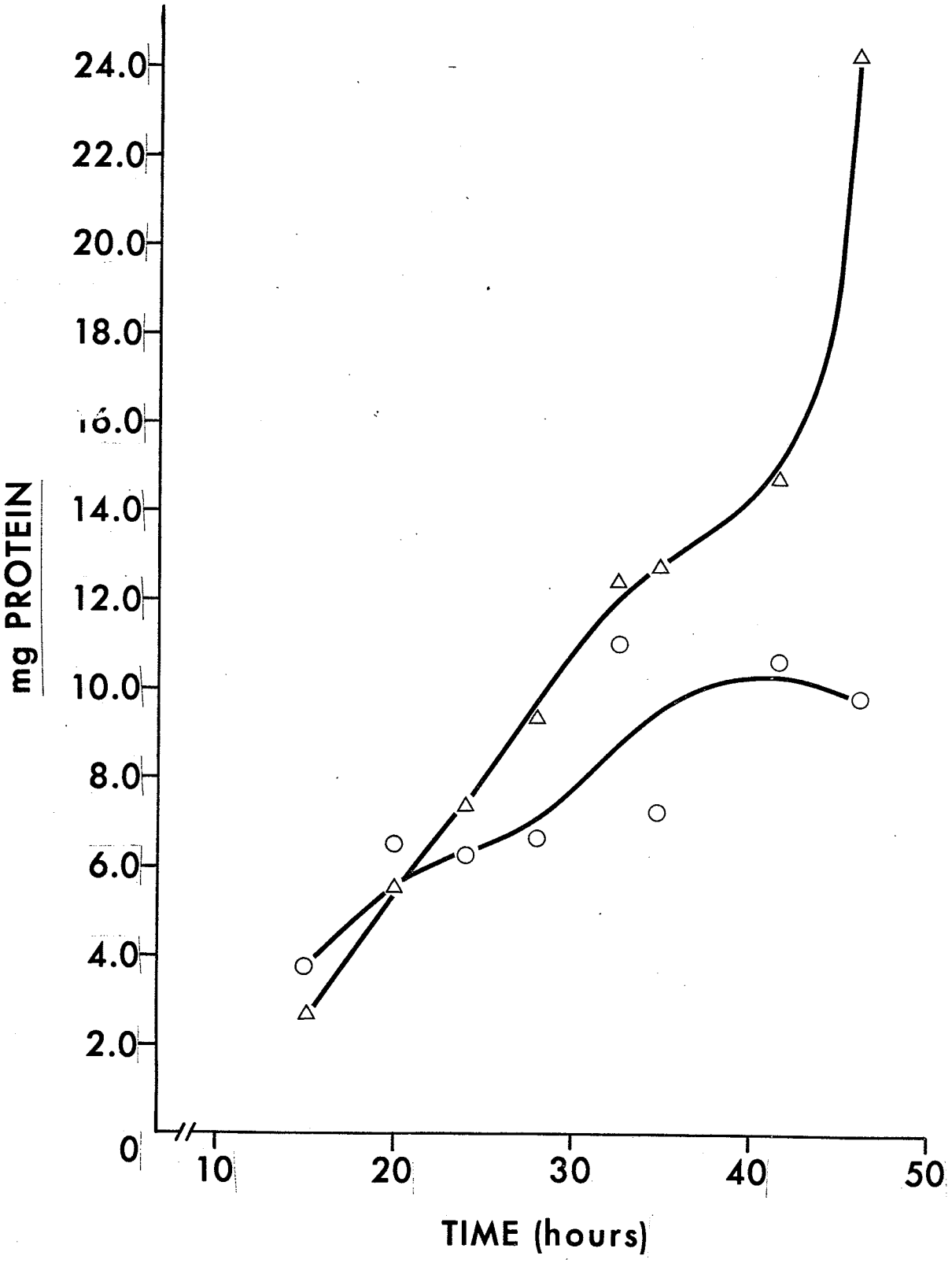

SUCROSE (mg/ml)
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
TIME (hours)

Figure 19. Average protein content of the mycelia of three flasks of control (0) and Zn (Δ) cultures of A. niger, as described in legend to Figure 12.


24.0
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
mg PROTEIN
10
20
30
40
50
TIME (hours)

The only similarity evident between the two growth forms with respect to protein production was the plateau seen between 32 and 40 hours; this might have been related to the change in rate of sucrose metabolism also seen at this time. It might represent induction of a new enzyme system and conversion to another metabolic pathway.

Dry weights of control and Zn cultures were normally similar until 25 to 30 hours (Fig. 10). Then control cultures approached stationary phase and Zn cultures continued to grow.

The RNA curves for Zn and non-Zn cultures were very similar (Fig. 20). Zn cultures had a slightly higher amount of RNA until approximately 40 hours, after which the RNA content of control cultures began to drop slightly while that of the Zn cultures continued to increase. This was likely due to the fact that Zn cultures were continuing to grow while control cultures were becoming stationary. There was a plateau in RNA content increase of both cultures between 32.5 and 35 hours.

The DNA content in each case was also approximately parallel until 32 hours, with the level being slightly higher in non-Zn cultures (Fig. 21). After the sharp rise which occurred in both cases between 28 and 32.5 hours, the levels dropped again rapidly in control cultures to a plateau level. The DNA level of the Zn culture continued to increase until 35 hours before dropping from a much higher level. The drop continued until about 40 hours, after which a slight increase resumed.

Figure 20. Average RNA content of three flasks of A. niger mycelium under citrate-accumulating (0) and non-accumulating (Δ) (i.e. in the presence of Zn) conditions. Experiment as described in legend to Fig. 12.

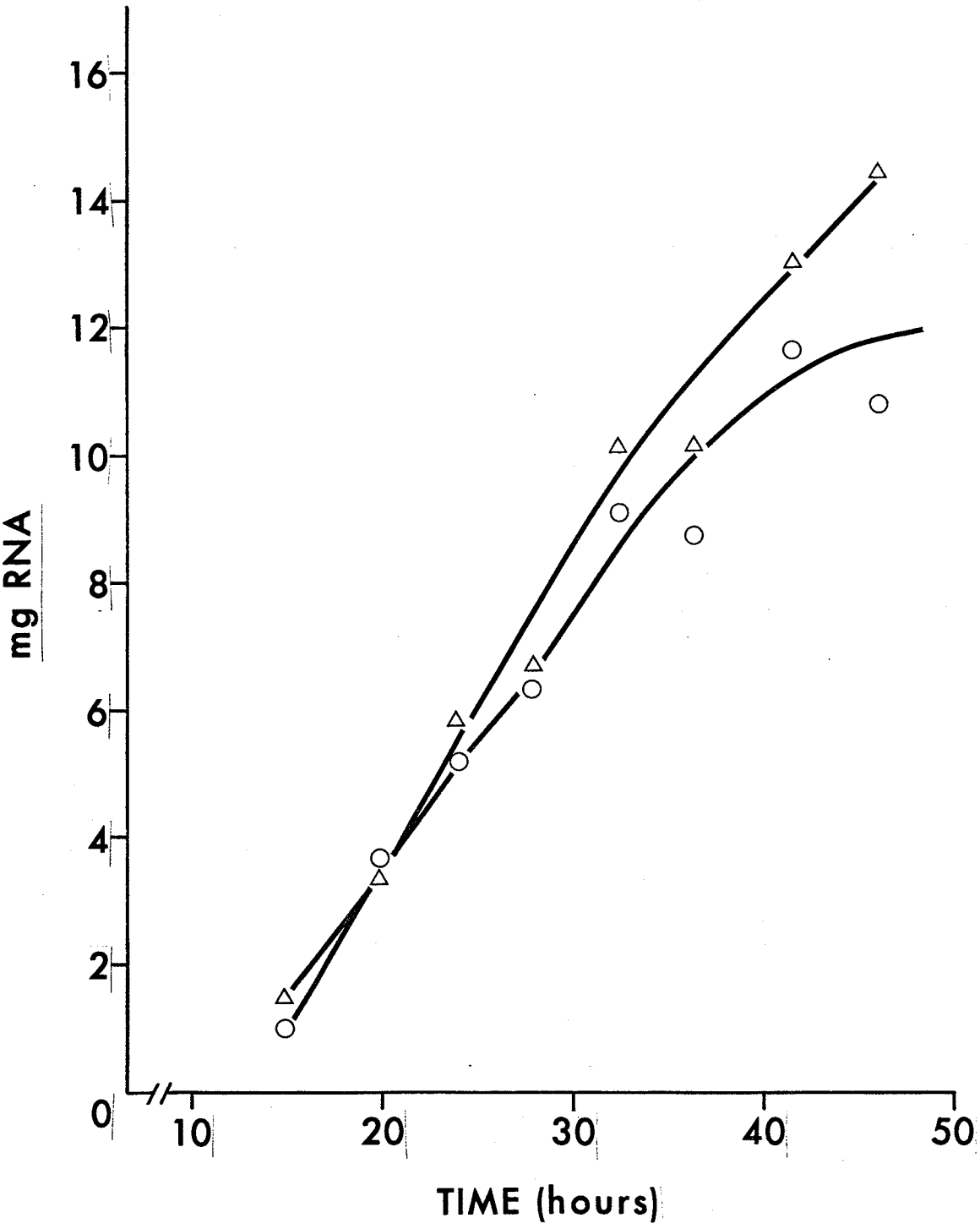
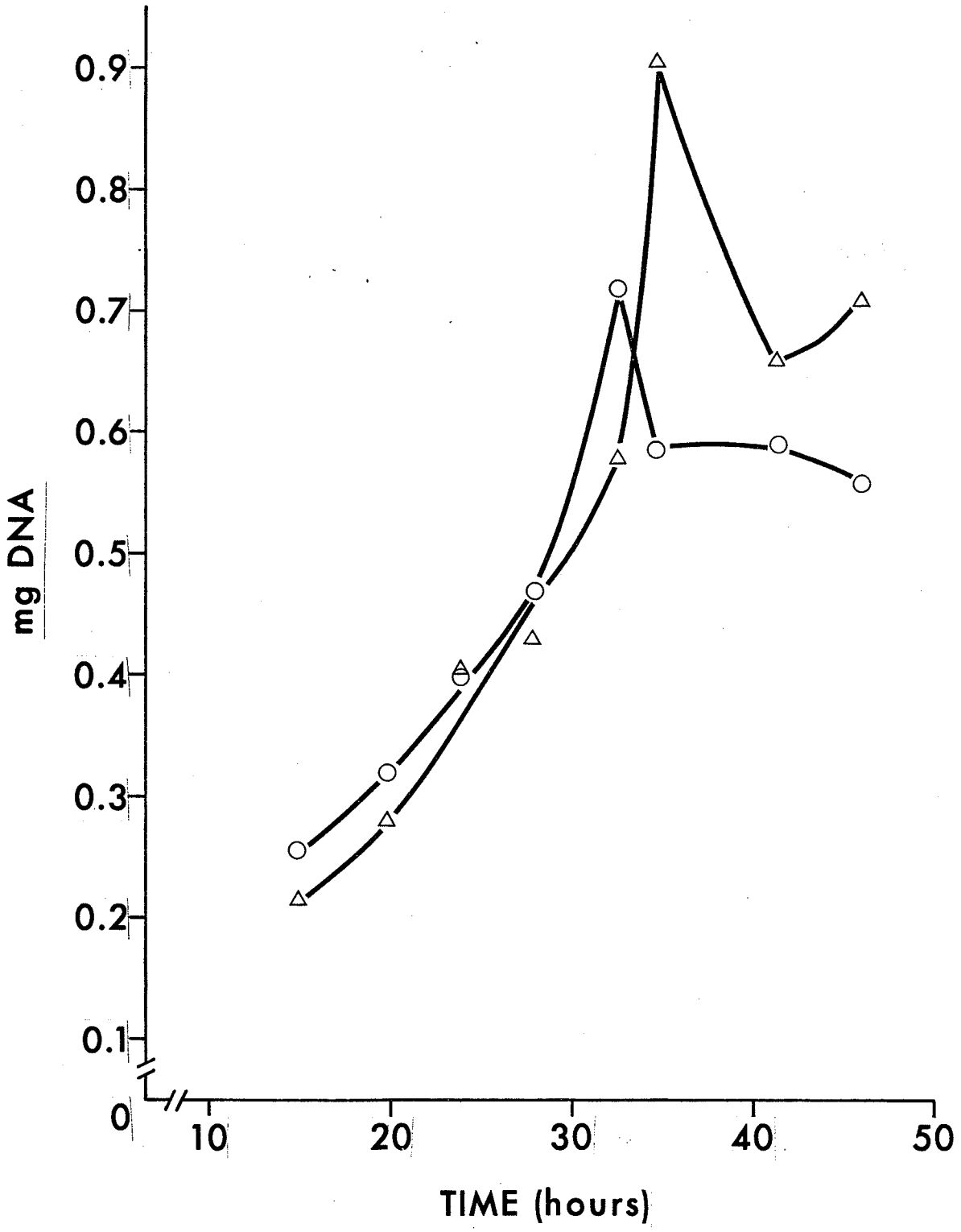

16
14
12
10
8
6
4
2
0
mg RNA
10
20
30
40
50
TIME (hours)

Figure 21. Average DNA content of three flasks of A. niger mycelium under citrate-accumulating (0) and non-accumulating (Δ) (i.e. in the presence of Zn) conditions. Experiment as described in legend to Fig. 12.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
mg DNA
10
20
30
40
50
TIME (hours)

BRANCHING RESPONSE

Endogenous branching factor

Branching is a normal adaptive response of A. niger and other fungi. Stale medium from A. niger cultures grown under citrate-accumulating conditions applied to the leading hyphae at the margin of a colony of A. niger grown on Park's medium plates caused temporary inhibition followed by regrowth via subapical and lateral branching. Occasionally attenuated regrowth of the hyphal tips occurred as well (Fig. 22).

Observation of treated colonies after twenty-four hours revealed that most regrowth occurred through subapical branching. New growth was also more highly branched than that of untreated mycelia and had a tangled appearance. More growth occurred in treated than non-treated mycelia, with hyphae being longer as well as more highly branched. Sporulation did not proceed beyond the region that had been treated, although untreated colonies continued to produce conidia as they grew.

These effects did not result from treatment of hyphae with Millipore-deionized distilled water or with sterile Park's medium or M-3 although lateral branching and constrictions of the primary hyphae occurred sometimes (Fig. 22).

The limiting dilution was defined as the highest dilution of a material in Park's assay medium that produced a positive effect, a positive effect being that described in the first paragraph of this section.

Figure 22. Patterns of growth of A. niger hyphae grown on Park's medium plates.

(a) Normal unbranched hypha .

(b) Normal, lateral branching.

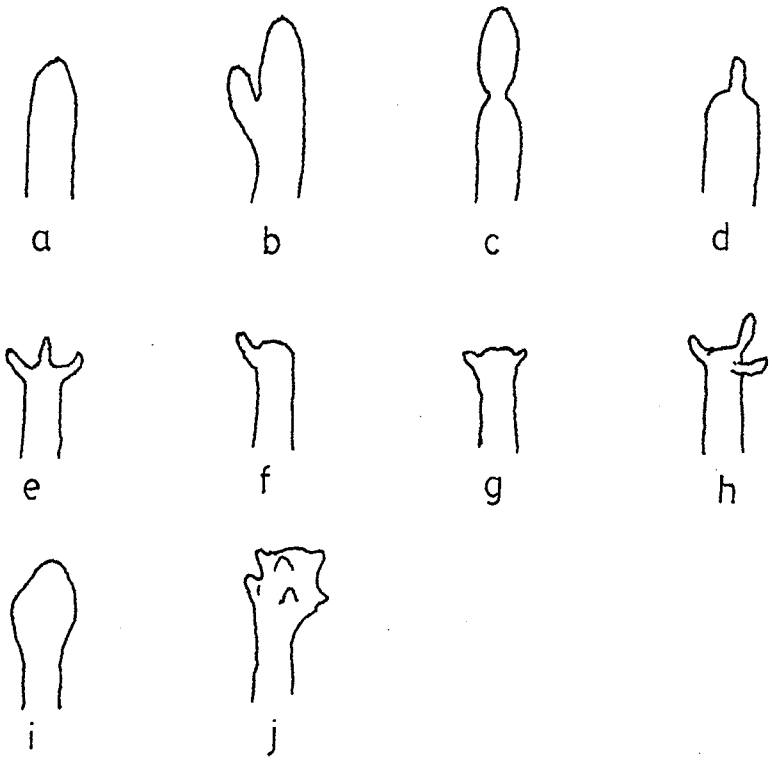
(c) Constriction and regrowth of primary hypha.

(d) Attenuated regrowth of primary hypha.

(e) Attenuated regrowth and subapical branching. (f), (g) and (h) Progressively increased subapical branching in the absence of regrowth of the primary hypha.

(i) Inhibited, bulging primary hypha.

(j) Regrowth of bulged hypha as in (i) through numerous subapical branches.


a
b
c
d
e
f
g
h
i
j

Stale medium from Zn-containing cultures produced these effects only to a limited extent. The limiting dilution achieved was inversely proportional to the amount of Zn in the medium, and was very low compared to that of stale Zn-poor medium.

The spontaneous branching of hyphae near the edge of a Petri plate or in close proximity to another culture, as well as the lack of response in these regions to staled culture medium suggested a "branching factor" as a normal metabolite of the fungus.

An attempt to purify the active compound or compounds was made. The branching factor was found in both the medium and the mycelium of citrate-accumulating cultures, and was usually found in association with the yellow pigment (YP) characteristic of these cultures. However, accumulating cultures with little or no YP also caused branching of test cultures. A soluble factor distinct from YP was therefore present as an active morphogen.

Activity in the YP fraction could be concentrated as an oily substance which separated upon freezing of liquid staled medium. This oily fraction floated at the tops of the tubes and could be collected in tiny amounts containing some water. Only small amounts were obtained, however. Because of this fact, and the fact that branching activity was also found in staled media with most YP removed, or in which YP had not been produced, further

attempts at purification were not made.

The branching activity from the medium with YP removed or isolated from the mycelium was soluble to some extent in ether, ethanol and water. Relative activity was tested as the degree of effect produced on test A. niger colonies on Park's medium plates. The factor was heat stable to boiling for 10 minutes and stable at -16°C indefinitely. It was active at pH 5.5 and 9.5, nearly as active at pH 2.5 and only slightly active at pH 7.5. It was bound by activated charcoal, was non-dialysable (24 A° membrane) in water, aqueous NaOH of pH 9.0 or aqueous HCl at pH 2.5. It was stable to treatment with $(NH_4)_2SO_4$, 10 N NH_4OH, concentrated HCl- if tested after pH was readjusted to pH 5.5 or less.

White precipitate or crystals formed occasionally but could not be recovered because of small amounts and high solubility in water. Very high activity was associated with this fraction as well as with the oily YP fraction.

Whether two different factors were actually present, or whether one active component was associated with both fractions was not determined.

Citric acid determinations by the enzymatic method indicated high concentrations in the YP fraction, the white precipitatate, and all other fractions which showed high branching activity.

Because of the possibility of various active factors which might interfere with one another, indicated experimentally as well as by the numerous reports of

fungal morphogens in the literature, isolation attempts were abandoned.

A direct means of identifying possible branching activators was then attempted, based upon the facts that branching resulted from media from citrate accumulating cultures but not from Zn-rich cultures, and that succinate had been found to be a fungal morphogen (Robinson, 1972).

Branching response to organic acids

In citrate-accumulating cultures the limiting dilution of the medium was found to be directly proportional to the concentration of citric acid produced, and represented a concentration of 10^{-16} M citric acid. The same effect was evident if pure citric acid (reagent) in Millipore-deionized distilled water or Park's assay medium was tested.

A "white control" was a culture grown under citrate-accumulating conditions, which for unknown reasons, had all the characteristics of a Zn culture. The culture attained a high growth rate and produced neither YP nor citric acid. This condition occurred randomly at a frequency of about one in a hundred growth flasks.

Cultures grown under citrate-accumulating conditions yielded staled media which induced a branching response in mycelia of plate cultures, when tested as early as fifteen hours after inoculation. Citric acid could not be detected by the normal enzyme assay - which was sensitive

to approximately 0.05 mM citric acid - at this time, and in fact could not be detected enzymatically until after twenty to twenty-four hours of growth. The limiting dilution at 24 hours corresponded with the detectable citrate by the enzyme assay (Table I); this value could be calculated and predicted from the measured amount of citrate and the limiting concentration of 10^{-16} M citric acid.

Other organic acids which gave a positive branching response at 10^{-15} M were succinic acid and glutaric acid (Table II). Oxaloacetate, cis-aconitate and pyruvate also produced branching. Notably, isocitrate did not, nor did malic, maleic,tartaric or oxalic acids. Ferrocyanide, which has been used to induce citric acid production (Clark, 1962) resulted in some lateral branching at 10^{-6} M, but had no effect at lower concentrations. Similarily, ethylenediamine tetraacetic acid (EDTA), a metal chelator, caused some lateral and a very little subapical branching at 10^{-3} M, but had no effect at lower concentrations.

The limiting dilution of 10^{-16} M citric acid defined the sensitivity of the test method. As shown in Fig. 22, increased concentrations of citric acid caused an increased branching response at low levels of citric acid. This effect was progressive until a concentration of 10^{-5} M citric acid was reached, after which the branching response was preceded by inhibition of hyphal growth.

TABLE I. Correlation between citric acid concentration and branching response.

Test substance	Limiting dilution	[Citric acid] (μmoles/ml)
M-3 medium	no response	
Park's assay medium	no response	
[a]Citric acid, 0.1 μmole/ml	9.09×10^{13}	
Citric acid, 5.0 μmole/ml	2.84×10^{14}	
Citric acid, 40.0 μmole/ml	3.55×10^{15}	
Culture filtrate from:		
M-3 (citrate-accumulating)	2.84×10^{14}	4.95
M-3 + 0.1 mg/l $ZnSO_4$	9.77×10^{4}	not detectable
M-3 + 0.2 mg/l $ZnSO_4$	3.91×10^{3}	not detectable
M-3 + 0.4 mg/l $ZnSO_4$	3.91×10^{3}	not detectable
M-3, white control	3.13×10^{3}	not detectable

[a]Citric acid was diluted in sterile Park's assay medium.

TABLE II. Activity of organic acids and other substances as branching agents.

Substance	Response[b]
Distilled water	-
Park's assay medium	-
M-3 medium	-
[a]Citric acid	+
Succinic acid	+
Glutaric acid	+
Oxaloacetic acid	+
Cis-aconitic acid	+
Pyruvic acid	+
Fumaric acid	+
Malonic acid	+
Malic acid	-
Maleic acid	-
Tartaric acid	-
Oxalic acid	-
Isocitric acid	-
α-Ketoglutaric acid	-
Gluconic acid	-
Glutamic acid	-
EDTA, 10^{-3} M	±
, 10^{-5} M	-
Ferrocyanide, 10^{-6} M	±
, 10^{-8} M	-

a. All acids were tested at 10^{-15} and 10^{-9} M.

b. +; subapical branching, slight inhibition

±; production of lateral and some subapical branching, no inhibition

-; some lateral branching only

Above threshold value for inhibition, increased concentrations increased the time lag before regrowth as subapical branching occurred. Above concentrations of about 10^{-4} M, bulging of the hyphal tips accompanied inhibition and regrowth occurred only as subapical branches.

The addition of 3', 5'-cyclic adenosine monophosphate (cAMP) at a concentration of 2×10^{-4} M to hyphae which had been treated with a culture filtrate or citric acid at the limiting dilution resulted in an enhanced branching response preceded by some inhibition before regrowth. At 2×10^{-4} M, cAMP itself caused some vacuolation, but little or no branching effect. At 2×10^{-3} M, however, some effect was evident, but no inhibition or swelling occurred, and branching was mostly lateral.

EFFECT OF ADDED CITRIC ACID

The logarithmic accumulation of citric acid shown in Fig. 15 suggested an autocatalytic mode of production. Therefore if the citric acid in the medium at a particular time was supplemented so as to equal the concentration expected at some later time, the entire curve of citric acid production should be shifted to the left by the time difference. When 1.0 or 0.5 mM citric acid was added to the medium of accumulating cultures production was not suppressed, but occurred normally at the earlier time period (Fig. 23). Maximum production occurred earlier by the time

Figure 23. Effect of supplementing the growth medium of accumulating cultures with exogenous citric acid on the medium total citric acid concentrations. Cultures were grown in M-3 with no addition (0), or with 0.1 mM (Δ), 0.5 mM (□), or 1.0 mM (∇) citric acid added. Each point is the average of three flasks.

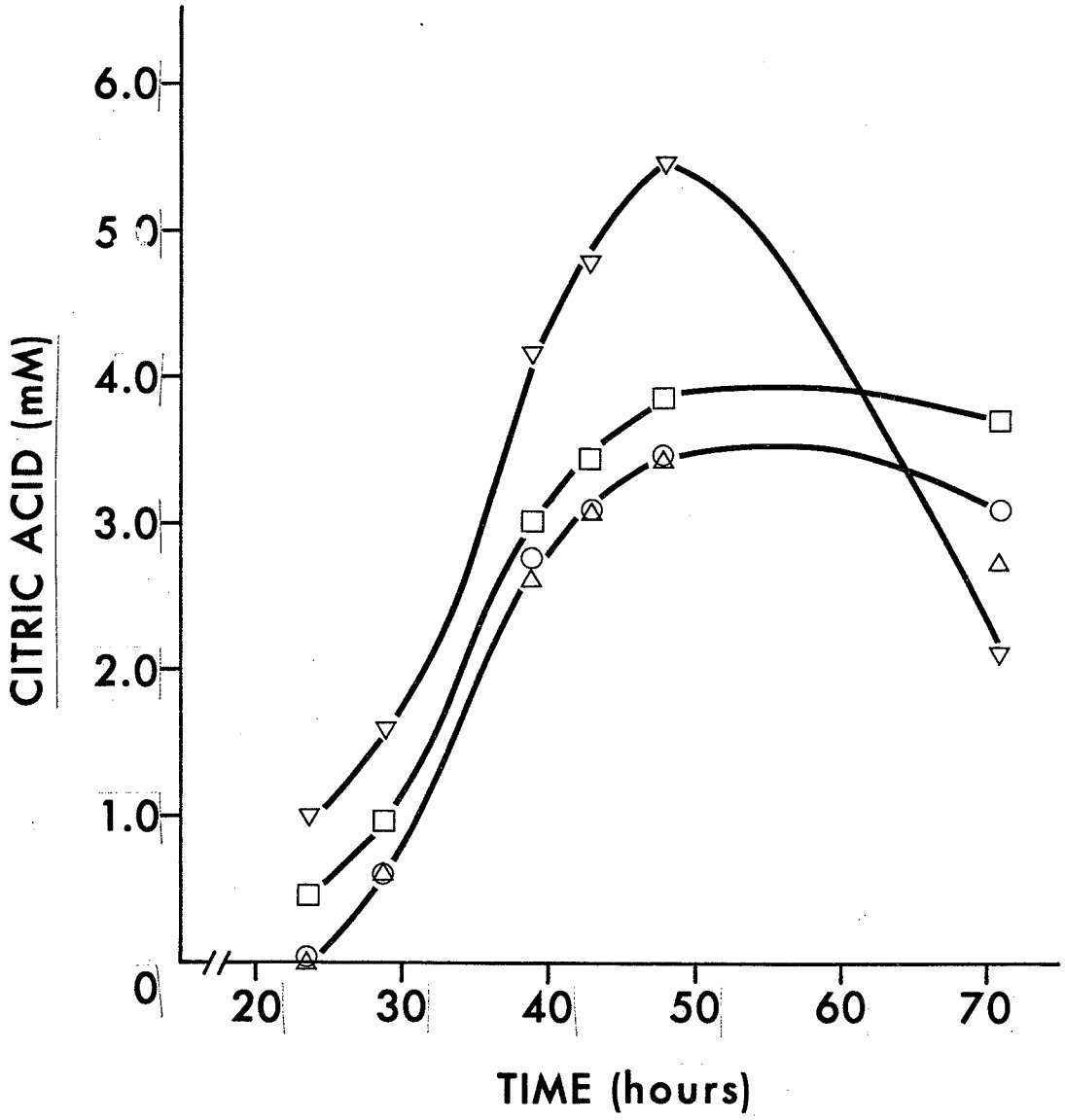

CITRIC ACID (mM)
6.0
5.0
4.0
3.0
2.0
1.0
0
20
30
40
50
60
70
TIME (hours)

Figure 24. Net production of citric acid by cultures grown in the presence of exogenous citric acid. Cultures were grown in M-3 with no addition (0), or with 0.1 mM (Δ), 0.5 mM (□) or 1.0 mM (∇) citric acid added. Each point is the average of three flasks.

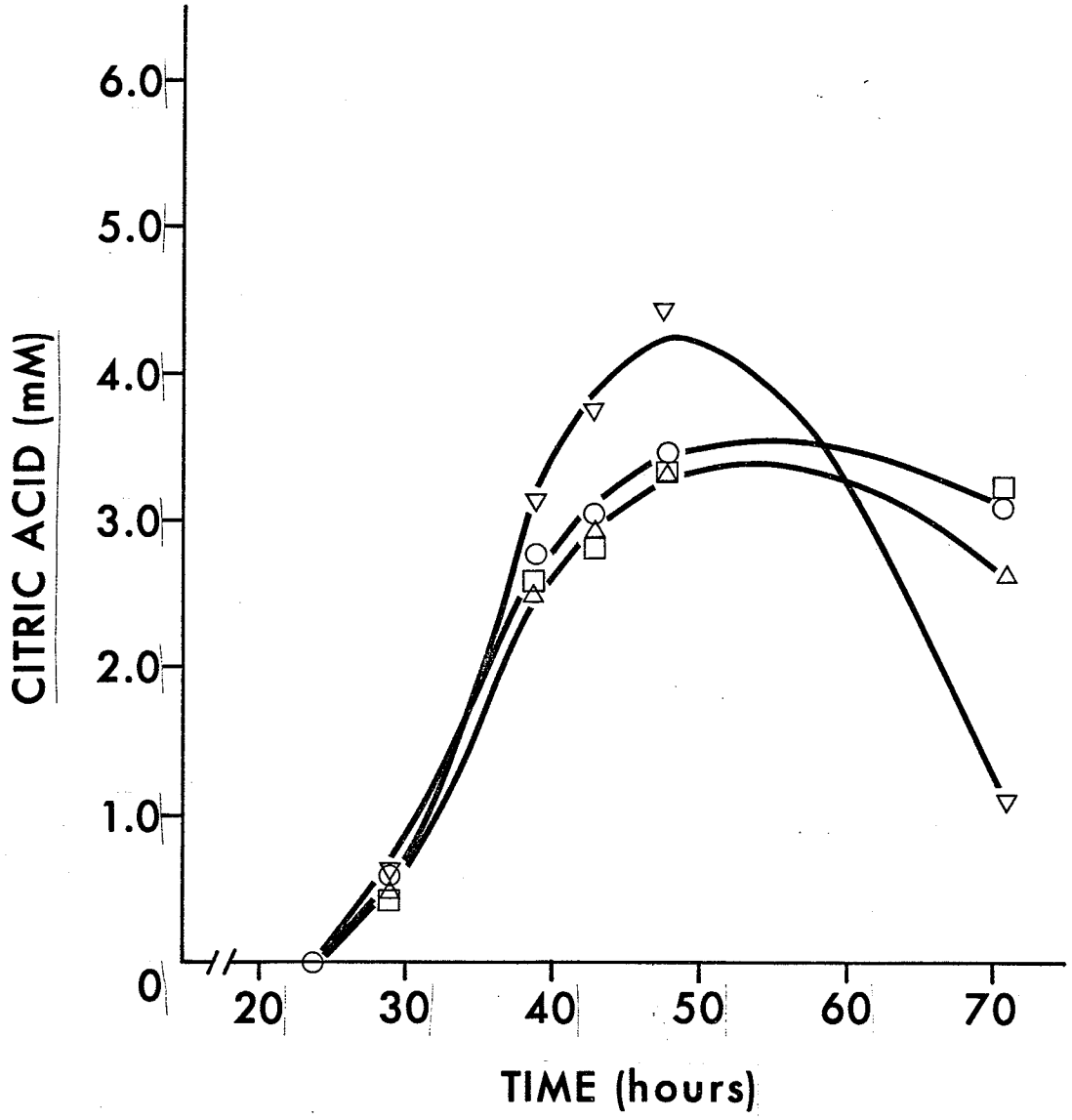

6.0
5.0
4.0
3.0
2.0
1.0
0
CITRIC ACID (mM)
20
30
40
50
60
70
TIME (hours)

Figure 25. Effect of the addition of exogenous citric acid on sucrose utilization by A. niger cultures. Cultures were grown in M-3 with no addition (0), or with 0.1 mM (Δ), 0.5 mM (□), or 1.0 mM (▽) citric acid added. Each point is the average of three flasks.

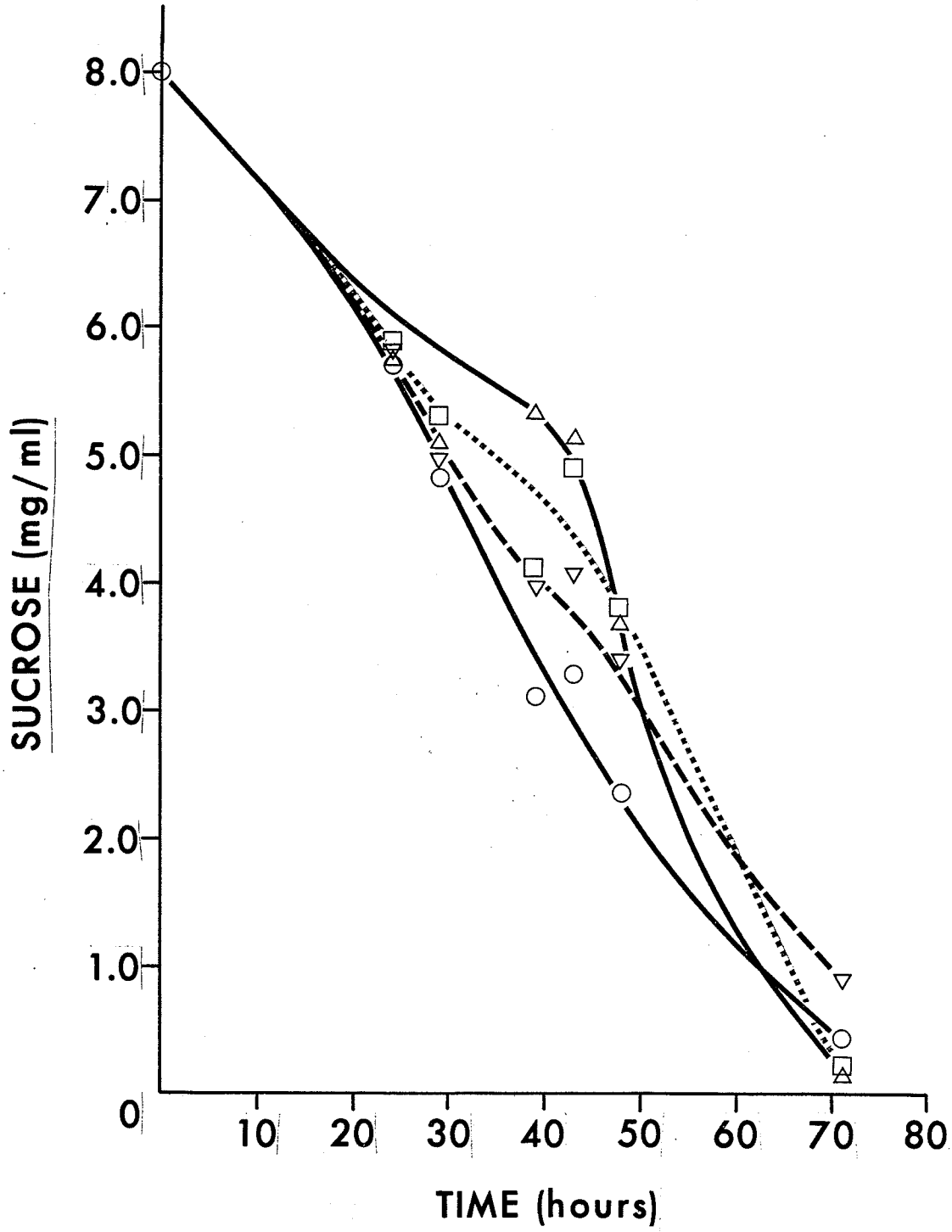
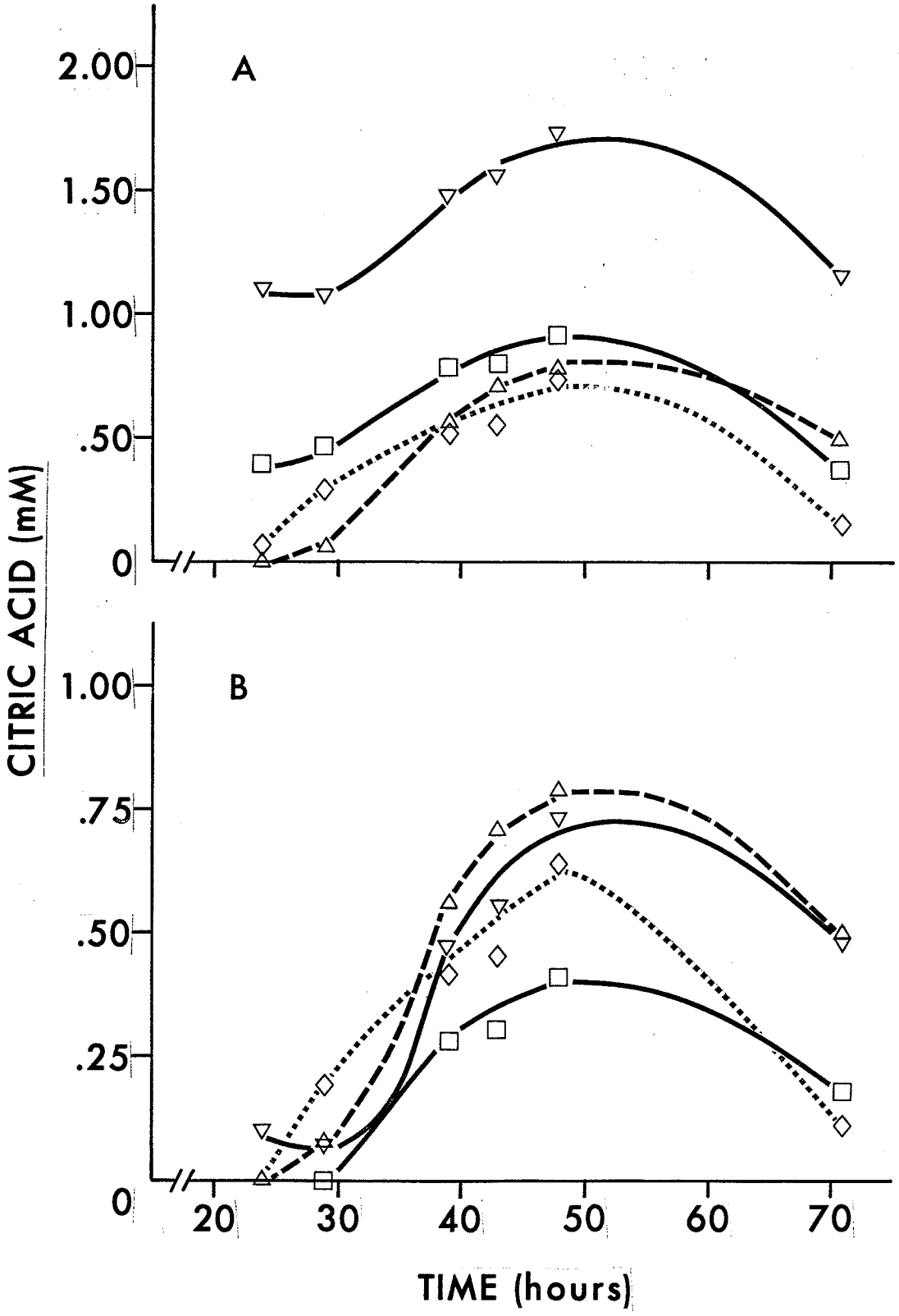

SUCROSE (mg/ml)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
10
20
30
40
50
60
70
80
TIME (hours)

Figure 26. Effect of the addition of exogenous citric acid on the medium levels (a) and net synthesis (b) of citric acid in Zn-grown cultures. A. niger was grown in M-3 containing 0.4 mg/l $ZnSO_4$ without further additions (Δ) or with 0.1 mM (◇), 0.5 mM (□) or 1.0 mM (▽) citric acid added. Each point is the average of three flasks.


A
B
2.00
1.50
1.00
.50
0
1.00
.75
.50
.25
0
20
30
40
50
60
70
CITRIC ACID (mM)
TIME (hours)

differential expected (Fig. 23, 24). The addition of 0.1 mM citric had very little apparent effect. With the addition of 1.0 mM citric acid, the amount of acid produced in excess of that added increased beyond control levels (Fig. 24). Reutilization of citric acid after 50 hours was also enhanced.

Initial sucrose utilization in cultures supplemented with citric acid was at a much slower rate than control cultures, so that the change in sucrose utilization rate at 35 hours became much more pronounced (Fig. 25). The rapid citric acid reutilization observed with 1.0 mM of the acid added may have resulted in the higher concentration of sucrose remaining in the medium.

In zinc-grown cultures, no real shift of the citric acid-accumulation curves was detected when exogenous citric acid was added to the medium (Fig. 26a). Perhaps some degree of shift could be detected for the cultures which had 0.1 mM of citric acid added. The added citric acid seemed to have been utilized from the medium immediately, as the concentration of the acid calculated by subtracting the amount added from the total determined enzymatically yielded negative values for 0.1 and 0.5 mM added citric acid at 24 hours, and for 1.0 mM at 29 hours also. In all cases, unlike the non-Zn controls, the amount of citric acid actually released from the cells was less than that from Zn cultures without added citric acid (Fig. 26 b). In these cultures the external citric acid seemed to have

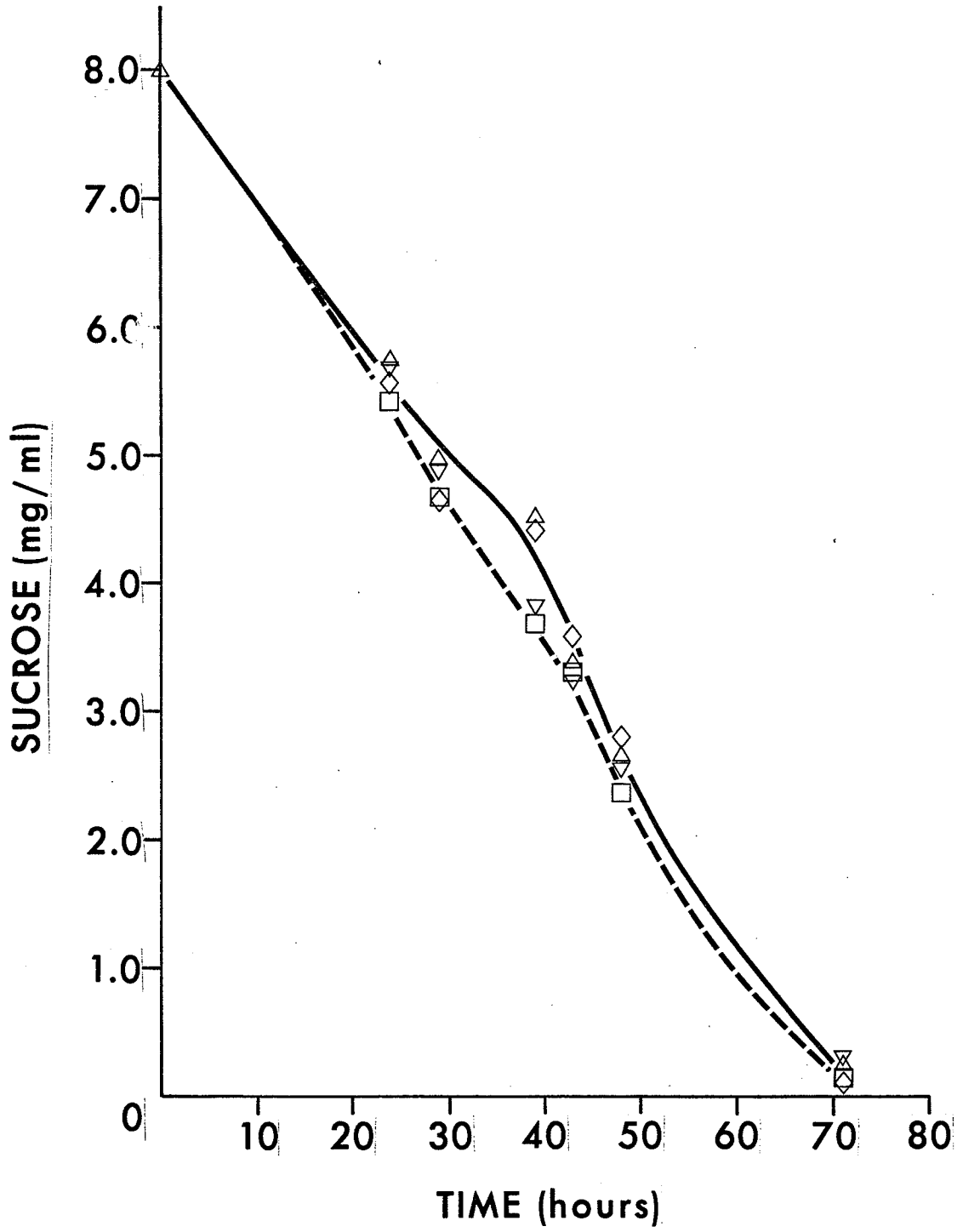
triggered further metabolism rather than increased release as in non-Zn cultures.

The sucrose utilization curves for Zn cultures with citric acid added (Fig. 27) approached linearity with increasing concentrations of added citric acid. The added citric acid appeared to enhance the rate of utilization of itself as well as to have abolished the shift in rate of sucrose utilization which occurred at 35 to 40 hours.

The abnormal cultures, described under general metabolism, which did not show normal citrate production under Zn-deficiency due to culture history (Fig. 2), did show stimulation by citric acid. To some extent the Zn cultures, but not the "controls", showed the autocatalytic mode of production (Fig. 28) while both culture types were significantly stimulated to increased citric acid production (Fig. 29) by the addition of exogenous citric acid.

The addition of 23.4 mM citric acid, which was the equivalent molar concentration to the normal sucrose concentration in the medium, and was in excess of the concentration of citric acid that was accumulated by the most active mycelia, did not repress citric acid accumulation (Fig. 30). Citric acid production was both reduced and delayed, but not inhibited.

Figure 27. Effect of the addition of exogenous citric acid on sucrose utilization by Zn-grown cultures. A. niger was grown in M-3 containing 0.4 mg/l $ZnSO_4$ without further additions (Δ) or with 0.1 mM (◇), 0.5 mM (□) or 1.0 mM (▽) citric acid added. Each point is the average of three flasks.


8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
SUCROSE (mg/ml)
10
20
30
40
50
60
70
80
TIME (hours)

The rate of sucrose utilization was much lower for mycelia grown in the presence of high levels of citric acid (Fig. 31). This might reflect the ability of the fungus to utilize citric acid as a carbon source even in the presence of sucrose and during active citric acid-accumulation.

The utilization of citric acid as a substrate in the absence of sucrose was quite slow until after 40 hours (Fig. 29), when reutilization of synthesized citrate would normally have occurred.

A comparison of citrate levels in the media (Fig. 30) and the sucrose utilization curves (Fig. 31) indicated a very efficient conversion of sucrose to citric acid when exogenous citric acid was present in the medium. At 43 hours, about 75% (on a molar basis) of the sucrose metabolized by cultures with exogenous citrate added was converted to additional citric acid, whereas the control cultures showed a 25% conversion.

ANTIBIOTIC EFFECTS

The production of citric acid by Aspergillus niger has remained essentially undefined, having been called at times secondary metabolism, at times shunt metabolism or at times referred to as an abnormality without a specific classification.

Figure 28. Effect of exogenously added citric acid on total medium citric acid concentration in media from abnormal (insensitive to Zn deficiency, Fig. 2) cultures of A. niger grown in M-3 without further additions (0) (dashed lines, open symbols) or with 0.4 mg/l $ZnSO_4$ (solid lines, solid symbols) added. Citric acid was added at zero time at concentrations of 1.17 mM (Δ) or 2.34 mM (□). Each point is the average of three flasks.

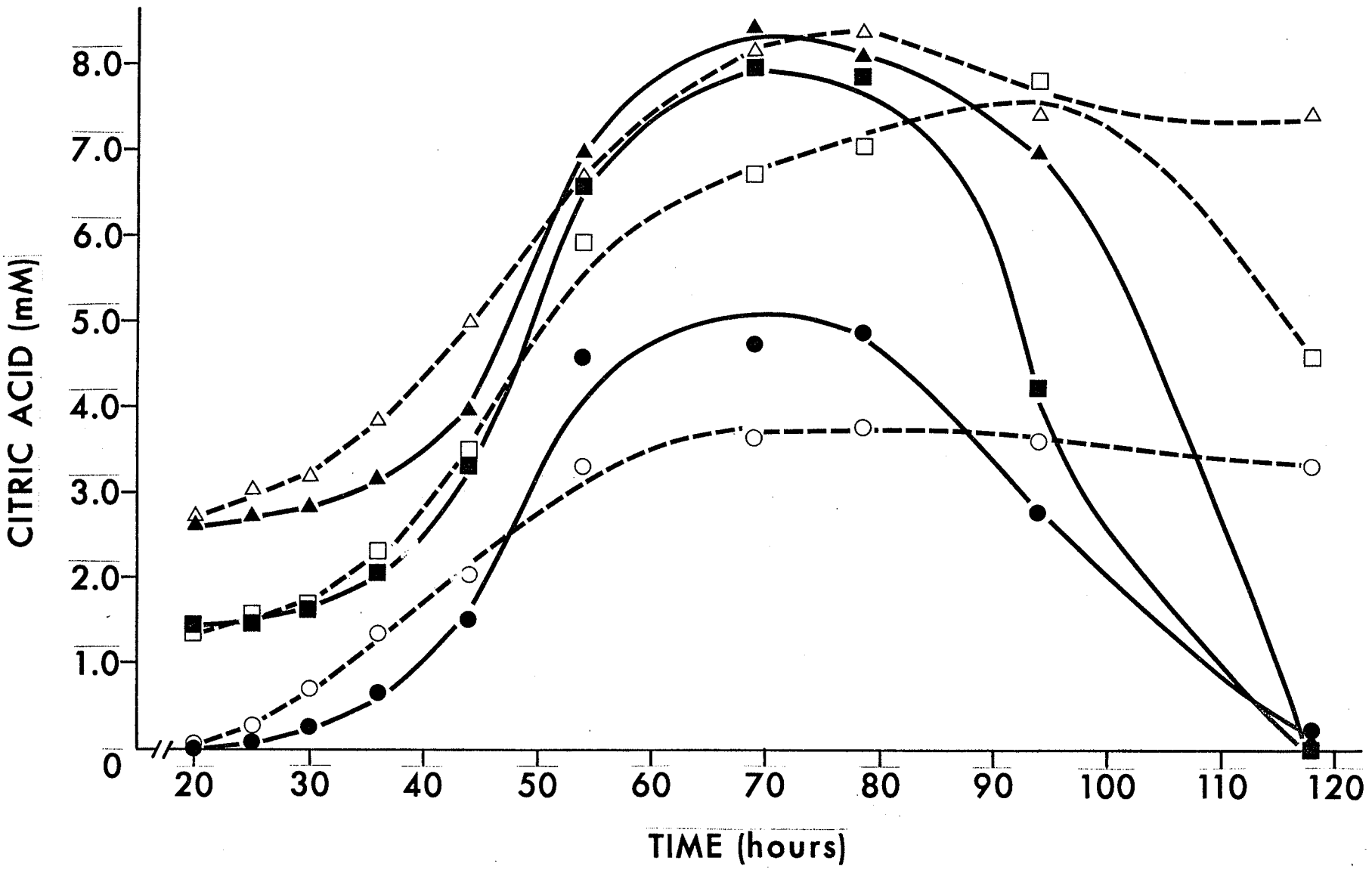

CITRIC ACID (mM)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
20
30
40
50
60
70
80
90
100
110
120
TIME (hours)

Figure 29. Citric acid production by cultures insensitive to Zn-deficiency (see Fig. 2) in the presence of exogenously added citric acid. A. niger was grown in M-3 without further additions (0) (dashed lines, open symbols) or with 0.4 mg/l $ZnSO_4$ (solid lines, solid symbols) added. Citric acid was added at zero time at concentrations of 1.17 mM (Δ) or 2.34 mM (□). Each point is the average of three flasks.

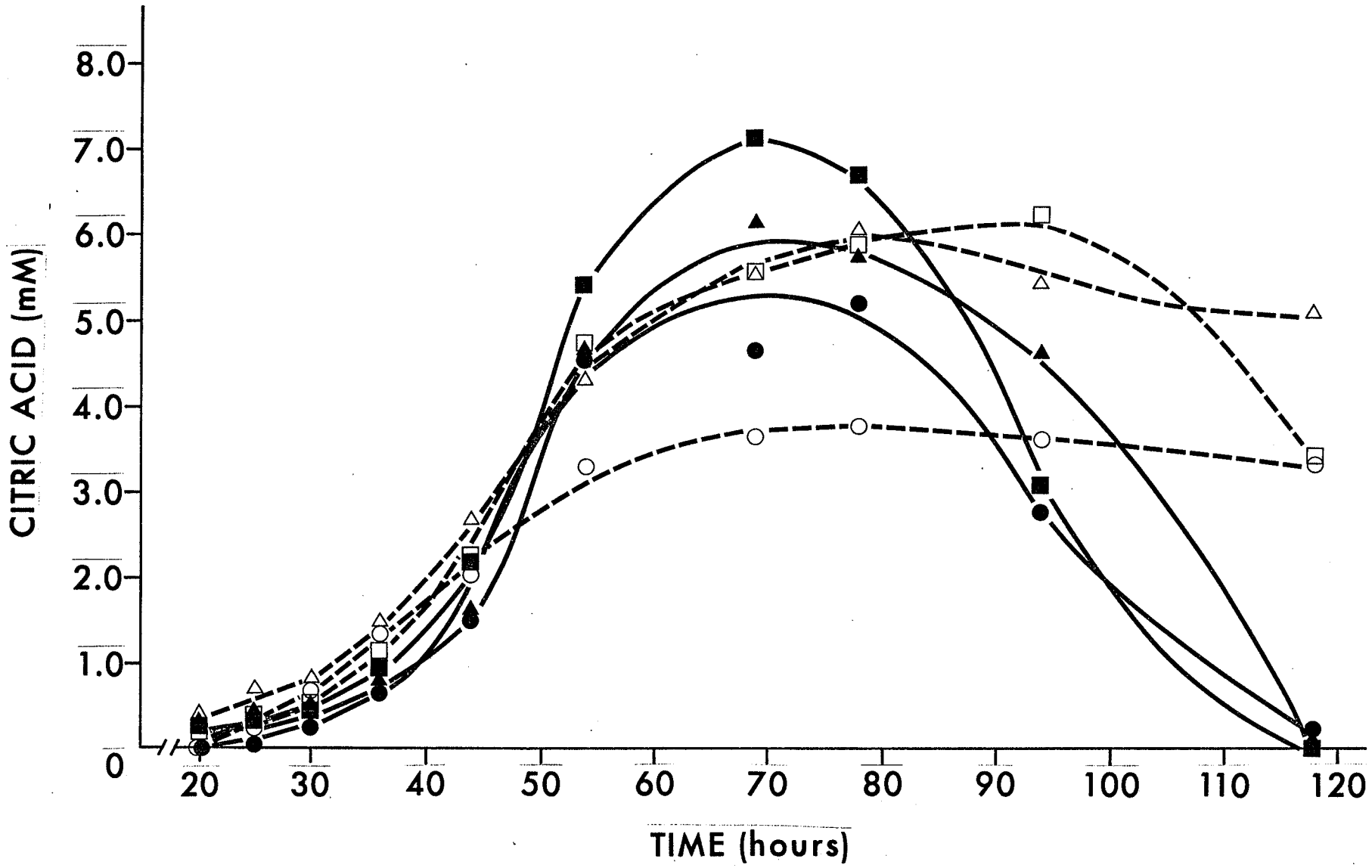

CITRIC ACID (mM)
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
20
30
40
50
60
70
80
90
100
110
120
TIME (hours)

Figure 30. Medium citric acid levels of A. niger cultures grown in M-3 (0), in M-3 with 4.9 g/l citric acid added (□) and M-3 with 4.9 g/l citric acid substituted for sucrose as the carbon source (Δ). Each point is the average of three flasks.

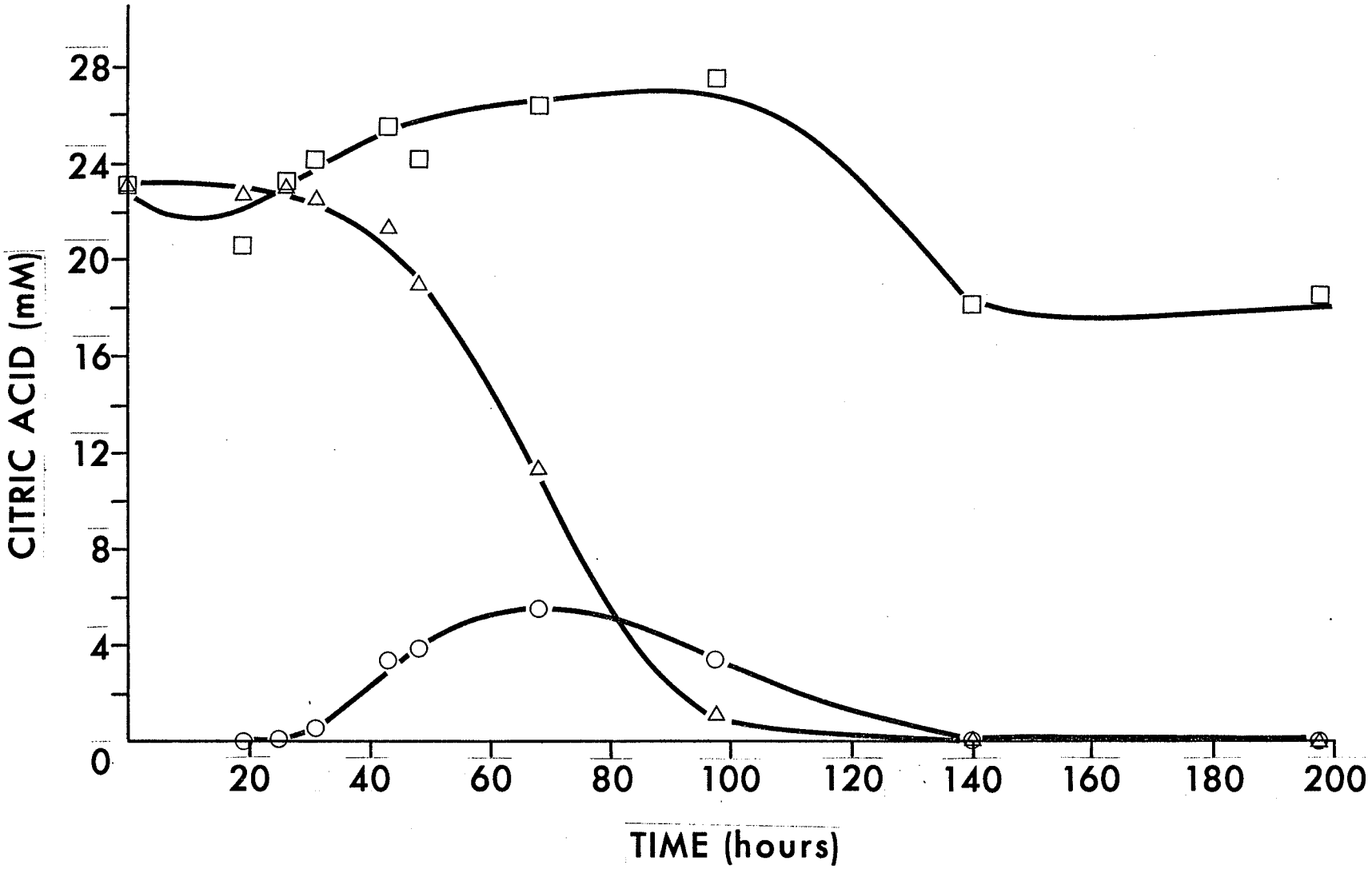
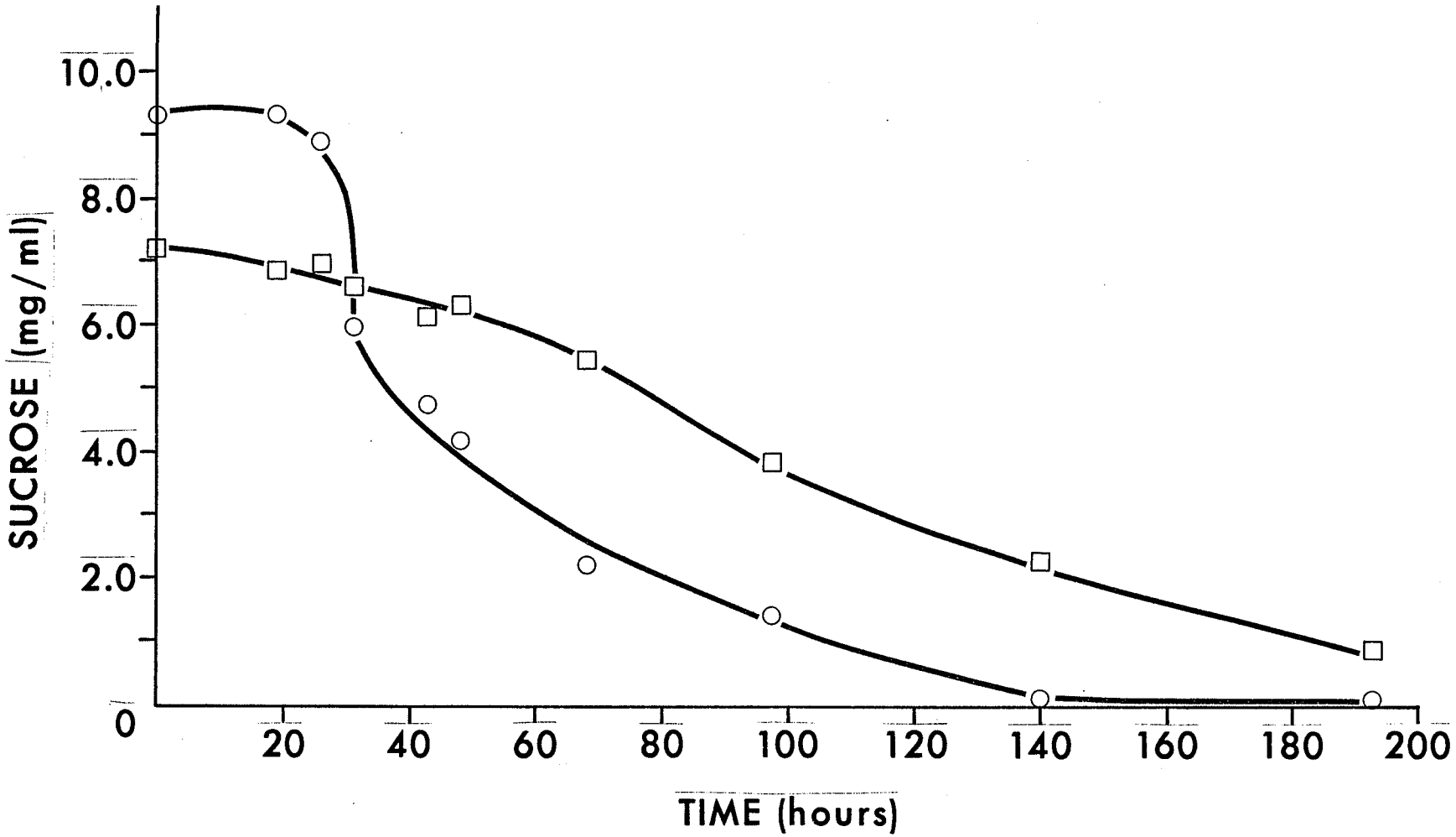

CITRIC ACID (mM)
28
24
20
16
12
8
4
0
20
40
60
80
100
120
140
160
180
200
TIME (hours)

Figure 31. Sucrose utilization by A. niger cultures grown in M-3 (0) or in M-3 with 4.9 g/l citric acid added (□). Each point is the average of three flasks.


10.0
8.0
6.0
4.0
2.0
0
SUCROSE (mg/ml)
20
40
60
80
100
120
140
160
180
200
TIME (hours)

The effects of various metabolic inhibitors which interfere with different stages of growth might help to define and lead to an understanding of this unique phenomenon of accumulation of a primary metabolite.

Mitomycin C is an inhibitor of DNA synthesis. At a concentration of 25 µg/ml, added at 24 hours after inoculation (the time at which active citric acid accumulation had just begun and cell growth was rapid), this inhibitor depressed citric acid accumulation and allowed increased dry weight above that of control cultures, as measured at 48 hours (Table III). The result was analogous to the non-citrate-accumulating Zn-containing cultures. A concentration of 30 µg/ml more effectively repressed citric acid production. Moreover, 30 µg/ml of mitomycin C added at 24, 28 or 32 hours was equally effective as an inhibitor of citric acid release and at promoting cell growth (Table IV), suggestive of a continuous requirement for DNA synthesis during this time for citrate accumulation.

Actinomycin D is an RNA inhibitor. At concentrations of 10, 20 or 25 µg/ml added at 24 hours this inhibitor was equally effective in reducing citric acid-accumulation as compared to controls at 48 hours (Table III). As well, 20 µg/ml added at 28 hours caused a similar loss of yield (Table IV). In all cases, except for 20 µg/ml added at 24 hours, the amount of growth remained equal to that of parallel control citric acid-accumulating cultures also determined at 48 hours.

TABLE III. Effects of metabolic inhibitors on citric acid production and dry weight.

Inhibitor[a]	Concentration (µg/ml)	Citric Acid	Dry Weight	Specific Activity
None		100%	100%	100%
Mitomycin C	25	37	141	26
	30	15	132	12
Actinomycin D	10	19	93	22
	20	20	132	14
	25	29	98	29
Chloramphenicol	200	24	149	16
	250	44	155	24
	300	45	154	28
Cycloheximide	1	55	69	80
	5	38	72	51
	10	39	93	34
	15	9	69	12
5-bromouracil	50	105	133	73
	100	72	165	44
	200	40	109	37

a. Inhibitors were added at 24 hours. Samples were taken at 48 hours; values are given as per cent of 48 hour control values.

b. Specific activity = mmoles citric acid/mg dry weight

TABLE IV. Effect of metabolic inhibitors on citric acid production and dry weight when added at different times.

Inhibitor	Time of addition (hr)	% of controls at 48 hours		
		Citric acid	Dry weight	Specific activity
None		100%	100%	100%
Mitomycin C	24	15	132	12
(30 µg/ml)	28	59	181	32
	32	27	131	21
Chloramphenicol				
(200 µg/ml)	24	24	149	16
	28	78	190	40
	32	60	132	46
Actinomycin D	24	20	132	14
(20 µg/ml)	28	19	100	19
Cycloheximide	24	9	69	12
(15 µg/ml)	28	8	83	7

Chloramphenicol is an inhibitor of procaryotic or mitochondrial protein synthesis. At 200, 250 or 300 µg/ml, added at 24 hours, assayed at 48 hours, it repressed citric acid-accumulation and promoted cell growth (Table III). This effect was also evident when 200 µg/ml was added at 28 or 32 hours (Table IV).

Cycloheximide is an inhibitor of eukaryotic protein synthesis. When 1, 5 or 10 µg/ml were added at 24 hours, or 15 µg/ml at 24 or 28 hours, growth was inhibited as was citric acid production as compared to 48 hour controls (Tables III and IV).

D I S C U S S I O N

DISCUSSION

METABOLISM ASSOCIATED WITH CITRIC ACID PRODUCTION AND THE EFFECTS OF ZINC

Citric acid is accumulated by A. niger only under very specific, carefully controlled conditions. These have been described in detail for many strains of A. niger, and specifically for the strain used in these studies by Wold (1974).

A very important factor in citric acid production is of course the strain used. As such, strains must be carefully selected and maintained to ensure that consistent information be obtained. To achieve this purpose, storage of conidia in a relatively inert state in sterile soil has proved successful in preventing the plague of strain deterioration which haunts workers in this field.

As shown by Wold and Suzuki (1976 a) the accumulation of citric acid is strongly affected by zinc. The addition of increasing concentrations of zinc (as $ZnSO_4$) to A. niger was shown to progressively lower citric acid yields (Fig. 3a, 4) and increase dry weights (Fig. 3b, 5) and sucrose utilization rates (Fig. 6) as compared to accumulating cultures. A concentration of 0.4 mg/l of

$ZnSO_4$ was normally used to inhibit citric acid production. Addition of 0.4 mg/l of $ZnSO_4$ to citrate-accumulating cultures at 20 hours (Fig. 8) and at successive 5 hour intervals (Fig. 9) after that showed that the effects were less as the time of addition became later. However, addition of Zn as late as 35 hours resulted in decreased synthesis of citric acid (Fig. 9) and increased dry weight (Fig. 10). The increase in utilization of sucrose caused by added Zn was also diminished with later times of addition of the metal ion (Fig. 11).

Some cultures lost the sensitivity to Zn-deficiency when transferred from soil stocks of spores grown on rich media (Fig. 2). This loss of sensitivity was explained as being due to an accumulation of Zn by the spores and a cyclic depletion and concentration effect. According to Dalby and Gray (1974), four of five fungi tested accumulated more Zn in the mycelium than in the spores; the exception was A. niger, which accumulated Zn in the spores rather than in the mycelium. Even so, the level of Zn found in the mycelium was higher than the levels in three of the other four fungi tested.

The patterns of internal metabolites of citric acid-accumulating cultures are quite distinct from those of the Zn-inhibited cultures. A high concentration of internal sucrose was built up in citrate-accumulating cultures, except at 20 hours when these cultures contained

less sucrose than at any other time (Fig. 12). The presence of $ZnSO_4$ (0.4 mg/l) in the growth medium prevented this internal build-up, except for an increased concentration at 20 hours, in direct opposition to the citrate-accumulating cultures. The same effects were evident in internal citrate concentrations (Fig. 13), including the inversion at 20 hours. Control cultures maintained high internal citrate levels but a depletion occurred at 45 hours in the presence of Zn.

Extensively washed mycelia showed the same patterns of internal citric acid accumulation for both control and Zn cultures, but the concentration extremes were attenuated as compared to the unwashed systems (Fig. 17). On the other hand, variations in sucrose levels were enhanced, although the concentrations were lower (Fig. 18). In these samples internal sucrose levels were higher in control cultures except at 10 hours and between 25 and 35 hours when they were higher in Zn-containing cultures. The values obtained for extensively washed mycelia probably more truly represent physiological values than those obtained from less extensively washed samples (Fig. 12 and 13).

Cell volumes of citrate-accumulating cultures were lower at all times after 20 hours, and especially at 45 hours when these cultures became stationary with respect to increases in dry weight (Fig. 10) and cell volume (Fig. 14), but Zn-cultures continued to grow.

Until 20 hours, both protein (Fig. 19) and RNA (Fig. 20) levels were higher in control than in Zn cultures, but thereafter the levels remained lower in citrate-accumulating cultures. On the other hand, control cultures had higher levels of DNA until 30 hours, after which the level dropped and a higher level was found in Zn cultures (Fig. 21).

In the absence of Zn, the fungal metabolism consisted mainly of the production of citric acid. As a result cell volumes, dry weight and protein content were lower, as was RNA content. The focus of metabolism on citrate production, and the channeling of carbon sources in that direction might explain the lowered cell size, mass and protein content. Since diverse protein synthesis was probably not occurring to a great extent, RNA production would not have been as important. The high DNA content in the first 35 hours was apparently necessary to maintain the differentiated state which the absence of zinc promoted.

The presence of Zn channeled the cells toward increased growth, as evidenced by higher cell volumes, dry weights, and protein content of the cultures. The higher RNA content, and for the first 35 hours, lower DNA content in Zn-cultures, may be due to a need for increased synthesis of varying proteins in the presence of a stable primary message.

EFFECTS OF CITRIC ACID ON THE MORPHOLOGY ASSOCIATED WITH CITRIC ACID ACCUMULATION

A distinct morphology is usually associated with secondary metabolite production in fungi. The production of citric acid by A. niger is associated with a highly branched non-filamentous structure (Martin and Waters, 1952; Clark, 1962) in the absence of conidiation (Currie, 1917; Snell and Schweiger, 1949).

The typical pellet mode of growth during citric acid accumulation has been reported for the strain of A. niger used in this study (Wold, 1974).

The addition of medium from citrate-accumulating cultures was found to inhibit linear growth and to promote high branch frequency in A. niger on solid media. This property was associated with the presence of YP, and more specifically with citric acid in the medium. Pure citric acid diluted in the assay medium could duplicate these effects, at a concentration as low as 10^{-16}M. Cyclic-AMP at 2×10^{-4}M enhanced the branching effect of dilute citric acid or medium samples. At 2×10^{-3}M c-AMP itself caused some lateral branching, but no inhibition or sub-apical branching.

Distinct inhibition of growth before regrowth was characteristic when 10^{-5}M citric acid was applied to hyphae of A. niger on solid media. The addition of 2×10^{-4}M c-AMP to citric acid samples at 10^{-15}M caused some inhibition.

Other organic acids tested at 10^{-15}M also caused some subapical branching. These were succinic, glutaric, oxaloacetic, cis-aconitic, pyruvic, fumaric and malonic acids. Acids which did not cause branching when applied at 10^{-15} or 10^{-9}M were malic, maleic, tartaric, oxalic, iso-citric, α-ketoglutaric, gluconic and glutamic acids. EDTA or ferrocyanide caused some branching at 10^{-3} and 10^{-6}M respectively, but not at lower concentrations.

Choudhary and Pirt (1965) found that addition of citrate - as a buffer - to the medium of A. niger resulted in pellet morphology as opposed to filamentous growth.

The restricted pellet mode of growth may therefore be a direct result of low concentrations of citric acid in the medium before the initiation of the idiophase and active citric acid accumulation.

EFFECTS OF CITRIC ACID ON CITRATE ACCUMULATION

Citric acid supplemented in the medium of cultures under accumulating conditions enhances the production of citric acid. This has been shown before (Bolcato, 1934).

With the addition of 0.5 and 1.0 mM citric acid to the medium, citric acid production occurred earlier (Fig. 23) and to a greater extent (Fig. 24). Citric acid reutilization was also promoted. Sucrose depletion was slower until about 40 to 45 hours (Fig. 25). The addition of citric acid to Zn-containing cultures could not induce added accumulation, and only at 0.1 mM added citric acid

was earlier production evident (Fig. 26 a, b). Citric acid did promote reutilization, however, which might account for lower net levels in the medium (Fig. 26 b). Sucrose utilization of Zn-cultures with added citric acid occurred without the usual lag found at about 35 hours (Fig. 27).

Cultures which had lost sensitivity to Zn-deficiency showed slightly earlier and enhanced citric acid production when given 1.17 or 2.34 mM exogenous citric acid, when grown in the presence or absence of Zn. They also showed an enhanced re-utilization rate (Fig. 28, 29).

The addition of as much as 23.4 mM exogenous citric acid to the growth medium did not repress citric acid production (Fig. 30). In these cultures, citric acid was found in the medium longer (Fig. 30) and sucrose utilization was slower (Fig. 31). Utilization of citric acid as a sole carbon source was also slower for the first 40 hours of growth (Fig. 30) than was normal sucrose utilization, but the final depletion of carbon source was more rapid.

Perlman (1951) found that the addition of 10 times this concentration, 0.26 M citric acid, to cultures did not repress accumulation. These cultures were sensitive to Fe rather than to Zn, and the addition of Fe enhanced utilization of the acid as well as repressing its synthesis.

Low levels of external citric acid thus seem capable of triggering, or enhancing, the accumulation of itself. Citric acid also promotes reutilization of

itself, which is characteristic of the Zn mode of growth. The fact that cultures insensitive to Zn-deficiency show the same effects, in the presence or absence of Zn, analogous to accumulating cultures, suggests that citrate may be the primary trigger for idiophase differentiation, which is allowed in the absence of the metal ion.

CELLULAR PROCESSES REQUIRED FOR CITRIC ACID ACCUMULATION

Citric acid has been called a shunt metabolite or a spill-over metabolite. Both of these terms imply that citric acid accumulates in the absence of growth, and that an active differentiated process and normal metabolic growth processes of the fungus are unnecessary; the fungus would merely spew out waste carbon through the use of existing, uncontrolled, enzyme systems.

The inhibitor studies (Tables III, IV), indicated that DNA synthesis was probably necessary, at least between 24 and 32 hours, for citric acid-accumulation but not necessarily for growth. In fact, citric acid-accumulation was repressed in favor of enhanced growth in the presence of mitimycin C. Similarly, mitochondria protein synthesis during this interval was probably necessary for citric acid synthesis but not for growth; growth was also promoted in the presence of chloramphenicol.

The inhibitor of DNA synthesis, mitomycin C, and mitochondrial protein synthesis inhibitor, chloramphenicol,

thus have the same effects on the fermentation as the addition of Zn. This correlates with the finding of a higher DNA content under citrate-accumulating conditions than in the presence of Zn.

The inhibitor of RNA synthesis repressed citric acid-accumulation, but had little effect on growth; i.e. growth continued as in control cultures in the presence of Actinomycin D. During the time interval studied at least, synthesis of RNA seems to be necessary both for citric acid production to be maintained and to cause reversion to the enhanced growth in parallel with Zn-containing cultures.

The partial inhibition of cytoplasmic protein synthesis by cycloheximide prevented both growth and citric acid accumulation. Cycloheximide inhibits Penicillium cyclopium at a concentration of 100 µg/ml (Nover and Müller, 1975), Aspergillus nidulans at 50 µg/ml (Sternlicht et al, 1973) and Saccharomyces carlsbergensis at 5 µg/ml (Kerridge, 1958).

These effects may in some way be related to the stage of growth of the cultures. The addition of either chloramphenicol or mitomycin C at 24 or 32 hours caused more inhibition of citric acid synthesis, and less promotion of growth than when added at 28 hours. In the case of chloramphenicol inhibition, however, the specific activities were similar at 28 and 32 hours but higher than at 24 hours. The effect of cycloheximide on citric acid synthesis was the same at 24 and 28 hours, but at

28 hours the inhibition of growth was less, so that the specific activity was somewhat lower.

The effects were greatest at 24 hours with mitomycin C or chloramphenicol, and similar at 24 or 28 hours with actinomycin D and cycloheximide. Thus the major differentiation process required for full idiophase development occurred by 24 hours, but remained sensitive throughout the period studied. That the effects of cycloheximide and actinomycin D were similar at 24 and 28 hours indicates that an active process, requiring continued protein and RNA synthesis is probably involved.

These studies were of a preliminary nature only. Further studies are necessary extending the time range studied, shortening the time between addition of inhibition and detection of effects, and monitoring internal metabolites levels as compared to parallel uninhibited and Zn-inhibited cultures to fully explain the effects observed. As well, incorporation of precursors into the putative target molecules should be determined to ensure that the antibiotics are indeed acting as expected.

CONTROL OF CITRIC ACID ACCUMULATION

According to Wold and Suzuki (1973 a, b; 1976 a, b), citric acid production is controlled by Zn, the effect of which is in some way mediated by c-AMP. They found that when c-AMP was added to citrate-accumulating cultures, it enhanced citric acid production in the idiophase and

enhanced growth in the trophophase. The change of response from growth stimulation to inhibition and citrate production occurred at 14 hours for an inoculum conidia density similar to that used in this study (Wold, 1974). The detection of sufficient citric acid in the medium (by plate assay) to promote hyphal branching at 12 hours correlates with the transition point expected from Wold's findings.

As in Wold's studies, the ability of a culture to produce citric acid was determined by the absence of Zn, while the presence of the metal ion directed the fungus toward growth. However, the finding of cultures insensitive to Zn-deficiency, but which could be stimulated by citric acid suggest that other controls might be more important.

Citric acid at low concentrations caused branching, characteristic of accumulating cultures, and the effect was enhanced by c-AMP. The acid also enhanced its own production and re-utilization; it promoted re-utilization but not synthesis in Zn-containing cultures.

These facts suggest that citric acid control over its own production is involved.

The activities of inhibitors suggest that citric acid production is not a spill-over process.

Thus an active, differentiated process, initiated by citric acid, and dependent upon the absence of zinc seems to occur. Both the morphology and the secondary metabolite production involved in this fermentation are

promoted, if not induced by citric acid.

The presence or absence of Zn determines what growth mode will be allowed. The presence of citric acid itself, in small quantities externally, triggers the active accumulation of the metabolite. Both the zinc and citric acid effects may be mediated by c-AMP.

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