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Manganese Deficiency Leads to Elevated Amino Acid Pools in Citric Acid Accumulating *Aspergillus niger*

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Abstract. Free amino acid pools have been investigated in a citric acid accumulating strain of *Aspergillus niger* during batch growth under manganese sufficient and deficient conditions by means of an improved chromatographic method. Studies on the mycelial content of several nitrogenous compounds under manganese sufficient and deficient conditions showed that manganese deficiency resulted in lower amino acid pool sizes during trophophase and considerable accumulation during idiophase, and in a reduction of the protein and nucleic acid contents. Addition of cycloheximide to mycelia grown with sufficient manganese also caused an elevation of free amino acid pool sizes, thus indicating that impairment of protein synthesis by manganese deficiency is responsible for the observed rise in amino acid concentration. Furthermore it was observed that the manganese deficient mycelia excreted high amounts of all amino acids suggesting that manganese deficiency may also affect membrane permeability.

Key words: Amino acids – *Aspergillus niger* – Manganese deficiency – Citric acid accumulation.

The filamentous fungus *Aspergillus niger* is known to accumulate high quantities of citric acid when grown under certain conditions, among which trace metal deficiency is a very important parameter (cf. Berry et al., 1977). Particularly the presence of manganese ions is very detrimental to citric acid production (Clark et al., 1966) and special care must be applied for the removal or antagonization of this metal ion during medium preparation.

A possible role for manganese in *Aspergillus niger* metabolism was found recently (Kubicek and Röhr, 1977): manganese deficiency was shown to result in a decrease in the concentrations of several enzymes connected with anabolism with the termination of growth phase. This was supported by the finding of severely reduced lipid levels under manganese deficiency (Orthofer et al., 1979).

A probable involvement of manganese in amino acid or protein metabolism had been suggested due to the findings that manganese deficient grown mycelia exhibit elevated intracellular concentrations of ammonia (Habison et al., 1979).

This paper presents results of an investigation indicating that a deficiency in manganese ions leads to impaired protein turnover and concomitant rise in the free intra- and extracellular amino acid pools of *Aspergillus niger*.

Materials and Methods

Aspergillus niger Strain. Aspergillus niger B 60 was used throughout these studies, which was selected from *Aspergillus niger ATCC 11 414* (Clark et al., 1966) by means of a paper culture technique (Röhr et al., 1976). The strain is kept on potatoe-dextrose agar slants and subcultured each month.

Culture Conditions. The composition of the medium and conditions for growing *Aspergillus niger* under pilot plant conditions have been given previously (Kubicek and Röhr, 1978). Sucrose was passed through a Dowex AG 50W-X8 cation exchange resin in order to remove metal ions. In the case of manganese supplementation 0.4 mg $MnCl_2 \cdot 4\ H_2O$ per liter were added to the medium.

Harvesting of Mycelium and Extraction of Amino Acids. Mycelia were harvested by suction filtration on precooled linen cloth. The cells were washed quickly with 20 ml tap water and then rapidly ground in a cold mortar chilled with liquid nitrogen. The total time for harvesting did not exceed one minute. The frozen and powdered mycelia were extracted by suspending 1 g of powder in 0.1 M lithium-citrate-buffer, pH 1.5, and homogenizing the suspension in an all glass homogenizer with a motor driven teflon pestle below 4° C. The homogenate was centrifuged at low speed to remove cellular debris and the opaque supernatant clarified either by high speed centrifugation (35,000 g, 20 min, room temperature) or ultrafiltration. The clear supernatant was kept at −20° C until determination.

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Free Amino Acids Released into the Medium During Cultivation. The culture filtrates obtained after suction filtration of the culture broth and the washing fluids were mixed with an equivalent amount of 0.1 M lithium citrate buffer, pH 1.5, and clarified as described above. The clear supernatants were kept frozen until use for amino acid analysis.

Amino Acid Analysis. Amino acid analysis was conducted by ion-exchange chromatography on spherical cation-exchange resins with an Unichrom Amino Acid Analyzer (Beckman Instruments, München, Germany). Neutral and acid amino acids were separated by means of the method described by Benson et al. (1967). Resin type Beckman M 72 was used in a 69 × 0.9 cm column (56 cm resin height) at an elution rate of 50 ml/h. For the separation of the basic amino acids, a modification of the physiological procedure described by Benson and Patterson (1965) was employed: buffer change (sodium citrate) was already performed at 30 min, and temperature was held constant at 55° C. Separation was conducted on resin Beckman M 81 in a 31 × 0.9 cm column (20 cm resin height) with an elution rate of 50 ml/h.

Extraction of Macromolecular Nitrogen Components. Mycelia were harvested, washed extensively with tap and distilled water, blotted between filter papers and then ground in a mortar under liquid nitrogen. The frozen powder was divided into two parts: one part was used for the extraction of nucleic acids with 0.5 N $HClO_4$ at 70° C after removal of the 0.25 N $HClO_4$ fraction at 4° C according to Kuboye et al. (1976). The other part was homogenized by means of a teflon homogenizer with 0.1 N NaOH. The homogenate was centrifuged and the supernatant used for the determination of soluble protein as described later.

Nucleic Acid Analysis. Total nucleic acid content was determined from measurements at 285 and 263 nm, using yeast RNA as standard.

Protein Analysis. The 0.1 N NaOH extract was dialyzed against water for several hours, made 0.1 N in NaOH again, centrifuged and the resulting clear superantant diluted in 0.1 N NaOH. The protein content of this solution was determined spectrophotometrically and calculated according to Warburg and Christian (1941).

Total Nitrogen. Total nitrogen of the dry mycelia was determined by Kjeldahl-digestion (Herbert et al., 1971) with subsequent determination of the formed NH_4^+ in an automatically operating apparatus according to the indophenole green method (Reardon et al., 1966). A factor of 6.25 was used to calculate protein from nitrogen measurement.

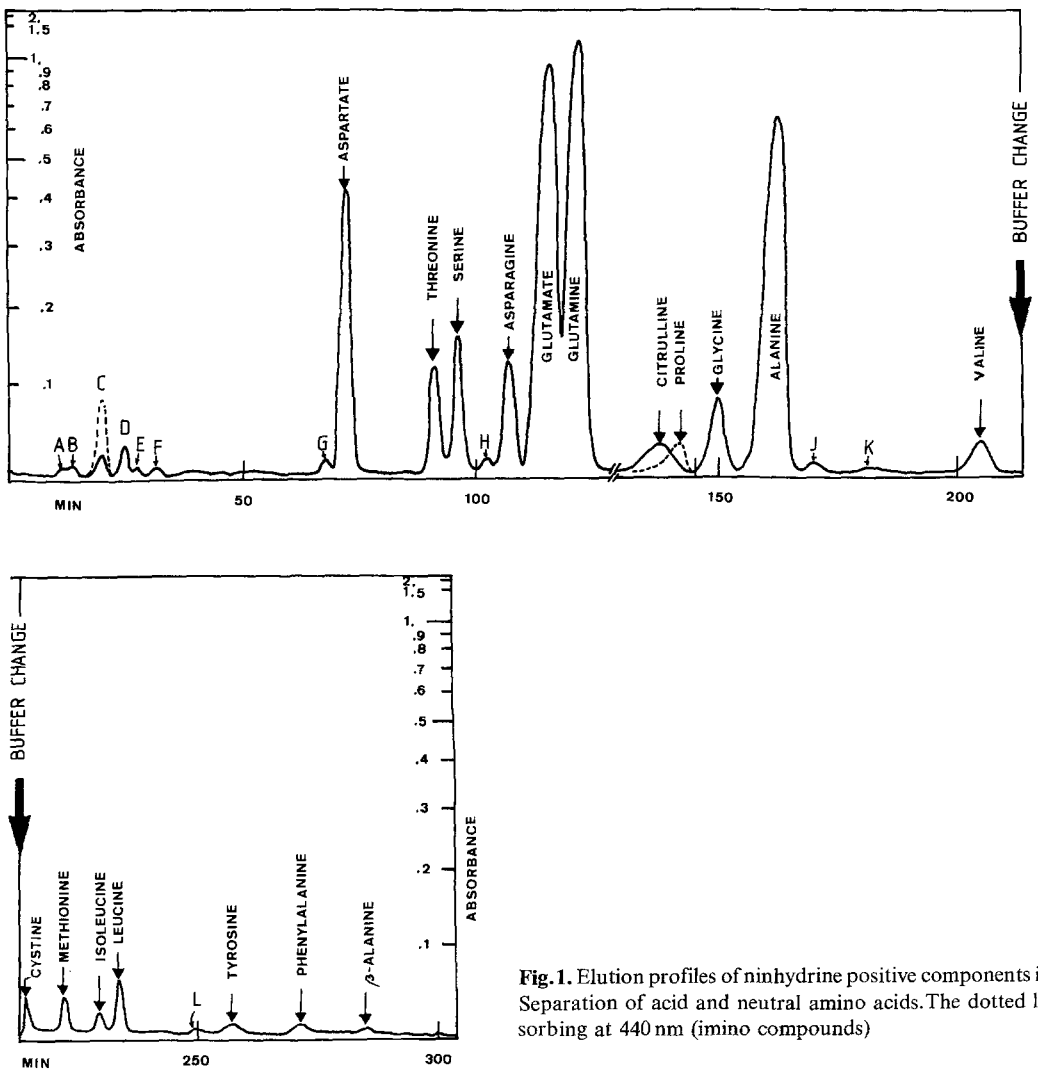

Fig. 1. Elution profiles of ninhydrine positive components in extracts of *Aspergillus niger*. Separation of acid and neutral amino acids. The dotted line represents substances absorbing at 440 nm (imino compounds)

Results

Separation of Free Amino Acids in Extracts of Aspergillus niger

In the attempt to study the influence of manganese ions on amino acid metabolism a convenient method had to be found which enabled the separation and the quantitative measurement of all common amino acids. The modified procedure described above resulted in a complete separation of all relevant amino acids. An example of the elution profiles is given in Fig. 1 for neutral and acid amino acids and in Fig. 2 for basic compounds. The elution times for the individual components differ sufficiently so that overlapping was prevented even when some of these components were present in excess.

As can be seen, in addition to common amino acids and amino acid amides several ninhydrine positive compounds were detected (named A – O), but only in minor amounts. Peak I may be identified as α-aminoadipinic acid, peak K as α-aminobutyric acid, and peak M as glucosamine; no attempts were made for the identification of the other components.

As some of the amino acids were present in very high amounts the validity of the sampling procedure was checked. If the metabolic activity of the mycelium was stopped by instant freezing at various times within 2 – 3 min after withdrawing from the bioreactor, the levels of the amino acids were fairly reproducible. More prolonged washing and filtering resulted in a decrease in the contents of easily metabolizable amino acids with a concomitant increase in ammonia. Only glutamine and glutamate showed some fluctuations even with the procedure described above; however, the value of the sum of both amino acids was fairly reproducible.

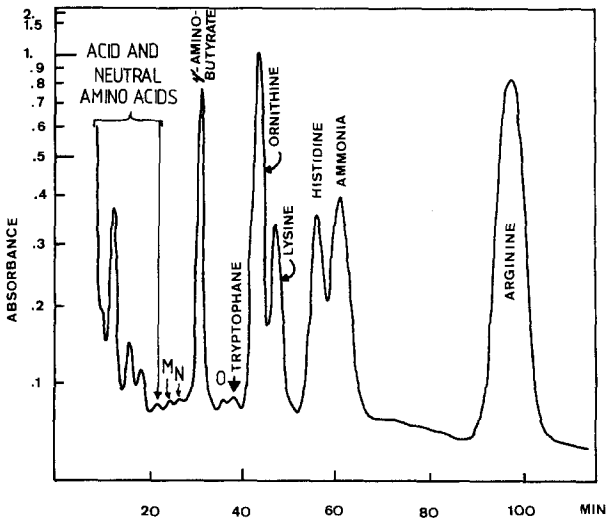

Fig. 2. Elution profiles of ninhydrine positive components in extracts of *Aspergillus niger*. Separation of basic amino acids

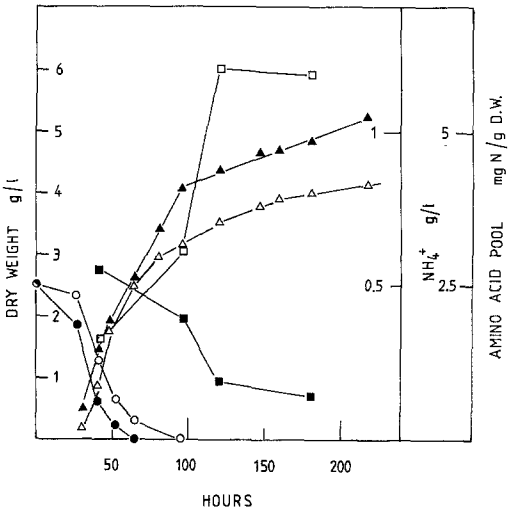

Fig. 3. Changes in mycelial dry weight (△), ammonium ion consumption (○) and intracellular amino acid levels (□) during cultivation of *Aspergillus niger* in the presence (*full symbols*) and absence (*empty symbols*) of manganese ions

Amino Acid Pools of Aspergillus niger Grown under Optimal and Suboptimal Levels of Manganese

The time course of several parameters of particular interest is shown in Fig. 3 for cultivations of *Aspergillus niger* on manganese supplemented (Mn +) and deficient (Mn −) media. The levels of the amino acid pool were measured at various times during growth on both media; the concentrations measured are given in Table 1 and 2.

The values for the free proteogenic pool amino acids fairly range among levels which were reported to be representative for moulds (cf. Schmit and Brody, 1975; Böhmer et al., 1978; Kleber and Aurich, 1968; Mora et al., 1978). Upon cultivation in manganese supplemented medium the concentrations of these pool amino acids reach a maximum during the period of unlimited growth (i.e. at 45 h of cultivation) or in the early stage of idiophase, and exhibit a declining tendency during further culture development. Manganese deficiency results in considerably reduced levels during unlimited growth, whereas after the shift to idiophase (i.e. at 70 h of cultivation) the amino acid pool increases significantly. A considerable amount of the total free intracellular amino acids must be allocated to compounds which are directly formed from glutamate: in these experiments, in particular glutamate, glutamine, γ-aminobutyrate, ornithine and arginine were present in higher amounts. In manganese supplemented medium these compounds resembled proteogenic amino acids in their concentration – time course, whereas with manganese deficiency during idiophase ornithine and arginine were continuously increasing, even after

Table 1. Pool amino acids by cultivation on manganese supplemented medium

Amino acids (μmol/g d.w.[a])	Cultivation time (h)			
	40	75	120	180
Aspartate	1.27	3.85	1.13	0.84
Asparagine	0.54	1.82	0.66	0.30
Serine	1.66	1.08	0.49	0.15
Threonine	3.54	3.78	0.68	1.00
Glutamate	12.23	8.10	2.25	0.89
Glutamine	7.29	3.62	1.23	0.73
Proline	–	1.25	–	–
Glycine	2.51	2.00	0.49	0.27
Alanine	12.09	5.33	2.40	1.31
Valine	3.31	1.72	0.61	0.48
Methionine	0.29	1.32	0.99	0.63
Leucine	0.66	–	0.32	0.18
Isoleucine	3.09	8.80	2.76	1.61
Tyrosine	0.91	2.53	1.03	0.67
Phenylalanine	0.97	2.53	1.04	0.69
γ-Aminobutyrate	10.40	5.67	2.00	1.34
Tryptophane	0.07	0.87	0.16	0.09
Ornithine	26.29	3.50	3.10	1.95
Lysine	4.11	8.07	2.90	1.08
Histidine	6.51	3.83	0.30	1.04
Arginine	46.66	15.38	5.75	3.17
Total amino acid-N (μgatom/g d.w.[a])	335.70	156.73	56.19	34.16
Ammonia	10.91	10.08	3.34	2.27

[a] d.w. = dry weight of the organism

Table 2. Pool amino acids by cultivation under manganese deficiency

Amino acids (μmol/g d.w.[a])	Cultivation time (h)			
	40	75	120	180
Aspartate	3.68	4.05	8.96	4.32
Asparagine	2.49	1.63	4.20	2.81
Serine	0.69	1.03	3.76	2.51
Threonine	2.00	1.95	3.21	2.11
Glutamate	7.59	15.39	40.89	34.78
Glutamine	33.65	17.90	32.90	20.19
Proline	1.18	–	3.66	2.75
Glycine	1.35	1.90	3.67	2.07
Alanine	13.82	18.88	22.11	14.73
Valine	0.47	0.83	2.03	1.18
Methionine	–	0.63	1.21	0.92
Leucine	0.39	0.29	1.34	0.76
Isoleucine	0.42	0.73	2.46	1.98
Tyrosine	–	–	1.29	1.49
Phenylalanine	–	–	0.96	0.93
γ-Aminobutyrate	7.00	5.66	2.71	2.88
Tryptophane	–	–	–	–
Ornithine	19.00	3.69	20.16	37.01
Lysine	1.76	3.05	7.44	3.98
Histidine	–	3.35	2.30	3.21
Arginine	11.82	24.98	45.76	60.91
Total amino acid-N (μgatom/g d.w.[a])	199.67	213.85	417.60	454.66
Ammonia	29.29	29.51	27.30	12.00

[a] d.w. = dry weight of the organism

120 h of cultivation, a time when all other amino acids were stable or slightly decreasing. This may be due to an elevated intracellular ammonia concentration; however, citrulline – another important intermediate in the urea cycle – was only detected in traces.

Excretion of Amino Acids into the Medium

To determine the total free amino acid production by the cell it is necessary to include the extracellular environment in measurements of the pools. Therefore the levels of free amino acids in the culture liquid were evaluated. Only the manganese deficient culture (Table 3) excreted measurable amounts of amino acids. The extracellular distribution of individual amino acids closely resembled the internal pool composition with the exception of β-alanine, which could only be found in the culture fluid and γ-aminobutyrate, which – together with arginine – amounted to 35 % of the total external pool. Excretion was observable predominantly after termination of growth.

Nitrogen Balance in Relation to Manganese Deficiency

In order to establish nitrogen balances further investigations on the mycelial protein and nucleic acid content were performed. The results shown in Table 4 clearly indicate that in the case of manganese deficiency the mycelium contains reduced amounts of protein and nucleic acids; furthermore the decrease in protein content during idiophase is more pronounced.

In an attempt to find a correlation between the increase in amino acid pool and the decrease in the protein content it was found that the amount of amino acids in fact equalled the differences in protein content between *Aspergillus niger* mycelia grown in manganese deficient and supplemented medium. This led to the conclusion that amino acid accumulation is caused by protein degradation rather than by enhanced amino acid synthesis. However, the accumulation of amino acids at 165 h exceeds the level which might be expected from protein degradation. The difference in nitrogen might possibly be accounted for by the observed degradation of nucleic acids during this period.

Possible Mechanisms Involved in Amino Acid Accumulation

In general two possible mechanisms could be responsible for the elevation of amino acid pools: autolysis during starvation, and inhibition of protein turnover.

Table 3. Excreted free amino acids into the manganese deficient culture medium

Amino acids (μmol/g d.w.[a])	Cultivation time (h)			
	48	95	120	165
Aspartate	TR[b]	2.64	6.30	6.33
Asparagine	–	1.62	2.98	2.75
Serine	–	1.32	1.36	1.36
Threonine	–	3.67	3.75	3.22
Glutamate	0.66	3.76	6.94	10.04
Glutamine	4.65	4.86	6.96	9.55
Proline	–	–	–	–
Glycine	TR[b]	1.88	1.53	1.33
Alanine	6.03	15.06	14.99	17.68
Valine	–	0.76	TR[b]	TR[b]
Methionine	–	TR[b]	1.51	1.65
Leucine	TR[b]	0.49	0.32	0.49
Isoleucine	TR[b]	3.06	3.19	3.19
Tyrosine	–	1.84	3.06	2.78
Phenylalanine	–	1.80	3.06	2.99
β-Alanine	–	TR[b]	2.46	2.52
γ-Aminobutyrate	12.33	31.61	65.76	73.16
Tryptophane	–	–	TR[b]	TR[b]
Ornithine	–	12.02	12.01	32.22
Lysine	–	12.34	12.22	11.22
Histidine	–	2.02	9.14	12.07
Arginine	TR[b]	13.16	86.69	122.94
Total amino acid-N (μgatom/g d.w.[a])	28.32	188.27	572.75	766.19

[a] d.w. = dry weight of the organism
[b] TR = only traces found

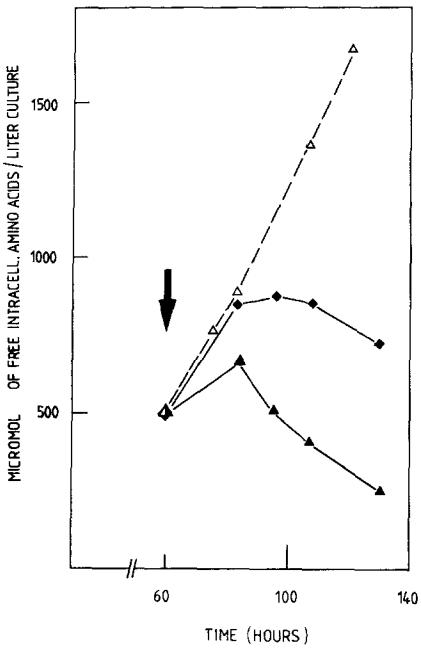

Fig. 4. Changes in intracellular amino acid contents during starvation: 200 ml samples of manganese sufficient grown *Aspergillus niger* culture broth were withdrawn from the fermenter after 60 h of growth (*arrow!*). The mycelia were separated by suction filtration under sterile conditions, washed twice and resuspended in 200 ml deionized water and incubated on a rotary shaker in 1 l shake flasks as described previously (Kubicek and Röhr, 1977). ▲ Amino acid contents after incubation in deionized water; ◆ amino acid contents after incubation in deionized water containing 10 μg cycloheximide per liter. The values of amino acid contents during manganese deficiency are given for comparison (△)

Table 4. Balancing of various nitrogenous compounds in relation to manganese deficiency during idiophase

Hours	95		120		165	
	Mn(+)	Mn(−)	Mn(+)	Mn(−)	Mn(+)	Mn(−)
Alkali soluble protein (mg N/g d.w.[a])	32.9	30.3	24.5	14.7	19.8	8.0
RNA + DNA (mg N/g d.w.[a])	10.8	8.1	7.8	6.5	8.4	4.7
Free intracell. AS (mg N/g d.w.[a])	1.9	3.2	0.8	6.2	0.7	5.9
Free extracell. AS (mg N/g d.w.[a])	0.15	2.8	0.1	8.0	0.1	9.9
Total measured nitrogenous cpd (mg N/g d.w.[a])	45.75	44.4	33.2	35.4	29.0	28.5
Kjeldahl-N (mg/g d.w.[a])	49.7	48.3	41.2	45.2	34.6	32.2
% of N found	92.0	91.9	80.6	78.3	83.8	88.5

[a] d.w. = dry weight of the organism

The first possibility was tested by incubating mycelia previously grown in manganese supplemented medium in deionized water for 70 h. The resulting amino acid pool sizes are given in Fig. 4 showing that even a decrease in the amount of free amino acids was observable. In addition extracellularly only ammonia was detectable. This disproves the speculation that autolysis may lead to an elevation of amino acid pools.

Investigating the second possibility it was found (Fig. 5) that addition of cycloheximide resulted in an

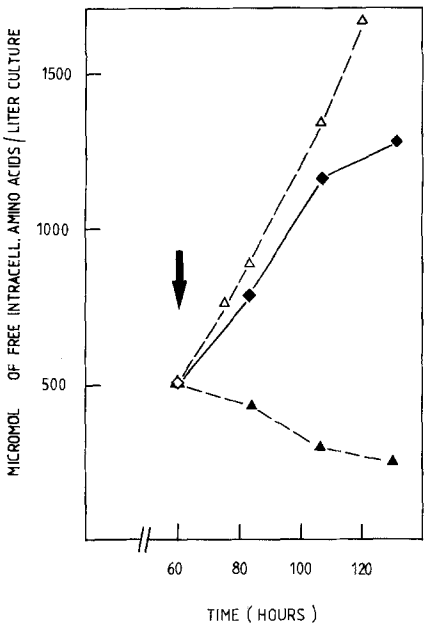

Fig.5. Influence of cycloheximide on intracellular amino acid pool size and composition: *Aspergillus niger* was grown in shake flasks under manganese sufficient conditions as described previously (Kubicek and Röhr, 1977). After 60 h of growth 10 µg cycloheximide were added per liter of culture (*arrow!*). ◆ Amino acid contents after addition of cycloheximide. The amino acid contents for manganese sufficient (▲) and deficient (△) cultivation are given for comparison

increase in the free amino acid pools of manganese sufficient grown mycelia. As it is shown in Table 5, the pattern of amino acid accumulation closely resembled the pattern observed under manganese deficient conditions. A direct relation between the concentration of cycloheximide added and the amino acid pool size could be observed, when the concentration of manganese in the medium was kept constant. Furthermore the effect of cycloheximide was strictly depending on the concentration of manganese (unpublished material). Thus it may be concluded that lower protein content and higher amino acid pool sizes are due to an impairment of protein synthesis caused by manganese deficiency.

Discussion

The results of the present investigation show that manganese deficiency as well as addition of cycloheximide lead to an accumulation of amino acids in *Aspergillus niger*. This was considered to be due to inhibition of protein synthesis, which also supports the view of the authors that manganese deficiency, by impairing anabolic reactions, also favours citric acid accumulation. The accumulation of amino acids in response to cycloheximide treatment and pyrimidine deprivation has also been reported in *Neurospora crassa* (Mora et al., 1978).

Table 5. Pool amino acids in relation to cycloheximide treatment[a]

Amino acids (µmol/g d.w.)	Conditions		
	10 µg CHM Mn(+)	2 µg CHM Mn(+)	Mn(−)
Aspartate	6.79	2.57	7.14
Asparagine	3.72	3.10	3.84
Serine	2.43	1.51	3.22
Threonine	2.91	1.68	3.00
Glutamate	35.42	17.56	37.44
Glutamine	23.67	11.35	22.90
Proline	0.91	0.36	2.10
Glycine	3.81	1.81	3.32
Alanine	23.92	7.65	20.94
Valine	1.88	1.08	1.73
Methionine	1.41	0.98	1.13
Leucine	1.57	0.67	1.27
Isoleucine	2.65	1.34	2.02
Tyrosine	0.74	0.76	0.83
Phenylalanine	0.47	0.52	0.86
γ-Aminobutyrate	2.31	2.64	3.68
Tryptophane	0.06	TR	–
Ornithine	15.37	6.41	18.59
Lysine	5.43	4.11	6.55
Histidine	1.97	0.78	3.17
Arginine	38.21	19.63	37.46
Total amino acid-N (µgatom/g d.w.)	302.92	151.19	311.16
Ammonia	35.32	13.82	30.96

Details of the experimental procedure have been given in the legend to Fig. 5. The values given for the amino acid pool composition refer to 40 h after the addition of 10 (or 2) µg cycloheximide per liter of culture (i.e. 100 h of total cultivation). Therefore values for cultivation under manganese deficiency at 100 h are also given for comparison

Abbreviations. TR = only traces detected; d.w. = dry weight of organism; Mn(+) = medium supplemented with manganese; Mn(−) = medium deficient in manganese

However, manganese deficiency also causes changes in the composition of the amino acid pools as well as in their excretion, thus indicating a multiple effect of manganese ions on *Aspergillus niger* amino acid metabolism.

Accumulation of amino acids was especially pronounced with amino acids derived from glutamic acid, which agrees well with findings of Kubicek and Röhr (1978) that the tricarboxylic acid cycle in *Aspergillus niger* is blocked at the α-oxoglutarate dehydrogenase step. As it is known that these basic amino acids are sequestered in vesicles, there is some possibility that they could represent end products of a mechanism by which cellular metabolism is protected against high NH_4^+ concentrations. The amounts of γ-aminobutyrate excreted into the culture fluid are surprisingly high. The fate of γ-aminobutyrate in fungi is as yet unknown. The only catabolic mechanism of γ-aminobutyrate has as

yet been described by Schmit and Brody (1975) in *Neurospora crassa* during conidial germination which leads to succinic semialdehyde, which – in the present case – would provide a mechanism for by-passing oxidative decarboxylation of α-oxoglutarate (cf. Kubicek and Röhr, 1978).

The particular role of manganese ions in these processes, however, could not be elucidated completely. Manganese deficiency has also been shown to lead to a decreased cellular content of protein, DNA and RNA in *Brevibacterium ammoniagenes* (Furuya et al., 1970), but its definite metabolic function could not be explained. Manganese ions have been known to be activators of many enzymes, but in most cases they can be replaced by magnesium. One exception is RNA-polymerase, which is a manganese requiring enzyme from bacteria (Stevens and Henry, 1964; Fox et al., 1964) as well as from fungi (Tellez de Inon et al., 1973). However, nucleic acid levels were not affected so severely as protein in *Aspergillus niger*.

The fact that manganese deficiency not only leads to accumulation of amino acids but also to their increased excretion deserves further interest. Although some of them, as γ-aminobutyrate and β-alanine, were predominantly present extracellularly, it was observed that in fact all amino acids were excreted. This situation has some parallelism to biotin deficiency in glutamate accumulating bacteria (Kimura, 1963). It may be possible that manganese deficiency may also cause altered membrane permeability as it has been suggested for glutamate producing bacteria. Recent studies (Orthofer et al., 1979) on *Aspergillus niger* lipid metabolism have shown that manganese deficiency is accompanied by a decreased synthesis of phosphatides which may in turn affect membrane structure.

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