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Role of glutamine synthetase in citric acid fermentation by *Aspergillus niger*

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Abstract. The activity of glutamine synthetase from *Aspergillus niger* was significantly lowered under conditions of citric acid fermentation. The intracellular pH of the organism as determined by bromophenol blue dye distribution and fluorescein diacetate uptake methods was relatively constant between 6·0-6·5, when the pH of the external medium was varied between 2·3-7·0. *Aspergillus niger* glutamine synthetase was rapidly inactivated under acidic pH conditions and Mn^{2+} ions partially protected the enzyme against this inactivation. Mn^{2+}-dependent glutamine synthetase activity was higher at acidic pH (6·0) compared to Mg^{2+}-supported activity. While the concentration of Mg^{2+} required to optimally activate glutamine synthetase at pH 6·0 was very high (≥ 50 mM), Mn^{2+} was effective at 4 mM. Higher concentrations of Mn^{2+} were inhibitory. The inhibition of both Mn^{2+} and Mg^{2+}-dependent reactions by citrate, 2-oxoglutarate and ATP were probably due to their ability to chelate divalent ions rather than as regulatory molecules. This suggestion was supported by the observation that a metal ion chelator, EDTA also produced similar effects. Of the end-products of the pathway, only histidine, carbamyl phosphate, AMP and ADP inhibited *Aspergillus niger* glutamine synthetase. The inhibitions were more pronounced when Mn^{2+} was the metal ion activator and greater inhibition was observed at lower pH values. These results permit us to postulate that glutamine synthesis may be markedly inhibited when the fungus is grown under conditions suitable for citric acid production and this block may result in delinking carbon and nitrogen metabolism leading to acidogenesis.

Keywords. Citric acid fermentation; glutamine synthetase; regulation by metal ions.

Introduction

Overflow of fungal metabolism forms the basis of citric acid industry (Maill, 1978). The mechanisms of citric acid fermentation are interesting not only because of their relevance to fungal physiology, but also in understanding metabolic regulation of cell growth and acidogenesis. Towards this objective, tricarboxylic acid cycle (TCA-cycle) (Mattey, 1977; Kubicek and Rohr, 1978), glycolysis (Kubicek and Rohr, 1978) and the enzymes therein (Kubicek and Rohr, 1977; Bowes and Mattey, 1980; Habison *et al.,* 1983) have received considerable attention. Inhibition by citric acid and the role of Mn^{2+} ions in citric acid production (Bowes and Mattey, 1979, 1980) have led to the

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Abbreviations used: γ-GHA: γ-Glutamylhydroxamate; His, L-histidine; Glu, L-glutamic acid; TCA-cycle, tricarboxylic acid cycle

implication of mitochondrial $NADP^+$-specific isocitrate dehydrogenase as an important enzyme for fermentation. Citrate, malate and 2-oxoglutarate levels were elevated during acidogenesis. Also, under conditions of Mn^{2+} deficiency, significant increase in concentrations of pyruvate and oxaloacetate was obtained. These studies led to the suggestion that 2-oxoglutarate dehydrogenase may have a role in acidogenesis by *Aspergillus niger* and that involvement of isocitrate dehydrogenase may be important only during later stages of citric acid production (Kubicek and Rohr, 1978). Regulatory aspects of citric acid fermentation have been recently reviewed by Rohr and Kubicek (1981).

Although it is generally accepted that citric acid fermentation is a nitrogen limited condition of the growth for the fungus (Berry 1975; Berry *et al.,* 1977), very little is known about the role of nitrogen metabolism during this process. The following observations: (a) increase in the intracellular NH_4^+ pool (Habison *et al.,* 1979) and elevated levels of TCA-cycle intermediates preceeding 2-oxoglutarate dehydrogenase step as well as (b) occurrence of large amounts of glutamic acid and metabolites derived from it (Kubicek *et al.,* 1979) under fermentation conditions, especially during Mn^{2+} deficiency, suggested to us that a block at glutamine synthetase step could be one of the important factors responsible for excessive production of citric acid. The results presented here provide some experimental support for this hypothesis.

Materials and methods

Chemicals

Imidazole, 2-mercaptoethanol, sodium arsenate, 2-oxoglutarate, carbamyl phosphate, bromophenol blue, fluorescein, ATP, ADP, AMP, EDTA and all the amino acids were obtained from Sigma Chemical Company, St. Louis, Missouri, USA. Glucose oxidase reagent (GLOX) was obtained from Kabi Diagnostica, Stockholm, Sweden. All the other chemicals were of analytical grade. Organic solvents were distilled before use.

Organism and growth conditions

A. niger (UBC 814) was maintained on potato dextrose-agar medium and an acid producing strain of *A. niger* (ATCC 9142) was obtained from National Chemical Laboratory, Poona, for comparative studies on citric acid fermentation.

A. niger (UBC 814 and ATCC 9142) were grown as a surface culture in one litre flasks containing 100 ml of the culture medium. The composition of the medium for normal growth of this fungus was essentially that described for *A. nidulans* by Pateman (1969), with minor modifications. One per cent glucose was used as carbon source. NH_4NO_3 (2·25 g/litre) was used as the nitrogen source. Addition of micronutrients (mg/litre): $FeCl_3 \cdot 6H_2O$, 20; $ZnSO_4 \cdot 7H_2O$, 10; $MnSO_4\ H_2O$, 3·0; $NaMoO_4 \cdot 2H_2O$, 1·5 and $CuSO_4\ 5H_2O$, 1·0; improved growth and yield of cells. The medium was buffered by using $Na_2HPO_4 \cdot 2H_2O$ (96 g/litre) and KH_2PO_4 (3 g/litre). The final pH of the medium was adjusted to 5·5-6·0. For citric acid fermentation in the laboratory, the following medium (1 litre) containing; glucose or sucrose, 140g; NH_4NO_3, 2·25g; KH_2PO_4, 1·0 g; $MgSO_4 \cdot 7H_2O$, 0·25g and $Fe(NH_4)(SO_4)_2 \cdot 12H_2O$, 0·1 mg and

sufficient HCl to decrease the pH to 2·3, was used (Perlman *et al.,* 1946). Fermentation being highly sensitive to trace metals (Kubicek and Rohr, 1977), especially Mn^{2+} ions, the media were prepared in double distilled water and also using analytical grade chemicals.

Uniform spore suspension (3 ml in double distilled water) obtained from 7-10 day old cultures was used as inoculum. The cells were harvested during the maximal growth phase (45 h after inoculation), washed and stored at — 20°C until further use.

Preparation of crude cell extracts and enzyme assays

Glutamine synthetase from *A. niger* (UBC 814) grown up to maximal growth phase (45 h) on 66 mM glutamate as nitrogen source was purified by ammonium sulphate fractionation, chromatography on DEAE-Sephacel and AMP affinity matrix and gel filtration on Biogel A5M and its homogeneity was determined by gel filtration and Polyacrylamide gel electrophoresis (Punekar *et al.,* 1984).

Crude cell extracts were prepared and the enzyme was assayed by the colourimetric determination of γ-glutamylhydroxamate (γ-GHA) formed (Rowe *et al.,* 1970). Experimental details were same as those described before (Punekar *et al.,* 1984). Specific activity was expressed as units/mg protein. Protein was estimated according to Lowry *et al.* (1951) using crystalline bovine serum albumin as the standard.

Inhibition studies

The enzyme was preincubated with appropriate concentration of the effectors (for 5 min) separately and the reaction was started by the addition of one of the substrates (usually NH_2OH). It was ensured that the pH of all the inhibitors used was adjusted to corresponding assay pH values. 2-Oxoglutarate was found to interfere with the colourimetric method for γ-GHA estimation (data not presented). In all these experiments the amount of γ-GHA formed was read against an appropriate blank containing the same amount of the interfering compound.

Citric acid estimation

Citric acid released into the medium during various stages of fermentation was monitored colourimetrically (Spencer and Lowenstein, 1967) by pyridine-acetic anhydride method.

Glucose estimation

Glucose remaining in the medium was estimated spectrophotometrically using glucose oxidase (GLOX) reagent (Bergmeyer and Bernt, 1974).

Preparation of fluorescein diacetate

Fluorescein diacetate was prepared by reacting fluorescein with acetic anhydride in pyridine. The product was recrystallized from 95% ethanol. The procedure was essentially similar to that described for fluorescein dibutyrate (Kramer and Guilbault, 1963).

Determination of intracellular pH

Internal pH of *A. niger* (UBC 814) mycelium was determined by two different methods. In both cases, fine mycelial suspension of the fungus grown up to 36 h as suspension culture in normal growth medium having NH_4NO_3 (28 mM) as nitrogen source was used. Buffers (0·1 M) of different pH values were prepared using citrate and potassium phosphate as buffer components, because such a buffer probably represents the environment the organism experiences during fermentation.

Bromophenol blue method: The dye, bromophenol blue, distributes across the plasma membrane in a pH dependent fashion (Kotyk and Janacek, 1975). The following equation was used to calculate the internal pH (pH_i):

$$pH_i = pH_e + \log\left[\frac{C_i}{C_e}(1 + 10^{pK - pH_e}) - 10^{pK - pH_e}\right]$$

where, pK value of the dye (bromophenol blue) was assumed to be 40; pH_e (external pH) was measured in the cell suspension; C_e (external concentration of the dye) was determined in the centrifugate; and C_i (intracellular concentration of the dye) was calculated from the difference between the initially added concentration and C_e. To calculate C_i , the intracellular water volume was approximated by haematocrit method (Kloppel and Hofer, 1976).

To a 3 ml cell suspension (about 4·5 mg dry wt/ml) maintained at predetermined pH values using 0·1 M citrate-phosphate buffer (at 25±3°C), 1 ml of bromophenol blue solution (0·1 mM) was added and the suspension was constantly stirred. Samples (1 ml) were withdrawn after 30 min at which time interval., the equilibration between the dye inside and outside was reached. The samples were centrifuged at 2,000 *g* for 10 min in a clinical table top centrifuge and the decrease in absorbance of the supernatant at 589 nm and at pH 7·5- was determined. The external concentration of the dye was obtained from a standard curve.

Fluorescein diacetate as α pH probe: By monitoring the monoanion-dianion transition of intracellular fluorescein (Slavik, 1982), the values of internal pH were calculated from a calibration curve prepared as follows: Fluorescein was dissolved in a series of buffers (citrate-phosphate, 0·1 M) from pH 2·0-7·5. At each pH the fluorescence intensity at 520 nm of the sample was recorded (in a Perkin-Elmer, model 203 spectrofluorometer with 150X xenon lamp source and a R212 photomultiplier tube) after excitation at 535 nm and 490 nm and the ratio of these two intensities (I_{490}/I_{435})was plotted against pH.

The cells grown as fine suspension in the growth medium in shake cultures at 28°C for 36 h were harvested, washed twice and resuspended in buffers of required pH. Prior to fluorescence measurements, the cell suspension (4·5 mg dry wt/ml) was incubated at 28°C with fluorescein diacetate (10 μM) for about 20 min. The cells were washed thoroughly and resuspended in the original volume of buffer before fluorescence measurements.

Results

Citric acid production, mycelial growth and morphology of A. niger on surface cultures

Before correlating changes in glutamine synthetase activity with acidogenesis, it was necessary to establish the fermentation characteristics of *A. niger* (UBC 841) strain. For this reason, the organism was grown on citric acid fermentation medium and modified Pateman medium (Materials and methods) separately and its growth characteristics were compared. Sugar uptake, citric acid released and the pH of the medium were monitored at different time intervals after inoculation and the results are shown in figure 1. Citric acid was not produced when the organism was grown on normal growth medium (figure 1a). However, when it was grown on fermentation medium, two distinct phases of growth and citric acid production were apparent. In the first growth phase (trophophase) there was an increase in the biomass (not shown), but release of citric acid into the medium was not observed. In the second *i.e*., fermentation phase (idiophase) increasing amounts of citric acid was excreted into the medium. Maximal amount of citric acid (about 22 mg/ml) was formed after about 10–13 days. These values are not as high as those encountered during industrial production, as traces of metal ions were not rigorously excluded by adding a chelator to the fermentation medium. Similar results were obtained when an industrially used acid-production strain of *A. niger* (ATCC 9142) (data not shown) was grown under the same conditions. Both the strains of *A. niger* (UBC 814 and ATCC 9142) sporulated within 72 h after inoculation into the normal growth medium, whereas no sporulation was apparent even after 9–10 days of growth on the fermentation medium. Both the strains produced non-sporulating, slimy, folded mats on the surface of the fermentation medium. As both the organisms had similar properties, we used *A. niger* (UBC 814) in these studies.

Glutamine synthetase levels in A. niger grown on fermentation medium

To determine the effects of composition of the media, pH, Mn^{2+} ions, on the levels of glutamine synthetase, the specific activities of the enzyme in crude extracts obtained from cells grown on normal growth medium (modified Pateman) and fermentation medium were determined. Both the strains (UBC 814 and ATCC 9142) showed approximately a two fold lowered levels of glutamine synthetase (specific activity 0·10 and 0·11 μmol/ min per mg of protein, respectively) when grown on citric acid fermentation medium as compared to the level of the enzyme in the mycelia grown on normal growth medium (0·20 and 0·21, respectively). The addition of $MnSO_4$ H_2O (200 mg/litre) to the fermentation medium resulted in an increase of glutamine synthetase levels in both the strains (0·10–0·18 μmol/min per mg protein for UBC 814 and 0·11–0·20 μmol/mm per mg protein for ATCC 9142). In addition to determining the glutamine synthetase levels at a fixed time interval (the maximal growth phase, 45 h), the activity of the enzyme was also estimated at different time intervals during growth and a reciprocal relationship with citric acid excretion was observed. The enzyme levels were high (0·22 units) until about 48 h at which time no citric acid accumulated and thereafter the levels decreased rapidly as citric acid started accumulating in the medium. When the medium was supplemented with different

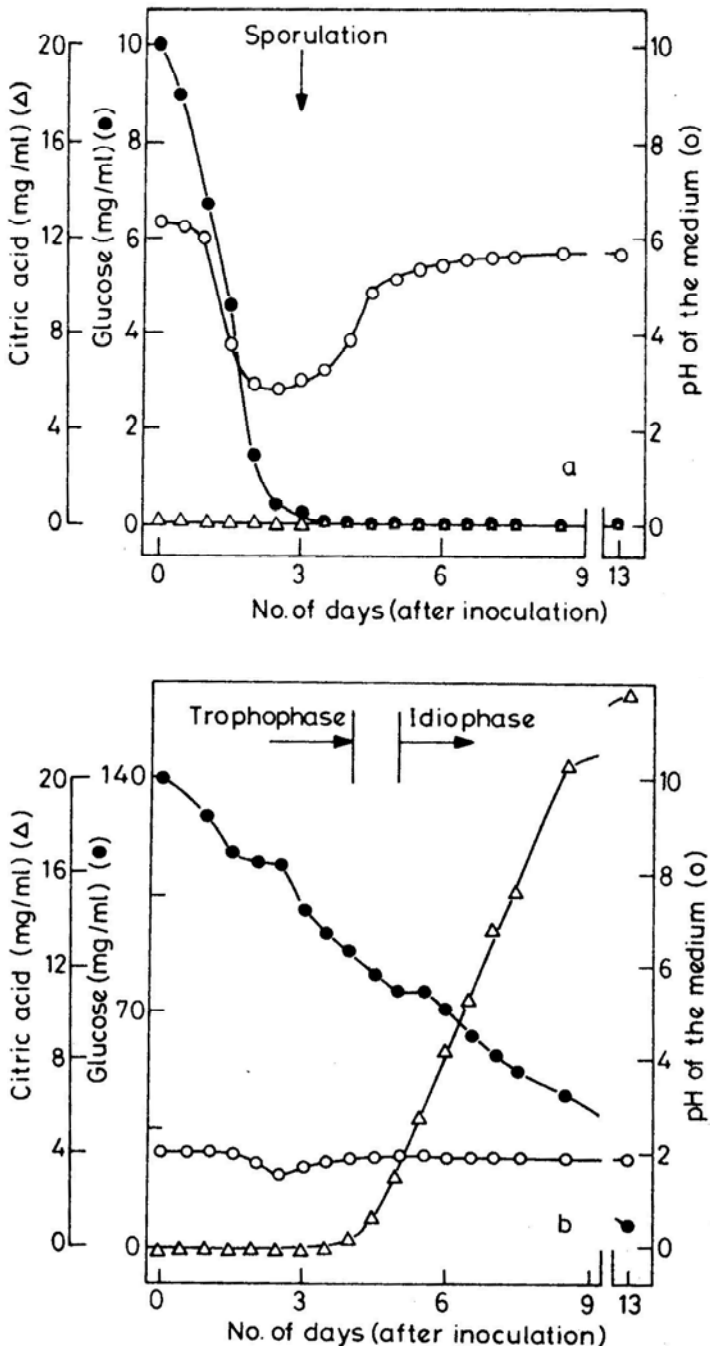

Figure 1. Fermentation characteristics of *A. niger* (UBC 814) strain. The organism was grown on (a) normal growth medium and (b) fermentation medium, by inoculating uniform spore suspension obtained from 6–8 day old cultures. Changes in the pH of the medium (O), glucose utilization (●) and citric acid released into the medium (Δ) were monitored at time intervals indicated.

amounts of Mn^{2+}, the increase in glutamine synthetase activity paralleled the increasing concentration of Mn^{2+} added. The ratio of the Mg^{2+} -dependent synthetase activity to Mn^{2+}-dependent γ-glutamyltransferase activity remained constant 0·15–0·17 under these conditions, suggesting that the activity was probably not being modulated by covalent modification as in *Escherichia coli.*

Effect of pH of the medium on internal pH of A. niger mycelia

Two different methods were employed to evaluate the internal (cytoplasmic) pH in UBC 814 strain of *A. niger.* Although the pH of the medium in which *A. niger* mycelia were suspended was varied by about 5 units in the range 2·3–7·0, the internal pH remained reasonably constant between 6·0 and 6·8, and infact was essentially unchanged until the external pH values were increased beyond 5·0 (figure 2a). At an external pH of 5·3, the intracellular pH was determined to be 6·4 using the fluorescent probe, fluorescein diacetate (figure 2b). Similarly, when the pH of the medium outside was 2·3, an internal pH of 5·9 was observed (figure 2b). In these studies, cells without the probe had negligible fluorescence at the wavelengths used. The results obtained by both the methods were in good agreement. Our data indicated that *A. niger* mycelia have an efficient *in vivo* buffering capacity and maintain their internal pH acidic within narrow limits.

Effect of pH on metal ion saturation

The pH optima for the Mg^{2+}- and Mn^{2+}-dependent synthetase reactions are 7·8 and 5·5, respectively (Punekar *et al.,* 1984). Figure 3 shows the saturation of glutamine synthetase by Mg^{2+} and Mn^{2+}, at two different pH values in the presence of 10 mM ATP. It is evident that at pH 6·0 the Mg^{2+}-supported synthetase activity was very low until the concentration of Mg^{2+} was increased beyond 20 mM. On the otherhand at pH 8·0, saturation pattern was sigmoid with a lag phase until about 5 mM Mg^{2+}, and maximum activity was observed around 20 mM Mg^{2+}. The saturation pattern of glutamine synthetase by Mn^{2+} was qualitatively similar at both the pH values with a maximum around 4 mM Mn^{2+} and concentrations beyond this value were inhibitory. For the same amount of enzyme protein (9·9 μg/assay) the maximum value for Mn^{2+}-supported synthetase activity at pH 8·0 was about half of that at pH 6·0.

pH inactivation of the enzyme

To further examine the role of glutamine synthetase in citric acid fermentation which is sensitive to changes in pH, the stability of the enzyme at various pH values was monitored. The sigmoid pH-stability profile suggested that the enzyme was more stable at neutral pH values than at acidic pH values with a mid point of inactivation around 5·5 (figure 4). Similar pH inactivation experiments were carried out in the presence of either Mn^{2+} (0·5 mM) or ATP: Mg^{2+} (25:10 mM) also. It can be seen that Mg ATP shifted the pH inactivation profile towards the acidic side with a mid point at 5·2, whereas Mn^{2+} caused a more significant shift with a midpoint pH of 4·8. These results demonstrated that Mn^{2+} and Mg· ATP protected the enzyme against inactivation at acidic pH values, as indicated by higher activity under these conditions.

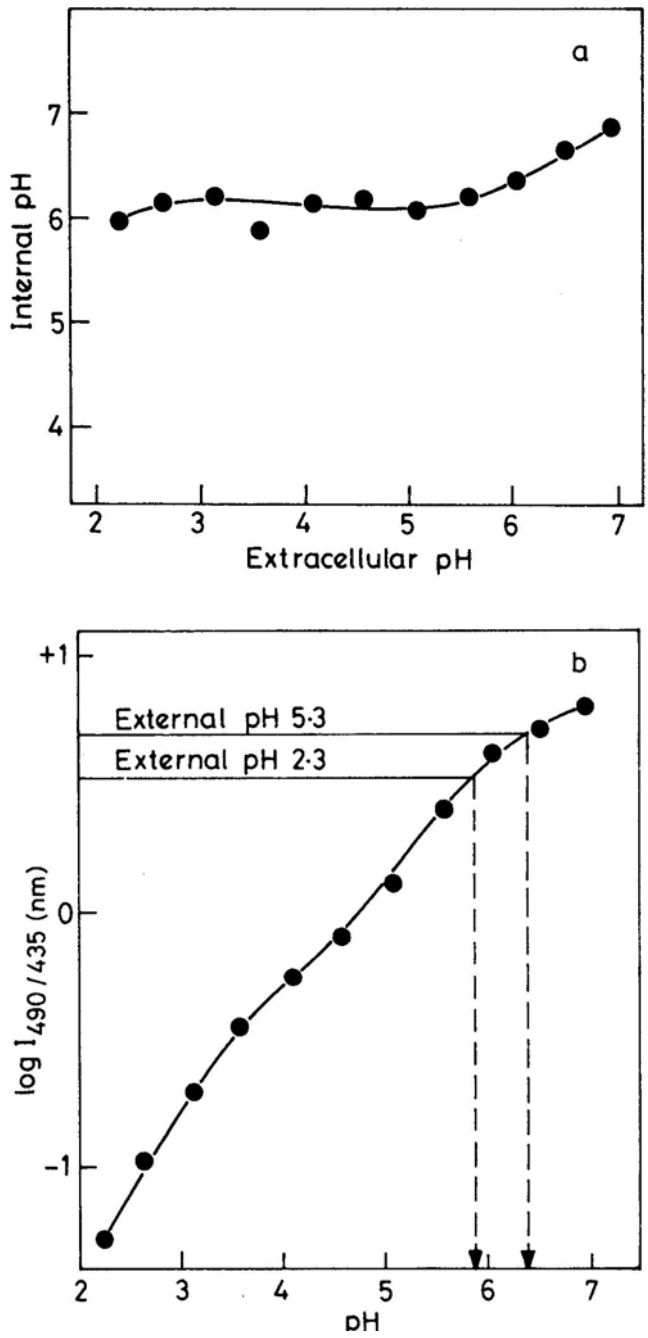

Figure 2. Correlation between extra- and intracellular pH of *A. niger* (UBC 814), (a) Bromophenol blue-dye distribution method, (b) Fluorescein diacetate method. Experimental details are given in Materials and methods.

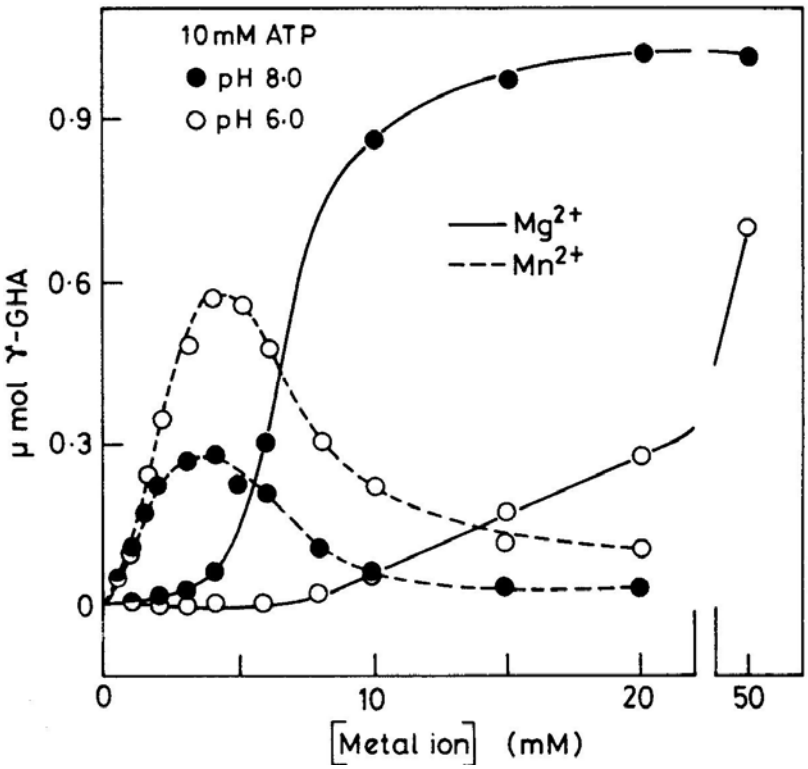

Figure 3. pH dependence of metal ion saturation of *A. niger* glutamine synthetase. The Mg^{2+}-dependent (solid lines) and Mn^{2+} dependent (broken lines) synthetase activity was assayed at pH 8·0 (●)and at pH 6·0 (O), in 100 mM imidazole hydrochloride buffer, using 10 mM ATP and the concentrations of Mg^{2+} (0-50 mM) or Mn^{2+} (0–20 mM) indicated in the figure. γ-GHA formed was estimated.

Is A. niger glutamine synthetase regulated by feed back inhibition ?

pH-Dependence of inhibitions: The effect of a number of possible feedback inhibitors (alanine, anthranilic acid, arginine, asparagine, γ-aminobutyric acid, glutamine, glycine, histidine, methionine, serine, tryptophan, AMP, ADP, GTP, NAD^+, $NADP^+$, carbamyl phosphate, DL-glucosamine-HCl, all at 20 mM) on the Mg^{2+} -dependent (at pH 7·8 and 6·7) and Mn^{2+}-dependent (at pH 5·5) synthetase activities was checked. Among all the compounds tested, alanine, glycine, histidine, serine, ADP, AMP, GTP and carbamyl phosphate caused some inhibition and hence the effect of these compounds was probed further. The results of such a study are shown in table 1. Alanine, glycine and serine had no effect on the Mg^{2+}-dependent synthetase activity (at both pH values), whereas Mn^{2+}-dependent synthetase activity was inhibited. Histidine significantly inhibited the Mg^{2+}-dependent activity at acidic pH (6·7) and also the Mn^{2+}-supported synthetase activity. It is also evident that GTP (at 5 mM) was without effect on the Mg^{2+}-supported reaction. AMP and ADP inhibited both the activities, while carbamyl phosphate inhibited the Mg^{2+}-dependent synthetase reaction only.

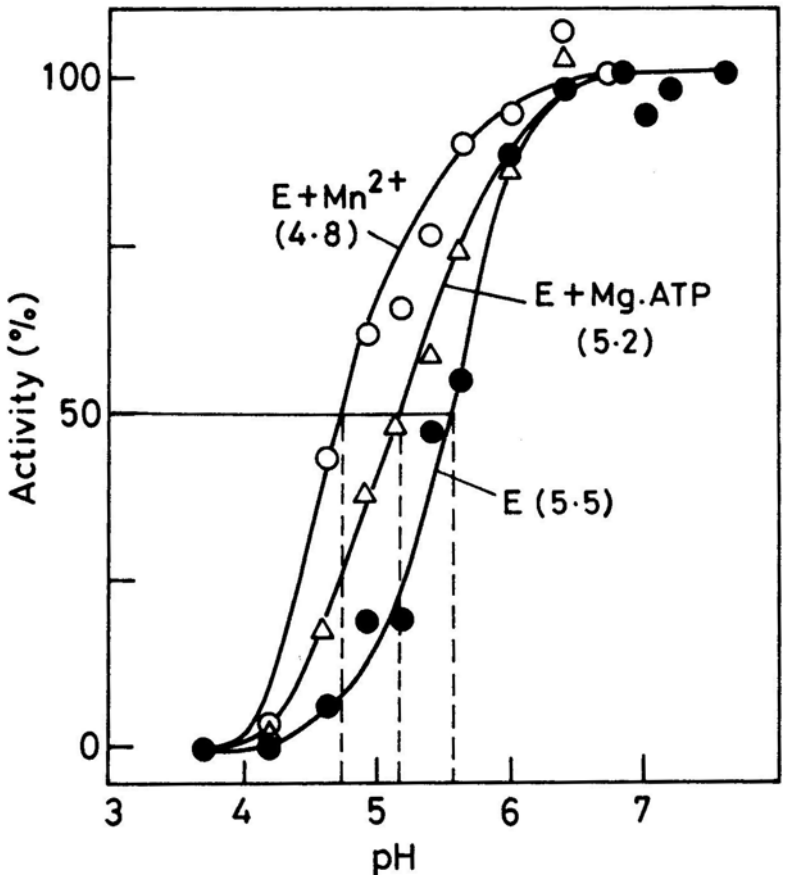

Figure 4. H inactivation of *A. niger* glutamine synthetase. The enzyme (14·3 μg) was incubated at 28°C for 30 min in the presence of 0·5 mM Mn^{2+} (O), 10:2, 5 mM Mg^{2+}: ATP (Δ) or without any additionally added metal ion (●)in 50 μl of buffer (100 mM) of different pH values indicated sodium acetate/acetic acid between 3·7–5·62 and imidazole hydrochloride between 6·0–7·7. At the end of this incubation, the residual activity was estimated by readjusting the pH to 6·0 and assaying Mn^{2+}-dependent γ-glutamyl transferase activity In each ease the activity at pH 7·5 was normalized to 100 as the enzyme was stable throughout the incubation period at this pH. The activity at other pH values were expressed as per cent of this normalized value.

Kinetics of inhibition by histidine, carbamyl phosphate and adenine nucleotides: In view of the inhibition of enzyme activity by these compounds, it was of interest to examine kinetic mechanism of this process in some detail. Fractional inhibition analysis, Dixon plots of inhibition and double reciprocal plots were drawn for histidine, AMP, ADP and carbamyl phosphate. These plots and the derived plots were used to analyze the nature of inhibition and obtain inhibition constants. The different inhibition constants obtained from kinetic experiments and the pattern of inhibitions are summarized in table 2.

Table 1. Inhibition of *A. niger* glutamine synthetase by amino acids, nucleotides and carbamyl phosphate[a]

Compound added (mM)	Activity		
	GS-S-Mg[b]		GS-S-Mn[c]
	at pH 8·0	at pH 6·7	at pH 5·5
None	100	100	100
Ala (20)	97	98	87
Gly (20)	97	95	85
Ser (20)	96	96	87
His (20)	99	83	59
GTP (5)	92	—	62
AMP (20)	44	18	18
ADP (5)	21	6	19
Carbamyl phosphate (20)	68	63	96

[a] The enzyme was preincubated for 5 min in the assay mixture containing compounds listed in the table and the reaction was started by the addition of NH_2OH. The reaction mixtures were incubated for 10 min at 28°C and γ-GHA formed was estimated. The enzyme activity in the absence of inhibitor was normalized to 100. The residual activity in the presence of added compound was expressed as a per cent of this normalized value.

[b] GS-S-Mg, Mg^{2+}-dependent glutamine synthetase reaction.

[c] GS-S-Mn, Mn^{2+}-dependent glutamine synthetase reaction.

Table 2. Kinetic inhibition constants and the nature of inhibition by end products of *A. niger* glutamine synthetase[a].

Inhibition	K_i (mM)	Competitive with	Nature of inhibition
His	8·0, 6·0[b]	Glu	Complete, linear
Carbamyl phosphate	7·0	Glu	Complete, linear
AMP	2·1	ATP	Complete, linear
ADP	—	(ATP)[c]	Complete, non-linear

[a] Mg^{2+}-dependent synthatase activity (pH 7·8) was monitored except in the case of histidine inhibition. Histidine inhibition was studied on Mn^{2+}-dependent synthatase activity (pH 5·5). The K_i values were computed from Dixon plots.

[b] Obtained from a slope replot of double reciprocal plots.

[c] Competitive nature of this inhibition was inferred from experiments using subsaturating concentrations of ATP.

Inhibition of glutamine synthetase by TCA-cycle intermediates

As there is a general increase in the concentration of the intermediates of TCA-cycle and glycolysis during fermentation (Kubicek and Rohr, 1978), it was of

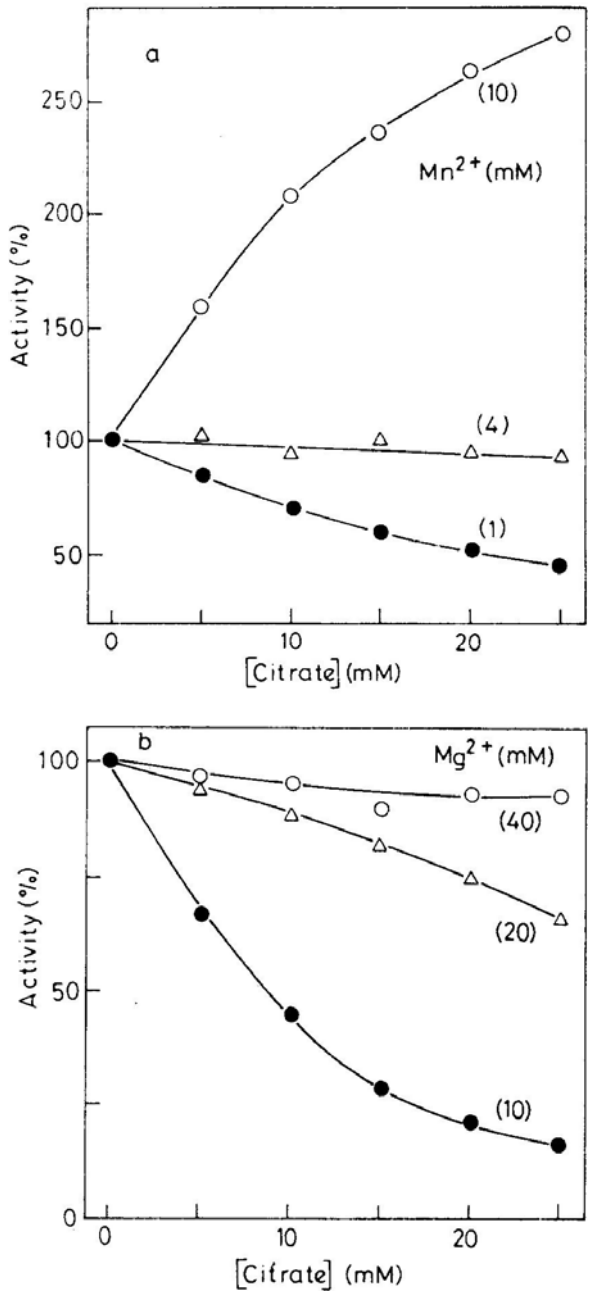

Figure 5 . Effect of increasing concentrations of citrate on glutamine synthetase activity, (a) Mn^{2+}-dependent synthetase activity was assayed at 1 (●)4 (Δ) and 10 (O) mM Mn^{2+} and 10 mM ATP in the presence of increasing concentrations of citrate. The activity in the absence of citrate was normalized to 100 for each Mn^{2+} concentration and the values in the presence of citrate were expressed as a per cent of this normalized value, (b) Mg^{2+}-supported synthetase activity determined at 10 (●), 20 (Δ) and 40(O)mM Mg^{2+} at 10 mM ATP and increasing concentrations of citrate. The activity in the absence of citrate was normalized to 100 for each Mg^{2+} concentration.

interest to examine the effect of some of these metabolites on glutamine synthetase activity. Except for citrate and 2-oxoglutarate, none of the other compounds tested (pyruvate, succinate, and oxaloacetate; at 25 mM) showed any appreciable effect. The effect of increasing concentrations of-citrate (0–25 mM) at three different concentrations of Mn^{2+} namely, 1 mM (subsaturating), 4 mM (optimal) and 10 mM (excess) and at a fixed concentration of ATP (10 mM) was examined (figure 5a). At 1 mM Mn^{2+} , about 50 % inhibition of Mn^{2+}-supported synthetase activity was observed in the presence of 25 mM citrate. There was no effect at 4 mM Mn^{2+}, whereas, at 10 mM Mn^{2+} , the normalized acitivity was enhanced considerably. Citrate, at 40 mM Mg^{2+} was without effect on the Mg^{2+}-dependent synthetase activity (at 10 mM ATP), whereas at 10 mM Mg^{2+} pronounced inhibition was observed and the effect of citrate at 20 mM Mg^{2+} was intermediate (figure 5b). The enzyme activity under similar conditions was marginally affected by 2-oxoglutarate at 1 and 4 mM Mn^{2+} (data not shown). The Mg^{2+}-dependent synthetase activity was not affected by the presence of 2-oxoglutarate at all concentrations of Mg^{2+} (10, 20 and 40 mM).

Table 3 summarizes the actual values of the glutamine synthetase activity obtained in the presence of citrate (25 mM) and 2-oxoglutarate (25 mM) and at different concentrations of either Mg^{2+} or Mn^{2+}. It is apparent from table 3 that excess Mg^{2+} was without effect. Under sub-optimal concentrations of Mg^{2+} (10 mM) and Mn^{2+} (1 mM), there was inhibition of glutamine synthetase activity by citrate and 2-oxoglutarate. However, when the Mn^{2+} was in excess, the same ligands had an activating influence on the Mn^{2+}-dependent synthetase activity. Taken together with the inhibition of the enzyme by excess Mn^{2+}, these effects could simply be due to the ability of citrate and 2-oxoglutarate to chelate divalent metal ions which are essential for activity and/or are also inhibiting the enzyme at higher concentrations (*e.g.* Mn^{2+}).

Table 3. Effect of 2-oxoglutarate and citrate on *A. niger* glutamine synthetase activity at different concentrations of Mg^{2+} or Mn2 + .

Activity[a]	Metal ion (mM)	No addition	+ Citrate (25 mM)	+ 2-Oxoglutarate (25 mM)
GS-S · Mg[b]	10	0·76	0·11	0·73
	20	0·96	0·64	0·95
	40	0·96	0·93	0·96
GS-S-Mn[c]	1	0·30	0·13	0·25
	4	0·62	0·58	0·76
	10	0·16	0·45	0·55

[a] The enzyme activity is expressed as μmol of γ-GHA formed in 15 min and using 10 mM ATP. About 9 μg of the enzyme was used per assay.
[b] GS-S-Mg, Mg^{2+}-dependent synthetase reaction.
[c] GS-S-Mn, Mn^{2+}-dependent synthetase reaction.

Effect of EDTA and ATP on the enzyme

To substantiate further that the effects of citrate and 2-oxoglutarate on the glutamine synthetase activity were probably due to chelation of divalent metal ions rather than as regulatory effectors, the action of a classical metal chelator (EDTA) and a physiological chelator (free ATP) on the enzyme activity was studied. When EDTA (0–25 mM) or ATP (0–25 mM, in addition to 10 mM ATP used in the assay) was varied at different fixed concentrations of metal ions, different patterns of changes in activity were observed (figure 6). Enzyme activity in the absence of EDTA and at 10 mM ATP was normalized to 100 and activities in the presence of different concentrations of EDTA and added ATP are expressed as per cent of this activity. At 10 mM Mg^{2+} inhibition was pronounced with almost no activity at 10 mM EDTA, whereas at 20 mM Mg^{2+}, very little inhibition was observed until 15 mM EDTA and thereafter a rapid decrease in activity was observed (figure 6a). On the other hand, at 40 mM Mg^{2+}, concentrations of EDTA upto 25 mM, had little or no effect. When 1 or 4 mM Mn^{2+} was used, there was a very rapid loss of activity with increasing concentrations of EDTA. Interestingly, when 10 mM Mn^{2+} was present, low concentrations of EDTA caused an apparent increase in activity, whereas higher concentrations as expected inhibited the enzyme activity (figure 6b). The profiles of ATP inhibition of Mg^{2+}- and Mn^{2+}-dependent glutamine synthetase activities were similar to those due to EDTA described above; marked inhibition at low concentration of metal ion and activation of Mn^{2+}-dependent activity at high metal ion concentration and no effect when Mg^{2+} as used to 40 mM (figure 6c, d). The effects of ATP were not as marked as that due to EDTA.

Discussion

A. niger is used extensively for citric acid production. Before assigning a role for glutamine synthetase in citric acid fermentation, it was necessary to establish that *A niger* UBC 814 used in this study was similar to the commercially used strain (ATTC 9142) for industrial production of citric acid. The similarity between the two strains was established by their identical characteristics with distinct trophophase and idiophase, citric acid production (figure 1) and absence of sporulation when grown on the fermentation medium. In addition, the effect of pH and Mn^{2+} ions on the specific activity of glutamine synthetase from both the strains grown under citric acid producing conditions were similar.

Fungi in general and *A, niger* in particular, are characteristically tolerant of very low pH values (as low as 2·0). Many have a pH optimum around 5·0-7·0 for growth (Berry, 1975). Information on the intracellular pH is important in assessing the significance of kinetic parameters of an enzyme studied *in vitro,* to that *in vivo.* It is to be expected that in *vivo* activity of the cytosolic glutamine synthetase (Punekar *et al.,* 1984) would be influenced by pH. An effective buffering system in *A. niger* maintains the internal pH within a narrow range (6·0–6·8) enabling the fungus to tolerate considerable changes in external pH. Intracellular acidic pH values are not unusual as many yeasts also

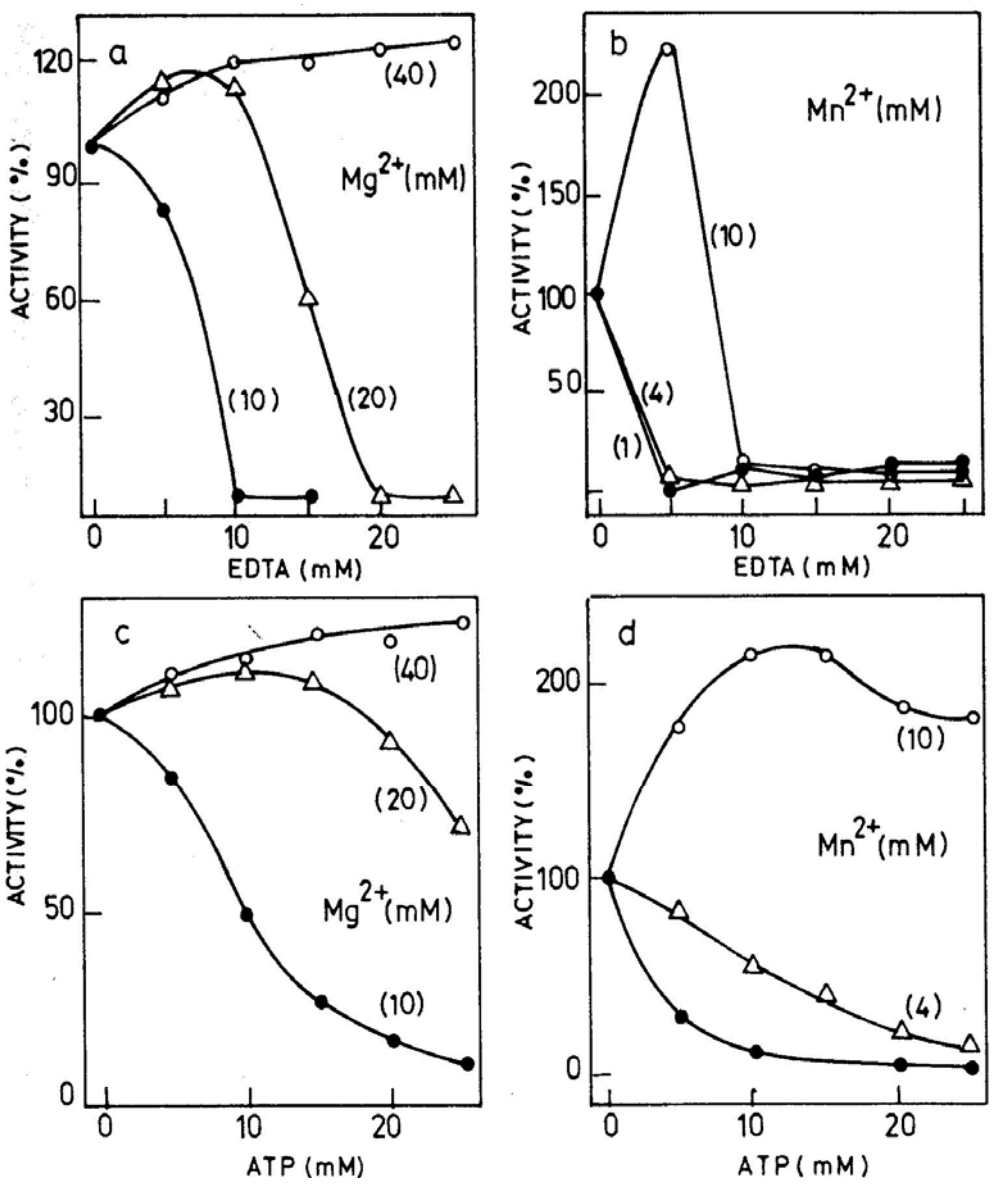

Figure 6. Effect of increasing concentrations of EDTA and added ATP on the *A. niger* glutamine synthetase activity. The Mg^{2+}-dependent synthetase reaction at 10 mM ATP was monitored in the presence of (a) EDTA or (c) ATP (added over and above the 10 mM concentration used in the assay), at 10 (●) 20 (Δ) and 40(O)mM Mg^{2+} . Similarly, Mn^{2+}-supported synthetase activity at 10 mM ATP was assayed in the presence of (b) EDTA or (d) ATP (added over and above the 10 mM concentration used in the assay), at 1 (●) 4 (Δ) and 10 (O) mM Mn^{2+}. In all these cases the activities were normalized to 100 at each concentration of the metal ion (either Mg^{2+} or Mn^{2+}) and the effects of EDTA and added ATP were expressed as per cent of these normalized values.

maintain the pH between 6·0-6·5 (Slavik, 1982; Nicolay *et al.,* 1982). Although, the methods employed in this study are sensitive, the internal pH values obtained (6·0–6·8) are at best average values, and could be a reasonable approximation to the cytosolic pH.

When intracellular pH was acidic (pH 6·0–6·5), the Mn^{2+}-dependent synthetase activity may be more meaningful as Mg^{2+}-dependent activity was very low as its optimal at pH was 7·8–8·0 (Punekar *et al.,* 1984). Under these conditions, very high concentrations of Mg^{2+} (greater than 50 mM) are required for acitivity. As high concentrations of Mg^{2+} are not normally present within cells, it would appear that Mg^{2+}-dependent activity may not be physiologically significant. Similar pH dependent Mg $^{2+}$ saturation was observed in the case of pig brain glutamine synthetase (Jaenicke and Jesior, 1978). The saturation of the Mn^{2+}-dependent activity of the enzyme by Mn^{2+} was not dependent on pH as maximal activation was observed at 4 mM Mn^{2+}, both that pH 6·0 and 8·0. These results support the suggestion made, that Mn^{2+}-dependent glutamine synthetase activity may be physiologically more relevant. High concentrations of Mn^{2+} ions are deleterious for the production of citric acid. However, during fermentation, the situation demands a major contribution by Mn^{2+}-dependent synthetase activity, but as Mn^{2+} ion deficient medium is used, a near total breakdown of glutamine synthetase activity ensues. This may lead to an apparent nitrogen starvation, although the medium contains adequate amounts of inorganic nitrogen supply.

Under fermentation conditions (pH 2·3) the half-life of glutamine synthetase was considerably decreased when compared to its stability under normal growth conditions, and was altered in the presence of ligands such as Mn^{2+} and Mg ATP. Another important effect of Mn^{2+} which could be of physiological relevance was the considerable protection it afforded glutamine synthetase and this was reflected by a shift in the pH inactivation (50 %) curve by about a unit to the acidic side. It was shown (Punekar *et al.,* 1984) that glutamine synthetase was a Mn^{2+}-dependent enzyme by purifying it to homogeneity and by characterizing its properties as well as by detailed metal activation kinetic analysis (Punekar, 1983). Fermentation being a Mn^{2+}-deficient state of growth of this fungus, glutamine synthetase becomes susceptible to inactivation at acid pH conditions in which the fungus is grown and cannot avail the Mn^{2+} protection. It was observed that a number of glycolytic and tricarboxylic acid cycle intermediates accumulate during fermentation (Kubicek and Rohr, 1978) and it could be postulated that these could specifically affect glutamine synthetase activity. From the results presented on the effect of citric acid (figure 5 and table 3) and 2-oxoglutarate (table 3), it was apparent that these metabolites were not exhibiting a specific regulatory effect but were merely complexing the essential metal ion required for activity. This suggestion was further substantiated by similar effects exhibited by a classical metal chelator like EDTA (figure 6a, b), or a physiological chelator of divalent metal ions such as ATP. The saturation of the enzyme by Mn^{2+} was bell-shaped both at pH 6·0 and 8·0 (figure 3) indicating that higher concentrations were probably inhibitory. The apparent activation observed when citrate or 2-oxoglutarate were added to reaction mixtures containing 10 mM Mn^{2+} support the conclusion that by complexing with excess metal ion they were releasing the inhibition. Under Mn_{2+} limiting conditions these TCA-cycle intermediates were inhibitory as they were complexing the metal ion and making it unavailable for interaction with the enzyme as well as the

nucleotide substrate. These conclusions were further supported by the effect of EDTA, a well known chelator of Mg^{2+} and Mn^{2+} (figure 6).

One major difference between the *A. niger* enzyme and the well characterized *E. coli* enzyme is the pattern of feedback inhibition (Hubbard and Stadtman, 1967; Woolfolk and Stadtman, 1967; Shapiro and Stadtman, 1970). While *E. coli* enzyme was partially inhibited by a number of feedback inhibitors. *A. niger* glutamine synthetase appeared to be inhibited only by histidine, carbamyl phosphate, ADP and AMP. All the inhibitions were complete and the inhibitors competed with either Mg ·ATP or L-glutamate. These results indicate that the predominant mode of regulation of *A. niger* glutamine synthetase is probably by the active site control rather than by allosteric regulation.

We have, in view of the above conclusion, examined alternative modes by which the activity of the enzyme could be altered under conditions of citric acid fermentation. Different metabolic pathways may be linked to each other by subtle but nevertheless far reaching regulatory interactions (Jensen, 1969). It is possible that a regulatory superstructure of sophisticated interactions exerted between metabolic pathways acts to reinforce and modify the basic allosteric controls operating within specific pathways. Glutamine synthetase *via* glutamate dehydrogenase (EC 1.4.1.3) links TCA-cycle (carbon metabolism) and the nitrogen metabolism (figure 7). It is likely that the activity of a key enzyme such as glutamine synthetase could be modulated when a profound change in the carbon metabolism has occurred as in the case of citric acid fermentation. During acidogenesis by *A. niger* elevated levels of TCA-cycle intermediates in addition to citric acid were observed (Kubicek and Rohr, 1978). This was accompanied by increased intracellular pool of NH_4^+ for glutamine biosynthesis Manganese deficiency is accompanied by elevated intra- and extra cellular-pools of amino acids, especially those derived from glutamate (Kubicek and Rohr, 1979). This was considered to be due to inhibition of protein synthesis. There was a relatively large increment of glutamate pool and the pools of amino acids derived from it *e.g.*, γ-aminobutyric acid, arginine and ornithine, when compared to the increment in glutamine pools. This analysis of amino acid pools suggests that a decreased flux through glutamine synthetase occurs in manganese deficiency. An inhibition of protein synthesis taken together with a decreased flux through glutamine synthetase explains most of the changes observed in the composition of amino acid pools as well as in their excretion. It becomes apparent from their data (Kubicek and Rohr, 1979) that a decreased rate of glutamine synthesis becomes more important during later stages of citric acid fermentation.

Based on the results presented in this paper and from supporting evidence obtained from literature, the following hypothesis can be made. A block in the activity of glutamine synthetase results in poor utilization of nitrogen available in the medium resulting in a delinking of nitrogen metabolism from carbon supply. This block results in accumulation of the intermediates of the cycle especially citric acid, which is excreted into the medium by the fungus. Although regulation at TCA-cycle and glycolysis may be an important aspect (Rohr and Kubicek, 1981), the contribution by glutamine synthetase block appears to be essential for substantial excretion of citric acid. The following observations presented in this paper strongly support this contention: (i)Low levels of glutamine synthetase (decreased specific activity) in

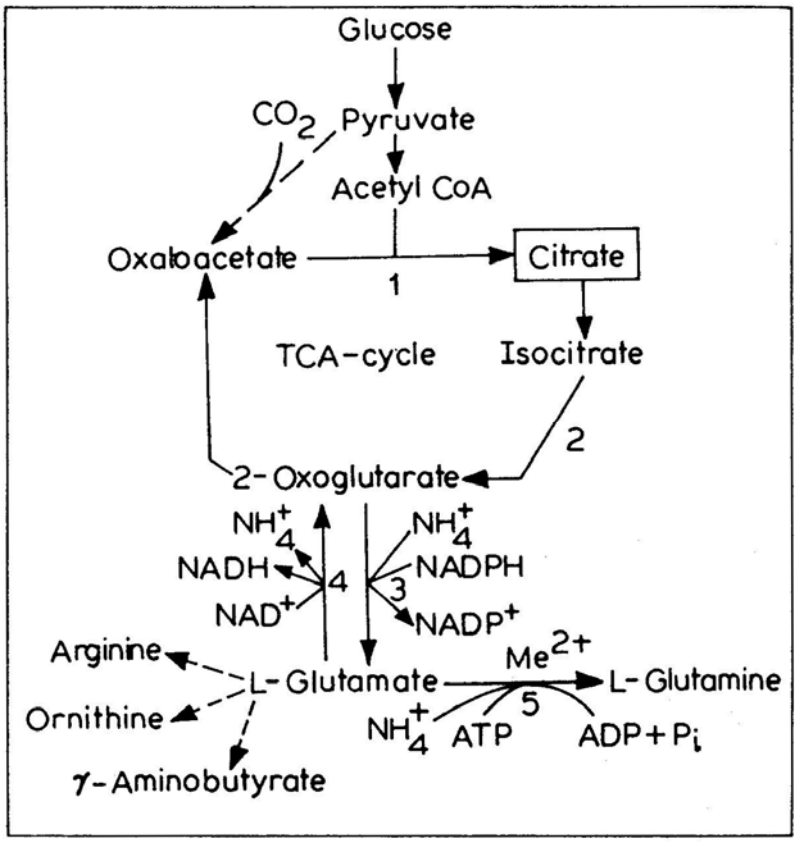

Figure 7. Interlocking of carbon (energy) metabolism with nitrogen metabolism. Important enzymes of this metabolic bridge are: (1), citrate synthase, (2), isocitrate dehydrogenase, (3), NADPH-glutamate dehydrogenase, (4), NAD^+-glutamate dehydrogenase and (5),glutamine synthetase.

fermentation medium and under conditions of low pH and manganese deficiency; (ii) *A. niger* glutamine synthetase is a manganese(II) enzyme (Punekar, 1983; Punekar *et al.,* 1984) and acidogenesis occurs essentially in a manganese-deficient state; (iii) In acidic intracellular pH favours the expression of Mn^{2+}-dependent synthetase activity rather than the Mg^{2+} supported synthetase activity; (iv) Glutamine synthetase is inactivated at acid pH values; and (v) Inhibition of the enzyme occurs by chelation of Mn^{2+} due to the high concentrations of citrate produced by the organism under these conditions.

It would, however, be interesting to probe further into the validity of this hypothesis by examining the excretion of TCA-cycle intermediates by a variety of glutamine synthetase mutants of *A. niger.* Recently, it has been hypothesised that free manganese(II) and iron(II) cations may cut as intracellular controls (Schramm, 1982; Williams, 1982). The integrated regulation of glutamine synthetase and acidogenesis in *A. niger* by manganese(II), may represent an in *vivo* example for such a control mechanism.

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