

## Regulation by Ammonium of Glutamate Dehydrogenase (NADP<sup>+</sup>) from *Saccharomyces cerevisiae*

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The activity of glutamate dehydrogenase (NADP<sup>+</sup>) (EC 1.4.1.4; NADP-GDH) of *Saccharomyces cerevisiae* is decreased under conditions in which intracellular ammonia concentration increases. A high internal ammonia concentration can be obtained (a) by increasing the ammonium sulphate concentration in the culture medium, and (b) by growing the yeast either in acetate + ammonia media, where the pH of the medium rises during growth, or in heavily buffered glucose + ammonia media at pH 7.5. Under these conditions cellular oxoglutarate concentrations do not vary and changes in NADP-GDH activity appear to provide a constant rate of oxoglutarate utilization. The following results suggest that the decrease in NADP-GDH activity in ammonia-accumulating yeast cells is brought about by repression of synthesis: (i) after a shift to high ammonium sulphate concentrations, the number of units of activity per cell decreased as the inverse of cell doubling; and (ii) the rate of degradation of labelled NADP-GDH was essentially the same in ammonia-accumulating yeast cells and in controls, whereas the synthesis constant was much lower in the ammonia-accumulating cells than in the controls.

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### INTRODUCTION

The ability to utilize ammonia as sole nitrogen source is widespread among micro-organisms. Ammonia is assimilated by incorporation into glutamine or glutamate. Glutamine synthetase, an ATP-requiring enzyme, catalyses the first of these reactions, and in many micro-organisms serves together with glutamate synthase as the main ammonia-scavenging pathway which is strongly induced under nitrogen limitation (Magasanik *et al.*, 1974; Vichido *et al.*, 1978). Glutamate dehydrogenase catalyses the second reaction [oxoglutarate + NH<sub>4</sub><sup>+</sup> + NAD(P)H → glutamate + NAD(P)<sup>+</sup>] and is usually regarded as a high capacity/low affinity enzyme, mainly functional under unlimited ammonia supply (Barratt, 1963; Burn *et al.*, 1974). In organisms having both aminating pathways, glutamine synthetase is drastically decreased upon an increase in ammonia supply, and amination proceeds via glutamate dehydrogenase (Stadtman & Ginsburg, 1974; Magasanik *et al.*, 1974).

In *Saccharomyces cerevisiae*, glutamate dehydrogenase (NADP<sup>+</sup>) (EC 1.4.1.4; NADP-GDH) is the main aminating enzyme and the glutamine synthetase/glutamate synthase pathway has only minor importance in ammonia assimilation (Tempest *et al.*, 1973; Roon *et al.*, 1974). However, the regulatory properties of NADP-GDH are poorly understood. Evidence for specific inactivation of this enzyme has recently been reported (Satrústegui & Machado, 1978;

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Abbreviation: NADP-GDH, NADP<sup>+</sup>-dependent glutamate dehydrogenase.

Bogonez *et al.*, 1979; Mazon, 1978; Mazon & Hemmings, 1979). In this paper we present results indicating that yeast NADP-GDH is also under the control of ammonia supply. Conditions in which the internal ammonia concentration is high bring about a considerable decrease of the enzyme activity. However, the decrease is not accomplished by a difference in the degradation rate of the enzyme as occurs during carbon starvation (Bogonez *et al.*, 1979), but rather by repression of synthesis.

## METHODS

*Organism and growth conditions.* A diploid strain (S18) of *Saccharomyces cerevisiae* isolated from commercial baker's yeast was used, as in our previous studies (Satrústegui & Machado, 1978; Bogonez *et al.*, 1979, 1983). Cultures were grown with aeration at 30 °C in a chemically defined medium containing yeast nitrogen base lacking amino acids and ammonium sulphate (Difco 0335-15), supplemented with carbon and nitrogen sources at the concentrations specified in the legends.

*Cell-free extracts for enzyme assays and immunoprecipitation studies.* Yeast cells were collected by centrifugation, washed twice with distilled water, and resuspended in three times their wet weight of disruption medium containing five times their wet weight of glass beads (0.5 mm diameter). The disruption medium was 0.9% (w/v) NaCl for enzyme assays and 20 mM-Tris, 500 mM-NH<sub>4</sub>Cl, 100 mM-MgCl<sub>2</sub>, 5 mM-2-mercaptoethanol, pH 7.4 (buffer A) for immunoprecipitation studies. Crude extracts were obtained by cell disruption in a MKS cell homogenizer (B. Braun). Cell debris was removed by centrifugation at 4 °C for 30 min at 27000 g. The supernatants were used for determination of enzyme activities and protein content.

*Enzyme and metabolite assays.* The assay for NADP-GDH has been reported elsewhere (Satrústegui & Machado, 1977). Protein was determined by the Lowry method, with bovine serum albumin as standard. One unit of enzyme activity is that amount of enzyme that transforms 1 μmol substrate in 1 min. Cell free extracts for metabolite assays were prepared as described earlier (Bogonez *et al.*, 1983). The methods used for the determinations of ammonia, 2-oxoglutarate and glutamate were essentially as described in Bergmeyer (1974). The term 'ammonia' as used in this paper does not define the state of protonation, but rather the sum of protonated and unprotonated forms present. The specific unprotonated and protonated states are described as NH<sub>3</sub> or NH<sub>4</sub><sup>+</sup>, respectively.

*Measurement of intracellular pH.* Internal pH was determined as described by Rottenberg (1979). Cultures in the early exponential phase were harvested by centrifugation and concentrated two- to threefold by resuspending the pellets in fresh medium buffered with 50 mM-PIPES at pH 5.1 and 7.2. [<sup>14</sup>C]Propionic acid (53 mCi mmol<sup>-1</sup>; 1.96 GBq mmol<sup>-1</sup>) was added to a final concentration of 17 μM. After reaching equilibrium (10 min) samples were collected on 1.2 μm filters (Millipore), washed twice with 5 ml fresh medium and counted for radioactivity. Results were corrected for non-specific binding after the abolition of ΔpH by 0.6 mM-carbonyl cyanide *m*-chlorophenylhydrazone.

The intracellular volume was determined by using <sup>3</sup>H<sub>2</sub>O for the total aqueous space and subtracting the space occupied by [<sup>14</sup>C]polyethylene glycol (Rottenberg, 1979). One mg of cells (dry weight) is equivalent to 5.42 μl of intracellular aqueous space.

*Immunoprecipitation of NADP-GDH.* NADP-GDH was labelled by growing yeast cells on different culture media in the presence of L-[4,5-<sup>3</sup>H]leucine (3 μCi ml<sup>-1</sup>). Duplicate samples of yeast cell-free extract containing 2.6 units of labelled NADP-GDH were incubated with an excess of antiserum obtained as previously described (Bogonez *et al.*, 1979) for 2 h at 37 °C and overnight at 4 °C. The antigen-antibody complex was pelleted by centrifugation at 5000 g for 45 min at 4 °C, on a discontinuous sucrose gradient containing 0.3 ml 1 M-sucrose and 0.15 ml buffer B [0.5 M-sucrose in 60 mM-Tris, 1 mM-leucine, 0.6% (w/v) Triton X-100, pH 7.4], and washed twice with cold buffer B. The washed immunoprecipitate was solubilized by heating for 10–20 min at 90 °C in 0.2 ml 60 mM-Tris, 4 M-urea, 1% (w/v) SDS, 1% (w/v) 2-mercaptoethanol, 10% (w/v) glycerol and 0.002% (w/v) bromophenol blue pH 7.4.

*Electrophoresis and counting procedures.* Solubilized immunoprecipitates were electrophoresed in the presence of urea and SDS as described by Maizel (1971). After staining of proteins with Coomassie blue, gels were sliced into 1 mm segments that were incubated overnight at 60 °C with 0.2 ml 30% (v/v) H<sub>2</sub>O<sub>2</sub>. Radioactivity in the gel slices was counted in 2 ml scintillation solution (5 g PPO, 0.5 g POPOP, 350 ml Triton X-100 and 650 ml toluene). The radioactivity corresponding to the position of NADP-GDH on the gel was totalled. The rates of synthesis and degradation were calculated from the decay in radioactivity of the NADP-GDH band, following the approach of Williams & Neidhardt (1969) and Schimke (1975).

*Chemicals.* L-[4,5-<sup>3</sup>H]Leucine (65 Ci mol<sup>-1</sup>; 2.4 TBq mol<sup>-1</sup>), [<sup>14</sup>C]polyethylene glycol and <sup>3</sup>H<sub>2</sub>O were obtained from Amersham. All other chemicals used were of the highest grade commercially available.

## RESULTS

*Effect of ammonia concentration in the culture medium*

Fig. 1(a) shows the variations of NADP-GDH activity in response to changes in the concentration of ammonia in the culture medium. Enzyme activity increased with increasing ammonium sulphate concentration up to 24 mM, and then decreased, reaching a level of  $550 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$  at 360 mM-ammonium sulphate. Fig. 1(b) shows that whereas intracellular 2-oxoglutarate concentrations remained constant in the different culture conditions, intracellular ammonia and glutamate concentrations underwent a gradual increase with increasing ammonium sulphate concentration.

*Effect of acetate*

The activity of NADP-GDH was constant, around  $2000 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$ , during growth on 10 mM-ammonium sulphate with glucose, galactose, ethanol or lactate as carbon source, but it decreased about 10-fold when acetate was used as carbon source (Table 1). The decrease in NADP-GDH activity did not correlate with changes in internal oxoglutarate or glutamate concentrations, but it was associated with a very large increase in internal ammonia concentration. This supports the results in Fig. 1, which suggest that an increase in external ammonium concentration associated with a rise in internal ammonia or glutamate levels results in a decrease in NADP-GDH activity.

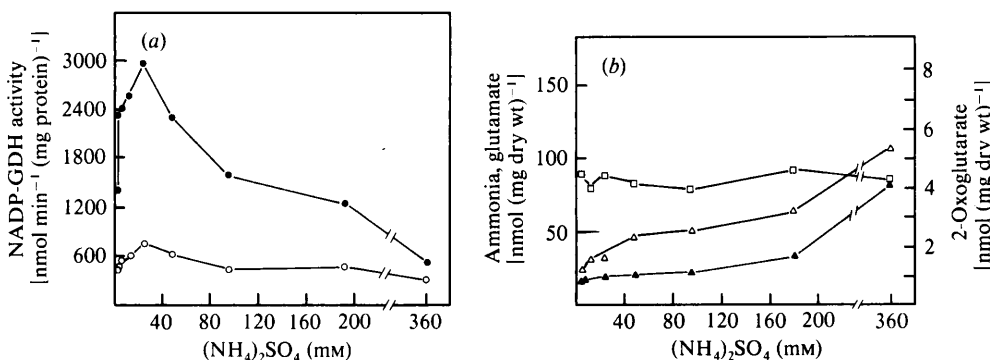


Fig. 1. Effect of ammonium concentration in the culture medium on (a) NADP-GDH activity (●), and (b) the intracellular concentrations of 2-oxoglutarate (□), glutamate (△) and ammonia (▲). Cultures were grown on minimal medium with 2% (w/v) galactose and  $(\text{NH}_4)_2\text{SO}_4$  at the concentrations indicated in the figure and harvested in mid-exponential phase. The results shown are representative values from a single experiment. The open circles in (a) represent theoretical estimates of the rate of oxoglutarate amination for the different internal ammonia concentrations. The values were obtained by substituting the measured internal substrate ( $\text{NH}_4^+$  and 2-oxoglutarate) concentrations and enzyme activities (NADP-GDH) into the Michaelis-Menten equation.

Table 1. NADP-GDH activity and intracellular concentrations of metabolites of yeast cells growing on different carbon sources with 10 mM-ammonium sulphate as nitrogen source

The results shown are means of three to nine determinations from individual experiments. Standard deviations were less than 10% of the mean value. The concentration of the carbon sources was 2% (w/v); lactate and acetate were provided as the sodium salts. Cultures were harvested in mid-exponential phase, and extracts for NADP-GDH and metabolite determinations were prepared as described in Methods. Enzyme activities are expressed as  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ , and metabolite concentrations as  $\text{nmol (mg dry wt)}^{-1}$ .

Carbon source	NADP-GDH	2-Oxoglutarate	Glutamate	Ammonia
Glucose	1814	0.66	14	4.7
Galactose	2537	0.46	18	4.6
Ethanol	2346	0.40	44	3.9
Lactate	1963	0.60	72	10.5
Acetate	185	0.73	52	185

Table 2. *Effect of external pH on NADP-GDH activity and intracellular metabolite concentrations*

The results shown are means of three to five determinations from individual experiments. Standard deviations were less than 10% of the mean value. The initial pH of the non-buffered media was adjusted to 5.5. Enzyme activities are expressed as  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ , and metabolite concentrations as  $\text{nmol} (\text{mg dry wt})^{-1}$ .

Growth medium composition	pH of medium at harvest	Ammonia	Glutamate	NADP-GDH	Specific growth rate ( $\text{h}^{-1}$ )	Internal pH
2% Acetate, 20 mM-NH <sub>4</sub> <sup>+</sup>	7.3	185	52	185	0.173	—
2% Acetate, 20 mM-NH <sub>4</sub> <sup>+</sup> , 0.20 M-PIPES, pH 5.5	5.7	33	79	500	0.189	—
2% Glucose, 20 mM-NH <sub>4</sub> <sup>+</sup>	3.5	4.7	14	1800	0.277	—
2% Glucose, 20 mM-NH <sub>4</sub> <sup>+</sup> , 0.2 M-PIPES, pH 5.5	5.1	56	94	1169	0.277	6.74
2% Glucose, 20 mM-NH <sub>4</sub> <sup>+</sup> , 0.2 M-PIPES, pH 7.5	7.2	105	152	289	0.277	7.13

#### *Modulation of internal ammonia concentrations by external pH*

Ammonium (NH<sub>4</sub><sup>+</sup>) transport in yeast is mediated by two active transport systems that operate over a narrow pH range (5.5–7.5) with maximal activity at pH 6.5 (Roon *et al.*, 1975; Dubois & Grenson, 1979). However, at higher pH values ammonia uptake is essentially a diffusion process (Bogonez *et al.*, 1983). We have previously shown that ammonia accumulation in cells growing on acetate results from a rise in the pH of the medium that follows the uptake of acetate. This process favours ammonia diffusion and pH-dependent accumulation by increasing the proportion of the uncharged species, NH<sub>3</sub>.

To test whether the rise in internal ammonia concentration that occurred in yeast grown on acetate or with high ammonium sulphate concentrations was responsible for the decrease in NADP-GDH activity found in these cells, the following experiment was performed.

The accumulation of ammonia in cells growing on acetate was reversed by the use of strong buffers that prevented the rise in the pH of the medium, and the effect on NADP-GDH activity was investigated. The prevention of the pH increase of the medium resulted in a sixfold decrease in intracellular ammonia concentration (Table 2). This decrease was accompanied by an increase in NADP-GDH activity from 185 to 500  $\text{nmol min}^{-1} (\text{mg protein})^{-1}$ . In contrast, the intracellular glutamate concentration remained constant.

When the acidification of the medium in glucose-growing cultures was prevented by buffering at pH 5.5 or pH 7.5, the ammonia content of the cells increased 10- and 20-fold, and the NADP-GDH activity decreased 2- and 7-fold, respectively (Table 2). These variations in NADP-GDH activity were unlikely to be the direct result of gross changes in internal pH, since this was similar at external pH values of 5.5 and 7.5 (Table 2). NADP-GDH from various sources is stable at neutral pH values (Smith *et al.*, 1975) and the pH range over which the stability is maintained is greater for the fungal enzymes (Barratt & Strickland 1963; Venard & Fourcade, 1972). Thus yeast NADP-GDH is very stable at neutral and alkaline pH values (pH 7.6 and above) where the mammalian enzyme becomes inactivated (Venard & Fourcade, 1972). On the other hand, the influence of a pH change from 6.3 to 7.1 on the amination reaction is a small increase (18%) in activity, which is unlikely to bear any relation to the drastic drop in NADP-GDH activity of cells growing in alkaline media (Barratt & Strickland, 1963; López-Quijada, 1963).

#### *Role of protein synthesis and degradation in the modulation of NADP-GDH activity by ammonia*

To ascertain the fate of NADP-GDH after the onset of the ammonia-dependent decrease in activity, we grew yeast cells under inducing conditions, i.e. low ammonium sulphate concentration. Then, when NADP-GDH activity was maximal, the cells were transferred to conditions in which the internal ammonia concentration was increased, either by increasing the ammonium sulphate concentration in the medium to 180 mM, or by raising the pH of the

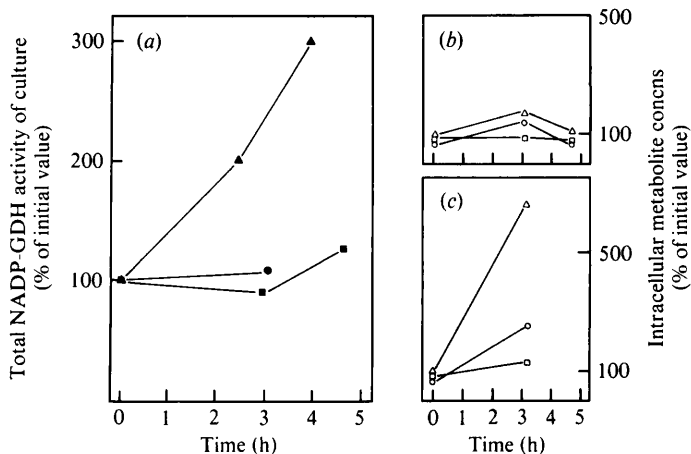


Fig. 2. NADP-GDH (a) and intracellular metabolite concentrations (b, c) in ammonia-accumulating yeast cells. (a) Yeast cells were grown in minimal medium with 2% (w/v) glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. At zero time a control sample was harvested, and the remaining cells were transferred to minimal medium with 2% glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (▲), 2% glucose + 180 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (■), or 2% glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 0.2 M-PIPES, pH 7.5 (●). At the times indicated, samples were harvested for assay. (b, c) Intracellular concentrations of 2-oxoglutarate (□), glutamate (○) and ammonia (Δ) after transfer to 2% glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (b), and after transfer to 2% glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 0.2 M-PIPES, pH 7.5 (c). In these experiments measurements were not made after 3 h in the cultures at high pH, because the buffering capacity of the media was gradually lost after that time.

medium to 7.5. NADP-GDH activity and intracellular metabolite concentrations were then measured at various times. There was a sevenfold rise in internal ammonia concentration 3 h after the pH shift (Fig. 2c), whereas total NADP-GDH activity in the culture after the shift to pH 7.5 had remained practically constant after 3 h of growth (Fig. 2a). These results might indicate that ammonia excess brings about repression of the synthesis of the enzyme and dilution of the pre-existing enzyme molecules in the newly formed cells. As indicated above, the low NADP-GDH activities observed during growth in acetate cultures were also associated with a rise in the internal ammonia concentration.

To investigate whether repression of synthesis is the main mechanism involved in the maintenance of low NADP-GDH activities during steady-state growth under ammonia-accumulating conditions, we studied the turnover rates of the enzyme during growth on acetate. In unbuffered acetate cultures, there was a continuous decay of NADP-GDH during exponential growth, which was possibly associated with carbon starvation of the cells, due to the pH being unfavourable for acetate utilization. However, in acetate cultures buffered at pH 5.5 the enzyme levels, although low, did not vary during exponential growth, allowing us to estimate the turnover constants of the enzyme under steady-state conditions.

Specific antibodies were used for immunotitration of extracts from acetate-grown and glucose-grown yeasts, and it was found that the activity of the extract correlated with the amount of immunoprecipitable material (results not shown), a result that rules out the existence of inactive, cross-reactive NADP-GDH molecules in acetate-grown cells.

To determine the rates of synthesis and degradation of NADP-GDH, the enzyme was labelled during growth on glucose (as control carbon source) or acetate and the loss of radioactivity per unit enzyme was followed after resuspending the cells in unlabelled medium. Fig. 3 shows the loss of labelled NADP-GDH during growth on 10 mM-ammonium sulphate and 2% glucose or 2% sodium acetate. The decrease in specific radioactivity of the enzyme protein results from the sum of two processes, dilution with unlabelled enzyme by growth, and the specific turnover of NADP-GDH. When the yeast was grown on glucose + ammonia, the apparent half life of the protein was 2.55 h, i.e. it had an 'apparent' degradation constant  $K'_d$  of 0.271 h<sup>-1</sup> [ $K'_d = \ln 2$  (apparent half life)<sup>-1</sup>]. Since the growth constant of these cultures was 0.277 h<sup>-1</sup>, the real

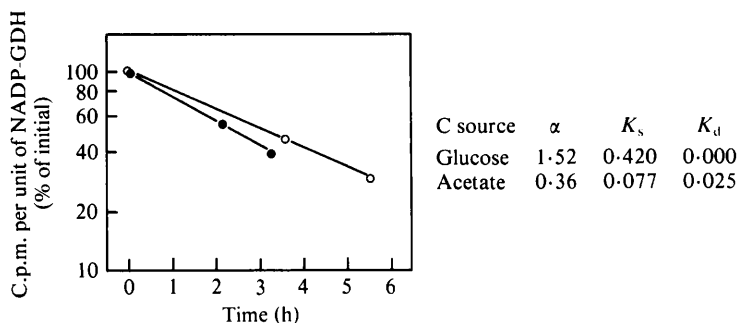


Fig. 3. Rate of loss of pre-existing NADP-GDH during exponential growth. Yeast cells were grown for four to eight generations in the presence of L-[4,5-<sup>3</sup>H]leucine (3  $\mu$ Ci ml<sup>-1</sup>) in minimal medium supplemented with 2% glucose + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (○), or 2% acetate + 10 mM-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> + 0.2 M-PIPES, pH 5.5 (●). At mid-exponential phase, cells were harvested, washed once with fresh medium containing 1 mM-unlabelled leucine and resuspended in the same medium. Samples were collected at the times indicated and processed as described in the text. The experiments were repeated twice with similar results. The amount of labelled enzyme in the immunoprecipitate (radioactivity incorporated in NADP-GDH per unit of enzyme activity) is expressed as a percentage of the value at zero time, and is plotted as a function of time after transfer to the unlabelled medium. Apparent degradation constants were calculated from the time needed to lose 50% of the radioactivity (c.p.m.) in NADP-GDH per unit of enzyme activity at zero time. The values of the synthesis ( $K_s$ ) and degradation ( $K_d$ ) constants of yeast NADP-GDH are shown to the right of the figure.  $\alpha$ , Specific activity of NADP-GDH expressed as units (mg protein)<sup>-1</sup>. The specific growth rate was 0.277 h<sup>-1</sup> in glucose cultures and 0.189 h<sup>-1</sup> in acetate cultures.  $K_s$  is expressed as units mg<sup>-1</sup> h<sup>-1</sup> and  $K_d$  as h<sup>-1</sup>.

degradation constant is below the detection limit of these experiments. Thus, in glucose + ammonia cultures, the decrease in specific radioactivity of NADP-GDH reflected only the dilution of labelled with unlabelled protein. This is essentially the same situation as found in cells growing on acetate, where the low levels of enzyme are due to a considerable decrease in  $K_s$  and little change in  $K_d$ .

#### DISCUSSION

The results reported here indicate that ammonia accumulation within cells of *S. cerevisiae* results in a decrease in NADP-GDH activity. The accumulation of ammonia can be brought about (a) by an increase in the external ammonium sulphate concentration, and (b) by a rise in the pH of the medium, which favours ammonia diffusion and accumulation (Bogonez *et al.*, 1983). This pH rise can be imposed on a glucose culture by heavy buffering at pH 7.5, but it also accompanies growth on acetate or lactate during the late exponential phase (not shown). Under all these conditions a decrease in NADP-GDH activity is observed (Table 2).

The physiological role of the variations of NADP-GDH activity with ammonia concentration can be explained on the basis of the results shown in Fig. 1. Over a wide range of external ammonia concentrations, the internal concentration of 2-oxoglutarate remained unchanged while those of ammonia and glutamate rose to a plateau that existed between 40 and 80 mM-ammonium sulphate, and increased further at higher concentrations. This suggests that the variations in NADP-GDH activity tend to buffer 2-oxoglutarate concentrations. A tentative theoretical calculation of the rate of utilization of 2-oxoglutarate and ammonia by NADP-GDH under each condition indicates that the flux through NADP-GDH might be relatively constant (Fig. 1a).

Taken together, these results strongly suggest that the maintenance of a minimum internal 2-oxoglutarate concentration is of primary importance for yeast cells and that changes in NADP-GDH, ammonia and glutamate levels tend to prevent any decrease in this concentration. These considerations are in agreement with the high  $K_m$  of the enzyme towards ammonia (10<sup>-2</sup> M) (Grisolía *et al.*, 1964), which could also represent a form of control of the amination pathway and avoidance of excessive loss of 2-oxoglutarate.

The results in this paper provide some information as to the mechanism of the decrease of NADP-GDH activity upon ammonia accumulation. Unlike the degradation process which NADP-GDH undergoes during carbon starvation (Bogonez *et al.*, 1979), during ammonia accumulation there is merely a repression of synthesis, with little, if any, degradation. Thus a shift from a low to a high ammonia concentration was not accompanied by loss of the pre-existing enzyme molecules, and the decrease in activity observed in acetate-grown, as compared with glucose-grown, cultures was the result of a pronounced decrease in  $K_s$  with only a minor increase in  $K_d$ . Moreover, the specific radioactivity of labelled NADP-GDH in growing cells was virtually unchanged during a shift to a high ammonium sulphate concentration in the culture medium (results not shown).

In contrast to a number of fast responses observed in ammonia or glucose catabolite repression in yeast where both repression of synthesis and enzyme inactivation operate (Funayama *et al.*, 1980; Legrain *et al.*, 1982; Grenson, 1983), the effect of ammonia accumulation on NADP-GDH level is clearly slower. If the functional switching from one mode of metabolism to another (i.e. a derepressed to a repressed condition) requires a loss of activity of around 60–80% in order to prevent excess removal of oxoglutarate, then we must conclude that two to three generations of cell growth are necessary to implement the repressed condition, and that possibly other mechanisms of fine control exist in order to prevent the exhaustion of oxoglutarate under ammonium excess.

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