The Regulation of Glutamine Metabolism in *Candida utilis*: Studies with $^{15}\text{NH}_3$ to Measure *in vivo* Rates of Glutamine Synthesis

By A. P. SIMS* AND A. R. FERGUSON

School of Biological Sciences, University of East Anglia, Norwich, NOR 88C

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SUMMARY

Experiments were carried out to establish whether glutamine synthesis in yeast is subject to control through the operation of cumulative feedback inhibition on the biosynthetic enzyme glutamine synthetase.

^{15}N isotope was used to measure the *in vivo* rate of amide synthesis in a culture growing on ammonia. The steady-state rate measured corresponded quite closely with a value calculated from a knowledge of the enzyme content of the yeast, and indicated that under these conditions yeast did not possess a large excess of biosynthetic capacity. In another experiment yeast was subjected to a brief period of nitrogen deprivation and rates of amide synthesis were determined immediately after addition of ammonia to the culture. This established that no significant increase in the rate of amide synthesis per unit of enzyme accompanied a reduction in the cell concentration of glutamine and other amino acids, and that the small increase in rate observed was caused by an increase in enzyme amount.

Measurements of the rate of glutamine synthesis in yeast with very high levels of enzyme confirmed that the enzyme content was of prime importance in determining the rate of synthesis in the organism. Thus when ammonia was added to yeast growing on glutamate, the rates of amide synthesis were initially nearly sixfold higher than ones observed on ammonia; this enhancement of rate almost exactly paralleled the increase in amount of enzyme in the yeast. Although the cell concentration of glutamine had greatly increased at this time and the concentration of some of its derivatives was unchanged, virtually no restraint operated to limit the synthetic activity of the enzyme in the organism. The rapid decline in the rate of glutamine synthesis which ensued occurred in response to a depletion of enzyme substrates as well as to enzyme inactivation, and the relative importance of these two factors in the control of glutamine synthesis was quantitatively assessed.

Studies using isotopically labelled ammonia have shown that in food yeast growing on ammonia, almost all the nitrogen taken up is first assimilated into two compounds, glutamate and glutamine. Balanced growth requires that nearly 75 % of the ammonia is incorporated into glutamate and much of the remainder into the amide group of glutamine (Sims & Folkes, 1964; B. F. Folkes and A. P. Sims, unpublished). Clearly controls must operate here to ensure the appropriate flow of ammonia into both glutamate and glutamine and also that rates of glutamate synthesis are sufficient to meet the demands for glutamate by the synthesis made of glutamine and by the transamination reactions leading to the synthesis of all other amino acids.

The regulation of the synthesis of glutamine has been studied in great detail in bacteria, particularly *Escherichia coli*, but it is uncertain whether similar mechanisms of control

* All communications should be addressed to A.P.S.

operate in the fungi (see the review of Shapiro & Stadtman, 1970). Earlier workers (Hubbard & Stadtman, 1967; Kapoor & Bray, 1968), however, concluded that because purified preparations of fungal glutamine synthetase were inhibited by mixtures of the end-products of glutamine metabolism, 'cumulative' inhibition played an important role in the regulation of glutamine synthesis in fungi as well as bacteria. However, physiological experiments using $^{15}\text{NH}_3$ to measure *in vivo* rates of glutamine synthesis in yeast (Sims, Folkes & Bussey, 1968) failed to reveal any inhibitory feedback upon the pathway and suggested instead that fluctuations in the cell concentrations of glutamate and ammonia were of great importance in the regulation of glutamine synthesis.

Further studies are reported here which were carried out to elucidate some of the factors which govern the rate of glutamine synthesis in food yeast. We have used isotopically labelled ammonia, $^{15}\text{NH}_3$, to measure the actual rates of glutamine synthesis in yeast cultured under different nutritional conditions and have then attempted to correlate changes of rate with variations in the level of glutamine synthetase, its substrates, and the end-products of glutamine metabolism.

METHODS

Organism and culture conditions. The strain of *Candida utilis* used was NCYC737; see Folkes & Sims (1974) for details of its origin and culture.

Harvesting and extraction of yeast. For accurate analysis and measurements of isotope incorporation it is essential that the harvesting and killing of cells be rapid. The large volume of yeast required for isotope analysis (200 ml) was removed from the culture vessel by using pre-evacuated pipettes fitted with stop-cocks at either end. The yeast was harvested by vacuum filtration on 9 cm Whatman GFA paper, washed with 10 ml 0.15 M-NaCl, and extracted by using 4×20 ml portions of 60 % ethanol (v/v). Extracts were taken to dryness on a rotary film evaporator at 30 °C.

Amino-acid analysis and estimation of ^{15}N content

(i) *Separation of amino acids.* This was as described by Ferguson & Sims (1974) except that the effluent from the Dowex 1 column, containing the neutral and basic amino acids, was freed of ammonia by passage through a column (2×1 cm) of Dowex 50 (sodium). Neutral amino acids were displaced from this column by eluting with 5 ml 0.1 M-sodium acetate buffer, pH 4.84. The column was regenerated by 10 ml 0.1 M-NaOH followed by 20 ml water. The effluent from the Dowex 50 column was incubated overnight at 25 °C with 0.2 units of glutaminase (Sigma Type IV, Sigma London Chemical Co., London). The resulting glutamate was recovered by passage through a Dowex 1 column, while the amide N was obtained by distilling the column effluent with 5 ml of 2 % NaOH in a Kjeldahl still.

(ii) *Estimation of amino acids.* Estimation of amino acids by the ascorbic-ninhydrin method was as described by Ferguson & Sims (1974).

(iii) *Preparation of samples for the mass spectrophotometer.* The blue complex formed by treating the amino acid with ninhydrin was hydrolysed by the addition of 0.25 ml of 3.6 M- H_2SO_4 (MAR quality) followed by heating to 100 °C for 5 min. The samples were transferred to a Kjeldahl distillation unit and the methoxyethanol removed by steam distillation (30 ml distillate collected). Good recovery of the $\alpha\text{-NH}_2$ of amino acids as NH_3 (about 85 %) was obtained by distilling the extract with 10 ml alkaline borate (0.15 M-sodium tetraborate, 0.15 M-trisodium phosphate, 0.6 M-sodium hydroxide). The distilled ammonia was trapped in 0.01 M- H_2SO_4 (MAR quality) and estimated by the phenol-hypochlorite

method (Weatherburn, 1967). To correct for any isotope dilution of the amino acids, sample blanks were prepared by putting 80 ml of 60 % alcohol through the complete procedure. The small amount of ammonia in the blank samples (approx. 3 µg N total) was estimated by isotope dilution.

The ammonia sample (taken to about 1 ml) was transferred to one arm of a Rittenberg tube and 1 ml sodium hypobromite reagent to the other. The tubes were evacuated according to the procedure of Sims & Cocking (1958).

(iv) *Measurement of isotope abundance.* Peak heights of mass 28 ($^{14}\text{N}^{14}\text{N}$), 29 ($^{14}\text{N}^{15}\text{N}$), 30 ($^{15}\text{N}^{15}\text{N}$) and 32 ($^{16}\text{O}^{16}\text{O}$) were routinely measured with an MS 3 Mass Spectrometer (A.E.I.). ^{15}N abundance of the samples was calculated from the equations:

$$^{15}\text{N abundance} = \frac{100}{2R + 1}$$

where R is the ratio of the peak heights of masses 28 and 29, or

$$^{15}\text{N abundance} = \frac{100}{\frac{1}{2}R' + 1}$$

where R' is the ratio of the peak heights of masses 29 and 30. Both methods of calculation gave satisfactory results since the isotope abundance used in these experiments allowed reliable estimates of the peak height at mass 30 to be made. Normally, measurements of abundance were based on 29:30 ratios; this minimized small errors due to air leaks after the conversion of ammonia to nitrogen. Calculations based on 28:29 ratios could give spuriously low estimates of isotope abundance if the sample contained more than trace amounts of methoxyethanol. This was shown to be due to the formation of another ion of mass of 28 (possibly CO^+). Measurements of abundance based on 28:29 ratios were normally 0.5 to 1 % lower than those based on 29:30 ratios. Rates of synthesis of glutamate and glutamine were calculated from the isotope data as described by Folkes & Sims (1974).

Measurement of enzyme activities

The preparation of extracts and the assay of enzyme activity were described by Ferguson & Sims (1971). The following enzymes were measured: glutamine synthetase [L-glutamate: ammonia ligase (ADP) (EC.6.3.1.2)]; NAD-specific glutamate dehydrogenase [L-glutamate: NAD oxido reductase (deaminating) (EC.1.4.1.2)]; NADP-specific glutamate dehydrogenase [L-glutamate: NADP oxido reductase (deaminating) (EC.1.4.1.4)].

In some experiments the activity of glutamine synthetase was estimated by ^{14}C -glutamine formation rather than by the production of L-glutamate-monohydroxamate. The two methods were compared; with ammonia, rates were 5 % higher. ^{14}C -glutamate was counted on a Scintillation Spectrophotometer Model 3310 by the method of Patterson & Greene (1965). Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Steady-state rate of glutamine synthesis in yeast growing on ammonia

Yeast, growing under steady-state conditions, was supplied with isotopically labelled ammonia, and rates of amide synthesis were estimated by following, with time, the labelling of glutamine amide; rates were calculated from the isotope data and the results of these calculations are plotted in Fig. 1(a). The steady-state rate of glutamine amide synthesis was 0.0641 mg N/min/g dry wt of yeast growing exponentially at 25 °C.

Fig. 1. Steady-state isotope incorporation into a yeast culture. Yeast was grown in a turbidostat at 25 °C. The culture volume was 1880 ml and the generation time 117 min. The input medium contained NH_3 (83.5 $\mu\text{g N/ml}$) and the cell density (0.705 mg dry wt/ml) was chosen so as to leave a nitrogen excess of about 14 $\mu\text{g N/ml}$ in the culture medium. After 22 h of growth under these conditions the experiment was started by replacing the input medium by one containing isotopically-labelled NH_3 (83.5 $\mu\text{g N/ml}$, 54.22 % ^{15}N excess); samples were removed at times indicated for analyses of medium, amino-acid pool and enzymes.

(a) Values for the turnover constant (b) were calculated at different times; the average value for b is 0.036/min. Estimates of the amount of soluble glutamate and glutamine per g.d.w. yeast at different times are also shown. ●, Glutamine; ▲, glutamate; and ○, individual values of turnover constant (b). Assuming 1 g.d.w. cells contained 2.448 ml H_2O (A. Wiemken, unpublished), the mean cell concentrations of glutamine and glutamate were calculated: glutamine = $51.7 \times 10^{-3} \text{ M}$; glutamate = $72.4 \times 10^{-3} \text{ M}$. The growth constant for the yeast was $a = 0.0062/\text{min}$, and hence the turnover number of the glutamine pool (b/a) = 5.9.

(b) Rate of ammonia uptake. The turnover constant for ammonia in the culture vessel was determined graphically from the slope of the graph and gave a value $b = 0.02769/\text{min}$. Since the ammonia excess in the medium = 14.6 μg and 1418 ml culture contained 1 g.d.w. yeast, the rate of uptake of ammonia into the yeast was 0.5732 mg N/min/g.d.w. at 25 °C. The other way for calculating ammonia uptake is as follows:

NH_3 content of input medium	= 83.5 $\mu\text{g N/ml}$
Steady-state NH_3 content of culture medium	= 14.6 $\mu\text{g N/ml}$
Amount of medium passing through the culture vessel in one generation	= 1316 ml
Therefore NH_3 taken up in one generation	= 90.67 mg N
Total weight of yeast taking up NH_3	= 1.326 g.d.w.
(i.e. 1.326 g.d.w. of yeast took up 90.67 mg N in 117 min)	
Therefore rate of uptake of NH_3	= 0.5819 mg N/min g.d.w. (25 °C)

The total uptake of ammonia into the yeast was estimated by two independent methods. First, as shown in the legend to Fig. 1, it could be obtained by measuring the input and exit concentrations of ammonia in the medium passing through the culture vessel, provided that the growth rate and cell density of the culture were also known. Secondly, rates of uptake could be estimated from measurements of the isotopic abundance of the ammonia in the culture medium. From the isotope data for ammonia in the medium a turnover constant, b , can be determined (B. F. Folkes and A. P. Sims, unpublished); since the steady-state concentration of ammonia is known the rate of ammonia uptake can be calculated (Fig. 1b). The values obtained by these two different methods were in good agreement ($\pm 1\%$)

and gave an uptake rate of 0.577 mg N/min/g.d.w. of yeast growing exponentially at 25 °C. It is also possible to show from these measurements that about 20 % of the ammonia assimilated by the yeast passed through the soluble pool of glutamine.

The rate of glutamine synthesis as measured *in vivo* by these methods can be compared with the potential rate of synthesis calculated from the enzyme content of the yeast. Glutamine synthetase was measured at 25 °C with saturating levels of ATP, glutamate, ammonia and magnesium at the optimal pH of the enzyme. The average specific activity of the enzyme was shown to be equivalent to the formation of 0.43 mg amide N/min/g protein at 25 °C, and since there was 0.325 g soluble protein/g.d.w. of yeast this means that the enzyme was capable of producing 0.140 mg amide N/min/g.d.w. at 25 °C. This value is probably an over-estimate, however, since activities were determined under optimal conditions, a situation not necessarily prevailing in the cell. In particular, the full expression of enzyme activity *in vivo* is likely to be limited by the restricted availability of ammonia. The ammonia content of yeast growing under steady-state conditions on ammonia was shown by Sims *et al.* (1968) to be approximately 50 µg N/g.d.w. (see Fig. 7, below). Since 1 g.d.w. of yeast contained 2.45 ml of cell water, the mean concentration of ammonia in ammonia-grown yeast was about 1.45×10^{-3} M. If it is assumed that ammonia was distributed throughout the cell uniformly, then the enzyme would have been able to function at approximately 90 % of its maximal rate (see Fig. 8*a*, below). Hence the potential rate of amide synthesis could at most have been only twice that actually measured *in vivo* (0.0641 mg amide N/min/g.d.w. at 25 °C). This closeness of the match between observed and potential rates of amide synthesis suggests that there can be only a limited increase in amide synthesis without a corresponding increase in enzyme level.

A close match between actual and potential rates of glutamine synthesis was also found in glutamate-grown yeast. (Because its generation time and total nitrogen content were very similar to ammonia-grown yeast, it is probable that the steady-state rates of glutamine synthesis were also similar.) Yeast growing on glutamate has a fivefold higher level of enzyme than yeast on ammonia (Ferguson & Sims, 1974; see also Fig. 5*a*, below), but the mean cell concentration of ammonia is only about 1.2×10^{-4} M (Sims *et al.* 1968). Hence, assuming the ammonia is distributed uniformly throughout the cell, the enzyme is unlikely to function at more than 14 % of its potential rate. Once again it would appear that growth of yeast on this source of nitrogen results in an accurately controlled increase in glutamine synthetase, one that is sufficient to compensate for the reduced availability of ammonia.

Rates of glutamine synthesis in yeast previously subjected to nitrogen depletion

A large excess of glutamine synthetase activity might be expected if the synthesis of glutamine were regulated by feed-back inhibition. Yeast growing on ammonia or glutamate, however, does not appear to contain a large excess of potential enzyme activity. We attempted to establish directly whether feed-back inhibition plays an important role in the regulation of the synthesis of glutamine in food yeast by observing whether, on the restoration of the ammonia supply after a brief period of nitrogen depletion, yeast was capable of synthesizing glutamine at rates much faster than those found under steady-state conditions. If feed-back processes operate, then a fall in the concentration of glutamine and products of its metabolism should result in enhanced rates of amide synthesis in the absence of an increase in enzyme level.

Yeast was grown in a turbidostat in a medium containing a slight excess of ammonia and the experiment was initiated by switching the input medium to one lacking nitrogen. As soon as the concentration of ammonia in the medium fell below the steady-state level

Fig. 2. The collected results from a nitrogen-depletion experiment carried out at 25 °C. □, Ammonia in culture medium; ▲, cell content of soluble glutamate; ●, cell content of soluble glutamine; and Δ, level of glutamine synthetase. The enzyme was assayed with saturating levels of all substrates at 25 °C and the data are expressed in terms of the potential synthetic capacity of the enzyme in the absence of any substrate limitation or feed-back control.

The arrows show the time when the inflow medium into the turbidostat was switched to one without nitrogen and when ammonia was restored to the culture.

Yeast was grown in a turbidostat at 25 °C. The input medium contained NH_3 (84 $\mu\text{g NH}_3\text{-N/ml}$) and the cell density (0.798 mg dry wt/ml) was chosen so as to leave a nitrogen excess of about 6 $\mu\text{g N/ml}$ in the culture medium. After 16 h growth under these conditions, the experiment was started by replacing the input medium by one free of nitrogen. Samples were removed at the times indicated for analysis of the culture medium and the level of enzyme and free amino acids in this yeast. After 36 min, NH_3 (32 mg NH_4Cl , 13.5 % ^{15}N excess) was added to the culture and the input medium was switched to one containing isotopically-labelled NH_3 (84 $\mu\text{g N/ml}$, 54.35 % ^{15}N excess). Further samples were removed at the times indicated. Rates of glutamine synthesis are plotted in Fig. 3a.

Fig. 3. Changes in the *in vivo* rate of amide synthesis (a) and in the level of glutamine synthetase (b) in yeast after a short period of ammonia deprivation. The enzyme activity data shown in (b) were used to establish the synthetic potential of the yeast at different times, assuming the enzyme operated in the absence of any feedback control or substrate limitation. The observed rate at a particular time was then expressed as a percentage of potential rate at that time to determine the extent to which its activity was realized. The dashed line in (a) shows the steady-state rate of glutamine amide synthesis on ammonia. ○, Rate of glutamine synthesis; ■, percentage realization of synthetic potential; and △, activity of glutamine synthetase.

there was a decrease in the pool of soluble glutamine (Fig. 2), indicating that the capacity of the yeast to synthesize glutamine had been previously adjusted to the available supply of ammonia; once the ammonia concentration had fallen, the steady-state rate of glutamine synthesis could no longer be sustained. Glutamate behaved quite differently under these conditions; the pool was maintained until the medium had been almost completely exhausted of ammonia. Glutamine and glutamate differed also in their response to the restoration of the ammonia supply. A rapid and sustained build-up of the glutamine pool occurred and after an hour its level was one and a half times that in steady-state yeast. The glutamate pool fell for some time after the addition of ammonia. This was presumably because initially there were abnormal demands made on it by the enhanced synthesis of glutamine; only later was it restored to its original level. A small but significant rise in the amount of glutamine synthetase was associated with the reduction of ammonia concentration in the medium and the decrease in the pool of glutamine.

The *in vivo* rate of glutamine synthesis was measured (Fig. 3a) after the addition of

ammonia to the culture. The rate increased only 30 % above the steady-state value. The kinetics of these changes differed from those observed with glutamate synthesis (Sims *et al.* 1968); here maximal rates (over 300 % above the steady-state rate) were realized immediately, and the subsequent decline in rate could be linked to an increase in the level of the total pool of amino acids and not to changes in the level of the enzyme. With glutamine synthesis maximal rates were delayed until about 8 min after the addition of ammonia to the culture, during which time enzyme activity fell (Fig. 3*b*). Throughout the latter phase of the experiment changes in the rate of glutamine synthesis could be correlated with changes in the level of enzyme. The absence of appreciable changes in the rate of synthesis independent of changes in enzyme level again makes it unlikely that control was being brought about by modulation of feed-back restraint on the enzyme.

The response of the organism to even a small reduction of ammonia concentration in the culture medium was very sensitive. Yeast was grown in a medium containing about 5 μg $\text{NH}_3\text{-N/ml}$. A comparison of pool and enzyme data from this with the previous experiment reveals that the concentration of glutamate was similar in the two experiments but that the concentration of glutamine was lower and the level of synthetase was higher. Measurement of the rate of glutamine synthesis at 60 min made at a time when the soluble pools and enzyme were returning to their characteristic steady-state values, indicated just how precisely this increase in enzyme could compensate for a reduction in external nitrogen supply. The rate measured (0.063 mg amide N/min/g.d.w.) corresponded almost exactly with the figure observed in the earlier steady-state experiment in which yeast was grown on ammonia at about 15 μg $\text{NH}_3\text{-N/ml}$. Calculations establish that here only about 30 % of the potential synthetic capacity of the enzyme was realized.

Rates of glutamine synthesis on addition of ammonia to glutamate-grown yeast

The absence of any mechanism of feed-back inhibition on the glutamine pathway should mean that the addition of ammonia to yeast having high levels of glutamine synthetase (GS) would result in enhanced rates of amide synthesis, increases that would be directly related to the level of enzyme. Comparison of the actual *in vivo* rates of synthesis with the levels of enzyme should then make it possible to elucidate the nature of the restraint operating on the enzyme. We have examined the effects of the addition of ammonia to yeast in which the adaptation to growth on glutamate has resulted in a considerable enzyme derepression.

Addition of ammonia to such yeast produced a rapid and extensive build-up in the pool of glutamine, so that at 15 min a sevenfold increase in its level was observed (Fig. 4). The fall in the pool of glutamate was equally dramatic, with its concentration falling to less than half that found in ammonia-grown yeast. Only after 2 h did the metabolite pools approach the levels characteristic of steady-state growth on ammonia.

Greatly accelerated rates of glutamine synthesis had also occurred (Fig. 5*a*); the maximal rate of amide formation which occurred 8 min after the addition of ammonia was 0.41 mg amide N/min/g.d.w. (25 °C), about six times the rate observed in steady-state yeast growing on ammonia. Clearly the derepression of glutamine synthetase in glutamate-grown yeast did lead to an increased synthetic potential, one that was fully realized when ammonia was added to the culture. Moreover, under these conditions control mechanisms were no longer able to ensure the appropriate flow of ammonia into glutamate and glutamine. The increased synthesis of glutamine resulted in an excessive diversion of ammonia, and subsequently also of glutamate, into glutamine. This high rate of amide synthesis was not sustained however. It subsequently fell to a value slightly below that found in ammonia-grown yeast and then rose again appreciably before falling to the rate characteristic of steady-state

Fig. 4. Collected results from a glutamate-to-ammonia transition experiment at 25 °C. □, Ammonia in culture medium; ●, glutamate in culture medium; ▲, cell content of soluble glutamate; and ●, cell content of soluble glutamine. The arrow indicates the time of addition of ammonia to the yeast growing on glutamate as its sole source of nitrogen. Cells (1880 ml) were grown in a turbidostat on glutamate (6 mM) at 25 °C. The cell density (0.975 mg dry wt./ml) was selected to provide the organism with about 4 μg N/ml excess in the culture media. The generation time of the cells was 122 min. After about 20 hours' growth at this glutamate concentration the experiment was initiated by adding NH_4Cl (32 mg N; 10.15 % ^{15}N excess) and replacing the inflow medium by one containing isotope (6 mM; 54 % ^{15}N excess).

growth on ammonia. During this period, the amount of glutamine synthetase fluctuated rapidly (Fig. 5*b*). The initial fall in activity was very rapid and it has been shown that this is due to inactivation (Ferguson & Sims, 1971). Oscillations in enzyme level closely matched the changes of *in vivo* rate; enzyme and amide synthesis increased when the pool of glutamate was being replenished and the pool of glutamine was contracting.

A pertinent feature of these results was the observation that, for a limited period, the full synthetic capacity of the enzyme could be realized. This establishes that conditions exist within the cell when the enzyme can operate in the absence of any form of restraint. Because the full realization of activity occurred at a time when the level of the pool of

Fig. 5. Changes in the *in vivo* rate of amide synthesis and in the level of glutamine synthetase after the addition of ammonia to yeast growing on glutamate. (a) Changes in the *in vivo* rate of glutamine-amide synthesis. The steady-state rate on ammonia is indicated by the dashed line. (b) Changes in the level of enzyme.

glutamine was very high, this establishes that glutamine itself can have no feed-back effect on the enzyme.

Relationship between GS activity and the in vivo rate of glutamine synthesis

Although the results so far described have shown that rates of amide synthesis were largely determined by the level of glutamine synthetase, it is equally clear that other factors must also have played an important part in determining the rate. Thus in the last experiment, the changes in the rate of amide synthesis could not be accounted for solely by changes in enzyme level. The rate of glutamine synthesis did not reach a maximum until about eight minutes after the addition of ammonia (Fig. 5a), a time lag during which appreciable inactivation of the enzyme had already occurred (Fig. 5b). If the *in vivo* rate of glutamine synthesis is expressed as a function of enzyme content (Fig. 6a), then it becomes clear that for much of the time some restraint is being imposed on glutamine synthetase activity: the curve shown is not linear over its entire range, it does not extrapolate through the origin, and at lower enzyme levels the rates of synthesis were appreciably lower than might have been expected. By plotting the change in the rate of amide synthesis per unit of enzyme against time (Fig. 6b) some indication of when the enzyme is being subjected to restraint or limitation can be obtained; changes in the level of the various pool intermediates may offer clues as to which type of effect is operating.

Fig. 6. Correlations between enzyme level and the *in vivo* rate of amide synthesis. (a) The correlation between the rate of amide synthesis and the level of enzyme. O, Data derived from the glutamate-to-ammonia transition experiment at 25 °C; and $\square$, a value derived from the ammonia steady-state experiment at 25 °C. The dotted line represents the potential rate of amide synthesis calculated from the enzyme data in the absence of any substrate limitation or feedback control. (b) Changes in the rate of glutamine synthesis per unit of enzyme with time. The *in vivo* rates of synthesis were expressed as a percentage of the potential rate calculated from the enzyme activity data. The broken line represents the percentage of the amide synthetic capacity realized in steady-state cells growing on ammonia as the sole source of nitrogen.

Feed-back effectors and the control of glutamine synthetase

The fact that the fall in rate of glutamine synthesis per unit of enzyme followed a large increase in glutamine prompted us to examine whether the concentration of other amino acids and nucleotides also increased, because these compounds have been implicated in the control of glutamine synthesis (Hubbard & Stadtman, 1967). In a separate experiment (Fig. 7) the pools of several amino acids were measured after the addition of ammonia to glutamate-grown yeast. During the period that the rate of amide synthesis per unit of enzyme fell most rapidly, there was actually a decrease in the concentrations of alanine, glycine and histidine, the amino acids that, on the basis of *in vitro* studies, have been suggested as possible inhibitors of glutamine synthetase. Moreover, our own studies (A.P. Sims & A.R. Ferguson, unpublished) with partially purified preparations of glutamine synthetase from *Candida*

Fig. 7. Changes in the pools of soluble amino acids in yeast after the addition of ammonia to a culture growing on glutamate as sole source of nitrogen at 25 °C.

Yeast was grown in a turbidostat on glutamate (6 mM) at 25 °C. The cell density (0.98 mg/g.d.w./ml) was selected to provide the organism with about 4 μ g N/ml excess in the culture medium. The experiment was initiated by adding NH₄Cl (1 g) and replacing the inflow medium by one containing 6 mM-NH₄Cl.

The results, with the exception of the ammonia data, (b), are expressed both as concentrations and in mg α NH₂-N/g.d.w. of cells. The ammonia data are plotted as mean cell concentrations; a water content of 2.448 ml/g.d.w. cells was used to calculate mean cell concentrations. The dotted line in (b) indicates the steady-state concentration of ammonia in yeast cells growing on this as a sole source of nitrogen. (a) ●, Glutamine; and ▲, glutamate. (b) □, Ammonia. (c) ■, Alanine; ◇, lysine; ◆, arginine; ▼, ornithine; ×, histidine; and ○, glycine.

utilis have shown that, whereas the enzyme assayed by transferase activity was susceptible to inhibition by some amino acids and nucleotides, the activity of the enzyme, when measured by means of the synthetase assay with Mg²⁺ as metal cofactor, was not inhibited by glutamine or by other amino acids and nucleotides, either separately or in combination. These results, together with those described earlier, seem positively to rule out the suggestion that the enzyme is regulated by 'cumulative' feed-back inhibition.

Substrate availability and the limitation of glutamine synthetase activity

Using a partially purified preparation of glutamine synthetase from *Candida utilis* we have determined the affinity of the enzyme for ammonia and glutamate (Fig. 8). The proper-

Fig. 8. The effect of varying the concentration of substrates on the activity of glutamine synthetase. All the rates are expressed relative to the maximal rate. Enzyme activity was estimated by [^{14} C]-glutamine formation, details of which are given in Methods. ○, Enzyme prepared from yeast growing on glutamate as the sole source of nitrogen; and ●, 'Inactivated enzyme' obtained from yeast by transferring it from glutamate to ammonia for 1 h. (a) The effect of varying the ammonia concentration. The two arrows indicate (left to right) the degree of saturation of the enzyme in yeast growing on glutamate and ammonia. (b) The effect of varying the glutamate concentration. The arrow indicates the value calculated for the mean cell concentration of glutamate in yeast grown on ammonia. The similarity in response of active enzyme and 'inactivated enzyme' indicates that no appreciable changes in the kinetic characteristics of the enzyme accompanied enzyme inactivation.

ties of the enzyme preparation confirm that the *in vivo* rate of glutamine synthesis is likely to be particularly sensitive to small changes in the concentration of ammonia: the enzyme exhibited co-operative responses to this substrate and the greatest changes in reaction rate occurred over the range of ammonia concentrations found in yeast. The enzyme had a much lower affinity for glutamate, but the cellular concentrations of this substrate under steady-state conditions of growth were normally sufficient to ensure that the enzyme was fully saturated. During the transition from glutamate to ammonia, however, the fall in the glutamate pool was such that enzyme activity, and hence the *in vivo* rate of glutamine

Fig. 9. A comparison of the relative rates of amide synthesis determined *in vivo* with values calculated from measurements of enzyme concentrations after allowing for changes in substrate availability.

Data obtained from the two separate glutamate-to-ammonia transition experiments at 25 °C have been used.

Estimates of the mean cell concentrations of ammonia and glutamate shown in Fig. 7 were used to calculate the extent to which glutamine synthetase was saturated with respect to the substrate at different times during the experiment (see Fig. 8). The enzyme activity data (Fig. 5) was then corrected using the appropriate saturation factors. The value calculated for 8 min was matched to the *in vivo* rate determined at this time and all the other values are shown relative to this point. Unfortunately the two experiments were not performed in exactly the same way. Changes in the mean cell concentration of ammonia were measured in yeast exposed to a somewhat higher concentration of ammonia (see Fig. 7); this could result in a more rapid build-up of ammonia in the cell and account for the deviation in the rate observed later in the experiment. ○, The *in vivo* rate of amide synthesis; and ---, calculated values.

synthesis, could be limited by the availability of glutamate. No measurements were made of the concentrations of ATP and Mg^{2+} during this experiment, but it seems unlikely that under the conditions used their availability would have limited amide synthesis (see Sols & Marco, 1970).

Estimates of the mean cellular concentrations of glutamate and ammonia have been used in conjunction with measurements of enzyme affinities to calculate the percentage saturation of the enzyme by these two substrates at different times during the glutamate-to-ammonia transition experiment. The values obtained, together with the corresponding measurements of enzyme level, were used to predict changes in the rate of glutamine synthesis after the addition of ammonia. These predicted rates were then related to rates actually measured *in vivo* (Fig. 9) by matching the one calculated for 8 min (maximum rate) with ones observed *in vivo* at this time. All other rates have been expressed relative to this. The closeness of the fit between the predicted and measured rates of synthesis during the nitrogen-transition experiment, a period which includes phases of increased saturation of enzyme by substrate, enzyme inactivation and substrate limitation followed by enzyme reactivation,

reveals the adequacy of these factors in explaining the large changes of rate of amide synthesis.

DISCUSSION

The fact that glutamine synthetase occupies a strategic branch point in cellular metabolism indicates that it is likely to be subject to a variety of metabolic controls.

The experiments described here were specifically designed to test whether cumulative feedback inhibition, a system of control formulated almost entirely from the results of studies with isolated enzyme, operated in the living cell. Results from three physiological studies where the actual *in vivo* rates of glutamine synthesis were measured lent no support to this idea but established instead that enzyme modulation was of prime importance. These observations confirmed earlier ones (Sims *et al.* 1968) that in the event of a sudden increase of amide synthesis the subsequent depletion of the metabolite pools of glutamate and ammonia can effect a substantial reduction in the level of enzyme activity. Our studies of the effect of possible end-products of glutamine metabolism on the activity of glutamine synthetase (as measured by transferase activity) were in part agreement with those reported earlier by Hubbard & Stadtman (1967) and illustrate the difficulty of formulating reliable systems of control when they are based almost exclusively on studies with purified enzyme.

These findings leave unanswered the question of how such precise enzyme modulation is achieved. Experiments of this type offer only scant clues to the nature of the mechanisms involved and this topic is the subject of other communications (Ferguson & Sims, 1974; Sims, Toone & Box, 1974). There is some circumstantial evidence to suggest that enzyme level might change in response to variation in the size of the glutamine pool. Thus enzyme inactivation (Fig. 5*b*) was associated with a sudden increase in the glutamine pool whilst the increase in the level of enzyme observed in the nitrogen-depletion experiment (Fig. 2) closely followed a reduction of the glutamine pool. Clearly the effect of such a mechanism would be to ensure that glutamine does ultimately inhibit its own biosynthesis. The speed at which changes in the amide pool took place after an alteration to the supply of nitrogen to the cell means that changes in glutamine concentration could provide a sensitive means to trigger such a mechanism. Thus even in yeast growing on ammonia, where the pool of glutamine is particularly large, the pool is completely replaced every 20 min. Even though most of the other pools of soluble amino acids are undergoing an equal or even more rapid turnover (Sims & Folkes, 1964) they do not appear to fluctuate greatly in size during changes of nutritional conditions (see Fig. 7, for example). Presumably this is because many of these compounds derive their nitrogen from, and maintain their pools at the expense of, glutamate and glutamine. In this respect the two primary pathways of assimilation behave very differently. Thus when yeast experienced a reduction of ammonia concentration in its culture medium (see Fig. 2), even a small reduction in the total pool of amino acids was sufficient to guarantee the continued synthesis of glutamate by removal of feedback restraint of the NADP-glutamate dehydrogenase. The lack of any such reserve of biosynthetic capacity on the glutamine pathway means that its synthesis is particularly sensitive to changes in ammonia concentration. The absence of direct control on this irreversible pathway provides a situation where even a small change in the rate will produce a large fluctuation in the cell concentration of glutamine which in turn could act as the signal to modulate enzyme level. Thus although this pattern of control may not be effective in ensuring pool homeostasis, it is particularly well adapted to exploit any sudden increase in the availability of ammonia, an essential nutrient normally in restricted supply.

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