Effects of D-Glutamate on Enzymes of Ammonia Assimilation in *Bacillus megaterium* NCIB 7581

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Enzymes possibly concerned in the assimilation of ammonia were assayed in *Bacillus megaterium* NCIB 7581 after growth with ammonium sulphate as sole source of nitrogen. Alanine dehydrogenase and aspartase were not detected, but both NADPH-dependent glutamate dehydrogenase and glutamate synthase (NADH- or NADPH-dependent) were found. Glutamine synthetase was also present, though differing methods of assay gave different values for its activity.

Glutamate dehydrogenase was competitively inhibited by D-glutamate, whereas glutamate synthase was insensitive to D-glutamate or D-glutamine (singly or together). D-Glutamate was a substrate for glutamine synthetase, and the use of L-glutamate by this enzyme was partly inhibited by a 10-fold molar excess of D-glutamate. L-Glutamine strongly inhibited glutamine synthetase, but D-glutamine had no effect.

A D-glutamate-resistant substrain (grown with ammonium sulphate as nitrogen source) contained 10 times more glutamate dehydrogenase activity than did the wild-type under the same conditions, and the enzyme showed altered affinity for 2-oxoglutarate and for D-glutamate. Activities of glutamate synthase and glutamine synthetase in the substrain were similar to those in the wild-type.

Glutamate dehydrogenase was absent from organisms grown with nitrate as sole nitrogen source, yet growth under these conditions was still inhibited by D-glutamate.

INTRODUCTION

Growth of *Bacillus megaterium* NCIB 7581 in a simple chemically defined medium was strongly inhibited by D-glutamate when ammonium sulphate was the nitrogen source (White, 1979). D-Glutamate was not inhibitory in the presence of L-glutamate or other L-amino acids that, like L-glutamate, could act as sole nitrogen sources for growth. D-Glutamate might therefore inhibit the assimilation of ammonia by *B. megaterium* 7581 and this paper describes a study of the enzymes (glutamate dehydrogenase, glutamate synthase, glutamine synthetase, aspartase and alanine dehydrogenase) that might take part in this process. The effect of D-glutamate on transaminases that use L-glutamate as substrate was also examined.

METHODS

Organisms. *Bacillus megaterium* NCIB 7581 and 7581 GRA-1 (a D-glutamate-resistant substrain) were described by White (1979); 7581 GRN-1 (another D-glutamate-resistant substrain) is described in this paper. *Escherichia coli* W (ATCC 9637) was described by White (1976). This organism was used to provide an extract with high enzymic activity to check the proper functioning of the assays for glutamine synthetase (four methods) and glutaminase.

Media. Medium A1 (supplemented with biotin and citrate) was described by White (1979). When NaNO_3 (3 g l^{-1}) was used as the sole nitrogen source, $(\text{NH}_4)_2\text{SO}_4$ was omitted from medium A1 and $\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$ 0022-1287/79/0000-8123 \$02.00 © 1979 SGM

(4 g l⁻¹) was added. The minimal medium of Davis & Mingioli (1950), with 0.2 % (w/v) glucose, was used for *E. coli*.

Conditions of growth. *Bacillus megaterium* (500 ml medium per flask) and *E. coli* (250 ml medium per flask) were grown at 37 °C in shaken 2 l flasks and turbidity was measured in an EEL colorimeter, as described by White (1972, 1979). The inoculum of *E. coli* was 0.1 ml per flask of a suspension of organisms (about 1 × 10⁸ ml⁻¹) from a fresh overnight culture on nutrient agar.

Harvesting and preparation of extracts. Organisms were centrifuged from the medium at 5000 g for 10 min at 2 °C, washed twice in 0.02 M-buffer (see below) and then resuspended, usually to about 30 mg dry wt ml⁻¹, in 0.1 M-buffer at a pH value appropriate for the enzymic assay in which the extract was subsequently to be used. This was most commonly Tris/HCl, pH 7.6. The suspension of washed organisms was disrupted in a pressure cell (Milner *et al.*, 1950) at 2 °C and 140 MPa. Debris was removed by centrifuging at 20000 g for 20 min at 2 °C and protein was measured in the supernatant liquid by the Lowry method, with bovine serum albumin as standard.

Assay of enzymes. Alanine dehydrogenase [EC 1.4.1.1; L-alanine:NAD⁺ oxidoreductase (deaminating)], glutamate dehydrogenase [EC 1.4.1.2 and 1.4.1.4; L-glutamate:NAD⁺ oxidoreductase (deaminating) and L-glutamate:NADP⁺ oxidoreductase (deaminating), respectively] and glutamate synthase [EC 1.4.1.13; L-glutamate:NADP⁺ oxidoreductase (transaminating)] were all assayed spectrophotometrically at 37 °C in anaerobic cuvettes with side-arms. The assay mixture (without one substrate and without extract) was placed in the cuvette and the extract in the side-arm. Nitrogen (O₂-free) was bubbled through the assay mixture for 2 to 3 min and, after bringing the contents of the cuvette to 37 °C, the extract was tipped from the side-arm. Elimination of air was necessary to prevent rapid oxidation of the reduced cofactor in the absence of substrate. After recording the blank activity (which was always low in the absence of air), substrate (NH₄Cl or glutamine) was injected through the rubber sealing cap and the change in absorbance was recorded. The assay system for alanine dehydrogenase was that of Yoshida & Freese (1970). The systems for glutamate dehydrogenase and for glutamate synthase were those of Meers *et al.* (1970), both at pH 7.6, which was the optimum for these enzymes in *B. megaterium* 7581. The graphical method of Lineweaver & Burk was used to determine apparent *K_m* values for these two enzymes. Initial rates of reaction were determined in the standard assay system with varying concentrations of one of the substrates (2-oxoglutarate or NH₄Cl or L-glutamine).

Glutamine synthetase [EC 6.3.1.2; L-glutamate:ammonia ligase (ADP-forming)] was assayed by four methods: the transferase assay, as described by Shapiro & Stadtman (1970); the assay based on release of inorganic phosphate from ATP, described by Shapiro & Stadtman (1970); the forward reaction assay of Bender *et al.* (1977); and the conversion of L-[U-¹⁴C]glutamate into [¹⁴C]glutamine. In this last method, imidazole/HCl buffer, pH 7 (50 μmol), L-glutamic acid (20 μmol), L-[U-¹⁴C]glutamate (2 μCi), ATP (Na salt, 7.5 μmol), MgCl₂ (50 μmol), MnCl₂ (5 μmol), NH₄Cl (50 μmol) and water (to give 1 ml total vol. after addition of extract) were mixed in a 100 × 10 mm tube and incubated at 37 °C before adding extract (250 μl, about 5 mg protein). Immediately after this addition, and then at intervals of 2 min, samples (100 μl) were removed and mixed with 300 μl ethanol at 2 °C. These mixtures were left for about 1 h while the protein settled out. L-Glutamine (1 μmol in 5 μl) was added to act as carrier of the [¹⁴C]glutamine in subsequent chromatography (see below). Four samples (each 50 μl) of the supernatant liquid were chromatographed on Whatman no. 1 paper using methanol/pyridine/conc. HCl/water (80:10:2.5:17.5, by vol.; Rhuland *et al.*, 1955) as solvent for about 16 h (descending) at room temperature. The glutamine spots were detected with ninhydrin and then the paper was left for 24 h until the spots had faded. The marked spots were cut into pieces and counted in the scintillation fluid of Sherman (1963). The external standard ratio was determined. Moving the positions of the paper in the fluid did not much alter the count or external standard ratio.

The 1-carboxylic acid group of glutamine (or glutamate) is lost as CO₂ on heating with ninhydrin. To relate measured counts to μmol glutamine, known amounts of L-[U-¹⁴C]glutamate (5 to 12.5 nCi per spot; with 0.05 μmol L-glutamate per spot, as carrier) were chromatographed, treated with ninhydrin and counted as above. The amount of glutamine formed was plotted against time of sampling and the initial rate of the reaction was determined.

As a semi-quantitative test for glutaminase [EC 3.5.1.2; L-glutamine amidohydrolase], extracts (about 1 mg protein) were incubated with L-glutamine (2 to 8 μmol) at 37 °C for 15 min in the assay mixture (without glutamate; 200 μl total vol.) used for measurement of synthesis of [¹⁴C]glutamine. Samples (50 μl) were chromatographed as described above, and the amounts of free glutamate and of remaining glutamine were assessed from the size and intensity of the spots with ninhydrin.

Aspartase [EC 4.3.1.1; L-aspartate ammonia-lyase] was assayed at 37 °C and pH 7 by the spectrophotometric method of Williams & Lartigue (1967).

Glutamate-oxaloacetate transaminase [EC 2.6.1.1; L-aspartate:2-oxoglutarate aminotransferase], glutamate-pyruvate transaminase [EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase] and glutamate-

phenylpyruvate transaminase [EC 2.6.1.5; L-tyrosine:2-oxoglutarate aminotransferase] were each assayed by similar two-step methods (Gunsalus & Stamer, 1955), in which the glutamate produced by transamination was assayed with bacterial glutamate decarboxylase (from Koch-Light).

Estimation of intracellular ammonia. Samples (5 ml) of bacterial cultures were filtered through Millipore discs (type HA, pore size 0.45 μ m, 25 mm diam.). Each wet disc plus organisms was boiled for 5 min in water (5 ml) to extract ammonia from the bacteria. The membrane and organisms were removed and ammonia was measured in samples (2 ml) of the solution, by the method of Tetlow & Wilson (1964). To correct for ammonia present in the wet disc (rather than in the organisms themselves), part of the original culture was centrifuged and the supernatant liquid (5 ml) was filtered through another disc from which ammonia was extracted and assayed. The volume of the intracellular 'pool' in the organisms (expressed in μ l) was taken as four times the dry weight (expressed in mg) of organisms collected (Tempest *et al.*, 1970), which was estimated from the turbidity of the culture (White, 1972). A further correction for ammonia present in the medium which occupied the interstices of the pad of organisms was estimated from the dimensions of the organisms, assuming that they packed as stacked rectangular blocks. Similar results were obtained if ammonia was extracted with HClO_4 or trichloroacetic acid at 2 °C.

Organisms collected on Millipore discs were washed once with 0.02 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7 before extraction and assay of ammonia. Even without any corrections, such assays gave lower values for the ammonia content of the organisms than were found from corrected assays of unwashed bacteria.

Chemicals. L-[U- ^{14}C]Glutamic acid was from The Radiochemical Centre, Amersham. All other chemicals were of the highest grade commercially available.

RESULTS

Internal concentration of ammonium in B. megaterium 7581

The internal concentration of NH_4^+ in *B. megaterium* 7581 (harvested during exponential growth from medium A1) was about 50 mM in unwashed organisms (after corrections) and 20 mM in washed organisms (see Methods). The internal concentration during the first stationary phase of growth after DL-glutamate was added to medium A1 was 70 mM and in the second phase of growth it was 60 mM (both unwashed organisms). Thus, uptake of ammonia is not prevented by D-glutamate.

Enzymes of ammonia assimilation in B. megaterium 7581

A number of enzymes have been implicated in the assimilation of ammonia in various organisms (for review, see Brown *et al.*, 1974). Since the concentration of NH_4^+ exceeded 30 mM in medium A1, glutamate dehydrogenase (NADPH-dependent) would probably be the principal enzyme of ammonia assimilation. This enzyme is known to be inhibited by D-glutamate in other organisms (e.g. Arkin & Grossowicz, 1970).

Glutamate dehydrogenase. This enzyme (NADPH-dependent) was present whether *B. megaterium* 7581 was grown in the absence or presence of DL- or L-glutamate (Table 1); NADH was ineffective as a cofactor. At the optimum pH of 7.6, the apparent K_m for 2-oxoglutarate was 0.37 mM and the apparent K_m for NH_4^+ was 21 mM. These values are both extremely close to those found by Hemmilä & Mäntsälä (1978) for the same enzyme from *B. megaterium* NCTC 10342. The enzyme was inhibited competitively by D-glutamate (K_i 3.3 mM; Fig. 1); almost complete inhibition occurred with 50 mM-D-glutamate in the assay system. This is the concentration present in the amino acid 'pool' during the first stationary phase of growth with DL-glutamate (White, 1979). At pH values that were not optimum for enzymic activity, the inhibitory effect of D-glutamate was slightly less pronounced; thus at pH 6.5 K_i was 6.3 mM.

Glutamate synthase. Alanine dehydrogenase (NADH or NADPH as cofactor) and aspartase were very weakly active, but considerable glutamate synthase was found (Table 1). Both NADPH and NADH were effective as cofactors, though NADPH gave about 10% higher enzymic activity and was used in all the assays described later in this paper. Glutamate synthase was not inhibited by 50 mM-D-glutamate or 5 mM-D-glutamate (separately or together) even when only 1 mM-L-glutamine was used in the assay system (cf. Miller &

Fig. 1. Determination of K_i (for D-glutamate) with NADPH-dependent glutamate dehydrogenase by the method of Dixon (1953): Δ , *B. megaterium* 7581; $\circ$ and $\bullet$, *B. megaterium* 7581 GRA-1 (5 and 1 mM-2-oxoglutarate, respectively, in the assay system).

Stadtman, 1972) nor was the enzyme repressed by growth with glutamate in medium A1 (Table 1), unlike the same enzyme in *B. megaterium* MA (Elmerich, 1972). Consequently, if this enzyme can really function *in vivo*, assimilation of ammonia ought not to be prevented by D-glutamate.

The assay systems for glutamate dehydrogenase and glutamate synthase differ only in that NH_4Cl is substrate for the former enzyme and L-glutamine for the latter. In *B. megaterium* 7581 the two enzymes are, however, clearly present. Glutamate synthase was completely inhibited by 1 mM-azaserine (an analogue of glutamine; Levenberg & Buchanan, 1957) in the assay system, while the dehydrogenase was unaffected. However, D-glutamate inhibited the dehydrogenase much more strongly than it did the synthase. The activity of the synthase was also less stable on storing at -15°C or at 2°C than was the dehydrogenase. Hydrolysis of glutamine (to liberate ammonia that might serve as substrate for glutamate dehydrogenase) could not account for the observed glutamate synthase activity. No glutaminase was detected (by the semi-quantitative test) in an extract of *B. megaterium* 7581, though an extract of *E. coli* had marked activity (see below). In any case, unless a high concentration of ammonia is available in the assay system, the measured activity of glutamate dehydrogenase should be very low owing to the high K_m value for NH_4^+ . The apparent K_m value of glutamate synthetase for L-glutamine was only 0.6 mM; D-glutamine was ineffective as a substrate. Meers *et al.* (1970) found a K_m value of 0.3 mM for glutamine with the enzyme from *B. megaterium* KM and Hemmilä & Mäntsälä (1978) found a value of 0.2 mM with *B. megaterium* NCTC 10342.

Glutamine synthetase. To be active *in vivo*, glutamate synthase requires 2-oxoglutarate, NADPH and L-glutamine, which is formed by glutamine synthetase. Activity of this latter enzyme was measured by the transferase assay, by release of inorganic phosphate (which proved unsatisfactory because inorganic phosphate was rapidly produced without glutamate in the assay system) and by a method which allowed a direct measure of the formation of $[^{14}\text{C}]$ glutamine from $[^{14}\text{C}]$ glutamate. The last method will only be valid if the $[^{14}\text{C}]$ glutamine is not hydrolysed during or after the incubation. No glutaminase was detected

Table 2. *Activities of glutamine synthetase under various conditions of assay*

Extracts of *B. megaterium* 7581 were prepared from organisms grown in medium A1 and harvested during exponential growth, as described in Methods. Details of assay procedures are given in Methods.

Assay procedure	Enzymic activity [nmol min ⁻¹ (mg protein) ⁻¹]
Transferase	10, 6
Incorporation of [¹⁴ C]glutamate	20, 3, 8, 5
'Direct' assay of Bender <i>et al.</i> (1977) with reaction mixture* containing:	
164 mM-L-glutamate	195
50 mM-L-glutamate	123
5 mM-L-glutamate	35
50 mM-L-glutamate without Mg ²⁺	5
50 mM-L-glutamate + 18 mM-Mn ²⁺ (in place of Mg ²⁺)	81
50 mM-L-glutamate + 5 mM-L-glutamine	10
50 mM-L-glutamate + 1 mM-L-glutamine	98
50 mM-L-glutamate + 5 mM-L-glutamine + 18 mM-Mn ²⁺ (in place of Mg ²⁺)	76
50 mM-L-glutamate + 20 mM-D-glutamine	130
164 mM-L-glutamate	90†
22 mM-L-glutamate	60†
11 mM-L-glutamate	30†
164 mM-D-glutamate	36†
39 mM-D-glutamate	15†
16 mM-D-glutamate	7†

* Containing 18 mM-Mg²⁺ unless stated otherwise, but no cetyltrimethylammonium bromide. Addition of this compound to the assay mixture or to the bacterial culture before harvesting did not alter the measured enzymic activity.

† All tested with the same extract, which had been kept for about a week at -15 °C. From these results, K_m for L-glutamate was about 10 mM and K_m for D-glutamate was about 200 mM. The ratio of V_{max} with L-glutamate to V_{max} with D-glutamate was 0.83. Similar K_m values and ratio of V_{max} values were obtained with an extract of substrain GRA-1.

in an extract of *B. megaterium* (see above) and the activity of glutamine synthetase measured by the radioisotopic method was in fair agreement with the transferase assay (Table 2). An extract of *E. coli* (which did contain glutaminase) had glutamine synthetase activities of 57 and 38 nmol min⁻¹ (mg protein)⁻¹ by the transferase and radioisotopic methods, respectively.

In contrast, the forward reaction assay (Bender *et al.*, 1977), even without cetyltrimethylammonium bromide, showed a much higher activity of glutamine synthetase in *B. megaterium* 7581 (Table 2). These values were in accord with predicted values (see Discussion) unlike the results of the other assays. The rate was proportional to the volume of extract added (up to 40 µl) and the reaction was entirely dependent on the addition of glutamate, ATP and Mg²⁺ (or Mn²⁺). L-Glutamine completely inhibited the assay when Mg²⁺, but not Mn²⁺, was the activator. D-Glutamine had no effect with either cation. These metal ions and L- and D-glutamine had similar effects on the enzyme from other *Bacillus* species in a different assay system (Deuel & Stadtman, 1970; Hubbard & Stadtman, 1967*b*). L-Proline (1 mM) in the assay system of Bender *et al.* (1977) had no effect on glutamine synthetase, so that proline-sensitive γ -glutamyl kinase, like that in *E. coli* (Baich, 1969), seemed to be absent. Furthermore, γ -glutamyl hydroxamate was formed with D-glutamate as substrate (see below) whereas γ -glutamyl kinase (in *Brevibacterium flavum*) uses only L-glutamate (Yoshinaga *et al.*, 1975).

With 50 mM-D-glutamate in the assay system (without L-glutamate), the rate of formation of product was 32% of the rate with the same concentration of L-glutamate. Deuel & Stadtman (1970) and Hubbard & Stadtman (1967*a*) found, with the enzymes from

B. subtilis and *B. licheniformis*, respectively, that D-glutamate gave about 20% of the rate seen with L-glutamate. This metabolism of D-glutamate and the lack of inhibition by D-glutamine are consistent with the prolonged excretion of D-glutamine by cultures of *B. megaterium* 7581 (White, 1979).

However, 50 mM-D-glutamate partly inhibited conversion of 5 mM-L-[U-¹⁴C]glutamate (1 μ Ci) to γ -[¹⁴C]glutamyl hydroxamate. These proportions of the two isomers were chosen because the concentration of L-glutamate in the amino acid pool of *B. megaterium* 7581 grown in medium A1 was 5 mM, and that of D-glutamate in the pool was 50 mM during the first stationary phase of growth in medium A1 plus DL-glutamate (White, 1979). The enzymic reaction was stopped immediately after adding the extract or after incubation for 30 min. Samples (2 μ l) were then chromatographed on thin-layer plates of silica gel G with ethanol/water (70:30, by vol.) at room temperature (Brenner *et al.*, 1965). The glutamate (R_f 0.60) migrated with little streaking, while the yellow-brown γ -glutamyl hydroxamate (iron salt) remained near the origin. Its radioactivity was measured in the toluene-based fluid of Snyder (1964) containing 4% (w/v) Cab-O-Sil (fumed silica). With D-glutamate in the assay system, utilization of L-[¹⁴C]glutamate was inhibited by 37%.

Transaminases

Transamination between 2-oxoglutarate and L-alanine (or L-aspartate or L-phenylalanine) was not inhibited by D-glutamate (Table 1).

Enzymes of the D-glutamate resistant substrain 7581 GRA-1

Extracts of this strain (grown with 1 mg D-glutamate ml⁻¹) had higher glutamate dehydrogenase activity than the parent strain, whereas activities of other enzymes tested were similar in both strains (Table 1). Even in the presence of 50 mM-D-glutamate (the concentration in the 'pool' during growth with D-glutamate), activity of glutamate dehydrogenase was still as high [280 nmol min⁻¹ (mg protein)⁻¹] as the activity of the wild-type enzyme without D-glutamate.

The glutamate dehydrogenase from the mutant and wild-type differed, the mutant enzyme having an apparent K_m (for 2-oxoglutarate) of 1.33 mM and a K_i for D-glutamate of 4.7 mM (Fig. 1). These values were determined with a crude extract, and so the mutant might make one enzyme with changed properties or might form two enzymes, the wild-type and an altered enzyme with properties more different than are yet apparent.

Growth of *B. megaterium* 7581 with nitrate as sole source of nitrogen

Organisms using nitrate as sole source of nitrogen must first reduce this anion, so that growth with nitrate should be a condition resembling growth with a low concentration of NH₄⁺, and glutamate dehydrogenase should not be functional. When *B. megaterium* 7581 grew in medium A1 with nitrate in place of NH₄⁺, no glutamate dehydrogenase was found, whereas glutamate synthase and glutamine synthetase were both present (Table 1). The increased activity of this latter enzyme in the nitrate-grown organisms may indicate that in *B. megaterium*, as in *E. coli* (Woolfolk *et al.*, 1966), NH₄⁺ causes partial repression of glutamine synthetase, while glutamate synthase, as in *E. coli* (Miller & Stadtman, 1972), is not repressed.

Even though glutamate dehydrogenase was absent, D-glutamate (0.01 mg ml⁻¹) prevented growth with nitrate, which meant that some other enzyme besides glutamate dehydrogenase must be sensitive. Resistant variants arose frequently; about 1 in 10⁵ organisms plated on medium A1 with nitrate and D-glutamate (1 mg ml⁻¹) grew into a colony $\geq$ 1 mm diam. after 48 h at 37 °C. From one such colony a substrain (called GRN-1, glutamate-resistant when growing on nitrate as nitrogen source) was isolated that was resistant to D-glutamate. Various enzymic activities of this substrain are given in Table 1. Substrain GRA-1 grew on medium A1 containing nitrate, but D-glutamate (0.02 mg ml⁻¹ or more) gave a transient inhibition (about 1 d) that was not seen when NH₄⁺ was used.

DISCUSSION

Growth tests strongly suggest that D-glutamate inhibits *B. megaterium* 7581 by interference with assimilation of ammonia. However, the entry of ammonia into the organisms is not prevented by D-glutamate. Studies with extracts of organisms grown as batch cultures in medium A1 imply that glutamate dehydrogenase (NADPH-linked) is important in the assimilation of nitrogen (from ammonia) into organic molecules and 50 mM-D-glutamate almost completely inhibits this enzyme. Glutamate dehydrogenase is much more active in the D-glutamate-resistant substrain GRA-1 and the enzyme of the resistant organisms is altered so that it is less sensitive to inhibition by D-glutamate.

A puzzling feature was the constitutive formation of glutamate synthase, that apparently did not function *in vivo* when D-glutamate was present in the medium. This enzyme was not inhibited by D-glutamate or D-glutamine, so that organisms would be expected to assimilate ammonia and grow in presence of D-glutamate, that is, unless D-glutamate also caused some inhibition in another area of metabolism.

Although glutamate synthase can act when only low concentrations of glutamine (< 0.5 mM) are present (Meers *et al.*, 1970), the rate of glutamate production by this enzyme cannot exceed the rate of supply of L-glutamine, and in *B. megaterium* 7581 the utilization of L-glutamate by glutamine synthetase is partly inhibited by D-glutamate. This inhibition does not appear striking at first sight (only 37% by a 10-fold excess of D-glutamate) but it may be enough to unbalance the flow of intermediates between glutamine synthetase and other enzymes. When production of L-glutamine is partly inhibited (by D-glutamate) then L-glutamate may not accumulate, because it should be continuously withdrawn as a substrate of other reactions (protein synthesis etc.) and its replenishment by glutamate synthase requires a supply of L-glutamine. Production of glutamine might stop completely if either (or both) of two conditions applied: (i) if the inhibition of glutamine synthetase by D-glutamate were competitive and therefore became greater as the ratio of L- to D-glutamate fell; (ii) if the demand for L-glutamate by other reactions did not quickly decrease when the supply of L-glutamate began to fall as a consequence of the lack of L-glutamine. In contrast, the partial inhibition of glutamine synthetase by D-glutamate should not be of serious consequence when a rapid supply of L-glutamate can be maintained in the presence of D-glutamate, as occurs in substrain GRA-1, by glutamate dehydrogenase action.

The 'natural' control mechanism for these two enzymes should operate quite differently. Excess L-glutamine inhibits further synthesis of itself but does not deplete the supply of L-glutamate, which can be generated from the surplus glutamine. When the concentration of glutamine falls to a non-inhibitory level (by conversion of glutamine to glutamate), a supply of glutamate will at once be available to resume the synthesis of glutamine.

Wohlhueter *et al.* (1973) calculated that 1.3 mol amide nitrogen are needed to form 1 kg dry wt *E. coli* when the organisms grow in a simple minimal medium. This amide nitrogen was assumed to be needed for synthesis of purines, pyrimidines, amino acids and amino sugars by those metabolic routes that normally use glutamine as nitrogen donor ('minor ports', see below). Almost 10 times more amide nitrogen is needed if it is used (via glutamate synthase) as the 'major port' of entry of nitrogen into organic compounds. If *B. megaterium* had similar quantitative needs for glutamine in 'minor ports' for entry of nitrogen (in fact the need will be slightly less than in *E. coli* because even in exponentially growing *B. megaterium* about 10% of the dry weight is poly- β -hydroxybutyrate), then the activity of glutamine synthetase needs to be about 20 nmol min⁻¹ (mg protein)⁻¹ in organisms growing in medium A1 at a doubling time of 90 min. In the same organisms, the activity of glutamate dehydrogenase ('major port') ought to be about 200 nmol min⁻¹ (mg protein)⁻¹. When the organisms grow with nitrate as sole source of nitrogen, glutamine synthetase becomes the 'major port' and, to maintain the same doubling time, an activity of about 200 nmol min⁻¹ (mg protein)⁻¹ would be necessary for both glutamine synthetase and glutamate

synthase, while glutamate dehydrogenase might disappear. Observed enzymic activities are in good accord with these predictions, though the activities of glutamine synthetase (when 50 mM-L-glutamate is provided in the assay system) and glutamate synthase are higher than needed for growth with 30 mM-NH₄⁺.

The pattern of nitrogen metabolism in *B. megaterium* 7581 grown with NH₄⁺ in medium A1 is strikingly different from that found by Elmerich (1972) in *B. megaterium* MA. This latter strain contained no glutamate dehydrogenase, but had high activities of glutamate synthase [110 nmol min⁻¹ (mg protein)⁻¹] and glutamine synthetase [340 nmol min⁻¹ (mg protein)⁻¹]. However, when grown with nitrate as nitrogen source, *B. megaterium* 7581 does resemble strain MA in the activities of these enzymes. Phibbs & Bernlohr (1971) also found no glutamate dehydrogenase in an unspecified strain of *B. megaterium*, but did not test for the presence of glutamate synthase. Hemmilä & Mäntsälä (1978) found both glutamate dehydrogenase [147 nmol min⁻¹ (mg protein)⁻¹] and glutamate synthase [60 nmol min⁻¹ (mg protein)⁻¹] in *B. megaterium* NCTC 10342 grown with 100 mM-NH₄⁺. The activity of glutamate dehydrogenase fell to 73 nmol min⁻¹ (mg protein)⁻¹ when only 3 mM-NH₄⁺ was supplied, while glutamate synthase activity rose to 110 nmol min⁻¹ (mg protein)⁻¹. Meers *et al.* (1970) found that *B. megaterium* KM contained very little glutamate dehydrogenase [10 nmol min⁻¹ (mg protein)⁻¹] when grown in a chemostat with limiting NH₄⁺, but the activity of glutamate synthase was 140 nmol min⁻¹ (mg protein)⁻¹. The species *B. megaterium* includes strains that differ considerably in their properties (White, 1972, 1977; Gordon *et al.*, 1973) and different methods of assimilation of ammonia may be further instances of this variability.

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