Inhibition by D-Glutamate as the Cause of Diphasic Growth of *Bacillus megaterium* NCIB 7581 with Glycerol plus DL-Glutamic Acid

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Growth of *Bacillus megaterium* NCIB 7581 in a simple chemically defined medium was inhibited by D-glutamate above 0.01 mg ml⁻¹; equimolar L-glutamate prevented this inhibition. When DL-glutamate (2 mg ml⁻¹) was present in the medium (with glycerol as the main carbon source), organisms grew exponentially until all the L-isomer had disappeared; growth then stopped for about 24 h during which there was a transient appearance of D-glutamine in the medium. Throughout the first stationary phase the concentration of D-glutamate in the medium fell continuously and when it was less than 0.01 mg ml⁻¹ there was a second phase of growth.

Exponential phase organisms growing without glutamate contained only 4 mM-glutamate in the free amino acid pool. During the first stationary phase with DL-glutamate added to the medium, the concentration of glutamate (all D-isomer) was 47 mM in the pool.

Of four other strains of *B. megaterium* tested, only one was sensitive to D-glutamate. From strain 7581 a D-glutamate-resistant substrain was easily developed. Among other amino acids added singly to the defined medium, only D- (and L-) serine was inhibitory to all five strains examined. Inhibition of *B. megaterium* 7581 by D-glutamate was prevented by single addition of several amino acids, each of which could act as a sole source of nitrogen for growth.

INTRODUCTION

Bacillus megaterium NCIB 7581 showed two distinct phases of growth in a simple chemically defined medium that contained only glycerol and DL-glutamate as sources of carbon (White, 1972). The cause of this diauxie has now been studied.

METHODS

Organisms. *Bacillus megaterium* NCIB 7581 and other strains of *B. megaterium* and their maintenance were described by White (1972). *Lactobacillus arabinosus* NCIB 6376 was grown overnight (and subcultured monthly) at 30 °C in stabs containing (g l⁻¹): Difco tryptone (10), Difco yeast extract (10), glucose (1.0), KH₂PO₄ (2.0), CaCO₃ (3.0) and agar (15). Stock cultures were kept at 2 °C.

Media. Medium A1 (glycerol as carbon source) and medium A2 (glucose as carbon source) both supplemented with biotin (0.2 µg l⁻¹) were used for *B. megaterium* 7581 (White, 1972); biotin was omitted for the other strains. Growth from small inocula in these liquid media was more consistent when trisodium citrate (dihydrate; 20 mg l⁻¹) was added.

Conditions of growth. Bacteria were grown in shaken 250 ml or 2 l conical flasks containing 50 ml or 500 ml medium at 37 °C (or 30 °C for strain 13632) and growth was measured turbidimetrically in an EEL colorimeter (White, 1972). The inoculum (1 ml for 500 ml medium) was a suspension in water of organisms from a fresh slope culture on nutrient agar. The suspension was adjusted to a colorimeter reading of 1.0 (0.35 mg dry wt ml⁻¹ with strain 7581) and used undiluted or diluted 1:10 or 1:100.

Assay of L-glutamate. This was done manometrically with bacterial glutamate decarboxylase, as described by Gale (1965).

Assay of D-glutamate. Samples were first treated with glutamate decarboxylase to remove the L-isomer, or to show its absence. The remaining D-glutamate was assayed by one or more of three methods.

(i) Chromatography. Four replicate paper chromatograms were developed with solvent A (see below) for about 8 h at room temperature. Amino acids were detected with 0.1 % (w/v) ninhydrin in acetone and heating at about 65 °C for 30 min. Each spot of glutamate was cut out and placed in 4 ml 0.005 % (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 75 % (v/v) aqueous ethanol. After 1 h at room temperature, A_{540} was determined (Giri *et al.*, 1952). The amounts of glutamate in test solutions were determined from a standard calibration curve; the four estimates for each sample were averaged.

(ii) Manometry. Warburg flasks (with two side-arms) contained in the main compartment: $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.5 (130 μmol), glutamate decarboxylase from vegetable marrow (about 4 mg), acetone-dried *L. arabinosus* (35 mg) containing glutamate racemase, pyridoxal phosphate (0.1 mg), EDTA (2.5 μmol) and water to 1.9 ml. D-Glutamate (about 5 μmol in 0.4 ml water) was in one side-arm and 0.2 ml 1 M-citric acid was in the other. The flasks were gassed with N_2 and equilibrated at 37 °C. Glutamate was tipped into the main compartment and incubation was continued until CO_2 evolution stopped, usually after 4 h. Citric acid was then tipped from the side-arm to release dissolved CO_2 . The total CO_2 released, after subtraction of controls (see below), was proportional to the D-glutamate added (1 μmol CO_2 per μmol glutamate) up to 10 μmol . Controls were: (a) without the acetone-dried *L. arabinosus*, to measure any L-glutamate; (b) without glutamate decarboxylase, to measure CO_2 released from substrates other than glutamate; (c) without any substrate, to measure CO_2 generated from the assay system alone. The rate of evolution of CO_2 was lower at pH values below 6.5. Although the optimal pH value for activity of the plant enzyme is about 5.8, it retains almost 50 % of its maximum activity at pH 6.5; it can thus be coupled to glutamate racemase (optimum pH 7.5), which has about 90 % of its maximum activity at pH 6.5.

(iii) Amino acid analyser. Samples of free amino acid pools (see below) contained too little glutamate for accurate manometric assay and separation of glutamate by paper chromatography was poor when several amino acids were present. Total glutamate was therefore measured with an amino acid analyser. Several extracts of pool amino acids were combined and the glutamate was separated on Dowex 50 (H^+ form, X4 cross-linking, 200 to 400 mesh, 15 ml column vol. in a 25 ml graduated pipette). After loading the sample, the column was washed with water (20 ml) and then eluted with 75 ml 1 M-HCl, followed by 30 ml water. The combined effluents were dried, taken up in water and chromatographed to confirm the presence of glutamate and absence of diaminopimelate. Isomers of glutamate in the effluent were then assayed manometrically.

Enzymes. Glutamate decarboxylase (EC 4.1.1.15) was isolated from large green vegetable marrows (*Cucurbita pepo*) as described by Schales & Schales (1955). Purification was taken as far as precipitation with $(\text{NH}_4)_2\text{SO}_4$ followed by dialysis of the redissolved solid. The non-diffusible residue was freeze-dried and kept as a powder at -15 °C. Enzymic activity remained almost unaltered after storage for a year. The powder was resuspended in 0.2 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.5 (30 mg powder ml^{-1}) immediately before an assay and centrifuged at 20000 g for 20 min at 2 °C. The clear, slightly green supernatant liquid was used.

Bacterial glutamate decarboxylase (acetone-dried *Escherichia coli* organisms) was obtained from Koch-Light.

Acetone-dried *L. arabinosus* was used as a source of glutamate racemase (EC 5.1.1.3). The organisms were grown overnight at 37 °C in stationary 2 l flasks, each containing 1 l of a medium modified from that of Glaser (1960) by replacing Difco nutrient broth with Oxoid Lab-Lemco and Oxoid peptone (each 5 g l^{-1}) and by increasing the NaCl concentration to 1.5 g l^{-1} . Organisms were harvested, washed in 0.02 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.5 and dried by the method of Gale & Epps (1944). The powder was kept at -15 °C and enzymic activity was stable for a year.

Glycerokinase (EC 2.7.1.30) and glycerol-1-phosphate dehydrogenase (EC 1.1.1.8) were from Boehringer; catalase (EC 1.11.1.6) and glutaminase (EC 3.5.1.2) were from Sigma.

Assay of glutamine. The procedure was similar to the chromatographic assay for D-glutamate. Standards were spots of 10 to 30 μl D-glutamine solution (0.5 mg ml^{-1}).

Assay of ammonia. The method of Tetlow & Wilson (1964) was used; glutamic acid or glutamine gave no interference.

Assay of glycerol. This was done spectrophotometrically with glycerokinase and glycerol-1-phosphate dehydrogenase, as described by Wieland (1965).

Oxidation of glutamic acid. *Bacillus megaterium* 7581, grown at 37 °C in medium A1, was washed twice in 0.02 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 6.8 and then resuspended in 0.1 M- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer to about 25 mg dry wt ml^{-1} . This suspension (0.5 ml) was incubated at 37 °C in a Warburg flask containing 0.2 ml 2 M-NaOH (with filter paper wick to absorb CO_2) in the centre well, and 0.1 M-buffer (as above) to give 2.5 ml total liquid volume (after tipping). After equilibration and measurement of the endogenous rate of O_2

uptake, L- or D-glutamate (2 mg in 0.2 ml of 0.1 M-buffer) was tipped from the side-arm and the new rate of O₂ uptake was measured.

Organisms were also harvested and washed, then dried with acetone as described above. The powder was suspended in 0.1 M-Na₄P₂O₇/NaOH buffer, pH 8.3 (50 mg powder ml⁻¹) and 0.75 ml of the suspension was put into a Warburg flask, to which was also added FAD (10 µg), catalase (1 µg) and 0.1 M-Na₄P₂O₇/NaOH buffer, pH 8.3 (to 2.5 ml final vol. after tipping); CO₂ was absorbed with 2 M-NaOH (as above). Either D-alanine or D-glutamate (each 2 mg in 0.2 ml of buffer) was tipped from the side-arm and the rate of O₂ uptake was measured at 37 °C.

Paper chromatography and electrophoresis. Chromatograms on Whatman no. 1 or 3MM paper were developed at room temperature with the following solvents: A, methanol/pyridine/water/conc. HCl (80:10:17.5:2.5, by vol.; Rhuland *et al.*, 1955); B, butan-1-ol/acetic acid/water (63:10:27, by vol.); C, phenol/formic acid/water [500 g:13 ml 90% (w/v) formic acid:154 ml water]. Electrophoresis was on Whatman no. 1 paper at 2 kV and about 50 mA for 1 h using either 90% (w/v) formic acid/water, pH 2.0 (80:2920, by vol.) or pyridine/acetic acid/water, pH 6.4 (200:9:2790, by vol.). Dried papers were developed with ninhydrin.

Isolation of walls. *Bacillus megaterium* 7581 was grown in medium A1 (without glutamate) and walls were isolated and hydrolysed as described by Day & White (1977).

Extraction and assay of 'pool' amino acids. Duplicate samples (each 20 ml) from cultures of *B. megaterium* 7581 and 7581 GRA-1 grown at 37 °C in medium A1 plus biotin (with or without added glutamate) were taken at intervals. 'Pool' amino acids were extracted with 0.25 M-HClO₄ at 2 °C as described by Tempest *et al.* (1970) and measured in the amino acid analyser. No correction was made for extracellular glutamate when this was present in the medium associated with a wet pad of organisms, since the correction would be small in comparison with the internal concentration. The dry weight of bacteria in the sample (i.e. mg per 20 ml) was determined from the turbidity at the time of sampling, and the volume of the internal 'pool' of amino acids (expressed in µl) was taken as four times this dry weight (cf. Tempest *et al.*, 1970).

RESULTS

Diphasic growth in medium A1

White (1972) reported diphasic growth of *B. megaterium* 7581 in a defined medium (White *et al.*, 1969) which contained DL-glutamic acid (1 mg ml⁻¹). In media A1 and A2, *B. megaterium* showed a single exponential phase of growth, but if DL-glutamate (usually 2 mg ml⁻¹) was added, growth again became diphasic. The growth pattern on medium A1 (containing glycerol) is shown in Fig. 1; that for growth on medium A2 (containing glucose) was similar. The concentration of NH₄⁺ remained unchanged at 30 mM after the first phase of growth and decreased to only 27 mM at the end of the second phase. At all stages of growth, the organisms had normal morphology without filamentous or swollen forms (cf. Al-ssum & White, 1977).

Identification of D-glutamine

Chromatograms (in solvent A) of the medium during the intermediate phase between the first and second periods of growth showed the transient appearance of a ninhydrin-positive spot in the position of a glutamine marker ($R_{\text{glutamate}}$ 0.75). The unknown compound (from about 2 ml medium) was eluted with water from several large chromatograms that had been developed with solvent A. Chromatography in solvents A, B and C and electrophoresis at pH 2 or 6.4 confirmed that it was glutamine. Hydrolysis (0.1 M-HCl at 100 °C) led to the formation of glutamate, which was identified as the D-isomer by its insensitivity to bacterial glutamate decarboxylase. The D-glutamine was not hydrolysed by glutaminase at pH 5.

The amount of D-glutamine appearing in the medium during the first stationary phase was measured by the chromatographic procedure (described in Methods) because a good separation from excess glutamate was not achieved by the amino acid analyser.

Inhibition of growth by D-glutamate

Events during the first phase of growth of *B. megaterium* 7581 in medium A1 with DL-glutamate (Fig. 1*a*) suggested that D-glutamate was inhibiting growth and that L-glutamate

Fig. 1. Events during growth of *B. megaterium* 7581 at 37 °C in medium A1 containing DL-glutamate (2 mg ml⁻¹). (a) First phase of growth; (b) second phase of growth: ○, L-glutamate; ●, D-glutamate; ▲, D-glutamine; ■, glycerol; □, culture turbidity (colorimeter reading); △, medium pH.

was preventing this inhibition. This was confirmed by the finding that addition of 2 µg D-glutamate ml⁻¹ to medium A1 increased the lag phase by 8 h. Larger amounts gave more prolonged inhibition: 0.02 mg ml⁻¹ inhibited growth for 24 h, and there was no visible growth for 3 d when 1 mg ml⁻¹ was added. D-Glutamine (1 mg ml⁻¹) did not inhibit growth. L-Glutamate (up to at least 2 mg ml⁻¹) was not inhibitory (growth of *B. megaterium* 7581 was monophasic and accelerated) and the inhibition by 1 mg D-glutamate ml⁻¹ was prevented when L-glutamate was also present, though now growth was diphasic. Adding 2-oxoglutarate (sterilized by filtration) at 1 mg ml⁻¹ to an exponentially growing culture neither caused inhibition nor prevented inhibition by D-glutamate (1 mg ml⁻¹) if added simultaneously.

The diphasic growth with DL-glutamate could be mimicked by adding D-glutamate to an actively growing culture in medium A1 (Fig. 2). If the addition was made early enough, there was a rapid inhibition of growth, followed by a stationary phase and finally a second phase of growth.

Since D-glutamate was inhibitory at such low concentrations, its effect was unlikely to be due to an impurity, and it would be surprising if L-glutamate could prevent an inhibition caused by a compound of unrelated structure. Samples of D-glutamate from three separate suppliers all had the same minimum inhibitory concentration. Each gave a single ninhydrin-

Fig. 2. Effect of adding D-glutamate (to 1 mg ml⁻¹ final concentration) to growing cultures of *B. megaterium* 7581 at 37 °C in medium A1. Turbidities of four separate cultures were measured at intervals: ○, no glutamate added; ●, D-glutamate added at 12 h; ▽, D-glutamate added at 15 h; □, D-glutamate added at 17 h. Arrows indicate times of addition of D-glutamate.

positive spot after chromatography with solvents A and B. After bioautography (with medium A2 seeded with *B. megaterium* 7581) the zone of inhibition of growth was in exactly the same position as the ninhydrin-positive material on chromatograms developed with solvent A.

Prevention of the inhibition caused by D-glutamate

When organisms were grown with D-glutamate (1 mg ml⁻¹) in medium A1 that also contained L-glutamate, the yield of *B. megaterium* 7581 in the first phase of growth was proportional to the amount of L-isomer added, i.e. about 0.15 mg dry wt μmol⁻¹ (Fig. 3). A similar yield of organisms per μmol L-glutamate was obtained when this amino acid was the sole source of nitrogen and (NH₄)₂SO₄ was replaced by Na₂SO₄·10H₂O (4 g l⁻¹). The yield of organisms per μmol NH₄⁺ was also similar when NH₄Cl was the nitrogen source (in the same modified medium). On the other hand, the yield of organisms per μmol L-glutamate (0.035 mg dry wt) was much lower when the amino acid was the sole source of carbon in medium A, containing (NH₄)₂SO₄ but without glycerol and citrate (Fig. 3), and resembled the growth yield on glycerol (0.037 mg dry wt μmol⁻¹) in medium A1. Thus D-glutamate might inhibit assimilation of ammonia and L-glutamate might provide an alternative source of nitrogen even in the presence of D-glutamate.

Of the other L-amino acids tested for ability to prevent the inhibition of growth by D-glutamate, most of those that served as nitrogen sources prevented inhibition by D-glutamate, while those that were ineffective as sole sources of nitrogen failed to prevent inhibition (Table 1).

Isolation of a D-glutamate-resistant strain

The second phase of growth, which occurred when the concentration of D-glutamate had fallen to a low level (Fig. 1a), did not represent growth only of D-glutamate-resistant bacteria (Fig. 4). Though some organisms with moderate resistance to D-glutamate were present these made up less than 0.1 % of the total population in the second phase of growth.

A D-glutamate-resistant strain of *B. megaterium* 7581 was developed. A single large colony that had grown after 2 d on solidified medium A1 plus D-glutamate (0.1 mg ml⁻¹) was transferred to solid medium A1 containing 0.8 mg D-glutamate ml⁻¹. Organisms now grew after overnight incubation and continued to grow rapidly in liquid medium A1

Fig. 3

Fig. 4

Fig. 3. Relation between extent of growth of *B. megaterium* 7581 and availability of nitrogen or carbon. Cultures were incubated in modified medium A1 [(NH₄)₂SO₄ omitted, see Results] with NH₄Cl (▽) or L-glutamate (○) as sole sources of nitrogen until growth stopped. Other cultures were grown in ordinary medium A1 plus D-glutamate (1 mg ml⁻¹) with various concentrations of L-glutamate (□) until the first stationary phase was reached. The extent of growth in medium A containing L-glutamate (●) or glycerol (▼) as sole sources of carbon was also measured.

Fig. 4. Viable counts during growth of *B. megaterium* 7581 at 37 °C in medium A1 plus DL-glutamate (2 mg ml⁻¹): ○, samples plated on medium A2; ●, samples plated on medium A2 containing D-glutamate (50 µg ml⁻¹); ▽, dry weight of organisms (from turbidity). After overnight incubation at 37 °C, the solid medium containing D-glutamate showed a background growth of minute colonies (equal in number to the normal-sized colonies growing on medium without D-glutamate) and a few larger colonies. Only the numbers of these partly D-glutamate-resistant colonies are plotted. No large colonies were seen on medium A2 containing 1 mg D-glutamate ml⁻¹.

containing 1 mg D-glutamate ml⁻¹ after a lag only a few hours longer than in medium without glutamate. When growth stopped, glutamate still remained in the medium. This sub-strain, named 7581 GRA-1 (D-glutamate-resistant, with ammonia as nitrogen source), did not show diauxie in medium A1 plus DL-glutamate (2 mg ml⁻¹) and reached 3 mg dry wt ml⁻¹ after 20 h incubation.

D-Glutamate in the peptidoglycan of the wall

D-Glutamate is a major component of bacterial peptidoglycans (see Schleifer & Kandler, 1972). Why a metabolite should inhibit growth is puzzling. D-Glutamate was confirmed to be present in walls of *B. megaterium* 7581 (hydrolysed in 6 M-HCl) by a manometric method. About 95% of the isolated glutamate was the D-isomer. A difficulty in this assay (when applied to the wall hydrolysate) was that the acetone-dried *L. arabinosus* contained diaminopimelate decarboxylase. Glutamate had therefore to be separated from diaminopimelate in the wall hydrolysate by ion-exchange chromatography (see Methods).

Oxidation of L- and D-glutamate

Exponential-phase organisms, harvested from medium A1 without glutamate, had O₂ uptake rates (above the endogenous rate) of 32 and 4 nmol min⁻¹ (mg dry wt)⁻¹ with L- and D-glutamate, respectively. D-Glutamate (2 mg) did not inhibit the uptake of O₂ with L-glutamate as substrate. Organisms, harvested at the end of the first phase of growth with DL-glutamate (2 mg ml⁻¹), had an O₂ uptake rate of only 8 nmol min⁻¹ (mg dry wt)⁻¹ above the endogenous rate with L-glutamate, and showed no increase above the endogenous rate with D-glutamate. Organisms from the same medium in the second phase of growth had a low endogenous rate of O₂ uptake which was not increased by L- or D-glutamate.

Table 1. *Abilities of L-isomers of individual amino acids to allow growth of B. megaterium 7581 in the presence of D-glutamate or to act as sole source of nitrogen for growth*

Cultures (50 ml) were grown at 37 °C in medium A1 plus D-glutamate (1 mg ml⁻¹) or in modified medium A1 without (NH₄)₂SO₄ (see Results), supplemented with individual amino acids at the concentrations indicated.

Amino acid	Concn (mg l ⁻¹)	Growth in presence of D-glutamate	Growth in medium without NH ₄ ⁺
Alanine	600	(+)	+
Arginine	1200	(+)	+
Aspartate	900	+	+
Cysteine.HCl	1100	—	(+)
Glutamate	1000	+	+
Glycine	500	—	—
Histidine.HCl	1400	—	—
Isoleucine	900	(+)	(+)
Leucine	900	—	+
Lysine	1000	—	—
Methionine	1000	(+)	(+)
Phenylalanine	1100	+	+
Proline	800	—	(+)
Serine	700	—	—
Threonine	800	—	—
Tryptophan	1400	—	—
Tyrosine	1200	—	(+)
Valine	800	—	+

+, Rate of growth similar to that in medium A1 without added amino acids; (+), slow growth; —, no growth.

Table 2. *Amino acids inhibiting growth of various strains of B. megaterium in medium A2*

Solutions of amino acids (D- and L-isomers separately; all at 1 mg ml⁻¹) were tested in wells (containing 100 µl solution) cut in solid medium A2 (1.5 %, w/v, agar) that was seeded with the different strains. Those amino acids that gave a zone of inhibition after incubation overnight at 37 °C (strain 13632 was at 30 °C) were next tested in liquid medium A2 (D- and L-isomers separately; each at 0.1 mg ml⁻¹). Only those amino acids that were confirmed as inhibitors in liquid medium are shown in the Table. Amino acids that were not inhibitory in the plate assay were not retested in liquid medium. Transient inhibitions are indicated by parentheses.

Amino acid	<i>B. megaterium</i> strain				
	NCIB 7581*	NCIB 8291	ATCC 13632†	KM	216
Aspartate	(D)	D	(D)	—	—
Cysteine	—	(D)	—	—	—
Glutamate	D	(D)	—	—	—
Leucine	D	(D)	—	—	—
Serine	D L	D (L)	D L	D (L)	D L
Threonine	—	D (L)	D L	D	—

No inhibition was found with L- or D-isomers of alanine, arginine, cystine, glycine, histidine, isoleucine, lysine, methionine, phenylalanine, proline, tryptophan, tyrosine or valine.

* Biotin added to medium A2.

† L-Glutamate (120 mg l⁻¹) and L-leucine (60 mg l⁻¹) added to medium A2.

No activity of D-amino acid oxidase (with either D-alanine or D-glutamate as substrate) was detected in acetone-dried organisms grown in medium A1 (exponential phase) or in medium A1 plus DL-glutamate (first stationary phase).

Glutamic acid in the free amino acid 'pool'

The internal concentration of free glutamic acid ('pool' glutamate) in *B. megaterium* 7581, during growth in medium A1, was 4 mM during the exponential phase and was thus higher than that of any other amino acid in the 'pool'. This concentration is, however only a tenth of that reported by Tempest *et al.* (1970) for *B. megaterium* KM grown in a chemostat. Some other amino acids were present in the 'pool' of strain 7581 but no proline was detected.

When L-glutamate (2 mg ml⁻¹) was added to the medium, exponential phase organisms contained 8 mM-glutamate with proline at 39 mM. Organisms from the first stationary phase with DL-glutamate (2 mg ml⁻¹) added to the medium contained 47 mM-glutamate (all D-isomer by manometric assay) but no detectable proline. The concentration of glutamate remained high (57 mM, but all L-isomer) during the second phase of growth; a small amount of proline (about 3 mM) appeared in the pool. Organisms at this stage of batch culture may more closely resemble organisms growing with nutrient limitation in a chemostat than do exponential phase organisms. The substrain 7581 GRA-1, when growing exponentially in medium A1 plus D-glutamate (1 mg ml⁻¹), contained 52 mM-glutamate (63% D-isomer) and no detectable proline in the 'pool'.

*Effects of other amino acids on growth of B. megaterium 7581
and other strains in medium A*

Other amino acids (D- and L-isomers) were separately tested as possible inhibitors of *B. megaterium* 7581 and four other strains (Table 2). D-Glutamate did not inhibit all the strains, though strain 13632 requires L-glutamate for growth and so an inhibition by D-glutamate would not be expected. Serine was inhibitory to all strains; addition of this amino acid to a simple chemically defined medium frequently leads to inhibition of growth of other bacteria (e.g. Cosley & McFall, 1973; Schleifer *et al.*, 1976).

DISCUSSION

Diphasic growth of *B. megaterium* 7581 in a chemically defined medium containing glycerol and DL-glutamate is a consequence of inhibition by D-glutamate. This inhibition is prevented by L-glutamate. In a medium containing both isomers of the amino acid, the organisms at first grow quickly. The L-glutamate rapidly disappears from the medium and further growth then becomes inhibited by the residual D-glutamate. The stationary population slowly metabolizes the D-glutamate, and when its concentration has fallen to below 0.01 mg ml⁻¹, a second phase of growth occurs, in which glycerol is presumably the source of carbon and energy. Coleman (1959) described a rather similar diphasic growth of *Rhodospirillum rubrum* with DL-glutamate, but no clear explanation was found for the behaviour of this organism.

D-Glutamate is present in the peptidoglycan of *B. megaterium* 7581 and is, therefore, a normal metabolite of this organism but it could not be detected in the amino acid pool. However, when the organisms were inhibited by D-glutamate, the 'pool' contained about 50 mM-D-glutamate. If the activity of glutamate racemase, or some other reversible mechanism for synthesizing D-glutamate, is too low, then inhibition may occur. Sensitivity to D-glutamate may be a consequence of *B. megaterium* 7581 taking up D-glutamate from the medium more rapidly than it can be metabolized.

The route of metabolism of the D-glutamate that disappears during the first stationary

phase is unknown. If it were directly converted to L-glutamate, then this product ought to allow further slow growth even in the presence of the remaining D-glutamate. D-Glutamate cyclotransferase, which has been suggested by Van der Werf & Meister (1975) as a means of removing D-glutamate, seems absent as there was no accumulation of 5-oxoproline. The rate of O₂ uptake with D-glutamate is consistent with oxidation to 2-oxoglutarate, but further metabolism of 2-oxoglutarate (to succinate etc.) should require a higher rate of O₂ uptake. Some of the D-glutamate is certainly converted to D-glutamine, which is excreted. D-Glutamine added to medium A1 did not inhibit growth of *B. megaterium* 7581, and no inhibition of the activities of several isolated enzymes by D-glutamine has been found (White, 1979).

The D-isomers of the amino acids that occur naturally as L-isomers in proteins are not generally regarded as being metabolically active (e.g. Rydon, 1948) and it has been common practice to include DL-amino acids in culture media. Nevertheless, many cases of inhibition of bacterial growth by D-amino acids have been reported (e.g. Lark & Lark, 1961; Tuttle & Gest, 1960; Bentley, 1969; Schleifer *et al.*, 1976). Usually it is wall synthesis that is impaired, though this does not seem to be the case in the present study. Since the inhibition of *B. megaterium* 7581 by D-glutamate can be prevented by several L-amino acids, the inhibition is obvious only when D-glutamate is added alone to a minimal medium. Possibly more examples of inhibition by D-amino acids might be found in other organisms if the D-isomers were tested alone in minimal media.

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