Physiology of Growth and Sporulation in *Bacillus cereus*

I. Effect of Glutamic and Other Amino Acids

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ABSTRACT

BUONO, F. (Syracuse University, Syracuse, N.Y.), R. TESTA, AND D. G. LUNDGREN. Physiology of growth and sporulation in *Bacillus cereus*. I. Effect of glutamic and other amino acids. J. Bacteriol. 91:2291–2299. 1966.—Growth and sporulation were studied in *Bacillus cereus* by use of an active culture technique and a synthetic medium. A high level of glutamic acid (70 mM) was required for optimal growth and glucose oxidation followed by sporulation even though relatively little glutamic acid was consumed (14 mM). Optimal growth occurred with a combination of 14 mM glutamic acid and 56 mM $(\text{NH}_4)_2\text{SO}_4$, aspartic acid, or alanine. Ornithine or arginine at 70 mM could replace glutamic acid in the synthetic medium without affecting the normal growth cycle. Glutamic acid was not replaced by any other amino acid, by $(\text{NH}_4)_2\text{SO}_4$, or by a combination of either α -ketoglutarate or pyruvate plus $(\text{NH}_4)_2\text{SO}_4$. Enzyme assays of cell-free extracts prepared from cells harvested at different times were used to study the metabolism of glutamic acid. Glutamic-oxaloacetic and glutamic-pyruvate transaminases were completely activated (or derepressed) during early stages of sporulation (period of 6 to 8 hr). Alanine dehydrogenase responded in a similar manner, but the levels of this enzyme were much higher throughout the culture cycle. Neither glutamic dehydrogenase nor α -ketoglutarate dehydrogenase was detected. Sporulation in a replacement salts medium was studied with cells harvested at different times from the synthetic medium. Cultures 2 to 6 hr old were unable to sporulate in the replacement salts medium unless glutamic acid (7.0 mM) was present. By the 6th hr, cells were in the early stages of sporulation, showing spore septa development. Cultures 8 hr old sporulated in the replacement salts medium. Other metabolic intermediates able to replace glutamic acid in the replacement salts medium were alanine, aspartic acid, and glutamine at equimolar concentrations. Also, ammonium ions in combination with pyruvic, oxaloacetic, α -ketoglutaric, or fumaric acid replaced glutamic acid. The likely role of these metabolites is discussed.

Spore formation is intimately related to metabolic phenomena, and is therefore an ideal system for studying the activities of enzymes involved in the formation of new structures. Recent investigations have shown that *Bacillus cereus* T (10, 14, 15) and probably other bacilli (7, 9, 28) undergo metabolic changes related to morphological changes during the course of sporulation. Of primary importance are those changes in the anabolism and catabolism of glutamic and other amino acids (1, 12, 21, 32). Glutamic acid has

been reported to serve as an amino donor in sporulating cells (32); a precursor to dipicolinic acid biosynthesis (24); and as a carbon and nitrogen substrate for the synthesis of other amino acids during the transition of a vegetative cell to a spore (1, 26).

This paper describes the ability of various amino acids, in particular glutamic acid, to support growth and sporulation of *B. cereus*.

MATERIALS AND METHODS

Organism and media. *B. cereus* ATCC 4342 was used throughout this investigation. The synthetic medium employed was a modified glucose-gluta-

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mate-glycine-salts (GGGS) medium (23), which contained: K_2HPO_4 , 3.0 mM; KH_2PO_4 , 3.5 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.8 mM; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 mM; NaCl , 0.2 mM; CaCl_2 , 0.2 mM; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.05 mM; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 mM; glycine, 1.3 mM; glutamic acid, 70 mM; glucose, 5.5 mM. The pH was adjusted to 7.0 with 40% NaOH before autoclaving.

A replacement salts medium, which was used to study sporulation in the absence of growth, contained: KH_2PO_4 , 3.5 mM; K_2HPO_4 , 3.0 mM; CaCl_2 , 0.2 mM; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.04 mM. The pH was adjusted to 7.5 with 4% NaOH before autoclaving. The ability of various L-amino acids and other metabolites to support sporulation was tested by adding them to the replacement salts medium at a concentration of 7.0 mM. Glutamine, cysteine, cystine, methionine, tyrosine, urea, and all Krebs cycle intermediates were filter-sterilized; the other amino acids and $(\text{NH}_4)_2\text{SO}_4$ were autoclaved.

Culture systems. The inoculum preparation and culture apparatus for use in the active-culture technique have been described (6). Briefly, the inoculum was prepared from heat-shocked spores (65°C for 15 min) transferred into 50 ml of GGGS medium containing 0.2% yeast extract in a 250-ml Erlenmeyer flask. The flask was shaken for 4 hr at 37°C, after which 10 ml of culture was transferred into each of two flasks containing 50 ml of fresh GGGS medium, and the flasks were shaken for 4 hr. A 75-ml amount of this culture was used to inoculate 1.5 liters of GGGS medium contained in a 2-liter glass solution bottle equipped with a glass sparger through which sterile humidified air passed at a rate of 4.5 liters per min. At each sampling time (usually every 2 hr), 15 to 20 ml of cell suspension was withdrawn into screw-capped tubes and stored at -20°C until used for glucose, glutamic acid, and poly- β -hydroxybutyrate (PHB) determinations. A second sample (6 ml) was taken for microscopic observations, growth, and pH determinations. Growth was measured turbidimetrically by use of either a Klett-Summerson photoelectric colorimeter equipped with a green filter (500 to 570 m μ), or a Beckman spectrophotometer (model DU at 540 m μ). The pH was measured by use of a Beckman Zeromatic pH meter. Sporangia were stained with malachite green according to the method of Schaeffer and Fulton (30), and fat inclusions (PHB) were stained by the method of Burdon (2). Quantitative measurements of PHB were made by use of the method of Law and Slepecky (22). The culture medium was assayed for glucose and glutamic acid by the method of Noelting and Bernfeld (27) and Zamir and Lichtenstein (38), respectively, and cell protein was assayed by the biuret reaction (11).

Sporulation in the absence of growth was investigated with cells harvested at different times from the GGGS medium. Cells were harvested by Millipore filtration and washed once with 10 ml of replacement salts. The cells (and filter paper) were then placed in a test tube (15 by 150 mm) containing 10 ml of the replacement salts medium and the test metabolite(s). Each test tube was fitted with a rubber stopper and a small glass tube through which sterile, humidified air passed. All culture tubes were incubated in a

water bath at 37°C. The cells were freed from the filter pad after a few minutes of aeration.

For isotope studies, 30 ml of medium was removed from the 2-liter solution bottles at 6 hr (end of growth). The cells were separated and washed as reported above, and were placed in a 50-ml gas washing bottle (Will Corp., Rochester, N.Y.) containing 30 ml of the replacement salts medium, 7.0 mM glutamic acid, and the isotope. Sterile, humidified air was forced through the suspension, and the expelled radioactive CO_2 was collected in 30 ml of a solution of ethanol-amine-ethylene glycol monomethyl ether (17). Either uniformly labeled L-glutamic acid- C^{14} (0.83 $\mu\text{mole/ml}$ of medium, 2.74×10^5 count/min) or DL-glutamic acid- I-C^{14} (0.80 $\mu\text{mole/ml}$ of medium, 2.3×10^5 count/min) was used. Samples (4 ml) were taken from the culture bottle every 2 hr until sporangia formation was complete (by 7 to 8 hr), and the samples were quick-frozen and stored at -20°C until analyzed. Samples (1 ml) from the CO_2 trap were also collected at the same time. To prevent isotopic saturation of the trapping solution, and to maintain a constant volume, the same amount of trapping solution was added back to the vessel.

The radioactive cells were chemically fractionated (29), and prepared for counting in a Nuclear Chicago dual channel liquid scintillation detector (3, 20).

Preparation of cell-free extracts and enzyme assays. At 2-hr intervals during culture growth in the GGGS medium, approximately 1,200 ml of culture medium was removed and centrifuged in the cold; the cell pellets were twice washed with phosphate buffer (pH 7.4), and resuspended in 35 ml of fresh buffer. For vegetative cell extracts, the heavy suspension of chilled cells was disrupted by a 5-min treatment with a Branson 20-kc Sonifier. The sporangia were broken by treatment for 30 min with a colloid Minimill (Gifford-Wood Co., Hudson, N.Y.). The cells were chilled during exposure, and the Minimill treatment was followed with a 30-sec exposure to sonic treatment. Microscopic observations of the treated vegetative cells and sporangia revealed complete breakage. The broken cells were centrifuged in the cold at $27,000 \times g$ for 30 min, and the supernatant fluids (crude cell-free extracts) were collected, frozen, and stored at -20°C until used. No appreciable loss in enzyme activity was noted after 3 weeks of storage. Extracted protein showed a linear response to cell concentration.

Glutamic dehydrogenase was assayed by the method of Strecker (36), and glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) by the Sigma-Frankel colorimetric procedure (Sigma Chemical Co., St. Louis, Mo., Technical Bulletin No. 505). Alanine dehydrogenase was assayed by the method of Zink and Sanwal (39) after the oxidation of reduced nicotinamide adenine dinucleotide (NADH_2) at 340 m μ in the presence of pyruvate and $(\text{NH}_4)_2\text{SO}_4$. The α -ketoglutaric dehydrogenase was assayed by three different methods (18, 25, 33).

Source of amino acids and other chemicals. Nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP),

NADH₂, reduced nicotinamide adenine dinucleotide phosphate (NADPH₂), α -ketoglutaric acid, L-alanine, and CoA were obtained from Sigma Chemical Co., St. Louis, Mo. The other amino acids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. C¹⁴ isotopes were purchased from New England Nuclear Corp., Boston, Mass. All other chemicals used were of analytical grade, and the glutamic acid was shown to be chromatographically pure.

RESULTS

Growth and sporulation curves of *B. cereus* grown in the GGS medium are shown in Fig. 1. Growth (measured by turbidity) ends by the 6th hr, at which time 95% of the glucose has been utilized, but only 10% (7.0 mM) of the glutamic acid has been consumed. Growth, expressed as turbidity, closely agrees with total cell counts, and is directly correlated to glucose oxidation. The increase in turbidity after the 6th hr (when cell division ceases) is characteristic for our system, and is related to forespore formation and spore maturation (refractility). Under phase optics, refractile sporangia appeared at about 10 hr, whereas, in thin sections observed in the electron microscope, the first stages of sporulation (axial chromatin filament) were seen at about 5.5 hr. The pH drop of the medium during the first 2 hr was due, in part, to the formation of acetic, pyruvic, and α -ketoglutaric acids; citric and lactic acids and acetylmethyl carbinol were not detected. The pH rose during sporulation as did the use of the metabolic intermediates. The glutamic acid level of the medium decreases by another 10% during sporulation.

The effect on growth, glucose oxidation, and sporulation of reducing the glutamic acid concentration in the GGS medium is shown in Fig. 2. At 35 mM glutamic acid, the rate of growth and of glucose oxidation was slightly decreased, delaying the appearance of sporangia (identified by phase optics or spore stains). However, at 14 mM glutamic acid, the amount normally consumed during growth and sporulation in the regular GGS medium, a much greater decrease in rate was noted. Here the culture time cycle was extended, but, once growth ended, sporulation began and was completed by 24 hr. This observation was consistently observed, and shows that a high concentration of glutamic acid is required for optimal growth and glucose oxidation even though relatively little glutamic acid was utilized.

With the lower level of glutamate (14 mM) used as a control, amino acids or (NH₄)₂SO₄ was added to see whether each would support growth and sporulation in a manner comparable to that of the normal GGS medium. The

FIG. 1. Growth, sporulation, pH, and chemical changes occurring during the culture cycle of *Bacillus cereus* grown in the GGS medium.

(NH₄)₂SO₄ (56 mM) addition (Fig. 3) supported growth, glucose oxidation, and sporulation in a manner comparable to that of the normal GGS medium. Aspartic acid (56 mM) or alanine (56 mM) was about equal to 70 mM glutamic acid, except that alanine delayed sporulation. Glutamine (56 mM) caused an initial lag in growth and glucose oxidation, and prevented sporangia formation at least up to 24 hr, at which time the experiment ended.

The ability of various metabolites (at 70 mM) to replace completely the high glutamate requirement in the normal GGS medium was studied. Arginine or ornithine replaced glutamic acid, whereas glutamine supported growth but extended the growth cycle time by 8 hr, and failed to support sporulation. Glutamic acid could not be replaced by aspartic acid, alanine, histidine, proline, hydroxyproline, (NH₄)₂SO₄, α -ketoglutaric acid plus (NH₄)₂SO₄, or pyruvate plus (NH₄)₂SO₄.

Table 1 shows the activities of some enzymes in extracts prepared from cells harvested at different times from the GGS medium. Both GPT and GOT were detected in extracts of vegetative cells and sporangia. Little enzyme activity was noted during vegetative growth, but the activity

FIG. 2. Effect on growth, glucose oxidation, and sporulation of lowering the initial concentration of glutamate in the GGS medium. The curves in the top graph represent per cent glucose remaining in the medium. The time axis is the same for both graphs. Symbols: $\times$, 70 mM glutamic acid (control); $\circ$, 35 mM glutamic acid; $\bullet$, 14 mM glutamic acid.

increased during the active period of glucose oxidation (4 to 6 hr), with sharp increases noted during the next 2-hr period. The latter period is the time of forespore development, active PHB degradation (Fig. 1), and the oxidation of exogenous acetate (*unpublished data*). The transaminase activities remained relatively high to the 10th hr, and decreased during the late stages of spore maturation. Under the conditions of the assay, no glutamic dehydrogenase was detected in any cell extracts by use of either NAD or NADP as coenzymes; nor was there any α -ketoglutaric dehydrogenase. Since no glutamic dehydrogenase was detected, the presence of a NAD-dependent alanine dehydrogenase was expected, because, in *Bacillus* species, this enzyme is found when glutamic dehydrogenase is absent (16). A relatively high alanine dehydrogenase activity was noted during vegetative growth (Table 1), and, as with the transaminases, a sharp increase in activity occurred between 6 and 8 hr, the period of forespore development.

Metabolite requirements for sporulation were checked by use of a replacement salts medium

FIG. 3. Effect on growth, glucose oxidation, and sporulation of 14 mM glutamic acid in the GGS medium supplemented with either $(\text{NH}_4)_2\text{SO}_4$, alanine, glutamine, or aspartic acid. The top graph relates per cent glucose remaining in the medium. The time axis is the same for both graphs. Symbols: $\bullet$, 14 mM glutamic acid; $\times$, $(\text{NH}_4)_2\text{SO}_4$ (56 mM); $\square$, aspartic acid (56 mmoles/ml); $\circ$, alanine (56 mM); $\blacktriangle$, glutamine (56 mM).

and cultures 6 to 10 hr old. Such cultures are metabolically active, and electron micrographs of thin sections showed cells from 6-hr cultures to be in the initial stages of sporulation; that is, to possess developing spore septa and forespore membranes. By 8 hr, the cells contain structurally identifiable forespores with a developing cortex. These morphological changes will be the subject of another publication. When cells from 6- or 7-hr cultures grown in the GGS medium were collected, washed, and placed in the replacement salts medium lacking glutamic acid, less than 5% sporangia formed after 8 hr of incubation (Table 2). However, 8-, 9- and 10-hr cultures from the GGS medium did form mature stainable sporangia (refractile bodies under phase optics) when placed in this replacement salts medium (8-hr cultures, 40 to 50% sporangia; 9-hr culture, 70 to 75% sporangia; 10-hr culture, 90 to 95% sporangia).

Sporangia (95%) formed in 6- or 7-hr cultures when glutamic acid (7.0 mM) was added to the replacement salts medium (Table 2). No de-

TABLE 1. Enzyme assays of cell extracts of *Bacillus cereus* grown in the GGS medium

Enzymes*	Cell age (hr)†					
	2	4	6	8	10	12
GPT	—	1.5	4.7	20.6	18.1	10.7
GOT	—	1.2	6.8	19.7	17.4	10.4
ADH	198.5	195.4	188.3	294.3	133.0	65.0

* GPT = glutamic pyruvic transaminase; measured as Sigma-Frankel units. One unit of enzyme forms 4.82×10^{-4} μ moles of glutamate per min at pH 7.5 and at 25 C. GOT = glutamic oxaloacetic transaminase; unit of activity was the same as GPT. ADH = alanine dehydrogenase. One unit of enzyme is equal to a change in OD at 340 m μ of 0.01 per min in a 3-ml volume under the conditions of Zink and Sanwal (39).

† The period between 2 and 4 hr is classified as vegetative growth; 6 to 8 hr, forespore formation; 10 to 12 hr, development of sporangia. Results are expressed as units per milligram of protein.

tectable cell division occurred, since the optical density (OD) readings were about the same during the first 3 hr of aeration. The OD did increase as spore refractility developed; the characteristic rise in pH of the medium was also evident. Even cultures removed from the GGS medium before 6 hr formed sporangia in the absence of cell division in the replacement salts medium containing glutamic acid. Such cultures (as early as 2 hr) appeared to be "induced" to sporulate by the addition of the glutamic acid. When glucose was added to these replaced cells, cell division (OD increase) occurred, and sporulation began only after the glucose was completely oxidized. Actively growing cells from the GGS medium harvested up to 6 hr and then resuspended in the replacement medium containing glutamic acid (7.0 mM) required the same time for complete sporulation; that is, 8 hr of aeration were required before 95% sporangia formed in 2-, 3-, 4-, 5-, or 6-hr cultures. However, older cultures (already containing complete forespores) completed sporangia development in less time under similar replacement culture conditions.

Glutamic acid could not be replaced by intermediates of the Krebs cycle (malate, fumarate, succinate, α -ketoglutarate, isocitrate, *cis*-aconitate, citrate, oxaloacetate, all at 7.0 mM), or by pyruvate or acetate (7.0 mM) added alone, together, or in combination to 6-hr cultures in the replacement salts medium. Fifteen different pairs of the aforementioned metabolites were tried. Urea or $(\text{NH}_4)_2\text{SO}_4$ at 7.0 mM was also ineffective. However, glutamic acid was effectively replaced by α -ketoglutarate and $(\text{NH}_4)_2\text{SO}_4$; pyruvate and $(\text{NH}_4)_2\text{SO}_4$; fumarate and $(\text{NH}_4)_2\text{SO}_4$; or oxaloacetate and $(\text{NH}_4)_2\text{SO}_4$ (all at 7.0 mM). Acetate plus $(\text{NH}_4)_2\text{SO}_4$ (7.0 mM) was effective only after a lag of 2 to 4 hr. At equimolar concentrations, the following were ineffective: citrate and $(\text{NH}_4)_2\text{SO}_4$; malate and $(\text{NH}_4)_2\text{SO}_4$; succinate

TABLE 2. Sporulation in a replacement salts medium of a 6-hr culture of *Bacillus cereus* grown in the GGS medium

Time*	Medium plus glutamic acid (7.0 mM)			Medium without glutamic acid		
	OD†	Per cent sporangia‡	pH	OD	Per cent sporangia	pH
hr						
0	.130	0	7.5	.130	0	7.5
2	.132	0	7.8	.128	0	7.5
4	.140	5	8.0	.130	0	7.6
6	.180	65	8.2	.120	5	7.6
8	.240	90	8.3	.110	5	7.7

* This refers to the number of hours the 6-hr culture taken from the GGS medium was in the replacement medium.

† OD at 540 m μ in a Beckman DU spectrophotometer.

‡ Determined by spore stains.

and $(\text{NH}_4)_2\text{SO}_4$; isocitrate and $(\text{NH}_4)_2\text{SO}_4$; isocitrate and $(\text{NH}_4)_2\text{SO}_4$; *cis*-aconitate and $(\text{NH}_4)_2\text{SO}_4$.

Several L-amino acids were tested for their ability to replace glutamic acid in the replacement salts medium. Aspartic acid, alanine, glutamine, or arginine, at an equimolar level (7.0 mM), was about as effective as glutamic acid. That is, 90 to 95% mature, stainable sporangia were observed after 8-hr aeration of 6-hr cultures taken from the GGS medium and placed into the replacement salts medium. At comparable concentrations, ornithine, histidine, and citrulline supported some sporangia formation (60%), whereas proline and hydroxyproline were less effective (30% sporangia in the same time period). The following, all at 7.0 mM, were unable to replace glutamic acid, even after an extended incubation period (up to 24 hr): glycine, serine

leucine, isoleucine, lysine, cysteine, cystine, methionine, asparagine, valine, tryptophan, phenylalanine, tyrosine, or threonine.

Table 3 shows the distribution as well as oxidation of radioactive glutamic acid in a 6-hr culture placed in the replacement medium. Glutamate was actively utilized with 73 to 80% of the activity found in the protein fraction. When glutamic acid-*l*-C¹⁴ was used as the substrate, there was no substantial rise in the alcohol-soluble fraction during sporulation; there was a rise with uniformly labeled glutamate. Millet and Aubert (26) had reported that the alcohol-soluble fraction contained, among other compounds, 80 to 90% of the dipicolinic acid (DPA) of the cells. The decrease in the hot trichloroacetic acid fraction with time is probably related to the fact that spores contain less deoxyribonucleic acid (DNA) than vegetative cells, and the decrease reflects the breakdown of the remaining vegetative DNA not enclosed by the spore septum. There was also a decrease in activity in the cold trichloroacetic acid fraction with uniformly labeled glutamate; this probably reflects a reduction in the low molecular weight metabolites of the cell during spore formation. The CO₂ evolution patterns were similar whether uniformly labeled glutamic acid or glutamic acid-*l*-C¹⁴ was used. In both cases, approximately 32% of the added glutamic acid was oxidized to CO₂.

DISCUSSION

The apparent relationship between sporulation and glutamic acid has raised many interesting problems relative to sporulation control. The

latter has received little attention, although a variety of conditions favoring sporulation are known. Schaeffer, Millet, and Aubert (31) have reported on catabolic repression of bacterial sporulation, and they stress the importance of an intracellular level of a nitrogen-containing catabolite in repressing spore genes during growth of *B. subtilis*. Szulmajster and Hanson (37) showed that the simultaneous presence of glucose and glutamic acid caused repression of aconitase synthesis in *B. subtilis*, which in turn led to the inhibition of spore formation.

B. cereus grown in the synthetic medium required a level of 70 mM glutamic acid in the medium for optimal growth and glucose oxidation, followed by fairly rapid and synchronous sporulation, even though only about 14 mM of the amino acid was actually utilized. Both the rates of growth and glucose oxidation were affected by the glutamate level of the medium. Since complete sporulation eventually occurred in the medium containing 14 mM glutamic acid, the concentration effect was directly related to growth and not to sporulation. Glutamic acid has been reported to influence the oxidative pathway of glucose in *B. subtilis* (8, 19). Moreover, Grelet (12) reported a similar reduction in growth of *B. cereus* var. *mycoides* upon reducing the normally high glutamate level of the medium. Grelet (13) proposed that glutamate was required to counteract the injurious effects of other amino acids. At present, we cannot explain this phenomenon in *B. cereus*.

The presence of GOT and GPT in cell extracts suggests that the metabolism of glutamic acid may be via transaminases. This has yet to be experi-

TABLE 3. Labeled glutamic acid distribution and oxidation during sporulation in *Bacillus cereus* in a replacement salts medium

Type of glutamic acid	Time*	Whole cells	Cold trichloroacetic acid	Alcohol-soluble	Hot trichloroacetic acid	Residue (protein)	CO ₂ evolution
	hr						
Uniformly labeled	0	—	—	—	—	—	—
	2	43 †	4.3	4.9	2.1	30.1	35,700 ‡
	4	50	3.0	7.7	2.2	33.5	22,206
	6	50	1.5	9.9	1.0	34.0	13,800
	8	50	0.8	12.0	0.25	33.0	13,400
Glutamic acid- <i>l</i> -C ¹⁴	0	—	—	—	—	—	—
	2	20	1.1	1.8	1.4	15.9	35,001
	4	21	1.1	2.2	1.1	15.5	18,010
	6	25	1.0	2.7	1.0	14.3	10,350
	8	21	0.9	3.0	1.0	14.0	13,632

* Number of hours that a 6-hr culture harvested from the GGS medium is in the replacement salts medium.

† $\times 10^{-3}$ μ moles/mg (dry weight).

‡ CO₂ evolution expressed as counts per minute per 2-hr interval.

FIG. 4. Diagram showing the interrelationship of glutamic acid with all the substrates supporting sporulation of *Bacillus cereus* in replacement salts medium.

mentally demonstrated, but it is supported by the inability of the present authors to find glutamic dehydrogenase and α -ketoglutarate dehydrogenase in the same extracts. These enzymes would be required if glutamate were metabolized via the Krebs cycle. Both GOT and GPT activities appear to be related to the exogenous level of glucose; that is, the activity of these enzymes increases as the glucose level decreases.

To study further the role of glutamic acid in sporulation, a replacement medium was used. This procedure has proven valuable in studying nutritional requirements for sporulation (4, 5). Young cells taken from the GGS medium would not form sporangia when replaced into a replacement salts medium. Older cells (8 hr and older) were capable of doing so, but these cells contained mature forespores. When glutamic acid (7.0 mM) was added to the replacement salts medium, sporulation occurred in 6-hr cells (and in younger cells), showing the need for nitrogen. The time for sporulation to occur in 2- to 6-hr cultures was independent of the age of the vegetative cells taken from the growth medium; that is, refractile spores (sporangia) were always seen after 8 hr in the replacement medium. When cells are removed from exogenous glucose, an activation (or derepression) of enzyme synthesis occurs for those enzymes involved in sporulation. Both GOT and GPT may be involved, based upon their presence in the cell extracts and their increase in specific activity during forespore development.

Figure 4 attempts to summarize the data for those metabolites which support sporulation in the replacement culture system. Since glutamic acid has an apparent importance in sporulation (1, 12, 21, 24, 26, 32) and has been shown in the present study to serve as the nitrogen requirement for sporulation, we have given it a central role. All the amino acids supporting spore formation in the replacement medium are capable of leading directly into glutamate via enzymatic reactions.

Alanine and aspartate can be converted into glutamate via the transaminases which are present in *B. cereus*. Glutamine can be converted to glutamate plus NH_3 if glutaminase is present; this enzyme has not been assayed for in our organism, but Krask (21) has reported the presence of glutamine synthetase in *B. cereus* T. In the replacement salts medium, ammonium ions in combination with pyruvate, fumarate, oxaloacetate, or α -ketoglutarate also supported sporulation. How ammonium enters into the metabolic "pool" of amino nitrogen in *B. cereus* is not known. Siegenthaler and Hermier (32) reported that the entrance is through amination of pyruvate in *B. subtilis* rather than through the amination of α -ketoglutaric acid, which is the more common way for bacteria. The high level of alanine dehydrogenase in *B. cereus* does support this view. No data are presently available to explain how amination occurs through fumarate or oxaloacetate; no evidence is available for the presence of aspartase in our organism. Although α -ketoglutarate plus NH_4 replaces glutamic acid, we cannot say that this occurred through direct amination since we were unable to detect glutamic dehydrogenase.

Results of the radioactive isotope work indicate that the bulk of glutamate may not be directly oxidized via the Krebs cycle, even though glutamic acid is readily assimilated into spore proteins. This is also supported by the failure to detect glutamic and α -ketoglutaric dehydrogenase. Both GOT and GPT are apparently important for converting glutamate into other amino acids needed for spore protein synthesis.

Mention should be made of the work of Srinivasan and Halvorson (35) and Srinivasan (34) identifying an endogenous factor in *B. cereus* T involved in the regulation of sporulation. Young vegetative cells grown in their "G" medium failed to sporulate in a replacement medium, but older granulated cells did. Extracts of sporulating cells "triggered" young vegetative cells to sporulate. The endogenous factor "sporogen" was reported to be ninhydrin-negative, and to be active at low concentrations. It seems unlikely that we are dealing with similar phenomena.

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