A GLUTAMIC ACID-PRODUCING BACILLUS[1]

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During an examination of microbial culture filtrates capable of reversing the inhibition of *Streptomyces aureofaciens* Strain NRRL B-1286 caused by the methionine analogue norleucine, one filtrate was found to possess striking reversing potency. Analysis showed this could not be due to methionine. The reversal properties of the filtrate were traced to glutamic acid which imparted high reversing activity to the filtrate by virtue of its unexpectedly high concentration. This paper deals with the study of a spore-forming bacillus which is capable of synthesizing extraordinary amounts of glutamic acid as the chief product of metabolism.

Synthesis of an individual amino acid by microorganisms was virtually unknown until the polyglutamyl peptides of *Bacillus anthracis* and *Bacillus subtilis*, consisting of D- and L-isomers of glutamic acid, were isolated (Ivanovics and Bruckner, 1937; Thorne, 1956). Distinctive accumulation of free amino acids first centered around the synthesis of α-ϵ-diaminopimelic acid by a mutant of *Escherichia coli* (Davis, 1952) and its adaptation to the commercial production of L-lysine by enzymatic decarboxylalation (Beesch and Tanner, 1958; Casida, 1956).

The fermentative production of high yields of α-ketoglutaric acid from hexoses (Lockwood and Stodola, 1946; Asai *et al.*, 1955) led to an efficient microbial conversion to L-glutamate (Otsuka *et al.*, 1957; Katagiri *et al.*, 1957; Smythe and Huang, 1956; Chas. Pfizer and Company, Inc., 1957). Later, a number of instances of direct fermentative production of L-glutamate from sugars were reported (Asai *et al.*, 1957; Kinoshita *et al.*, 1957*a*; Kita, 1957). The extensive survey by Kinoshita *et al.* (1957*a*) showed that about one fifth of the 650 bacterial cultures tested, one third of the 372 streptomycetes, one third of the 468 yeasts, and one tenth of the 475 fungi produced as much as a few mg of glutamate per ml of medium initially containing 10 per cent glucose. Only one organism, *Micrococcus glutamicus* n. sp., (Kinoshita *et al.*, 1958*a*) produced very high yields of glutamate, the highest reported being 0.25 moles per mole of glucose consumed. Selected mutants of this organism are capable of direct fermentative formation of L-ornithine and L-lysine from glucose, both in molar yields of 0.36 (Kinoshita *et al.*, 1957*c*; Kinoshita *et al.*, 1958*b*). It is of interest that the outstanding glutamate-forming organism described by Asai *et al.* (1957) was also a micrococcus, *Micrococcus varians*.

The work reported here was in progress when the several Japanese studies on direct amino acid fermentations were published. Although different, our organism is similar to *M. glutamicus* and *M. varians* in its remarkable ability to synthesize L-glutamic acid and, at the moment, these two kinds of bacteria are unique in this respect.

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MATERIALS AND METHODS

Culture methods. Stock cultures of the bacillus (strain 14B22), which was isolated from Austin, Texas, soil, were maintained on nutrient agar slants; the slants were incubated for 18 to 24 hr at 30 C and then stored in a refrigerator. Stocks were transferred weekly. Inoculum for physiological experiments was obtained by cultivation for 18 to 24 hr in the following liquid medium: glucose, 10 g; $(NH_4)_2SO_4$, 2.64 g; KH_2PO_4, 2.38 g; K_2HPO_4, 5.65 g; $MgSO_4 \cdot 7H_2O$, 1.0 g; $FeSO_4 \cdot 7H_2O$, 1.1 mg; $MnCl_2 \cdot 4H_2O$, 7.9 mg; $ZnSO_4 \cdot 7H_2O$, 1.5 mg; yeast extract (Difco), 0.25 g; precipitated $CaCO_3$, 1.0 g; deionized water, 1 L. Sufficient bromthymol blue was added to permit a detectable color change indicative of the pH of the culture. The glucose and the $CaCO_3$ were sterilized separately, then added to the medium. Most experiments were carried out in 250- or 500-ml Erlenmeyer flasks containing 50 or 100 ml of medium. A 2 per cent 18-hr liquid

inoculum was used. Liquid cultures were incubated on a mechanical shaker operating at 76 4-in reciprocations per min.

Accumulation of glutamic acid and residual sulfate in the medium lowered the pH to the point of inhibition of further growth of the bacillus, in spite of the presence of $CaCO_3$. Inhibition of growth was noticeable at about pH 6.5 and was marked at about pH 6.0. Cultures were, therefore, periodically adjusted to pH 7.0 to 7.5 by addition of 28 per cent NH_4OH. Bacterial growth was measured turbidimetrically on a Klett-Summerson photoelectric colorimeter (blue filter) after eliminating the $CaCO_3$ with a drop of HCl.

Chemical procedures. All analyses were performed on clear supernatants obtained by centrifugation. Quantitative determination of glutamic acid was performed by chemical and enzymatic procedures used interchangeably. For small numbers of assays, the enzymatic method was preferred for its accuracy, simplicity, speed, and specificity for dextrorotatory glutamic acid.

(1) Ninhydrin colorimetric method:—Good separation of glutamic acid from the small amounts of other ninhydrin-reacting materials in the culture filtrates was achieved by developing the chromatographs descendingly for 20 to 24 hr with buffered phenol (see footnote to table 1) (Block *et al.*, 1955). Five levels of a standard glutamic acid solution were run on the same paper, to obtain a calibration curve. The spots were located with ninhydrin, cut out, and the glutamate quantitated colorimetrically in a *n*-butanol extract (Smith and Agiza, 1951).

(2) Enzymatic method:—The glutamic acid was decarboxylated by an enzyme prepared from fresh white squash (Schales and Schales, 1946). This enzyme is specific for the L (+)-isomer; also, it did not attack any other amino acid encountered in the filtrates. The CO_2 was measured manometrically; final readings were made after release of bound CO_2 by addition of H_2SO_4.

Bound glutamic acid was measured by glutamic acid determinations before and after acid hydrolysis. Autoclaving at 121 C for 3 hr in 3 N HCl gave maximal release of glutamate. The HCl was removed by evaporation to dryness.

Residual glucose was determined by an anthrone method (Morris, 1948). Filter paper electrophoresis was carried out in a Williams hanging strip electrophoresis cell (Block *et al.*, 1955), with phthalate buffer, pH 5.9, as the electrolyte. A current of 1.5 ma per paper strip (30 mm wide) was applied for a period of 2 to 3 hr. The dried papers were developed by spraying with ninhydrin reagent.

RESULTS

Identification of glutamic acid produced by strain 14B22. A sample of culture filtrate was applied as a band on filter paper and chromatographed in buffered phenol. After location of the glutamate on an end guide strip, the main portion was cut out and eluted with water. This material was rechromatographed one-dimensionally in 4 different solvent systems. The ninhydrin positive material had the same R_f values as authentic glutamic acid. A two-dimensional chromatogram, using buffered phenol and *n*-butanol-acetic acid-water as solvent systems, failed to distinguish between the unknown and authentic glutamic acid applied as a mixture.

Chemical and enzymatic analysis of a great many culture filtrates of strain 14B22 established that glutamic acid accounted for approximately 90 per cent of the total free amino acid content.

Infrared spectrum. The KBr pellet technique was employed with a 0.2 per cent suspension of glutamic acid hydrochloride crystals prepared as described below. The bacterial sample had an infrared absorption spectrum identical with that of authentic glutamic acid hydrochloride.

Optical rotation. Values for glutamic acid calculated from manometric CO_2 production by action of the L (+)-glutamic decarboxylase on the bacterial product usually agreed to within 5 per cent of the values obtained by the ninhydrin procedure; hence, the bacterial product probably consisted of the L (+)-isomer exclusively. This was conclusively established by optical rotation measurements. Culture filtrate acidified with HCl was treated with the ion exchange resin Amberlite 1R4B[2] in the basic form, to absorb the glutamic acid. The glutamic acid was eluted by shaking the resin in successive portions of 0.25 N HCl until the pH of the liquid remained below 2. The extract was concentrated to a syrup *in vacuo* and concentrated HCl added. Crystals of glutamic acid hydrochloride formed upon

[2] Product of Rohm and Haas Company, Philadelphia, Pennsylvania.

standing in the refrigerator overnight. After recrystallization from the charcoal decolored solution, the crystals were washed with ethanol and then with ether. A solution of the crystals had an $[\alpha]_D^{25} = +22.5$. Authentic glutamic acid hydrochloride prepared in the manner of the bacterial product had an $[\alpha]_D^{25} = +23.1$. The bacterial product was, therefore, practically pure dextrorotatory glutamic acid.

Bound glutamic acid. Strain 14B22 characteristically formed capsular material abundantly. The viscous material was partly associated with the cells and partly in solution. Copious precipitates of whitish amorphous aggregates were formed when saturated $CuSO_4$ or excess ethanol was added to the culture filtrate. These observations led to the suspicion that strain 14B22 produces polyglutamyl peptides (Thorne *et al.*, 1954). This seemed to be supported by a 7 to 12 per cent increase in L (+)-glutamic acid after acid hydrolysis of the filtrate (determined enzymatically). However, acid hydrolysis of the $CuSO_4$ precipitable material (Bovarnick, 1942) failed to release any glutamic acid. Furthermore, all the hydrolyzable glutamic acid in the filtrate was dializable through cellophane; the polyglutamyl peptides are nondialyzable (Bovarnick, 1942). Hence, the bound form of glutamic acid was not polyglutamyl peptide. Hydrolysis of the eluates from different R_f bands of a one-dimensional paper chromatogram developed in buffer and phenol indicated that all of the bound glutamic acid occupied an area between R_f 0.5 and 0.7.

Figure 1 shows the appearance of two-dimensional paper chromatograms of the culture filtrate before and after acid hydrolysis. By far the largest and most intense ninhydrin reacting spot was glutamic acid (spot *B*). Spot *D*, much smaller and fainter, was identified as alanine by R_f values in several one-dimensional and two-dimensional chromatograms. Valine, aspartic acid, and an unidentified spot (*F*) were present in very small quantities. The conspicuous difference resulting from acid hydrolysis was the disappearance of spot *C* concomitant with an increase in size and color intensity of the glutamic acid spot.

When a water eluate of the area corresponding to spot *C* was autoclaved (at neutrality) at 120 C for 15 min, it no longer reacted with ninhydrin.

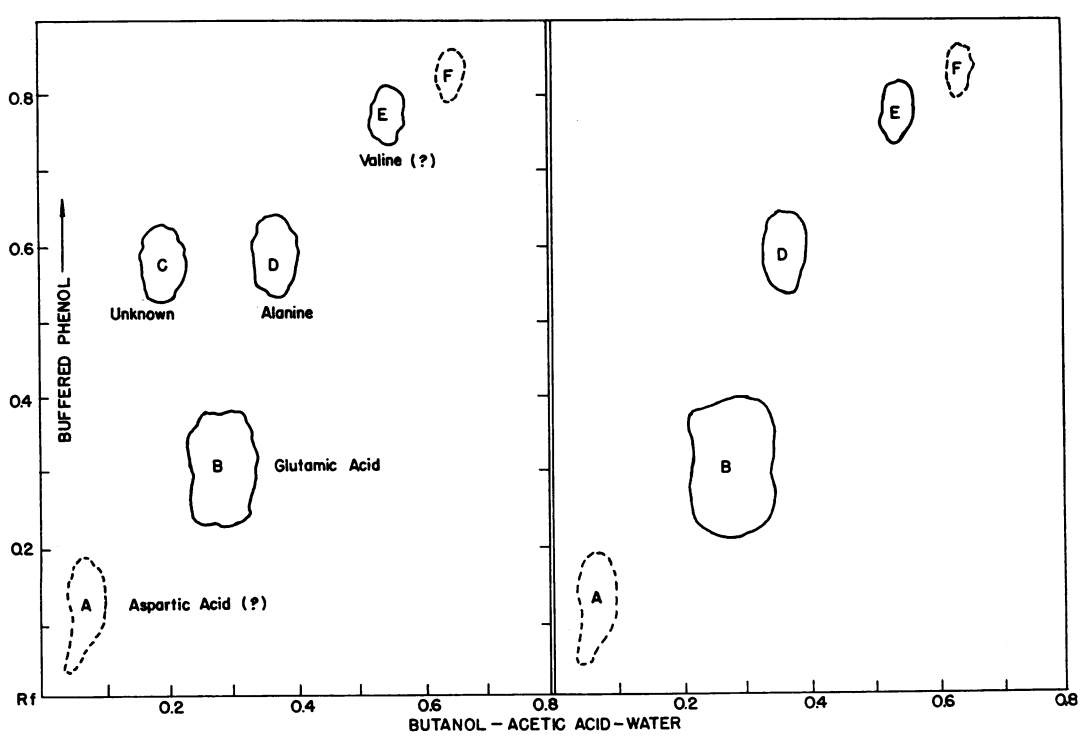

Figure 1. Two-dimensional chromatograms of strain 14B22 filtrate before and after acid hydrolysis

The heat instability at neutral pH values, and the acid hydrolysis to yield glutamate, indicated that the substance in question probably was glutamine. This was confirmed by paper chromatography and by paper electrophoresis.

Pyrrolidone-5-carboxylic acid. Minor amounts (up to 20 μg per ml) of this anhydride of glutamic acid were found in the culture filtrates. It was extracted at pH 1 with ethyl acetate. The acid in the extract had R_f values (0.53 in *n*-butanol-acetic acid-water; 0.63 in buffered phenol) similar to those of authentic pyrrolidone-5-carboxylic acid synthesized from glutamic acid according to the procedure of Wilson and Cannon (1937). Also, the eluate of the pyrrolidone-5-carboxylic acid spot from the chromatogram of the ethyl acetate extract yielded glutamic acid after acid hydrolysis. It is not known whether this acid is produced enzymatically or by a chemical equilibrium from glutamic acid of glutamine.

Formation of extracellular glutamic acid by Bacillus strain 14B22. (1) Neutralization:—Under conditions of glutamic acid formation, the pH of the medium ordinarily drops to about pH 5.4 within 24 hr; as seen in figure 2, pH values below approximately 6 prevent growth, sugar utilization, and glutamic acid synthesis. The striking influence of neutralization is illustrated in table 1. The presence of $CaCO_3$ and periodic neutralization with NH_4OH was superior for glutamate formation. NH_4OH also supplies the nitrogen required for the abnormally large amount of amino acid synthesized. Urea also serves as a satisfactory nitrogen source and neutralization agent, via urease.

(2) Carbon dioxide tension:—The decided increase in glutamate yield when $CaCO_3$ was used simultaneously with NH_4OH indicated that the effect of $CaCO_3$ is not attributable solely to its buffering action. The universal involvement of CO_2 in biosynthesis prompted examination of this factor as a possible explanation of the stimulating influence of $CaCO_3$. Dissolution of $CaCO_3$ by the developing acidity in the culture would automatically elevate the CO_2 concentration. A marked CO_2 stimulation of polyglutamyl peptide formation by virulent strains of *B. anthracis*, with selective incorporation of $C^{14}O_2$ into the α-carboxyl group of the peptide glutamic acid, has been described (Thorne *et al.*, 1952).

The effect of added CO_2 was determined in an experiment involving two sets of triplicate flasks

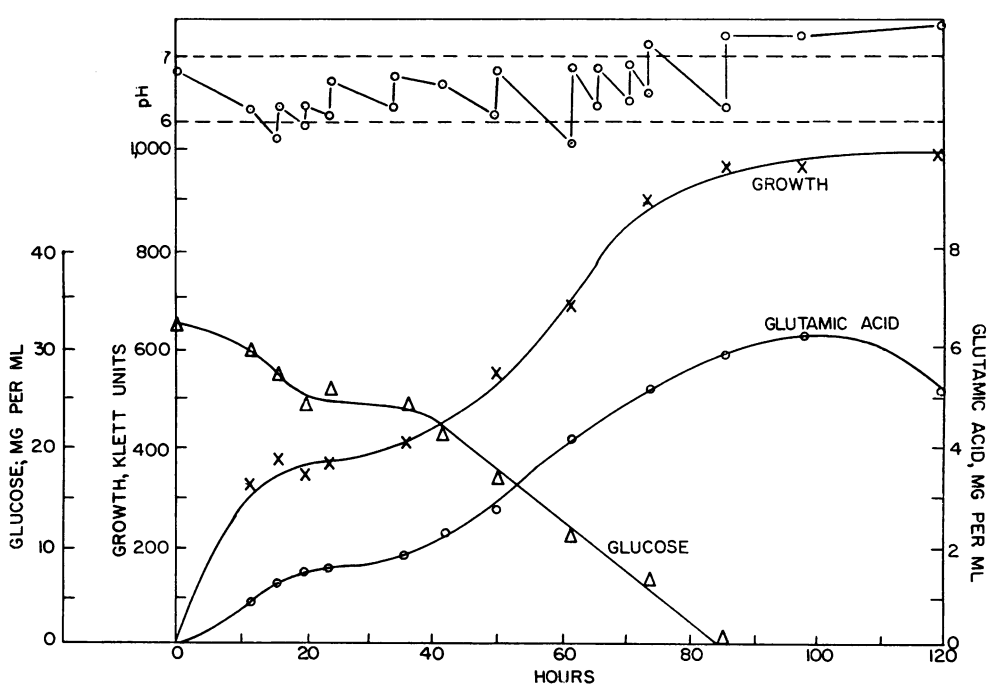

Figure 2. Changes during growth of Bacillus strain 14B22 in shaken culture. Medium: glucose 3 per cent; yeast extract (Difco), 0.01 per cent; $CaCO_3$, 0.1 per cent; mineral salts.

TABLE 1

*Influence of neutralization on glutamic acid formation by Bacillus strain 14B22**

Neutralizing Agent	Free Glutamic Acid† Synthesized
	mg/ml
None	0.86
0.1% $CaCO_3$	1.05
KOH (periodic)‡	1.39
NH_4OH (periodic)	3.36
0.1% $CaCO_3$ and NH_4OH (periodic)‡	5.70

* Three per cent glucose-salts medium containing 0.01 per cent yeast extract (Difco). Incubated with shaking for 3 days.

† Paper chromatography elution-ninhydrin method.

‡ Every 3 to 6 hr, as judged by color of bromthymol blue in the medium.

in two 10-L desiccators. One desiccator was gassed with air containing 5 per cent CO_2, and the other was gassed with ordinary air as a control. Both were incubated on the shaking machine. Periodic neutralization with NH_4OH was carried out as usual. The desiccators were regassed each time they were opened for the neutralization. Analysis showed that added CO_2 definitely stimulated glutamic acid formation during the first 24 hr, but thereafter had little effect on the rate of glutamate synthesis. In repeated experiments, the final glutamate content was always 10 to 15 per cent higher in the presence of added CO_2.

The influence of calcium ion on growth and glutamic acid synthesis was not investigated.

(3) Carbon and nitrogen sources:—Starch, lactose, and sucrose were utilized effectively for growth and glutamic acid formation. Various kinds of molasses supported excellent growth of Bacillus strain 14B22, but glutamic acid formation was negligible.

The organism grew in concentrations of glucose up to 9 per cent, the highest tested; however, under the relatively limited aeration and agitation conditions of our experiments, the optimal concentration for glutamic acid formation was 3 per cent. At higher concentrations, sugar utilization was incomplete.

Urea and $(NH_4)_2SO_4$ were satisfactory sources of nitrogen for glutamate formation in a basal glucose-salts medium containing 0.01 per cent yeast extract. $NaNO_3$ was unsatisfactory.

Changes during growth. The development of a growth culture of Bacillus strain 14B22 was followed analytically, with the results charted in figure 2. The experiment was carried out in a 2-L low-form flask containing 1 L of medium. The large volume was dictated by the need for frequent samplings for the various analyses. Poorer aeration under these conditions probably caused the lower than usual maximal yield of glutamic acid. Figure 2 shows that growth and acid production are accompanied by the utilization of glucose and a continual fall in pH. This indicates that the production of extracellular glutamic acid by strain 14B22 is primarily the result of active assimilation and synthesis, and not an autolytic or degradation process involving a polypeptide intermediate.

The sensitivity of the organism to pH is illustrated by the leveling out of growth between the 15th and 40th hr; this was associated with a noticeable drop in glutamic acid production and in the utilization of sugar. The pH during that period was 5.5 to 6.5. Since this degree of acidity is known to be harmful to strain 14B22, the interruption of growth and metabolism probably was caused by inadequate neutralization during that sensitive interval. This is evidenced by the resumption of growth, sugar consumption, and glutamic acid formation after the medium was readjusted and maintained at a pH higher than 6.5.

This experience also provides a logical explanation for the fluctuation in maximal glutamate yields from experiment to experiment and between replicates in any one experiment. These may range from 6 to 13.5 mg of glutamic acid per ml of 3 per cent glucose medium (0.24 to 0.55 moles of glutamic acid per mole of glucose consumed). The practice of periodic manual adjustment of the pH obviously is quite crude, and in a culture of this type, where the pH changes very rapidly, it is not surprising that the final glutamate levels were influenced by the difficulty of maintaining an optimal pH continuously. Partly because of the retardation between the 15th and 40th hr, the completion of growth and glutamic acid synthesis in this experiment (figure 2) took a longer time than usual. Ordinarily, maximal yield was obtained on the 3rd day, at which

time active growth and glucose consumption were finished.

Complex organic extracts. These are essential for growth and glutamic acid synthesis in glucose-salts medium, but the concentration is extremely critical. Various types of extracts are suitable, in quite low concentrations. For example, yeast extract (Difco) was optimal at 100 μg per ml of medium, resulting in 10.5 mg of glutamic acid per ml. Twice this concentration stimulated growth markedly, but virtually abolished glutamic acid formation. Addition of extra yeast extract to an actively synthesizing culture at once caused cessation of glutamic acid synthesis.

From these and other experiments it is concluded that an inverse relation exists between gross cell synthesis of strain 14B22 and gross accumulation of extracellular glutamic acid. A not unlikely interpretation of this relationship is that, under the proper nutritional conditions, that portion of the substrate carbon, that would be converted to glutamic acid, becomes incorporated into whole cells (and probably to CO_2). This relationship is similar to organic acid production and growth of filamentous fungi (Foster, 1949).

It is evident that one or more constituents of yeast extract play a determinative role in this metabolic shunt. A preliminary search for the active factor(s) was performed by adding Casamino acids (Difco), yeast extract (Difco), ribonucleic acid, deoxyribonucleic acid (all at 500 μg per ml), and a mixture of B vitamins[3] individually and in combinations to 3 per cent glucose-salts-0.01 per cent yeast extract medium. Each treatment reduced glutamic acid formation from 10.4 mg per ml in the unsupplemented control medium to 1 mg or less.

The active component of the B vitamin mixture proved to be biotin; it is believed that biotin is an impurity in the Casamino acids and nucleic acid preparations tested. This vitamin is essential for growth of Bacillus strain 14B22 in glucose-salts medium and apparently in the principal growth stimulating agent contributed by yeast extract. However, the latter allows a higher maximal growth than biotin itself, and evidently contains other growth stimulating factors.

[3] Thiamin, riboflavin, *p*-aminobenzoic acid, calcium pantothenate, nicotinic acid, pyridoxal, pyridoxine, pyridoxamine, each at 1 μg per ml; folic acid, 0.002 μg per ml; and biotin, 0.001 μg per ml.

Influence of biotin concentration on metabolism of strain 14B22. Fifty-ml quantities of 3.5 per cent glucose-salts (containing 0.1 per cent $CaCO_3$) medium received graded concentrations of biotin. Duplicates were run at each concentration. Periodic neutralization with NH_4OH was performed in the usual way. When a flask did not require NH_4OH neutralization for two 5-hr intervals, it was removed and held in the refrigerator until all the flasks could be analyzed simultaneously. The data are presented in figure 3. It is clear that a reciprocal relation exists between glutamic acid synthesis and growth as influenced by biotin and that the level of biotin is extremely critical for maximal synthesis of glutamic acid. For example, maximal growth required 10 $\mu\mu$g of biotin per ml but glutamic acid formation was only a fraction of what it was at 1 $\mu\mu$g biotin where growth was only one third as great.

Physiological efficacy of glutamic acid synthesis was at its maximum in cells of an even lower biotin content. Thus, weight for weight, cells from the 0.3 $\mu\mu$g biotin medium were twice as efficient in synthesizing glutamic acid as the 1.0 $\mu\mu$g biotin medium cells were, although the latter accumulated the largest concentration of glutamic acid.

The efficiency per unit of glucose consumed was greatest in 1 $\mu\mu$g of biotin. All the available glucose had been consumed in this and in the higher biotin treatments; only half the available glucose was consumed in the 0.3 $\mu\mu$g biotin treatment.

The content of biotin in molasses undoubtedly explains the poor production of glutamic acid in the earlier experiments with molasses as the carbon source, and the adverse effect of complex organic extracts.

Characterization of the glutamic acid-producing Bacillus strain 14B22. Morphology. The organism is a facultatively aerobic, sporeforming rod. Young cultures from nutrient agar (figure 4, *a* and *b*): rods, 0.9 to 1.2 μ by 2.0 to 5.0 μ; occur singly or in chains of a few cells; ends rounded in stained preparations; cells stained with basic fuchsin have beaded or mottled appearance; encapsulated, nonmotile; no flagella seen in electron photomicrographs; gram-negative (in 6- to 12-hr cultures).

On glucose nutrient agar the rods were larger and longer and contained many large refractile globules. Old cultures from both media (figure 4,

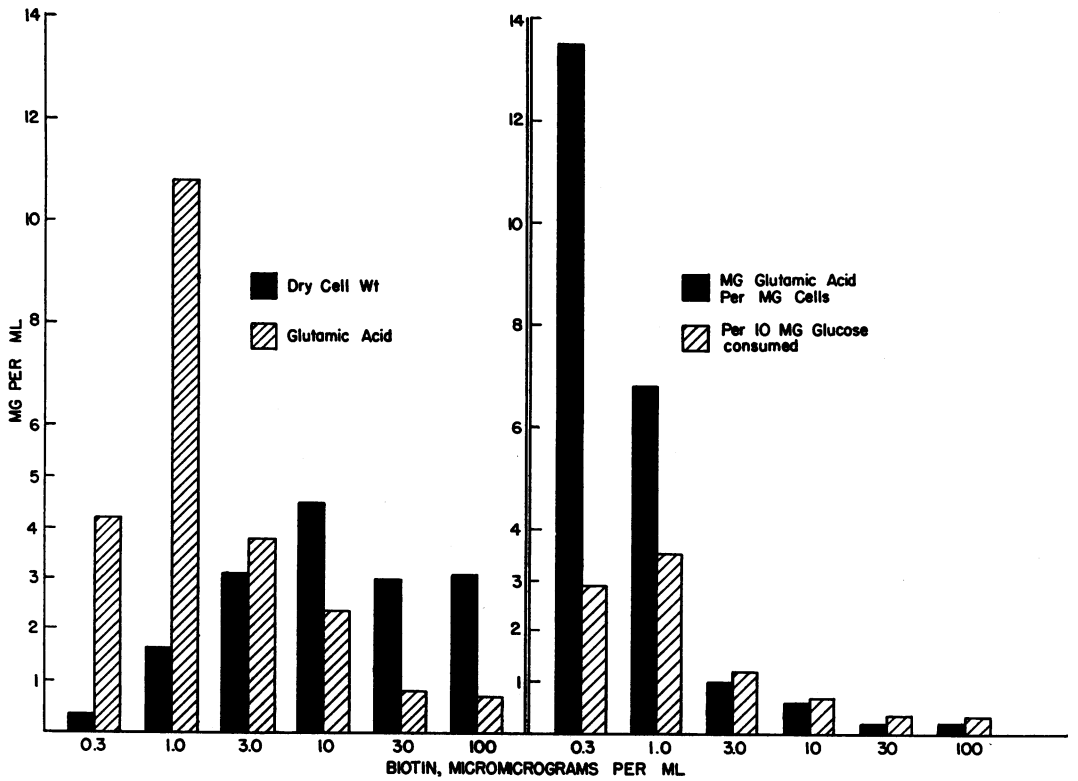

Figure 3. Metabolic response of Bacillus strain 14B22 to concentrations of biotin. Data represent averages of duplicate cultures.

c, *d*, and *h*) contained filaments, chains, and spores. The cells were irregular in shape, some with pointed ends.

On glucose-yeast extract-salts medium, liquid or agar, the young cells were larger and highly vacuolated, containing large refractile globules. The morphology in the liquid medium is distinctive, and is quite different from the morphology of the one reference strain each of *Bacillus megaterium* and of *Bacillus cereus* studied concomitantly. Bacillus 14B22 in this medium is typically pleomorphic, the majority of cells being distorted in size, and misshapen. They were quite unlike the normal, compact rods characteristic of the genus *Bacillus*, which was also displayed by the reference species studied under the same conditions. The cells were considerably enlarged or swollen, and were reminiscent of yeasts, even to the extent of the possession of budlike protuberances (figure 4, *e* and *f*). These bizarre forms are not restricted to "old" cultures in the maximal stationary phase; they are the typical form in the logarithmic phase of growth in the glucose-yeast extract-salts medium (figure 4, *i*).

Sporangia. Not distinctly swollen (figure 4, *c*).

Spores. 0.8 to 1.2 μ by 1.2 to 2.2 μ; cylindrical or oval; central or paracentral; abundant formation after 48 hr on nutrient agar. Majority were resistant to heating for 1 hr at 60 C.

Colonies. On nutrient agar after 48 hr at 30 C: smooth; circular (1.5 to 2 mm); entire; convex; nonspreading; translucent; glistening; creamy white; ammonia odor.

Nutrient broth. Turbid, with sediment; no pellicle.

Glucose-yeast extract-salts slants. Growth abundant; butyrous; raised; moist; glistening; opaque; greyish-white.

Glucose-yeast extract-salts broth. Turbidity abundant; sediment and ropy capsular materials; no pellicle.

Glucose-salts medium. No growth.

Physiological and biochemical characteristics. See table 2.

Since the sporangia are definitely swollen by

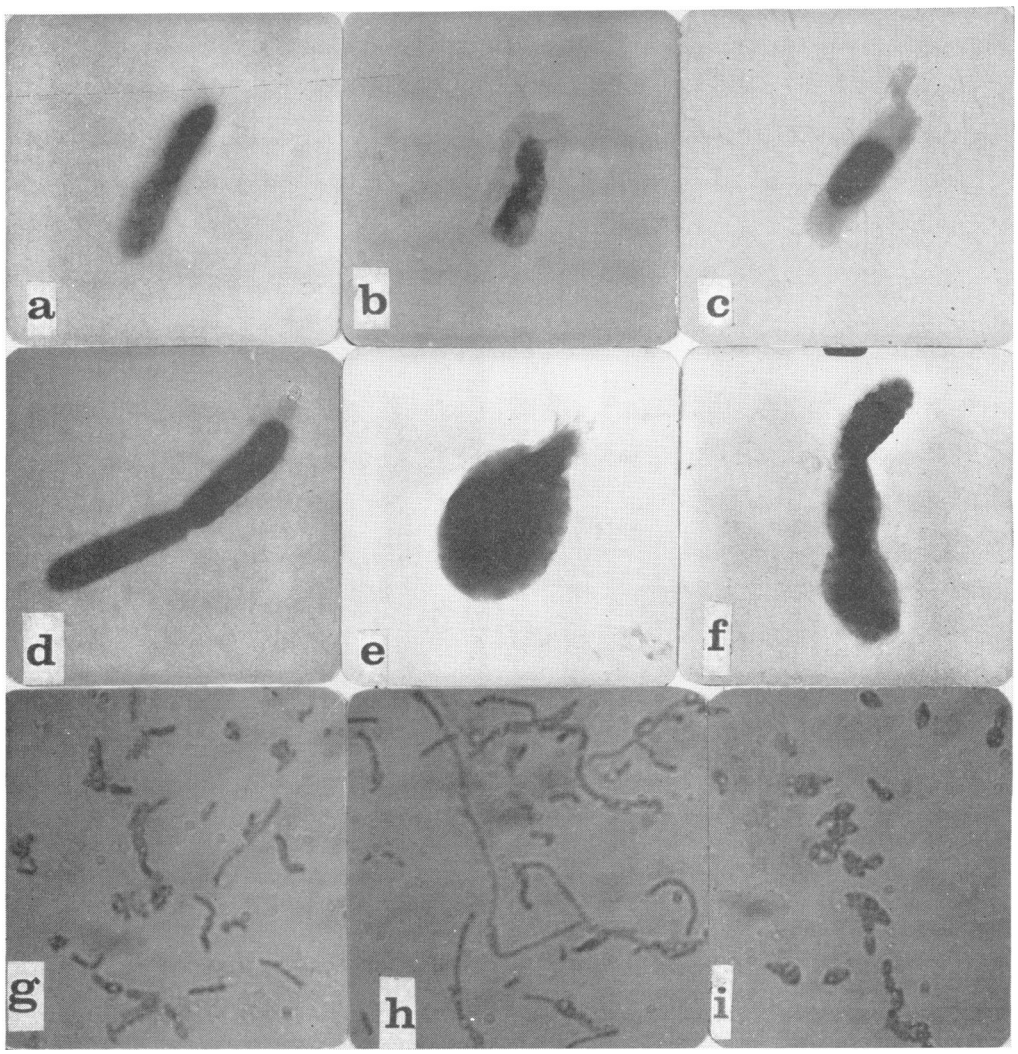

Figure 4. Bacillus strain 14B22; *a* to *f*, electron photomicrographs; *g* to *i*, oil immersion photomicrographs, stained with basic fuchsin. *a* and *b*, from 24-hr nutrient agar culture, ×6100; *c*, from a 5-day nutrient agar culture, showing sporangium and endospore, ×7500. *d*, from a 4-day glucose-nutrient agar culture, ×6200; *e* and *f*, from a 36-hr shaken culture in liquid glucose-yeast extract-salts medium, ×7500; *g*, from a 24-hr glucose-nutrient agar culture, ×950; *h*, from a 3-day glucose-nutrient agar culture, ×950; and *i*, from an 18-hr shaken culture in liquid glucose-yeast extract-salts medium, ×950. (*Note:* All figures reduced 9 per cent in reproduction here.)

the spores, strain 14B22 belongs to the Smith, *et al.* (1952) group I of the species of *Bacillus*. The gram-negative property of strain 14B22 is not regarded as important enough to except it from group I. The vacuolated character of the stained vegetative cells, together with their size, places them in the *B. megaterium-B. cereus* section of group I. For accurate diagnostic purposes, comparative studies of unknown and authentic species conducted side by side under identical conditions are indispensable in resolving with confidence the taxonomic disposition of new isolates. When studied in this manner (table 2) strain 14B22 has points of resemblance to *B. megaterium* and to *B. cereus*, but is not identifiable as either one of these species as defined by Smith *et al.* (1952). Classification of strain 14B22 is considered under Discussion.

TABLE 2

Physiological and biochemical characteristics of Bacillus megaterium, Bacillus cereus, and Bacillus strain 14B22

Character	*Bacillus megaterium*	*Bacillus cereus*	Strain 14B22
Growth on			
Glucose-nitrate agar	+	−	−
Potato slant	+	+	−
Acid formation from			
Glucose	+	+	+
Sucrose	+	±	+
Mannitol	+	−	−
Arabinose	−	−	−
Xylose	+	−	+
Glycerol	+	+	−
Lactose	−	−	+
Salicin	−	+	+
Voges-Proskauer reaction*	−	+	+
Hydrolysis of			
Starch	+	+	+
Gelatin	+	+	+
Casein	+	+	+
Nitrate reduction	−	+	+
Urease formation	+	+	+
Citrate utilization†	+	+	+
Anaerobic growth in glucose-yeast extract-broth	−	+	+
Anaerobic gas formation from nitrate	−	+	−
Egg yolk‡ reaction	−	+	−
Nutritional requirements in glucose-salts broth	NH_4^+-N	Amino acids	NH_4^+-N and biotin

* Clark and Lubs medium fortified with 0.2 per cent yeast extract (Difco).

† Citrate agar fortified with 0.2 per cent yeast extract (Difco).

‡ McGaughey and Chu (1948).

DISCUSSION

The differences between Bacillus strain 14B22 and *B. megaterium* and *B. cereus*, respectively, namely, Gram staining reaction, pleomorphism, glutamic acid formation, and certain physiological and biochemical characteristics, are greater than those representing the basis for differentiating *B. megaterium* and *B. cereus* (Smith *et al.* 1952). Knight and Proom (1952) classified as intermediates of *B. megaterium* and *B. cereus* those closely related organisms which were aberrant in the Voges-Proskauer reaction and in the production of acid from arabinose and xylose. They further suggested that the intermediate types might be assigned to either *B. megaterium* or *B. cereus* if the egg-yolk reaction and the anaerobic growth property corresponded to either authentic species. Strain 14B22 behaves like *B. megaterium* in the former reaction and like *B. cereus* in the latter reaction. Likewise, its nutritional requirements are different from those of the two authentic species. *B. megaterium* uses ammonia-N and has no exogenous vitamin requirements. *B. cereus* cannot use ammonia-N, requires amino acids, and does not require exogenous vitamins (Knight and Proom, 1952). Strain 14B22 can use ammonia-N, but requires biotin. This nutritional pattern was displayed only by *Bacillus pumilis* in the group I species of *Bacillus*; however, other properties of strain 14B22 rule out an identity with *B. pumilis*.

The nutritional character is not distinctive enough to warrant creation of a new species for strain 14B22. As more and more members belonging to the *B. megaterium-B. cereus* group are collected, undoubtedly more intermediate forms will be described. It is to be expected that additional nutritionally aberrant strains will be encountered. Conceivably, a diagnostically valid new species name could be assigned to this organism, but adoption of this practice by investigators making detailed studies of new isolates of group I bacilli would lead to an unworkably large number of "species." In cases like the one described here, the interests of bacterial taxonomy are served best by adopting the viewpoint of the "lumpers" rather than the "splitters." Hence, we prefer to designate strain 14B22 as a biotin dependent strain of the *B. megaterium-B. cereus* intermediates.

The striking regulation of cell synthesis versus glutamic acid synthesis mediated by biotin suggests an important role for this vitamin in one or more "switching" reactions in this organism. The importance of this effect is underscored by a similar biotin regulation of growth versus glutamic acid formation by biotin-requiring micrococci (Australian Patent Applications 23,556 and 23,557, cited in Beesch and Tanner, 1958). Additional studies on the metabolism of Bacillus strain 14B22 will be published later.

Enzymes of *M. glutamicus* have been studied by the Japanese workers (Kinoshita *et al.*, 1957*b*), who concluded that the reductive amination of α-ketoglutaric acid by glutamic dehydrogenase is the main pathway of glutamic acid synthesis in that organism. The metabolism of *M. glutamicus* evidently can be dislocated so that the pathway can be shifted either to α-ketoglutaric or to glutamic acid accumulation. Bacillus strain 14B22 produces very small amounts of α-ketoglutaric, succinic, and acetic acids, but the amounts were not significantly increased under conditions unfavorable for glutamic acid accumulation.

SUMMARY

A biotin dependent, facultatively aerobic, gram-negative, sporeforming rod belonging to the *Bacillus megaterium-Bacillus cereus* intermediates has been described. In suitable media the bacterium is distinctively pleomorphic and produces extraordinary amounts of free L-glutamic acid. In 3 per cent glucose medium, up to 12.5 mg of glutamic acid were synthesized per ml of medium (0.55 moles of glutamic acid per mole of glucose utilized). The concentration of biotin is critical. In limiting biotin concentrations, glutamic acid synthesis occurs at the expense of cell synthesis; in nonlimiting biotin concentrations, cell synthesis takes place at the expense of glutamic acid synthesis.

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