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Changes in Amino Acid Permeation During Sporulation

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Changes in amino acid uptake in *Bacillus licheniformis* and in the amino acid pools of three *Bacillus* species were investigated, by use of cells from different stages of the life cycle. *B. licheniformis* contains catalytic uptake systems for all of the 10 amino acids studied. The apparent maximal velocities of uptake decreased during sporulation but did not fall below the range observed for other microorganisms. In sporulating cells, the apparent affinity constants of the uptake systems for individual amino acids remained about the same as in growing cells, i.e., from 2×10^{-7} M to 7×10^{-6} M, whereas, in some cases, the apparent maximal velocities decreased significantly. Because the velocity of uptake showed an atypical dependence on substrate concentration, it was postulated that these cells contain two or more uptake systems for each amino acid. Only one of these systems appeared to be operative at a substrate concentration below 10^{-6} M. Working at these low substrate concentrations, catalytic activities producing a net efflux of amino acids were demonstrable in vegetative cells in the presence of chloramphenicol, but these exit systems were lost during sporulation. A pool formed by the addition of radioactive algal hydrolysate will exchange with the external medium in vegetative cells but not in sporulating cells. Glutamic acid and alanine comprise at least 60% of the amino acid pool of *B. licheniformis* A-5, *B. subtilis* 23, and *B. cereus* T during all stages of growth and sporulation. The concentrations of the other amino acids in the pool varied extensively, but reflected, in general, the amino acid turnover known to occur during sporulation.

During sporulation in the absence of exogenous nutrients, it is assumed that the turnover of vegetative-cell protein provides sufficient quantities of the free amino acids for the biosynthesis of spore protein (6, 13). In addition, it is generally accepted that bacteria contain specific uptake systems, termed permeases (7, 10), that appear to be constitutive for amino acid uptake, and are distinct from internal metabolic enzymes. Catalytic activities producing an efflux of endogenous amino acids (termed exit permeases, 10) also are present in bacteria.

The goal of this study was to determine the rate of protein turnover during sporulation, by use of the following reasoning. Amino acids released from vegetative protein during turnover should exchange with extracellular amino acids. Thus, it should be possible to measure the rate and quantity of turnover during sporulation of endogenous amino acids derived from prelabeled vegetative-cell protein by equilibrating the pool with a large excess of exogenous amino acid. If it is assumed that the permeases are not rate-limiting, the measurement of the rate and extent of release of radioactive amino acid from the cells should allow a calculation of the amount of turnover occurring during sporulation. However, in preliminary studies with sporulating *Bacillus licheniformis* cells, it was shown that the addition of an excess of unlabeled amino acid to the medium did not cause the release of endogenous amino acids (4). Consequently, the amino acid pool is not exchangeable with the extracellular medium, and it is demonstrated in this report that some of the amino acid uptake systems decrease in activity and that the net efflux systems disappear entirely during sporulation.

MATERIALS AND METHODS

Culturing of the microorganisms. *B. licheniformis* A-5 was used for all of the uptake studies. Cells were grown in a "minimal" medium that consisted of (per liter): $MgSO_4 \cdot 7H_2O$, 1.0 g; $MnCl_2$, 5.5 mg; $FeSO_4 \cdot (NH_4)_2SO_4 \cdot 6H_2O$, 25 mg; NaCl, 0.4 g; KCl, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.1 g; H_3PO_4, 0.45 g; and citric acid, 0.312 g. The *p*H was adjusted to 7.2 with KOH, and

the medium was sterilized by autoclaving at 18 psi for 15 min. Prior to use, the medium was adjusted to 20 mM glucose and 50 mM ammonium lactate by adding the appropriate sterilized solutions. Other additions were made as noted for each experiment. Spores were prepared by growing cells for 3 days in the minimal medium. The spores were then sedimented and washed several times by centrifugation at 10,000 × *g* for 20 min, suspended in distilled water (10^{10} per ml), pasteurized at 65 C for 90 min, and stored at 0 C. All culture inocula (8 to 10%) consisted of vegetative cells prepared by germination of about 2×10^7 spores per milliliter and growth in the minimal medium (total time was 16 hr) at 37 C. Microscopic observations were performed by use of a Leitz phase-contrast microscope, and counts of cells and sporangia were made with a Petroff-Hausser chamber.

In testing for the constitutive nature of the uptake systems, the above minimal medium was supplemented with 1% Casamino Acids (Difco). Quantitative determinations of the amino acid pools were made on extracts of cells grown in 1-liter lots in the minimal medium or in an enriched medium. The enriched medium was prepared from the minimal salts medium with the addition of a final concentration of 0.05% yeast extract (Difco), 0.5% Casamino Acids, 0.1 mM L-tryptophan, 0.05% NH_4Cl, and 20 mM glucose. *B. licheniformis* A-5, *B. subtilis* 23 (courtesy of J. Spizizen), and *B. cereus* strain T (courtesy of B. Church) were used for these studies.

Uptake studies. B. licheniformis cells were grown in either 10- or 25-ml lots (depending on the number of samples needed) at 37 C in an Eberbach water-bath shaker adjusted to 120 strokes [1-inch (2.54-cm)] per minute. The minimal medium (with 20 mM glucose and 50 mM ammonium lactate) was used exclusively with additions as noted for each experiment. Uptake studies were initiated by the addition of the radioactive amino acid. Samples were taken at appropriate times (for amino acids with high apparent maximal velocities, samples were taken at 15 and 30 sec at low substrate concentrations) by removing 0.4 ml of suspension and immediately pipetting 0.2 ml into 1.8 ml of 0.1 M phosphate buffer (*p*H 7.0) at 0 C and the other 0.2 ml into 1.8 ml of 5% trichloroacetic acid (w/v) at 80 C. No more than a total of 10% of any culture was removed by sampling. As soon as possible (between 10 and 180 sec) the diluted suspensions were filtered on 25-mm membrane filters (pore size, 0.45 μ; Millipore Filter Corp., Bedford, Mass.) and washed with either the phosphate buffer or 5% trichloroacetic acid. In some experiments, only buffer-washed samples were taken. Loss of accumulated amino acid has not been observed. The filters were then placed in plastic counting vials, and 10 ml of a scintillation solution composed of 70% toluene-30% ethyl alcohol containing 0.4% PPO (2,5-diphenyloxazole) and 0.01% POPOP [1,4-bis-2-(5-phenyloxazolyl)benzene] was added. The radioactivity of the samples was determined in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 314, and the millimicromoles of radioactive amino acid added and recovered in the samples were calculated, by use of the specific activity of the original amino acids.

It should be stressed that kinetic studies were performed with cells either in the early exponential phase of growth or in the sporulative stage. Additions were made to the cultures and samples were removed from them without stopping the shaker or removing the flasks from it. Samples were taken for turbidity measurements immediately after the 2-min uptake samples were processed. Reproducible results were not obtained when this procedure was altered.

For studies on the constitutive nature of the uptake systems, suspensions were prepared from cells growing in either the minimal medium or in the minimal medium plus 1% Casamino Acids. The cells were sedimented at 10,000 × *g* for 30 min, suspended in the original volume of 0.1 M phosphate buffer (*p*H 7.0), and sedimented as above. The cells were finally suspended in one-half the original volume of 0.1 M phosphate or tris(hydroxymethyl)aminomethane (Tris) chloride buffer at *p*H levels varying from 6.0 to 8.5. The suspensions were then returned to the water-bath shaker at 37 C, and the uptake experiments were performed.

A mixture of 18 of the following 19 L-amino acids was used in competition studies: alanine, arginine, aspartic acid, cysteine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. The mixture of 18 amino acids omitted the single radioactive amino acid that was being used in the particular experiment, and this mixture is referred to as the "other 18 amino acids."

Pool studies. Samples of cells from 200 or 300 ml of culture were removed at the indicated times, sedimented by centrifugation at 10,000 × *g* for 20 min, and washed two times by suspension in the minimal salts medium at 3 C followed by centrifugation. After washing, the cells were suspended in distilled water and placed in a boiling-water bath for 30 min. The cells were separated from the boiled-water extract by centrifugation at 10,000 × *g* for 10 min. The extract was adjusted to a *p*H of about 12 with 1 M KOH and placed in a boiling-water bath for 15 min to remove NH_3. After adjusting the *p*H to 7.0 with 1 M HCl, the amino acid content was determined by the method of Trool and Cannan (12).

The extracted cells were washed two times with distilled water and suspended in 25 ml of water with the aid of a glass homogenizer and a Teflon plunger. Triplicate samples (5.0 ml) were placed in weighed aluminum foil planchets, dried at 105 C, and weighed on a Sartorius model 2500 balance. With these data, a dry weight-turbidity standard curve was prepared, and, in the uptake studies, the data were calculated and are presented on a dry weight basis.

Quantitative amino acid analyses of pools, prepared as above, were performed on a Beckman model 120B Amino Acid Analyzer. The elution procedure was modified from the standard Beckman method to allow the separation of methionine from diaminopimelic acid and serine from muramic acid. Diaminopimelic acid and the hexosamines, present in small amounts in all cell extracts, are ignored in presenting the data.

Chemicals. All chemicals were of reagent grade

purity. All of the radioactive L-amino acids were uniformly labeled with ^{14}C and were purchased from either New England Nuclear Corp., Boston, Mass., or Tracerlab, Waltham, Mass. They had the following specific activities (microcuries per micromole): L-alanine, 123; L-arginine, 194; L-aspartic acid, 162; L-glutamic acid, 204; L-histidine, 243; L-leucine, 243; L-lysine, 243; L-isoleucine, 239; L-phenylalanine, 360; L-tyrosine, 191; L-valine, 200; and algal protein hydrolysate, 179 (assuming an average molecular weight of the amino acids as 125). The radioactive amino acids were chromatographed on Whatman no. 1 paper in (i) *n*-butanol-water-acetic acid (5:4:1) and (ii) methanol-pyridine-water (40:10:1). The strips were scanned on a Baird Atomic 4 pi instrument, and, in all cases, only a single radioactive spot was observed at the expected position. The unlabeled L-amino acids were of the highest purity available (either crystalline or chromatographically pure) from Sigma Chemical Co., St. Louis, Mo., or Mann Research Laboratories, Inc., New York, N.Y. Chloramphenicol was a product of Parke, Davis & Co., Detroit, Mich., and the actinomycin D was a gift from H. B. Woodruff, Merck, Sharp & Dohme, West Point, Pa.

RESULTS AND DISCUSSION

Kinetics of uptake in growing cells. To determine why the amino acid pool in *B. licheniformis* is not exchangeable with the external medium during sporulation, an examination of the kinetics and characteristics of the uptake and exit systems was performed.

Linear nature of the system. As is the case for other bacteria, the uptake of amino acids from the extracellular medium by *B. licheniformis* appeared to be mediated by a group of "permease" systems, the characteristics of which are reported subsequently. The kinetics of uptake of individuals of a representative group of amino acids were quite similar. Uniformly labeled amino acids were added at various concentrations to cells growing exponentially on the salts-glucose medium. The rate of uptake was determined by sampling the culture at 0.5, 1, 2, 5, and 15 min and measuring the amount of uptake by isotopic methods. Initial velocities were calculated from velocity versus time graphs and plotted against amino acid concentration. Figure 1 shows a plot of a typical experiment with L-phenylalanine at concentrations ranging from 2.8×10^{-9} M to 9.1×10^{-5} M, a 32,500-fold range. The curve can be divided into two parts. At low phenylalanine concentrations, the rate of uptake was linear and consequently was dependent on external concentration up to about 10^{-6} M. Above this, the rate of uptake was not directly proportional to concentration but was still increasing at about 10^{-4} M. The shape of the curve is not consistent with that of a single enzymelike activity, and it is possible that two overlapping uptake systems are responsible for such a broad curve.

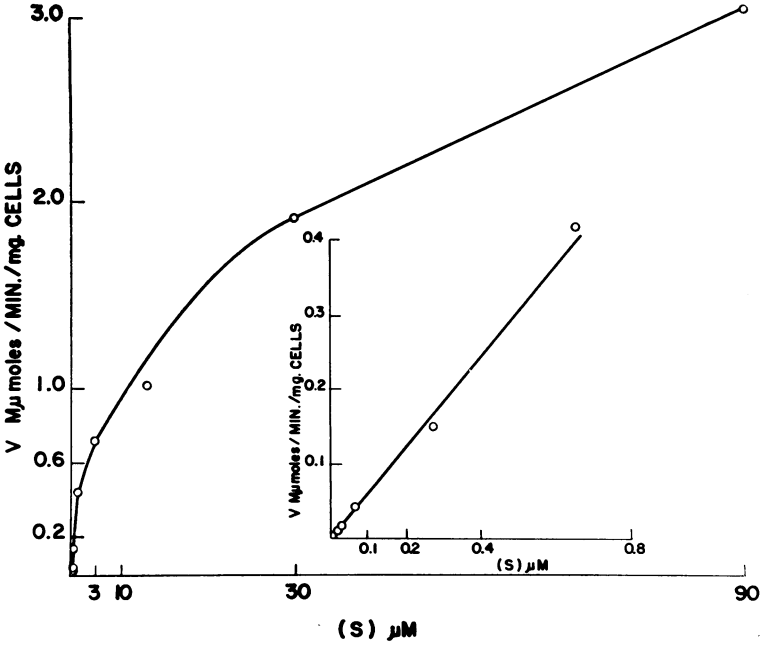

FIG. 1. *Effect of the concentration of* L-*phenylalanine on the uptake into growing cells. The concentration range was from* 2.8×10^{-9} M *to* 9.1×10^{-5} M. *The insert replots only those data below* 0.8×10^{-6} M *on a 10-fold expanded scale.*

The insert in Fig. 1 replots the initial velocities of the uptake of phenylalanine at concentrations below 0.8 μM. Here it is clear that uptake was directly related to amino acid concentration, even at 2.8×10^{-9} M. At concentrations below 10^{-6} M, the rate of endogenous synthesis of amino acid was not affected, and the pool size did not change. Also, the kinetic data should all relate to the specific permease and not be disturbed by the possible activity of a second low affinity constant permease.

Because of the considerations just mentioned, it was concluded that the rate-limiting reaction being examined in these studies was the uptake system. If this is the case, then the rate of utilization of endogenous amino acids would be greater than the rate of uptake, and the newly acquired amino acids should be incorporated into protein immediately after entering the cells. Under these conditions, it should be impossible to demonstrate a pool of radioactive amino acid. This is, in fact, the case as will be shown subsequently. By the use of these low substrate concentrations in kinetic studies of amino acid uptake, it is felt that values are derived that are not affected by any of the other activities of the cell, since the incorporated amino acids have no effect on the pool size.

All of the other amino acids tested (see Table 1 for the entire list) were removed from the extracellular medium by growing cells in a similar fashion. Initial velocity versus substrate concentration plots produced curves with the same shape as that on Fig. 1, although absolute values differed.

Kinetic constants. To determine apparent maximal velocities (or apparent dissociation constant for the binding site) and apparent affinity constants for the individual uptake systems, double reciprocal v versus substrate concentration figures were prepared. Since values were desired only for the low substrate concentration systems, only those data obtained at amino acid concentrations below 10^{-6} M were used. Figure 2 shows a typical plot of the data for the uptake of aspartic acid. Two points can be made here. First, a straight line can be drawn through all of the points covering a concentration range from 6×10^{-9} to 6×10^{-7} M. Therefore, the single permeation system that was active at about 10^{-6} M amino acid concentration was also the same system that was responsible for amino acid uptake at concentrations near 10^{-9} M. A possible second permease would be operating only at high substrate concentrations.

The second point to be made is that the straight line in Fig. 2 apparently goes through the origin.

TABLE 1. *Kinetic constants for the uptake systems in vegetative and sporulating cells*

Amino acid	Apparent affinity constants[a]		Apparent maximal velocity[b]	
	Vegetative cells	Sporulating cells	Vegetative cells	Sporulating cells
Alanine	3.1	2.9	3.8	0.8
Arginine	0.3	1.1	0.9	0.9
Aspartic acid	1.3	0.8	1.3	0.1
Glutamic acid	3.4	3.5	0.6	1.3
Isoleucine	0.9	1.0	4.4	0.4
Leucine	2.5	1.2	0.6	0.4
Lysine	1.1	1.9	7.1	2.1
Phenylalanine	0.5	0.9	1.0	0.006
Tyrosine	3.3	2.3	1.3	0.3
Valine	2.3	0.5	3.0	0.2

[a] Variation among replicate values was about 20% in vegetative cells and 50% in sporulating cells. Results expressed $\times 10^{-6}$ M.

[b] Variation among replicate values was about 30% in vegetative cells and 100% in sporulating cells. Results expressed as micromoles per minute per gram of cells.

Similar plots have been seen for all of the amino acids studied. However, when the four values at the higher end of the substrate concentration scale were replotted in a 10-fold expanded form (insert in Fig. 2), an intercept was always seen and apparent maximal-velocity values could be determined. By use of this apparent maximal velocity and the slope of the line over the entire concentration range, apparent affinity constants can be calculated, when it is assumed that the slope equals the affinity constant divided by maximal velocity.

The apparent affinity constants and maximal-velocity values obtained in the above manner for a representative group of amino acids are listed in Table 1. The apparent affinity constants ranged from 2.7×10^{-7} M for arginine to about 3.3×10^{-6} M for tyrosine and glutamic acid. This range is the same as that for the uptake of histidine by *Salmonella* (1) and for diaminopimelic acid and cystine in *Escherichia coli* (8). The differences are significant but do not prove that there are separate systems for each amino acid.

A surprising aspect of the entire investigation involved the relationship between the uptake of the substrate amino acid at low concentrations and the concentration of the same amino acid inside the cell. Since the endogenous concentrations were approximately 10^{-4} M (shown subsequently), the uptake of amino acid at 5×10^{-9} M was against a 20,000-fold gradient. The magnitude

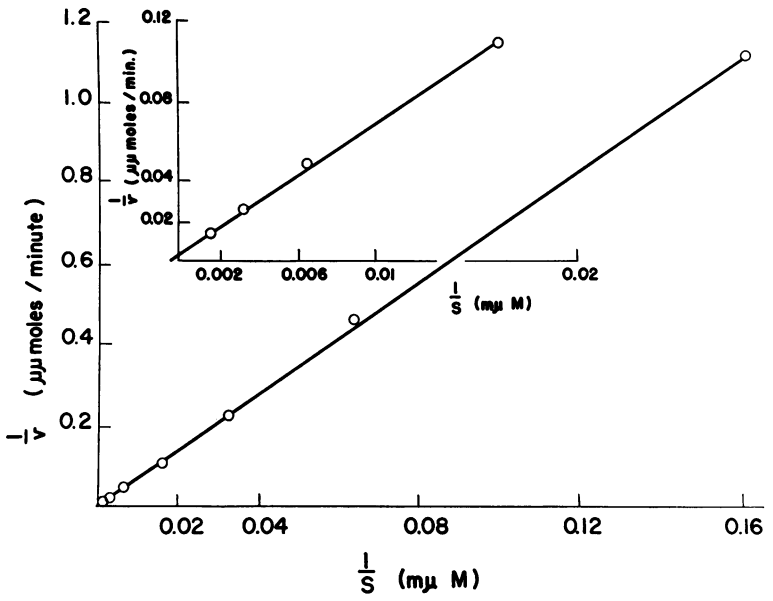

FIG. 2. *Reciprocal v versus substrate concentration (S) plot for the uptake of L-aspartic acid. Highest concentration used was 0.625 × 10⁻⁶ M.*

of this difference assured that the concentration in the pool was not affected by the addition of exogenous amino acid. Since amino acid uptake is linearly proportional to substrate concentration, it follows that endogenous amino acid has no effect on the uptake of exogenous amino acid in this *B. licheniformis* system.

Growing *B. licheniformis* cells contain uptake systems that remove at least 10 amino acids from the external medium at concentrations ranging from 10^{-9} to 10^{-6} M. The lower value may not be a lower limit, as it is presently impossible to measure uptake at lower concentrations.

The data led to several conclusions about the uptake systems in *B. licheniformis*. The reaction was first-order at substrate concentrations as low as 10^{-9} M, and the rate was directly proportional to substrate concentration in this low range. Also, because of the presence of a catalytic system with a high affinity for substrate, the cell seemed to be favorably endowed to compete ecologically in an environment that was not as plentiful in a nutritional sense as that usually provided in the laboratory. Values of apparent maximal velocity that were derived by measuring uptake rates at these low concentrations were all in the range of 1.0 μmole per min per g of cells, and apparent affinity constants varied from 2.7×10^{-7} M to 7×10^{-6} M. Because these values were easily reproducible and because the highest substrate concentration used in each experiment was at or above the apparent affinity constant, it was felt that real uptake systems were being examined and that the data were not altered by "high substrate concentration" systems. It is suggested that these latter "permeases" exist in *B. licheniformis*, as uptake rates at 10^{-4} M substrate were several times greater than the calculated apparent maximal velocity for the "low substrate concentration" system.

It has been shown that, at 10^{-6} M or below, the rate-limiting reaction in the overall conversion of extracellular amino acids to intracellular metabolites or proteins is the uptake of the amino acid. Under these conditions, the metabolic fate of the intracellular labeled amino acid is function of pool sizes and turnover rates and not the amounts and maximal velocites of enzymes of a metabolic pathway.

Kinetics of uptake into sporulating cells; kinetic constants. In the preceding section, cells in the early stages of the logarithmic phase had to be used because variable results were obtained otherwise. Thus, the uptake rates were 50% lower in late exponential-phase cells and in many cases 90% lower in presporulating cells. Because of this variation, it was possible to study amino acid permeation only in sporulating cultures, not in presporulating cells.

Under normal cultural conditions, growth is complete at about 6 to 7 hr and sporangia become visible under phase-contrast at about 13 hr, at

which time uptake studies were performed in a manner identical to that used for growing cells. Table 1 shows the apparent affinity constants and maximal velocities for 10 different amino acids.

In general, the apparent affinity constants of the amino acids for binding sites on both vegetative and sporulating cells were of the same order of magnitude. In contrast, apparent maximal velocities of uptake of at least seven amino acids into sporulating cells were 10- to 100-fold lower than those of growing cells. Because of this, apparent velocities of the uptake of these amino acids at constant concentration decreased markedly in sporulating cells when compared with growing cells, but it is clear that sporulating cells were capable of taking up amino acids from the extracellular medium and incorporating these amino acids into cell protein.

Characteristics of the uptake systems; general nature. Since an explanation for the inability to observe amino acid exchange between the intracellular pool of sporulating cells and the extracellular medium was not provided by the foregoing kinetic studies, further experiments on the general nature of the uptake systems were performed.

A typical time course of uptake of L-isoleucine-^{14}C is shown in Fig. 3. The uptake rate is about sevenfold slower than the maximal velocity. The arithmetic difference between the two curves (buffer-washed and trichloroacetic acid-washed) produced values indicating the size of the pool of radioactive amino acid at any time period. Very little radioactive L-isoleucine was detected in the pool (Fig. 3), showing that the pool of isoleucine in *B. licheniformis* turned over at an extremely rapid rate. It is assumed that endogenous and exogenous amino acids are perfectly mixed in a single metabolic pool. As will be shown later, the amount of isoleucine in the pool was about 2 μmoles per g of cells. Since *B. licheniformis* has a doubling time of 1 hr, it can be calculated that 1 g of cells requires and synthesizes 3.2 μmoles of isoleucine per min for growth. Therefore, the entire pool was turned over every 37 sec, and it was not possible for the isoleucine to be accumulated to a measurable degree when it was being taken up into this pool at a rate of 0.15 μmole/min (Fig. 3). However, when chloramphenicol was added to the culture at 50 μg/ml, this large requirement for isoleucine greatly diminished and a pool of radioactive isoleucine was seen (Fig. 3). The minimal inhibitory concentration of chloramphenicol under these growth conditions was about 25 μg/ml. Actinomycin D at 1.6 μg/ml (about three times the minimal inhibitory concentration) produced the same effect although not as pronounced in this case (Fig. 3).

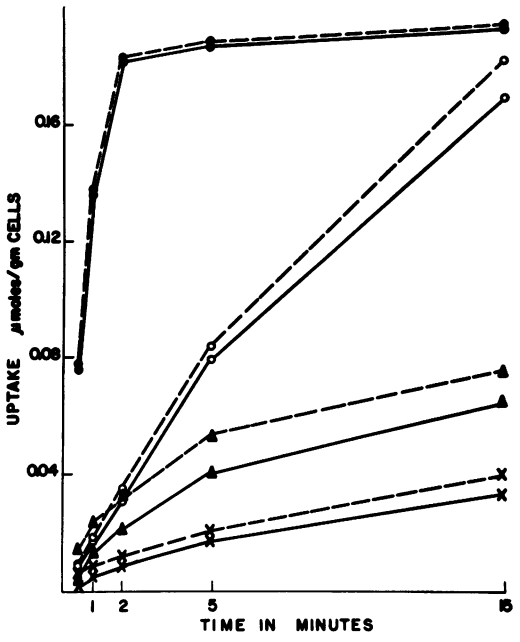

FIG. 3. *Uptake into undisturbed and washed cells. L-Isoleucine-^{14}C was added at zero-time (164 μμmoles/ml, final concentration) to exponential-phase cells (0.51 mg/ml). Symbols: control, ●; plus 50 μg of chloramphenicol per ml, ▲; plus 1.6 μg of actinomycin D per ml, ×, both added 5 min before zero-time. L-Isoleucine^{14}C was added (○) to washed cells (0.9 mg/ml) at a final concentration of 410 μμmoles/ml. The dashed lines indicate buffer-washed samples; the solid lines, samples washed with hot 5% trichloroacetic acid. The addition of chloramphenicol or actinomycin D to washed suspensions produced at least a 95% inhibition of uptake.*

To characterize the uptake system more completely, this experiment was altered in several ways. First, washed cells suspended in phosphate or Tris chloride buffer at different *p*H levels were substituted for the undisturbed growing cells. The rate of uptake of amino acids into such cells was greatly diminished. For example, L-isoleucine-^{14}C was taken up about nine times slower (Fig. 3) at *p*H 7.0 in phosphate buffer at 37 C. Similar results were obtained at *p*H levels ranging from 6.5 to 8.0. No uptake was demonstrated at 10 C, and only a slow rate occurred at 30 C.

Glucose addition to cells resuspended in buffer at *p*H 7.0 did not cause an enhancement in the rate of amino acid uptake. This does not indicate that energy is not required, as the cells have a high rate of endogenous respiration. When the cells were allowed to incubate for several hours, the uptake rate decreased. Also, 10^{-3} M sodium azide inhibited uptake at least 90%. Thus, *B. licheniformis* cells have uptake systems that are energy-

dependent and accumulate amino acids against a large gradient.

The uptake systems for at least a few of the amino acids are constitutive. Cells that had grown for several hours on the minimal medium plus 1% Casamino Acids were washed and suspended in 0.1 M phosphate buffer (*p*H 7.0). The uptake of L-isoleucine-^{14}C followed the same curve (Fig. 3) as was seen with cells grown in the absence of amino acids. The uptake of L-leucine and L-arginine was the same in cells grown with or without casein hydrolysate.

Thus, the uptake systems for at least these three amino acids are constitutive, and the levels are not altered by growth on amino acids.

Some variability is seen in the uptake of different amino acids. Apparent affinity constants and maximal velocities span a 20-fold range. Figures 4 and 5 show some additional differences. In all cases, less than 80% of the added radioactivity was found in the cells, with the remainder still in the culture medium. Since the amount of amino acid in the pool was much greater than the amount added (see *Amino acid pools*), it is unlikely that the cessation of uptake was due to an internal saturation. Because of the lack of 100% uptake the following experiments were performed. L-Isoleucine-^{14}C was added at concentrations from 4.2×10^{-8} M to 4.2×10^{-7} M (specific activity, 239 $\mu c/\mu$mole), and 60 $\pm$ 3% of each sample was taken up. The L-isoleucine-^{14}C was then diluted with chromatographically pure L-isoleucine to a specific activity of 79.7 $\mu c/\mu$mole and was added at concentrations from 1.26×10^{-7} M to 1.26×10^{-6} M. Again, 56 $\pm$ 4% was taken up in five tests. The L-isoleucine-^{14}C, purchased as chromatographically pure, was resolved by paper chromatography in two sol-

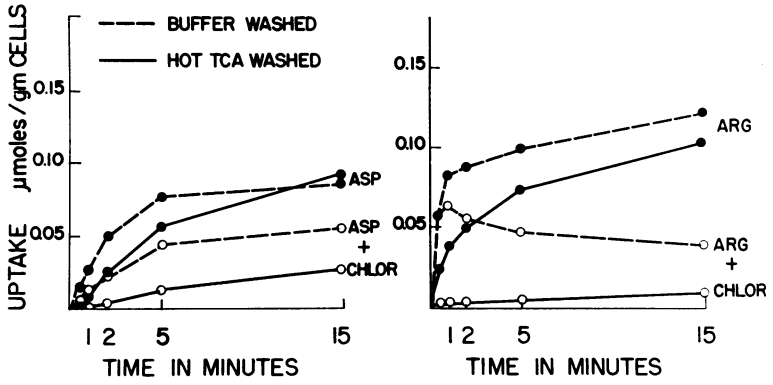

FIG. 4. *Uptake into undisturbed cells. L-Aspartic acid-^{14}C (123 $\mu\mu$moles/ml) or L-arginine-^{14}C (103 $\mu\mu$moles/ml) was added at zero-time to growing cells (0.50 to 0.52 mg/ml). Chloramphenicol at 50 μg/ml was added 1.0 min before zero-time.*

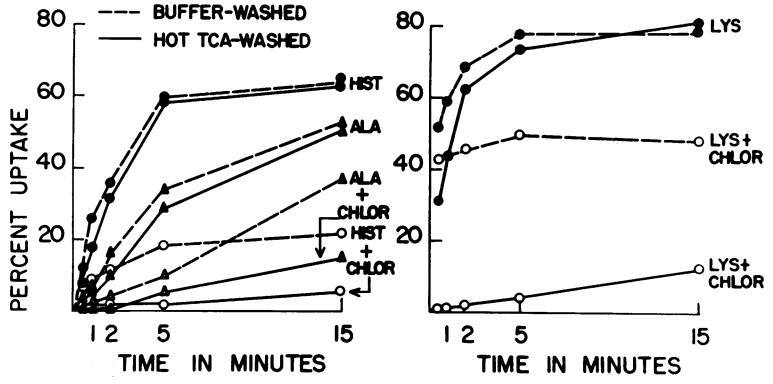

FIG. 5. *Conditions the same as in Fig. 3. L-Histidine-^{14}C (82.5 $\mu\mu$moles/ml), L-alanine-^{14}C (162 $\mu\mu$moles/ml), or L-lysine-^{14}C (82.5 $\mu\mu$moles/ml) was added to growing cells at 0.6 mg (dry weight) per ml. The figure is plotted as percentage uptake instead of micromoles per gram of cells to permit the inclusion of multiple curves on one figure.*

vents, and at least 98% of the radioactivity migrated as a single component with pure L-isoleucine. The D-isomers, at concentrations equal to those of the L-amino acid, did not inhibit the uptake of the L-amino acids.

It had been shown previously (4) that a fair amount of amino acid oxidation occurs in *B. licheniformis* and that $^{14}CO_2$ is produced immediately after adding labeled amino acids. In acidic media, the radioactive CO_2 would not be evolved. Thus, it seems likely that all of the amino acid is taken out of the medium and into the cells, some of it being oxidized and the remainder incorporated into protein.

Several of the amino acids formed significant pools in the presence of chloramphenicol, lysine being the most pronounced (Fig. 5). Also, 50 μg of chloramphenicol per ml inhibited incorporation of amino acid into trichloroacetic acid-insoluble material 96% with arginine (Fig. 4), but only 70% with alanine (Fig. 5). Finally, it should be pointed out that chloramphenicol had a minimal effect on the initial rate of amino acid uptake. This was most clearly demonstrated with arginine (Fig. 4) and lysine (Fig. 5).

Specificity. Figure 6 shows the results of some preliminary experiments on the competition among amino acids for uptake into *B. licheniformis* cells. The carbon-labeled amino acids were added at concentrations of 10^{-7} to 2×10^{-7} M. In each case, a 10-fold excess of each of the other 18 "protein" amino acids was added 10 sec before zero-time. Hence, the unlabeled competitors were added at concentrations near the affinity constants of the labeled amino acids and just in the range when a small but reproducible inhibitor effect should be seen.

The uptake of the labeled amino acid was inhibited by the presence of the other amino acids in all cases except L-lysine (Fig. 6). The uptake of several other amino acids, in addition to the five shown in Fig. 6, was also inhibited by the "other 18 amino acids." L-Lysine uptake is not inhibited by a 100-fold excess of L-ornithine or L-arginine, so further studies must be performed on the lysine uptake system.

Since the apparent affinity constants are not grossly altered in sporulating cells, it could be proposed that the binding sites for the amino acids on the surface of the sporulating cells are still intact and apparently the same as in the vegetative cells. However, this did not seem to be the case. When competition studies identical to those described for growing cells were performed with sporulating cells, the data shown in Fig. 7 were obtained. Here, essentially no competition between the labeled amino acid and the "other 18 amino acids" was evident. Thus, it would appear that some change took place in the structure or activity of the uptake systems, even though very little change was seen in the apparent affinity constant.

Figure 7 also demonstrates several other points. First, as documented in Table 1, the initial rate of

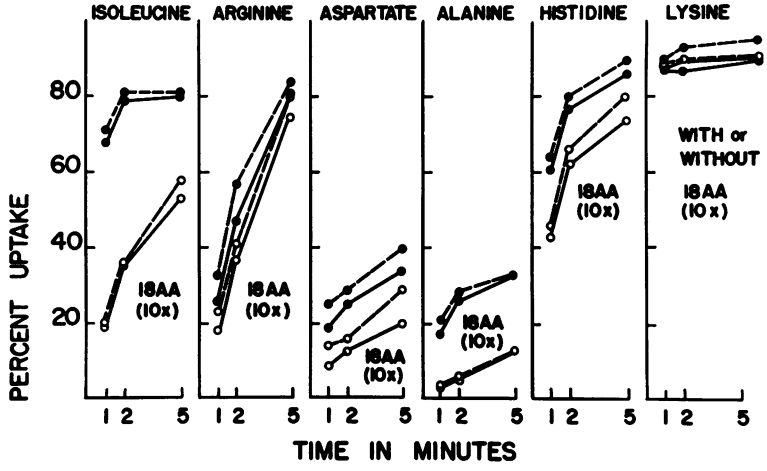

FIG. 6. *Effect of the "other 18 amino acids" (18AA) on the uptake of a single radioactive amino acid by growing cells.* ^{14}*C-labeled* L*-amino acids were added at zero-time as follows:* L*-isoleucine (210 $\mu\mu$moles/ml) to 0.33 mg of cells per ml;* L*-arginine (129 $\mu\mu$moles/ml) to 0.36 mg of cells per ml;* L*-aspartic acid (133 $\mu\mu$moles/ml) to 0.38 mg of cells per ml;* L*-alanine (202 $\mu\mu$moles/ml) to 0.41 mg of cells per ml;* L*-histidine (103 $\mu\mu$moles/ml) to 0.43 mg of cells per ml; and* L*-lysine (103 $\mu\mu$moles/ml) to 0.46 mg of cells per ml. Symbols: ●, controls; ○, uptake into cultures to which the "other 18" (see Materials and Methods) amino acids were added at 10 sec before zero-time. Dotted lines are buffer-washed cells; solid lines are cells washed with hot trichloroacetic acid.*

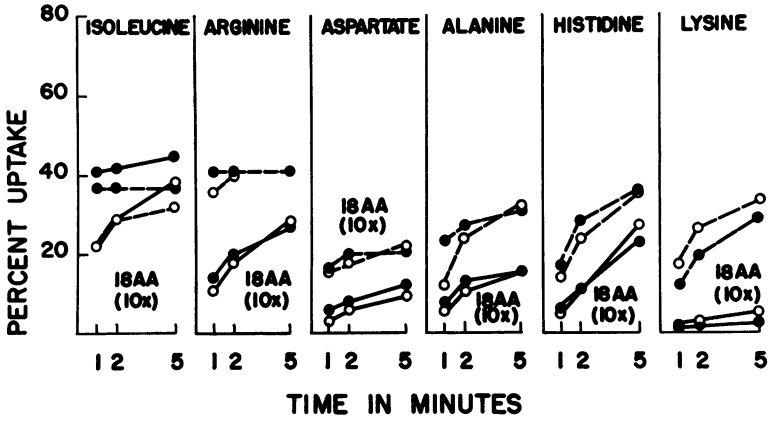

FIG. 7. *Effect of the "other 18 amino acids" (18AA) on the uptake of a single radioactive amino acid by sporulating cells. Same as Fig. 6 except that the cells were in the postexponential phase (12- to 13-hr cultures; 1.5 mg/ml).*

uptake was greatly diminished in sporulating cells. Since samples were not taken at times less than 1 min for these studies, initial velocities cannot be calculated, but a comparison of the uptake values in Fig. 6 and 7 shows clearly that the sporulating cells were less active than the growing cells. It should be emphasized that sporulating cultures contained four to five times more dry weight than the growing cultures.

Another change seen in the sporulating cells was the demonstration of a radioactive pool. Here, the difference between the radioactivity measured in buffer-washed cells and hot trichloroacetic acid-washed cells was quite pronounced and easily measured. Since the cells are not growing, it is assumed that the rate of utilization of the amino acids from the pool for protein synthesis is slow, and amino acids incorporated into the pool remain there long enough to be measured.

Although these studies are not at all comprehensive, they are consistent with the general picture of uptake in bacteria (7). Thus, there appear to be relatively characteristic, active, uptake systems for amino acids in vegetative and sporulating cells of *B. licheniformis*.

Net efflux systems. The original question that prompted this investigation has not yet been answered. It is not possible to equilibrate the pools of amino acids with an excess of exogenous amino acid. Thus, the amount of amino acid released from vegetative-cell protein via turnover during sporulation cannot be measured. As was shown above, the amino acid uptake systems are functioning, albeit some at a reduced rate, during sporulation, so exogenously added amino acids do get into the cells to dilute the pools. However, if exchange with the external medium is to be measured the amino acids must get out of the cells. This led to an examination of the "exit systems" for amino acids in *B. licheniformis*.

Kepes and Cohen (10) consider the exit of amino acid from a metabolizing cell as a carrier-mediated process and not simply diffusion. Thus, the addition of an excess of a C^{12} amino acid to cells containing a pool of the ^{14}C isomers has been shown to cause a loss of the radioactive amino acid from the cell by processes with kinetics different from those of the uptake system. These types of experiments have been performed on growing and presporulating *B. licheniformis* cells.

The addition of a number of amino acids to cells incubated in the presence of 50 μg of chloramphenicol per ml produced a significant pool of the amino acid. Of the 13 amino acids tested, all accumulated a pool in vegetative cells and only isoleucine and histidine failed to accumulate a pool large enough from which to measure a net efflux in sporulating cells. Figure 8 demonstrates the kinetics of the "exit systems" of arginine, alanine, histidine, and lysine in vegetative cells. The pools were labeled by the addition of 10^{-7} to 2×10^{-7} M amino acid to cells incubated in 50 μg of chloramphenicol per ml. After 6 min, a 100-fold excess of the same (unlabeled) amino acid was added to the culture, and a rapid loss of radioactive amino acid from the cell was observed. The rate of loss appeared to be less than the initial rate of uptake, suggesting that the exit system has kinetics different from that of the uptake system.

However, when this same experiment was performed with sporulating cells, no loss of the radioactive amino acid from the pool was observed. Figure 9 depicts results with radioactive arginine, glutamic acid, and aspartic acid, demonstrating that the labeled amino acid is not removed from the pool after the addition of an excess of un-

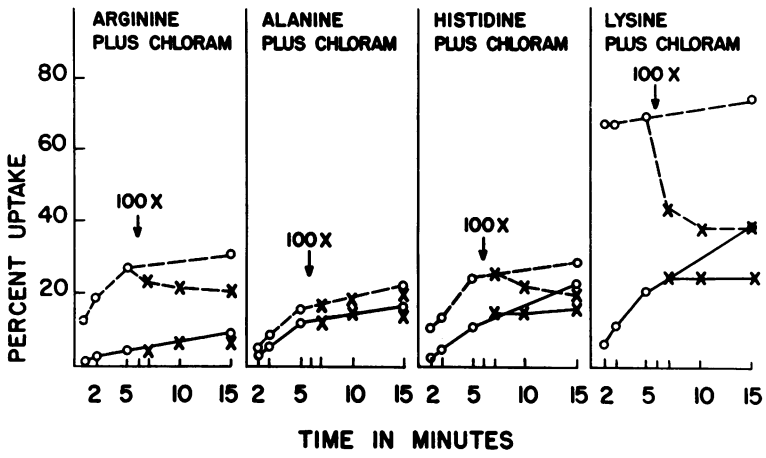

FIG. 8. *Net efflux system in vegetative cells.* ^{14}C*-labeled* L*-amino acids were added at zero-time to duplicate, chloramphenicol-treated (50 μg/ml at 1 min before zero-time), 10-ml, exponential-phase cultures as follows:* L*-arginine-*^{14}C *(0.25 μc, 1.29 mμmoles) to 0.33 mg of cells (dry weight) per ml;* L*-alanine-*^{14}C *(0.25 μc, 202 mμmoles) to 0.38 mg of cells (dry weight) per ml;* L*-histidine-*^{14}C *(0.26 μc, 1.03 mμmoles) to 0.33 mg of cells (dry weight) per ml; and* L*-lysine-*^{14}C *(0.25 μc, 1.03 mμmoles) to 0.38 mg of cells (dry weight) per ml. At 6 min, 0.129 μmole of* L*-arginine, 0.202 μmole of* L*-alanine, 0.103 μmole of* L*-histidine, and 0.103 μmole of* L*-lysine were added to one of the two radioactive arginine, alanine, histidine, and lysine cultures, respectively. Symbols: ○, control cultures; ×, radioactivity in the treated (chased) cultures. Dotted lines are buffer-washed cells and solid lines are cells washed with hot trichloroacetic acid.*

labeled amino acids. Similar results were obtained with alanine, histidine, and lysine. Finally, by use of ^{14}C algal hydrolysate, net efflux systems were again demonstrated in vegetative cells (Fig. 10A) but not in sporulating cells (Fig. 10B). Thus, in addition to the changes in the kinetics of amino acid uptake, it is apparent that the activity of the efflux systems was lost or greatly diminished when the cells changed from vegetative to presporulating metabolism.

Because the mechanism of amino acid permeation is not known, it is impossible to speculate on the reasons for the decrease of uptake activity and the efflux activity in sporulating cells. The absence of the exit systems hinders attempts at determining the rate and extent of amino acid turnover during sporulation. A previous report (4) showed that vegetative cells, labeled with amino acids, lose a significant portion of this radioactivity during sporulation, primarily as carbon dioxide. However, the addition of large amounts of unlabeled amino acid to the extracellular medium does not enhance the loss of the radioactive amino acid in question from the cells. Therefore, sporulating *B. licheniformis* cells will not exchange amino acids either recently accumulated, as reported here, or from protein turnover (4) with the medium.

It should be emphasized that, under the conditions reported here, the pools were being labeled with an insignificant quantity of amino acid compared with the amount present in the pool during growth. The pool sizes are not changed and there is no evidence that the recently accumulated labeled amino acid resides in a pool separate from the "metabolic pool" (9). Thus, it is unlikely that release from an expandable pool in growing cells can explain the results on the "exit permease activity."

Halvorson (6) suggests that the spore protein is derived both from de novo synthesis and from the entrapment of vegetative-cell protein within the initial spore membrane. Protein synthesis during sporulation has been demonstrated by many workers, primarily by measuring radioactive amino acid incorporation into spore protein. However, the entrapping hypothesis is based on changes in specific activities of amino acids from prelabeled cells. This hypothesis demands that no metabolism and interconversion of amino acids occur during sporulation. Nevertheless, a previous report (4) has shown that a great deal of amino acid metabolism is possible during sporulation, and, since these metabolites are not lost from the sporangium, evidence that intact vegetative-cell protein is used in the construction of a spore is lacking.

Amino acid pools; pools in B. licheniformis grown on minimal medium. Because *B. licheniformis* cells appear to lose the "exit permease" on converting to presporulative metabolism, the decrease in the rate of amino acid uptake

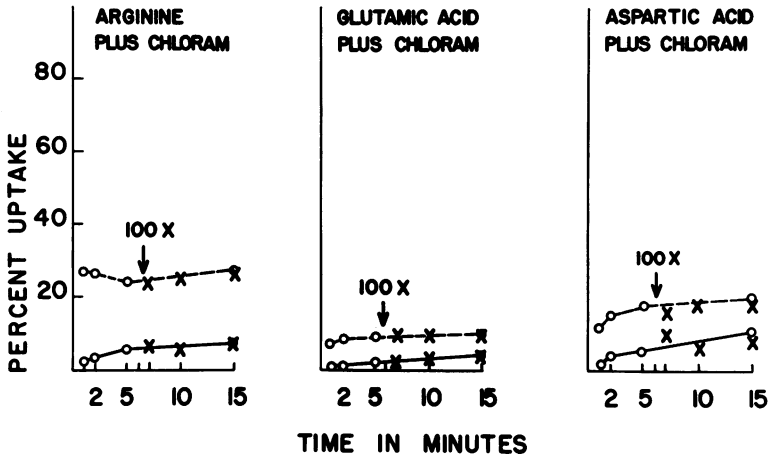

FIG. 9. *Absence of efflux systems in sporulating cells. Same as Fig. 7 except that the cells were postexponential [13-hr cultures, about 1.4 mg of cells (dry weight) per ml] and the amounts of amino acids used were as follows: L-arginine-^{14}C (0.25 μc, 1.29 mμmoles) followed by 0.129 μmoles of L-arginine, L-glutamic acid-^{14}C (0.25 μc, 1.22 mμmoles) followed by 0.122 μmole of L-glutamic acid, and L-aspartic acid-^{14}C (0.25 μc, 1.54 mμmoles) followed by 0.154 μmole of L-aspartic acid.*

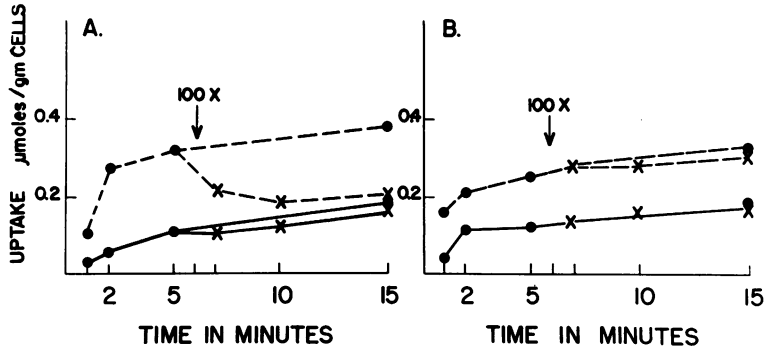

FIG. 10. *Efflux systems of Bacillus licheniformis. Algal hydrolysate-^{14}C (0.5 μc, 2.80 mμmoles) was added at zero-time to duplicate, chloramphenicol-treated (50 μg/ml at 1 min before zero-time), 10-ml, exponential-phase cultures (A) and postexponential-phase cultures (B). At 6 min, 0.280 μmole of casein hydrolysate was added to one of the two cultures in each experiment. The symbols are the same as in Fig. 7.*

may be a result of increases in the total pools of the individual amino acids. The pool of each amino acid was determined by preparing a boiling-water extract of cells at a number of stages of growth and sporulation. The mixture of amino acids was resolved and quantitatively measured on a Beckman Amino Acid Analyzer. Table 2 presents a compilation of the results of about 30 such determinations. The age of the cultures is designated as a variation from T_0, the approximate time when the cells completed vegetative growth and initiated presporulation. Thus, T_{-2} is just after the middle of exponential growth and T_8 cultures contain about 5 to 15% sporangia observable by phase-contrast.

The total quantity of amino acids in the hot-water extract from vegetative cells was consistently in the range of 150 to 200 μmoles per gram of cells (dry weight). As growth began to cease, this total rose about 50%, dropped somewhat, and then rose again to about 400 μmoles/g during the middle of presporulation ($T_{2.5}$ to T_5). The pool then dropped as sporangia formation ensued. Similar but somewhat higher values were obtained with cold 5% trichloroacetic acid or 5% perchloric acid extracts. It should be emphasized that the above values are the sum of the quantities of the individual amino acids eluted and measured by the Amino Acid Analyzer. Quantitative ninhydrin analyses (12) of the original extracts fall in the range of 400 to 700 μmoles per g of cells and do not show the variations seen above. In

TABLE 2. *Changes in the pools of individual amino acids during sporulation*

Amino acid	Culture age							
	T_{-2} (171)[a]	$T_{-3/4}$ (227)	T_0 (290)	T_1 (241)	$T_{2.5}$ (426)	T_5 (416)	$T_{6.5}$ (347)	T_8 (300)
Lysine	6.6[b]	3.7	4.1	5.4	5.3	6.2	4.0	4.4
Histidine	1.2	0.3	0.4	0.6	0.4	0.4	0.6	0.6
Arginine	none	0.1	0.4	0.4	0.6	0.4	0.2	none
Aspartic acid	0.7	1.9	1.2	0.5	1.0	1.9	1.7	1.4
Threonine	0.2	1.2	1.5	1.2	2.0	2.7	1.8	1.5
Serine	4.5	4.5	2.1	3.7	4.6	4.5	3.4	3.1
Glutamic acid	28.9	36.5	29.8	25.0	27.5	22.6	28.0	37.7
Proline	none	none	none	0.8	0.9	1.0	1.6	1.9
Glycine	1.8	3.6	2.2	4.0	4.1	4.8	3.6	3.1
Alanine	44.5	36.6	50.7	45.0	39.7	37.3	41.4	36.8
Valine	4.5	2.8	2.0	2.7	4.9	4.6	3.6	3.5
Methionine	1.4	1.2	0.8	1.4	1.4	1.5	1.2	0.7
Isoleucine	1.1	1.0	1.0	1.5	2.0	2.7	2.2	1.8
Leucine	2.0	2.3	1.7	4.9	3.7	3.5	3.1	2.7
Tyrosine	0.5	1.9	0.9	1.1	1.7	2.8	1.5	0.2
Phenylalanine	0.9	1.9	0.9	1.1	1.7	2.8	1.5	0.2

[a] Numbers in parentheses indicate the sum of the quantities of the individual amino acids from the Beckman Amino Acid Analyzer data, expressed as micromoles per gram (dry weight).

[b] Results expressed as moles per 100 moles.

fact, vegetative cells usually contain about 700 μmoles of ninhydrin-reacting "amino acid" per g of cells. Because of this, the sum of the individual amino acids has been used in ascribing physiological significance to differences in the amino acid uptake studies.

With the assumption that 1 g of cells (dry weight) contained 4.0 ml of water in the viable state, the concentrations of the amino acids in the pool of growing cells can be calculated. These values ranged from about 10^{-4} M (threonine and tyrosine) to 2×10^{-2} M (glutamic acid and alanine). Several aspects of the changes of the individual amino acids of the pool are noteworthy. Most showed both a maximal value at about T_5 and a transient minimum at T_0 to T_1. There did not seem to be any relationship between the vegetative pool (T_{-2}) and the presporulative pool (T_5), or any logical trend in the pools when compared with the quantity of amino acid that will subsequently be polymerized to form spore protein (4). A *B. licheniformis* protease activity, previously characterized (3), did appear in presporulative cells and could be responsible for the degradation of vegetative protein, but the relative increases of the individual amino acid pools from T_0 to T_5 did not correlate with the relative content of the protein amino acid in the vegetative cell. Therefore, it is impossible to relate the pool size of the amino acids to any specific physiological event of sporulation. It is not unexpected, however, to observe an increase in the total amino acid pool during a period of no net growth but presumed high turnover. The absence of the net efflux systems would then allow a pool to accumulate.

Pools of amino acids in cells grown on an enriched medium. The size of the combined alanine-glutamic acid pool was extremely large, comprising 60 to 75% of the total pool at all stages of the life cycle. This unexpected result prompted a brief examination of the composition of the pools of several other bacilli. *B. licheniformis*, *B. subtilis* 23, and *B. cereus* strain T were grown on an enriched medium containing casein hydrolysate and yeast extract, and were centrifuged and washed at T_{-2}, T_0, and $T_{2.5}$. The pools were extracted into boiling water as before, and the concentrations of the amino acids were determined in the Amino Acid Analyzer. These data are presented in Table 3.

Again, the pools of alanine and glutamic acid were quite large, accounting for 45 to 65% of the *B. licheniformis* pool, 70 to 80% of the *B. subtilis* 23 pool, and 70 to 73% of the *B. cereus* pool. In addition, the quantities of the individual amino acids and the total pools changed extensively, but, as with *B. licheniformis* grown on the minimal medium, no direct relationship to any event in the physiology of sporulation was evident.

When glutamic acid and alanine were omitted from consideration, the quantity of amino acids in the pools was quite small; growing cells con-

TABLE 3. *Amino acid pools of Bacillus licheniformis, B. subtilis 23, and B. cereus T grown on an enriched medium*

Amino acid	*B. licheniformis*			*B. subtilis*			*B. cereus*		
	T_{-2} (679)[a]	T_0 (361)	$T_{3.5}$ (422)	T_{-2} (260)	T_0 (380)	$T_{3.5}$ (500)	T_{-2} (75.9)	T_0 (73.8)	$T_{3.5}$ (296)
Lysine	8.8[b]	8.2	6.8	4.6	6.7	5.1	13.4	6.5	1.5
Histidine	1.9	1.2	0.8	0.4	0.3	—	0.8	0.2	0.1
Arginine	trace	0.1	0.2	trace	—	—	—	—	—
Aspartate	2.7	2.2	2.1	0.9	1.3	0.9	1.0	2.6	4.2
Threonine	3.1	3.1	2.1	1.7	1.8	0.6	1.3	1.0	0.7
Serine	2.5	1.9	3.9	4.2	1.3	1.1	0.9	1.5	1.5
Glutamate	21.0	22.9	36.5	47.7	47.2	53.1	23.5	18.8	51.9
Proline	10.5	6.0	0.7	8.5	2.7	14.9	—	2.8	8.9
Glycine	3.4	1.5	3.8	1.8	0.3	1.3	1.2	1.9	1.3
Alanine	22.4	38.6	28.8	25.1	34.9	16.5	46.7	54.5	22.3
Valine	6.9	5.0	4.3	1.3	1.3	1.7	4.2	2.4	1.9
Methionine	1.4	0.4	1.4	0.4	0.2	0.8	0.7	1.8	0.7
Isoleucine	3.5	1.4	2.4	0.9	0.4	0.8	1.7	1.4	0.8
Leucine	7.0	3.7	3.2	1.2	0.6	1.2	1.9	2.1	1.2
Tyrosine	0.3	0.3	1.1	0.2	0.1	0.4	0.5	0.8	0.7
Phenylalanine	3.9	2.8	1.5	0.5	0.1	0.9	1.1	1.0	1.8

[a] Numbers in parentheses indicate total amino acid pool, expressed as micromoles per gram (dry weight).

[b] Results expressed as moles per 100 moles.

tained 80 to 100 μmoles per g of cells, a value that is at the lower end of the range found in other bacteria (8). The large proportion of glutamic acid and alanine is surprising, although similar data have been presented by Bent (2) on the amino acid pools of several fungi. The possibility that large quantities of these two amino acids have a unique function in sporulating microorganisms is being pursued further. Because of the methods used, it was impossible to determine the configuration of glutamic acid and alanine. The possibility of a large proportion of the D-isomers should be considered.

In general, pool sizes change during sporulation. For this reason, care must be taken in interpreting data on amino acid incorporation into spore components, as intracellular specific activities will vary with time.

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