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Differential Amino Acid Requirements for Sporulation in *Bacillus subtilis*

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The amino acid requirements for sporulation were studied by use of auxotrophic mutants of *Bacillus subtilis* 168. Cells were grown to T_0 in medium containing the test amino acid and were then transferred to a minimal medium lacking that amino acid. Omission of leucine caused no reduction in sporulation. Omission of methionine, lysine, and phenylalanine appeared to cause reduced levels of sporulation, and sporulation was completely inhibited when isoleucine, tryptophan, and threonine were omitted. The amino acids in this third class showed a sequence of requirements, with tryptophan required earlier than isoleucine, which in turn was required earlier in the sporulation process than threonine. Isoleucine omission did not affect the early sporulation functions of extracellular protease formation or septum formation, but prevented the increased levels of protein synthesis and oxygen consumption that normally accompany early sporulation stages. Isoleucine did not appear to be metabolized to other compounds in significant amounts during sporulation. The role of isoleucine in the sporulation process remains unclear.

In recent years, simple, model systems of intracellular differentiation have been sought for use in studying the basic concepts of developmental biology. One such system that has become increasingly popular is the process of endospore formation in *Bacillus* species. Our investigation of this system has been concerned with the change in the role of certain metabolites during the time when sporulation is first initiated; of special interest in the present study was the role of amino acids.

It has been known for some time that extensive protein turnover is initiated at the time sporulation begins. Foster and Perry (8) showed that shortly after cells of *Bacillus* species initiated spore formation in medium, they could be suspended in distilled water and complete the process normally. They termed the process endotrophic sporulation and used it as evidence that essentially all of the amino acids necessary for spore synthesis were capable of being derived from proteins existing in the cells at the time of the initiation of that process. Direct measurements showed that 76% of the ^{35}S of labeled methionine in vegetative cells was liberated during sporulation. Monro (16) calculated that at least 50% of the cellular proteins were involved in turnover during sporulation; the extent of recycling was not calculated. Halvorson (9) estimated that, at a minimum, 80% of the spore proteins were synthesized de novo. Allowing for recycling and redistribution of radioactivity from one amino acid into another, he proposed that the value could approach 100%.

The rationale behind our experiments has been as follows. Because a large proportion of the protein synthesis coupled to spore formation is actually derived from turnover of vegetative proteins, the amino acid requirements of *B. subtilis* auxotrophic mutants might be expressed only during vegetative growth. If the requirements for any amino acids were also expressed during spore formation, or if a differential requirement for some amino acids was observed between vegetative growth and sporulation, the effect might be exploited as a tool to explore newly induced pathways for amino acid utilization unique to spore synthesis.

A previous publication by Jicinska (11) described research based on a similar rationale. She reported that, with various auxotrophic mutants of *B. subtilis*, growth-limiting concentrations of some amino acids could support vegetative growth and subsequently provide for induction of sporulation. The extent of

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sporulation in some cases was related to the concentration of free amino acid present. If the amino acid concentrations were too low, no sporulation occurred. Although a lysine auxotroph would grow vegetatively in suboptimal concentrations of the amino acid, it would not sporulate unless grown at the optimal concentration.

In her studies, sporulation was not initiated synchronously, but rather in the gradual manner of a cell population ending exponential growth in complex medium. We have sought a better method to determine which amino acids are essential at the time sporulation is initiated. The system chosen was that described by Sterlini and Mandelstam, in which sporulation is initiated synchronously by first growing *B. subtilis* in a complete growth medium and then transferring to a minimal medium to induce stationary-phase metabolism more synchronously (23).

In our experiments, this double-media technique was used to study the effects of several amino acids on sporulation. Use of the chemically defined resuspension medium has the advantages of making it possible to control the amount of free amino acid present when sporulation is initiated, of not interfering with vegetative growth, and of synchronizing the induction of sporulation. Some auxotrophic requirements are shown to function only during vegetative growth; others are essential for early spore synthesis but function over different time periods.

MATERIALS AND METHODS

Bacteria. *B. subtilis* 168 U1 biotin$^-$SM^R was a *leu*$^-$, *ile*$^-$, *met*$^-$, *bio*$^-$ auxotroph resistant to 200 μg/ of streptomycin/ml. It was derived from the Mu8u5u1 strain of Sueoka by K. Bott. *B. subtilis* strains GSY 254 (*lys*$^-$, *trp*$^-$), GSY 225 (*phe*$^-$, *trp*$^-$), and GSY 261 (*thr*$^-$) were all obtained from C. Anagnostopoulos.

Media. The growth and sporulation (resuspension) media used in this work were those described by Sterlini and Mandelstam (23). In each case, they were supplemented with appropriate amino acids when necessary to satisfy an auxotrophic requirement. An overnight culture of the bacteria shaken in 5 ml of the growth medium at 37 C was started from a stock of spores that had been heat-shocked for 10 min at 80 C. Enough of the overnight culture was added to 95 ml of the growth medium (in a 1-liter Erlenmeyer flask) to bring the initial absorbancy to approximately 0.1 as measured with a Bausch & Lomb Spectronic-20 colorimeter at 500 nm. This culture was then incubated on a reciprocal shaker at 37 C (120 to 130 rev/min). Samples of 3 ml were removed every 0.5 hr to follow the absorbancy. When the growth reached 0.7 at A_{500} (late exponential phase), 10- to 15-ml samples of the culture were filtered through sterile filters (47-mm HA, 0.45 μm; Millipore Corp.). The filters with the trapped cells were then placed in 250-ml flasks containing 10 to 15 ml (equal volume) of the resuspension medium, with or without the appropriate amino acids (depending on the experiment) at 40 μg/ml or biotin to 0.1 μg/ml. Alternatively, the samples were centrifuged at 10,000 rev/min for 10 min, and the bacteria were resuspended in an equal volume of the resuspension medium with or without amino acids. The time at which the medium was switched to induce sporulation is referred to as T_0. These sporulating cultures were then shaken for 18 to 24 hr at the same speed and temperature as before to allow completion of the sporulation process. Some variability in sporulation efficiency of the control cultures was noted between experiments; consequently, a control was run with each experiment. In evaluating the data, we compared the results within a particular experiment rather than making direct comparisons between experiments.

Amino acid pulse technique. When a pulse of a particular amino acid was tested, the cells underwent the same treatment as before, but upon resuspension they were first placed in medium containing the amino acid being tested, for 1 or 2 hr, and were then filtered or centrifuged again and resuspended in an equal volume of resuspension medium lacking the test amino acid. During and after the pulse, the cultures were shaken as usual and, after 18 to 24 hr, the amount of sporulation was determined.

Determination of sporulation. Total viable units in the cultures at the end of the experiments were determined by suitable dilution in 2× Davis medium without glucose (Difco; hereafter referred to as 2× Davis salts) and plating on Tryptose blood agar base (Difco). Heat resistance was determined by heating a sample of the undiluted culture at 80 C for 10 min and plating appropriate dilutions in 2× Davis salts on the Tryptose blood agar base. Sporulation was expressed as the percentage of total colony-forming units present which survived heating at 80 C for 10 min. Since each strain used had a different efficiency of sporulation in the presence of all of its required amino acids, and because of slight variations in sporulation efficiency from experiment to experiment, controls were always run simultaneously with each starvation experiment. This variability in sporulation efficiency was not alleviated by use of isogenic strains, so the research was continued without converting all markers to the same strain.

Oxygen uptake measurement. Experiments involving the oxygen electrode proceeded as above, except that after the 3-ml samples were used for absorbancy readings, their respiratory activity was determined with a YSI 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio), either directly or after appropriate dilution of highly active samples into 2× Davis salts. This modified polarographic method of monitoring oxygen consumption compares the rate at which oxygen is removed from medium containing cells to that of a blank of uninoculated diluent. Samples were taken every 0.5 hr during growth and at 0.5-hr intervals for 3 hr after resuspension. Results were expressed as microliters of dissolved oxygen utilized per minute

by the culture, with the necessary correction made for dilution.

Protein labeling technique. At the time of resuspension, 5 μCi (specific activity, 15 Ci/mmole) of tritiated leucine was added to each 20-ml sample of the resuspension medium used. Samples of 0.5 ml were taken every 15 min after resuspension and added to 0.5 ml of 10% trichloroacetic acid. The samples were placed in an ice bath for 30 min and then in a water bath at 90 C for 10 min. The precipitated protein in the samples was filtered through 25-mm filters (HA, 0.45 μm; Millipore Corp.) which had been presoaked in 5% trichloroacetic acid. The filters were washed with 5% trichloroacetic acid several times and finally with 95% ethanol; they were then dried and placed in scintillation fluid (100 mg of 1,4-bis-2-[5-phenyloxazolyl]benzene and 3 g of 2,5-diphenyloxazole per liter of toluene) for counting in a Packard Tri-Carb scintillation spectrometer. The amount of radioactivity was assumed to be proportional to the amount of protein synthesized during the time of labeling.

Radioactive pulsing and protein electrophoresis. Cells were grown to T_0 as usual. Three 30-ml volumes were filtered and resuspended in an equal volume of resuspension medium. To one was added 50 μCi of ^{14}C-isoleucine (specific activity, 300 mCi/mmole) plus the normal components of a control culture with isoleucine at 40 μg/ml. To a second was added isoleucine as above but in the presence of 50 μCi of ^{14}C-leucine. The third resuspension medium was devoid of isoleucine but contained 50 μCi of tritiated leucine. At 2 hr after resuspension, all except 4 ml of culture was removed from the growth flask for experimental determinations; the remaining 4 ml was shaken for 24 hr as a control to monitor the efficiency of sporulation. Previous experiments had shown that the differences in aeration between a 30-ml culture and a 4-ml culture did not affect the final sporulation frequency. A 5-ml amount of each culture was centrifuged, and the pellet was used for cell fractionation experiments and chromatograms (see below); 1 ml of each was centrifuged to provide cells for electron microscopy. The remaining 20 ml was centrifuged at room temperature for 10 min at 10,000 rev/min, resuspended in 2 ml of resuspension medium lacking amino acids, and then sonically disrupted in the cold with 2-min pulses by an MSE sonic oscillator (Measuring & Scientific Equipment Ltd., London). The soluble and particulate portions of the extract were separated by centrifugation for 30 min at 30,000 × *g*. The supernatant fluids were dialyzed against Kornberg buffer (7) and stored at −20 C until solubilization for electrophoresis. The solubilization of the samples and the sodium dodecylsulfate (SDS) gel electrophoresis were carried out according to the methods of Laemmli (14). The acrylamide gels were frozen at −70 C and cut with stacked razor blades into 1-mm slices; each slice was dissolved with 0.7 ml of Biosolve (Nuclear-Chicago Corp.) for 1 hr at 70 C. Toluene-based scintillation fluid and quantitation of radioactivity were the same as described above.

Cell fractionation and chromatography. The cell pellets from 5 ml of culture, grown in the presence of radioactive amino acids as described above, were fractionated according to the procedure of Roberts et al. (19). The radioactivity in each fraction was determined.

The protein fraction was hydrolyzed with 1 ml of 6 N HCl at 100 C in a sealed vial. The neutralized hydrolysates were mixed with cold carrier amino acid corresponding to the radioactive amino acid present, spotted on separate thin-layer cellulose chromatography plates, and subjected to two-dimensional chromatography. The first solvent was butanol-acetone-diethylamine-water, 70:70:14:35, and the second was isopropanol-formic acid-water, 40:2:10 (22). The plates were then placed in a cassette in contact with a photographic film to expose the spots corresponding to the location of radioactivity.

RESULTS

Amino acid starvation after exponential growth. Starving *B. subtilis* U1 bio$^-$ at T_0 for its three required amino acids simultaneously caused a block in the sporulation process such that only 2% sporulation was observed after 24 hr. The starved cultures did not lyse extensively and could be induced to continue the sporulation process efficiently even up to 8 hr later if the amino acids were readded to the culture. When this organism was starved for each of its amino acid requirements singly, the data summarized in Table 1 were obtained. A test of the number of spores present at the time of resuspension (an indication of asynchronous background sporulation in the growth medium) showed 7% relative to the control, so that all values shown, except those for the "no isoleucine" experiments, were significantly above this background level. The change from growth medium to sporulation medium by either the filtration or centrifugation method

TABLE 1. *Percent sporulation for the U1 strain of Bacillus subtilis when various components of resuspension medium were withheld*[a]

Conditions	Expt no.					Avg
	1	2	3	4	5	
Control	92	82	75	73	94	83
No isoleucine	1	1	10	2	10	5
No methionine	31	66	86	51	63	59
No leucine	68		121	74	79	86
No biotin	63	53	32	34	126	61

[a] Control values (sporulation in the presence of isoleucine, methionine, leucine, and biotin) are expressed as number of heat-resistant colony-forming units divided by number of colony-forming units times 100. Experimental values are expressed as number of heat-resistant colony-forming units divided by number of heat-resistant colony-forming units in the control times 100.

had no significant effect, as can be seen from Table 1. The data in the column for experiment 1 were obtained by the centrifugation method; those in other columns, by the filtration method.

The results clearly show that the omission of different amino acids during sporulation had very different results. Whereas the omission of leucine reduced sporulation an average of 14% or less (if one considers that starvation slightly increases the susceptibility of cells to autolysis), the omission of methionine or the vitamin biotin caused an average reduction of approximately 50%.

The reduction in frequency of sporulation among the second group of metabolites is real, but, because of variability in sporulation efficiency from experiment to experiment, many more repetitions would be required to make the actual percentage reduction a statistically accurate figure. For the present, we have chosen to postpone an interpretation of this type of effect. Suffice it to note that withholding certain metabolites causes a partial reduction in sporulation. Most interesting, however, is the complete blockage of sporulation in the absence of isoleucine (no significant sporulation above the background level).

Supplying isoleucine as a pulse before starvation. Subsequent experiments employed pulses of isoleucine to determine whether there was a particular time in the sporulation process when isoleucine was needed (Table 2). All results in Table 2 came from the use of the filtration method of resuspension so that as little time as possible was taken in making the transfers, thus keeping the actual length of the pulse more exact. For this reason, in all further experiments discussed in this paper only the filtration method of transfer was used. That this method of transfer is crucial was evident from one experiment with the centrifugation method, which showed a 1-hr pulse of isoleucine giving 65% sporulation (relative to control), which is much different from the results of Table 2.

TABLE 2. *Percent sporulation for the U1 strain of Bacillus subtilis when isoleucine was present in the resuspension medium for only the first stages of sporulation*[a]

Conditions	Expt no.							Avg
	1	2	3	4	5	6	7	
Control	123	127	102	118	83	70	94	102
No isoleucine	0.2	1	0.5	20	9	14		7
1-hr isoleucine	0.6	0.5	0.5	21	29	28	7	12
2-hr isoleucine		83	52	171	126	55	54	77

[a] Percentages for control and experimental conditions are expressed as in Table 1.

Values for sporulation greater than 100% in this and subsequent experiments may result from the fact that *B. subtilis* tends to grow in chains. Such results are not uncommon in experiments with sporulating organisms (17). A chain only appears as one colony in a viability count, but, when heat-shocked, the chains are broken, liberating several sporulated cells and thus making it appear that more spores exist than there are viable cells. Although withholding a particular amino acid always resulted in the same relative effect, the exact frequency of sporulation can be seen to vary from experiment to experiment. This variability is currently unexplained, because it was even noted when samples of the same batch of medium were used in successive experiments.

Control cultures were 80% viable (viable-cell count after 24 hr with respect to viable-cell count at T_0), whereas cultures that had been starved for amino acids were, on the average, 50% viable. Foster and Perry noted that lysis accompanied transfer in their experiments with endotrophic sporulation (8). Ramaley and Burden (18) noted similar effects when they used the replacement technique to study sporulation. This loss of viability during the sporulation process prompted the method of expressing sporulation in all experimental cultures as a percentage of the control sporulation. Such results seem to indicate that when cells do not sporulate, they slowly lyse as one would expect in a normal death phase of cell growth.

Table 2 clearly indicates that a 2-hr pulse of isoleucine immediately after T_0 restored the sporulation level to nearly that of the control. Since a 1-hr pulse had no such effect, this amino acid requirement must be exerted some time later than the first hour after T_0 or for the entire 2-hr period. That this time of isoleucine requirement is a fairly narrow interval is evident from the result mentioned above that was obtained with the centrifugation method of resuspension. That method showed sporulation to be restored by a 1-hr pulse, in contrast to the 2-hr pulse shown to be necessary with the filtration method. This can be explained by the fact that, during the 15 min required to transfer the cells to the "no isoleucine" medium after the pulse, the time of isoleucine requirement is passed with the isoleucine still present during the centrifugation. However, in the 5 min required by the filtration transfer

method, the requirement period would be passed with the isoleucine already absent after the 1-hr pulse. This tends to indicate that isoleucine is necessary for sporulation processes at a time shortly after the first hour following resuspension. Isoleucine seems to be involved in some process occurring at this time that is vital to sporulation, and either isoleucine is not needed after this for sporulation to be completed or it is required only in the smaller amounts that protein turnover supplies. The size of the isoleucine pool was not estimated in our experiments, but Bernlohr (5) has shown that between vegetative growth and sporulation of *B. licheniformis* there is a 44% reduction in the size of the isoleucine pool. Coincidentally, he showed a similar 44% reduction in the pool size of leucine, an amino acid whose absence we have shown presents less drastic alterations in the sporulation pattern of *B. subtilis*.

Other auxotrophs starved and pulsed before starvation. Table 3 summarizes the experiments with GSY 254, a lysine and tryptophan auxotroph. Lysine deprivation resulted in a slightly reduced sporulation frequency. However, the omission of tryptophan resulted in levels of sporulation barely above background, similar to those seen with isoleucine. Pulses of tryptophan produced patterns of sporulation different from those obtained with pulses of isoleucine. Although a 2-hr pulse restored full sporulation, a 1-hr pulse of tryptophan restored a great deal more sporulation than a 1-hr pulse of isoleucine did (Table 2). This may indicate that the time of requirement for tryptophan in the sporulation process is slightly earlier than for isoleucine, that tryptophan acts more efficiently, or that it penetrates the cell more readily.

Table 4 summarizes the experiments with GSY 225, a phenylalanine and tryptophan auxotroph. Phenylalanine can be put into the same category as methionine and lysine with respect to the "reduced" level of sporulation resulting from its omission. The fact that the tryptophan marker in this strain is the same as that in GSY 254 serves only to confirm the results for tryptophan found with the previous strain.

With GSY 261, a threonine auxotroph, control levels of sporulation were too low to give the results great significance, but they do suggest that threonine is in the category of isoleucine and tryptophan as an amino acid whose presence is required for significant levels of sporulation (Table 5). Moreover, the pulse data suggest that threonine is required at a time in the sporulation process different from either isoleucine or tryptophan, since the full 2-hr pulse restored less than half of this strain's ability to sporulate.

Detailed analysis of the effects of isoleucine starvation. Once these differences between the various amino acids were noted, isoleucine was chosen as the single amino acid for more detailed study in an attempt to learn more about its specific role in the sporulation process. It was chosen because the previous experiments done with the isoleucine auxotroph had been found to be highly reproducible, because the efficiency with which the isoleucine auxotroph sporulated in the presence of all amino acids was high, and because com-

TABLE 3. *Percent sporulation for the GSY 254 strain of Bacillus subtilis*[a]

Conditions	Expt no.							Avg
	1	2	3	4	5	6	7	
Control	106	110	99	66	119	89	103	99
No lysine	91	52	103	69	65	21	69	67
No tryptophan	20	0	25	20	7	5	17	11
1-hr tryptophan			66	82	54	41	52	59
2-hr tryptophan			125		61	125	110	105

[a] Percentages for control and experimental conditions are expressed as in Table 1.

TABLE 4. *Percent sporulation for the GSY 225 strain of Bacillus subtilis*[a]

Conditions	Expt no.			Avg
	1	2	3	
Control	115	107	106	109
No phenylalanine	52	51		52
No tryptophan	17	9	1	9
1-hr tryptophan		53	24	39
2-hr tryptophan		132	70	101

[a] Percentages for control and experimental conditions are expressed as in Table 1.

TABLE 5. *Percent sporulation for the GSY 261 strain of Bacillus subtilis*[a]

Conditions	Expt no.					Avg
	1	2	3	4	5	
Control	37	23	76	13	34	37
No threonine	6	2	0.5	3	1	3
1-hr threonine	1	2		2	2	2
2-hr threonine		13		21	8	14

[a] Percentages for control and experimental conditions are expressed as in Table 1.

plete blockage of sporulation resulted when isoleucine was omitted from resuspension media.

Protein synthesis. To determine whether protein synthesis was necessary for the effect of isoleucine on sporulation to occur, chloramphenicol was added during the duration of the isoleucine pulse. The results in Table 6 clearly show that when protein synthesis was shut off by the drug in the presence of isoleucine during the first several hours of sporulation, the normal restoration of sporulation by a 2-hr pulse was prevented. The data may be interpreted to mean that the isoleucine effect is mediated through protein synthesis at some point.

The role of isoleucine in the total protein synthesis of the cell during the period right after resuspension is shown in Fig. 1. Although the amount of protein synthesis seems to be roughly the same in the presence or absence of isoleucine during the first hour, the control continued to rise, reaching a value more than 1.5 times that achieved in the other two cultures. It is obvious that, even in the absence of exogenous isoleucine, significant protein synthesis occurs, likely made possible by protein turnover, but the presence of isoleucine allows for the much greater synthesis apparently needed after the first hour to give sporulation. The presence of isoleucine for only the first hour had no significant effect. The plateau reached does not necessarily mean that protein

TABLE 6. *Percent sporulation for the U1 strain of Bacillus subtilis when isoleucine and chloramphenicol were both present*[a]

Conditions	Expt no.		Avg
	1	2	
Control (2.5-hr isoleucine)	63	68	65
No isoleucine		15	15
Chloramphenicol (50 μg/ml) + isoleucine	62		62
Chloramphenicol (100 μg/ml) + isoleucine	0.3	3	2
Chloramphenicol (200 μg/ml) + isoleucine	0	0.5	0.3

[a] Cells were grown to T_0 as usual and filtered into the five cultures indicated, with the chloramphenicol added 15 min before the isoleucine to allow the drug time to stop all protein synthesis before isoleucine could act. At the end of 2.5 hr, the four cultures containing isoleucine were filtered and resuspended in an equal volume of medium with no isoleucine. Percentages for control and experimental conditions are expressed as in Table 1.

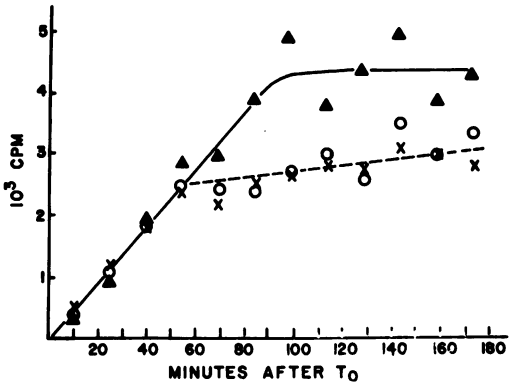

FIG. 1. *Total cell protein synthesis in strain U1 bio⁻ during the first 3 hr after resuspension. Cells were placed in normal resuspension medium plus radioactive leucine and treated as described in Materials and Methods. A control (Δ) had isoleucine present throughout, another culture (×) had no isoleucine present, and a third culture (O) had isoleucine present for only the first hour after resuspension.*

synthesis has stopped or that the radioactivity cannot enter the cell, but rather that total protein degradation and synthesis are in balance after the initial burst of synthesis. We know that amino acids can enter the cell because a pulse of isoleucine between 1 and 2 hr will restore sporulation.

Table 7 shows that when isoleucine was present during the first 2 hr after resuspension radioactive isoleucine was significantly (71%) incorporated into the protein fraction. Relatively less of the isotope was found in other cellular fractions (12% in the cold trichloroacetic acid-soluble fraction and 21% into the alcohol-soluble fraction). By contrast, when radioactive leucine was used in the presence of isoleucine, much less protein became labeled (41.5%) and more incorporation into the alcohol-soluble fraction was detected (47.2%).

In control experiments in which isoleucine was withheld, radioactive leucine was incorporated to the same extent into the protein fraction, suggesting that about the same relative amount of protein was synthesized, but slightly less alcohol-soluble fraction material was made. These results indicate that the presence of isoleucine slightly stimulates leucine incorporation into the alcohol-soluble fraction. Possibly this reflects nonspecific stimulation or general protein incorporation into alcohol-soluble proteins and lipids. However, isoleucine appears to be important for the synthesis of some portion of the protein fraction.

TABLE 7. *Cell fractionation according to method of Roberts et al. (19)*

Fraction[a]	Total radioactivity/fraction (counts/min)		
	Control with isoleucine		No isoleucine + ^{14}C leucine
	+ ^{14}C-isoleucine	+ ^{14}C-leucine	
1. Original whole cells	551,260 (100%)	72,236 (100%)	63,930 (100%)
2. Cold trichloroacetic acid-soluble	66,760 (12.1%)	3,792 (5.4%)	4,612 (7.2%)
3. Alcohol-soluble	117,050 (21.3%)	34,068 (47.2%)	26,100 (40.8%)
4. Alcohol-ether-soluble	8,210 (1.5%)	492 (0.7%)	836 (1.3%)
5. Hot trichloroacetic acid-soluble	25,460 (4.6%)	3,884 (5.3%)	4,680 (7.3%)
6. Protein (resuspended in acidified alcohol)	392,220 (71.1%)	30,000 (41.5%)	27,702 (43.3%)

[a] 2. Transient cell intermediates; 3. Lipid and alcohol-soluble protein; 4. Lipid and alcohol-insoluble protein; 5. Nucleic acids; 6. Proteins.

Radioautographs of the protein hydrolysates showed only a single radioactive spot corresponding to the amino acid added. Therefore, during the labeling period isoleucine was not significantly converted into other amino acids.

Figure 2 shows the electrophoretic mobility of soluble proteins in extracts of cells that had been labeled for 2 hr after resuspension. In the absence of isoleucine, labeled leucine was incorporated almost as efficiently as in its presence. Several distinct differences in the electrophoretic band pattern were observed when these extracts were compared to those of cells labeled with leucine in the presence of isoleucine. The most significant differences between the two are marked by arrows on Fig. 2. The isoleucine appears to stimulate incorporation into more than one small-molecular-weight soluble protein during the 2 hr following T_0, whereas these proteins are either not made in the absence of isoleucine or are complexed in such a manner that they have a different electrophoretic mobility.

Spectrophotometric assays for protease activity in the culture supernatant fluids suggest that in the absence of isoleucine nearly as much activity was produced as in the presence of the amino acid. The induction of protease activity, recognized as an early, spore-specific process (9), appears to be unaffected by the absence of isoleucine. Electron microscope studies in which whole cells were stained with phosphotungstic acid according to the procedure of Ryter (20) showed no detectable differences in morphology between starved and unstarved cultures. Prespore septa were observed both with and without the amino acid present.

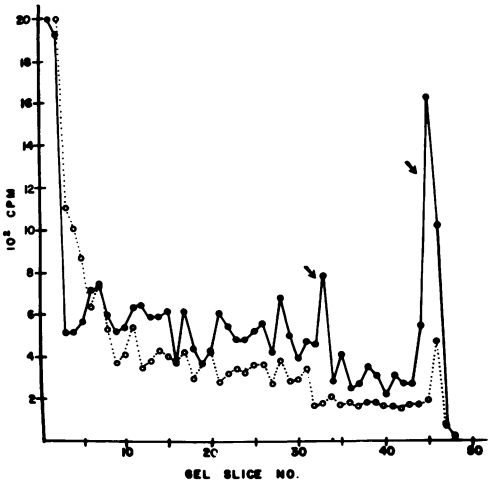

FIG. 2. *Electrophoretic mobility of proteins in dialyzed supernatant fractions of cells extracted after 2 hr in resuspension medium. Bands are revealed by radioactivity measurements of 1-mm slices of gels after SDS gel electrophoresis. Cells were resuspended in either normal resuspension medium containing isoleucine plus 50 μCi of ^{14}C-labeled leucine (●) or in medium lacking isoleucine and containing 50 μCi of ^{3}H-labeled leucine (○). After samples were treated as described in Materials and Methods, the two samples were mixed and run on the same gel; the radioactivity due to each label in each 1-mm slice of gel was determined.*

These two results suggest that isoleucine has no effect on the very earliest sporulation functions.

Respiratory activity. Accompanying the early induction of spore-specific enzyme in

wild-type sporulating cells is a dramatic increase of oxygen consumption (17). A typical result for an oxygen consumption experiment is shown in Fig. 3. After rising steadily during the growth phase, at T_0 when the cells were transferred to resuspension medium, the consumption of oxygen dropped sharply. In the control, oxygen uptake again rose, reaching a peak between 1 and 2 hr after resuspension. In the absence of isoleucine, consumption declined to a stable level after apparently beginning the rise normally. In the pulsed cultures, the uptake of oxygen rose as long as isoleucine was present, but dropped sharply after it was removed. The oxygen consumption peak between 1 and 2 hr after resuspension may, therefore, be caused by, or be essential for, isoleucine to exert its restoration of sporulation with a 2-hr pulse. The smaller peak seen in the "no isoleucine" culture is unexplained but could be caused by residual isoleucine from the growth medium that can be used to start a rise in oxygen uptake. If this interpretation is correct, the residual isoleucine must be quickly depleted so that there is none present to give the peak in oxygen uptake that accompanies

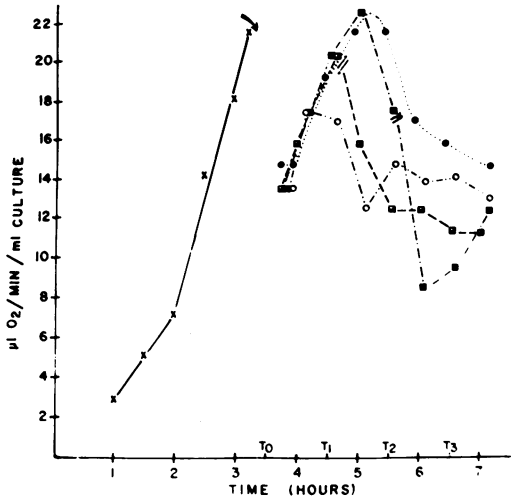

FIG. 3. *Rate of oxygen consumption of whole cells in growth medium and subsequent early stages of sporulation in resuspension medium. The solid line* (×) *indicates vegetative growth. At the arrow, the culture was divided into four equal fractions, and each was filtered and replaced in a different resuspension medium.* (●···●) *Control, isoleucine present;* (O···O) *no isoleucine present;* (◪– – –◪) *isoleucine present for first hour after resuspension (break in line, -/ /-, indicates removal);* (■ -·-·- ■) *isoleucine present for first 2 hr after resuspension (break in line, -/ /-, indicates removal).*

sporulation between the first and second hours.

Oxygen uptake experiments with the other amino acid auxotrophs indicate that, for the amino acids which are not absolutely required for sporulation (methionine, leucine, lysine, and phenylalanine), the peak of oxygen consumption occurred between the first and second hours after resuspension, as in the control cultures. In similar studies utilizing amino acids which are required absolutely for sporulation (isoleucine, tryptophan, and threonine), no such peak occurred. This supports the correlation between sporulation and oxygen consumption established for isoleucine.

DISCUSSION

Our results suggest that the metabolites tested might be divided into three categories according to the effect their absence has on sporulation. Leucine falls into the first category, causing no reduction in sporulation when absent. Methionine, lysine, phenylalanine, and the vitamin biotin may fall into a second category for which absence causes a reduced level of sporulation. Lysine might be expected to be required in large amounts for sporulation because of its role in dipicolinic acid synthesis for the spores (2, 3). However, the GSY 254 lysine mutation is at a late step in the pathway such that dipicolinic acid synthesis would not be altered (C. Anagnostopoulos, *personal communication*). In reality, the significance of the partial reduction caused by this entire group cannot presently be interpreted. Isoleucine, tryptophan, and threonine are in a distinctly different third category, since absence of any of these amino acids causes complete inhibition of sporulation.

These results show the requirements for amino acids above the levels that the cell can supply itself by protein turnover. Thus, of all the amino acids tested in this study, leucine appears to be the only one which can be adequately supplied by turnover. The amino acids which are absolutely required for sporulation appear to be required at levels far above what turnover supplies during early stages of the process.

At this point, it is not known whether the more significant observation is that a leucine auxotroph can complete normal sporulation if its auxotrophic requirement is not met, or that omission of isoleucine, tryptophan, and threonine causes a subsequent block in normal spore development.

Several suggestions can be made as to the role of isoleucine in sporulation from the data

gathered in these experiments. The effect of isoleucine must be mediated by protein synthesis, as the chloramphenicol experiments demonstrated. Gel electrophoresis revealed that, although soluble intracellular proteins were made in a range of molecular weights without isoleucine, it is possible that at least one or two classes of proteins could be made in greater abundance in its presence. Total protein synthesis appears to be greatly inhibited by the absence of isoleucine, although leucine incorporation is not drastically altered. It is evident that isoleucine exerts its effect between the first and second hours after resuspension (Table 2), which is its period of requirement. This is also supported by observations that the early sporulation functions of septa formation and extracellular protease activity were unaffected by the absence of isoleucine.

This is the same period that the peak in oxygen consumption is seen—a result consistent with the work of Murrell (17). Ramaley and Burden (18) used a similar double-media technique to show that the peak specific activity for aconitase, fumarase, and isocitric dehydrogenase (enzymes of the Krebs cycle) occurs between 1 and 2 hr after resuspension in *B. subtilis* 168. That observation also agrees with the time for the peak of oxygen consumption noted in this study.

Several explanations have been considered for the cause of blockage in sporulation induced by a lack of amino acids in the isoleucine group, but, on the basis of data already discussed, none seems to provide an adequate interpretation of the data.

It seems unlikely that the different amino acids would permeate the cells to a different degree, since all amino acids in our study except for lysine are neutral. The permease which does exist (5) would not be expected to discriminate among them differentially.

It is possible that some proteins made during early stages of sporulation may be richer in certain amino acids than vegetative proteins, creating the differential amino acid requirements for spore formation seen here. The existence of such unique proteins has not been seriously considered before (9, 10, 21, 24) but certainly warrants closer scrutiny. It is well known that peptide antibiotics are produced by *Bacillus* species during early spore formation, although they have never received special attention as molecules controlling sporulation. Our analyses for antibiotic activity in supernatant fluids of isoleucine-blocked cultures to date have been inconclusive. The *B. subtilis* group of organisms produces several widely known antibiotics (1, 4, 17, 21, 24, 25). One is bacitracin, which contains three residues of isoleucine in a total of 12 amino acids per molecule (but no tryptophan or threonine); others are the cyclic peptides subtilin and mycobacillin (1, 4, 25). Subtilin contains isoleucine and tryptophan residues, but is equally rich in leucine. Mycobacillin contains no isoleucine, tryptophan, or threonine. In addition, the cyclic peptides may not be synthesized by the normal route of protein synthesis (1, 4) and may be insensitive to the action of chloramphenicol. It has been reported that the amino acid composition of antibiotics produced by the *B. subtilis* group varies depending on the strain and the growth medium (1). Consequently, the exact structure of any antibiotics being produced under our conditions is unknown, as is the relevance of antibiotic production to the block induced by withholding amino acids.

In this connection, it is worthy of note that Majumdar and Bose (15) found no correlation between the uptake of amino acids from the growth medium of *B. subtilis* and the production of an antibiotic against *Aspergillus niger* (mycobacillin). Their studies did reveal a preferential uptake of some amino acids depending on the phase of antibiotic fermentation, but no relation could be observed between the uptake during various time intervals and either the amount of antibiotic or the amino acid composition. These authors did not relate antibiotic production to the synthesis of spores.

Buono et al. (6) have shown that high concentrations of glutamic acid are required for sporulation of *B. cereus*, although very little is actually used. Bernlohr used the rate of carbon dioxide release as a measure of amino acid oxidation in *B. licheniformis* (5). His studies suggest that sporulating cells develop increased ability to oxidize glutamic acid, isoleucine, threonine, valine, and several other amino acids concomitant with the cessation of growth. It is, therefore, possible that the amino acids in our third category are serving as energy sources during sporulation. However, if the amino acids were being used as energy sources, one would expect to see the degradation of labeled isoleucine into other compounds in a significant amount, which our chromatograms or cell fractionation did not reveal. It is conceivable that, if direct oxidation to carbon dioxide were occurring, our assays would not have detected it. Further, our resuspension medium contains a fairly high concentration of glutamic acid; consequently,

one would expect it to be preferentially oxidized as the energy source. Further work is needed in this area, for isoleucine seems related to oxygen consumption by the fact that the consumption drops immediately after isoleucine removal.

Kane et al. (12, 13) have extensively documented a "metabolic interlock" in *B. subtilis* whereby enzymes of the aromatic pathway (including vitamins and amino acids) and the histidine pathway regulate synthetic activity in the terminal branches of these metabolic sequences to exhibit influence on apparently unrelated biochemical pathways. If isoleucine participates in such interactions to the same extent as tryptophan, or if all of the amino acids in our third category are related through metabolic interlock, the eventual inhibition of sporulation may be due to the same reaction.

At present, one can say that isoleucine absence prevents protein synthesis and the increased oxygen consumption that occur between the first and second hours after resuspension, which appear essential for sporulation to proceed. The relation between these two and the actual action of isoleucine remains to be elucidated. It is possible that isoleucine acts as a regulator of total protein synthesis, and, when this synthesis does not occur in the absence of isoleucine, the enzymes for the peak of oxygen consumption are not made. Following the labeled isoleucine's location in the cell right after entry may determine whether such regulation is the case.

The results shown here suggest that the period of requirement for each amino acid in the third class may be different. The experiments suggest that tryptophan is required earlier than isoleucine, which in turn is required at an earlier time in the sporulation process than threonine. Although the results of these experiments do not agree in some respects with those of Jicinska (11), the discrepancies may be accounted for by a difference in experimental technique. Her procedure may have interfered with normal vegetative growth, thereby altering the sporulation process and thus the amino acid requirements for that process.

The striking difference between the effects of the amino acids suggests the possibility that at least some of the early events of sporulation may be regulated at the substrate level by the abundance of essential amino acids.

Considerable work remains to trace the mode of action of isoleucine in sporulation, as well as that for tryptophan and threonine. The methods used here can prove of considerable value in those studies. Auxotrophic mutants used with the double-media technique allow one to obtain information on how requirements for different nutrients, that a wild type could synthesize itself, change during sporulation. This technique also permits one to control accurately the amount of each nutrient and to synchronize sporulation so that the temporal order of events and their relation to the times of action of the nutrients can be accurately determined. Such information can give much greater insight into the complex processes occurring during the early stages of this differentiation.

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