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Macromolecular Syntheses During Germination and Outgrowth of *Bacillus subtilis* Spores

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Alanine and glucose used jointly are known to be necessary and sufficient for spore germination in *Bacillus subtilis* 168. By testing them separately, we have verified that alanine provokes optimal phase-darkening of the spores but inhibits macromolecular syntheses, while glucose is specifically needed for initiating those syntheses. By using them in succession we obtained evidence suggesting that: (i) sporal modifications which lead to phase-darkening must occur before macromolecular synthesis can start; (ii) the amino acid pool, on which the early protein synthesis is solely dependent, expands during incubation in alanine which allows degradative but prevents synthetic activities; and (iii) progression of degradations in alanine not promptly followed by syntheses in glucose produce a metabolic imbalance in the germinating spore. A sharp transition in the origin of building blocks was shown by using a tryptophan-defective mutant. At first the synthesis of proteins depended on pre-existing amino acids from turnover of sporal material since it occurred in the absence of any exogenous amino acid and its rate remained unaltered by supplying either all amino acids except tryptophan or tryptophan alone. Eventually, protein synthesis became dependent strictly on exogenous tryptophan and strongly on the supply of several other amino acids, not required later during vegetative growth. Clearly, by the start of outgrowth, all building blocks must be provided either by endogenous de novo synthesis or by exogenous supply.

The degradative processes characterizing germination of bacterial spores are independent of macromolecular syntheses since they take place even in the presence of inhibitors of ribonucleic acid (RNA) and protein synthesis, such as chloramphenicol, puromycin, or actinomycin D (3, 17, 30, 34). Moreover, they occur even in a medium unable to carry out the complete development of a spore into a vegetative cell (6), provided that few factors, specific for each type of spore and designated as germinating agents, are present. On the other hand, the occurrence of macromolecular syntheses can be demonstrated very early in germinating spores, (1, 2-4, 11, 18, 28, 29, 30, 32, 35, 39) even in media lacking growth factors later required for cellular growth (2, 3, 18, 33). For instance a culture of *Bacillus cereus* T needs the external supply of several amino acids, yet its spores synthesize a limited amount of proteins without external supply of these amino acids within a few (2-5) minutes after being placed in buffer containing only the germinating agents. This is shown by their ability to incorporate a labeled amino acid, used as a tracer, into trichloroacetic acid-insoluble material. Gel electrophoretic analysis of extracts from these labeled spores showed that only a few species of proteins are produced unless complete medium is added (18, 32, 33, 36). This suggests the existence of an early phase of synthetic activity, which is self-sustaining as far as supply of building blocks is concerned and which is restricted, possibly specifically controlled, as far as genome expression is concerned (5, 30, 32). It has been surmised that this synthesis occurs by turnover, since the amino acid pool in spores is very small (only glutamic acid, arginine, and lysine were found; references 20, 21) and since the presence of germinating factors which trigger the degradative process is necessary. Similar conclusions were reached by studying RNA synthesis in spores of *B. megaterium* (27), *B. cereus* (32), and *B. subtilis* (2, 3, 30). To better define the self-sustaining nature of these early syntheses, we present in this paper data concerning: (i) the time relationship between early syntheses and phase-darkening (one of the earliest mani-

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festations of spore germination); (ii) the individual role in the early syntheses of each of two agents, alanine and glucose, needed for germination of *B. subtilis* 168; and (iii) the time at which external supply of tryptophan becomes necessary for the development of spores of a tryptophan-defective mutant into vegetative cells. Furthermore, we show that proteins synthesized late, but before the first division, are strongly dependent on exogenous supply not only of tryptophan but also of several other amino acids. This contrasts with the completely autonomous early syntheses and with those occurring during vegetative growth which are dependent on tryptophan alone.

MATERIALS AND METHODS

B. subtilis 168 Trp^- was the strain used in these experiments.

Media. The sporulation medium (26) contained in grams per liter: Difco nutrient broth, 8; $MgSO_4 \cdot 7H_2O$, 0.25; and KCl, 1. The pH was adjusted to 7.0 to 7.2 with NaOH. Added after sterilization were: $Ca(NO_3)_2$ to 10^{-3} M, $MnCl_2$ to 10^{-5} M, and $FeSO_4$ to 10^{-6} M.

TTK buffer. TTK buffer consists of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer at pH 7.4, containing 0.05% Tween 80 and 1% KCl.

Basal medium TG20 (7) contains in grams per liter: NaCl, 4.68; KCl, 1.5; NH_4Cl, 1.08; $MgCl_2$, 0.2; Na_2SO_4, 0.35; $CaCl_2$, 29×10^{-3}; $FeCl_3$, 0.5×10^{-3}; $ZnCl_2$, 0.27×10^{-3}; Tris base, 12. The pH was adjusted to 7.4 with HCl. After sterilization, KH_2PO_4 (6.4×10^{-4} M) and 0.5% glucose were added.

LB agar contains in grams per liter: tryptone, 10; yeast extract, 5; NaCl, 5; agar, 10; and 1 ml of 1 N NaOH.

Supplements. Supplements, as indicated for each experiment, were used at the following concentrations: glucose (GLC), ribose, and L-glucose (Calbiochem), 2 mg/ml; L-alanine (Ala), D-Ala, α-aminoisobutyric acid (AIBU) (Calbiochem), and α-amino-*n*-butyric acid (ABU) (Sigma Chemical Co.), 340 μg/ml; tryptophan (Trp), and all other amino acids (except those above) 40 μg/ml; casein hydrolysate (Nutritional Biochemicals Corp. and Difco), 500 μg/ml.

Preparation of spores. Growth and sporulation occurred in "sporulation" medium. Spores were then washed and cleaned by discontinuous density gradient centrifugation in Renografin (31). A 20-ml suspension of spores (no more than 300 mg) in water was centrifuged over a 5-ml layer of 62% Renografin (considering commercial Renografin as 100%) in a Spinco SW25 rotor for 60 min at 15,000 rpm. The pellet contained the refractile spores, while cells, debris, and phase-dark spores formed a layer at the interface. The refractile spores were washed five times with water to eliminate the Renografin. We have also used Renografin for separating phase-dark from phase-brilliant spores in a population only partially darkened. However, Renografin-treated phase-dark spores were no longer viable.

Clean spores suspended in water (1 mg/ml) were activated by heat shock, 60 min at 65 C for batch 12 and 20 min at 80 C for batch 19, then washed twice in water, and suspended at 10 mg/ml in ice-cold Tris buffer, pH 8, 0.2 M. Unless otherwise specified, the spores were diluted to 0.2 mg/ml in the appropriate media. Germination was performed at 41 to 42 C.

Incorporation of radioactive compounds. The radioactive compounds used were: ^{14}C-amino acid mix (1.5 μCi/mg; New England Nuclear Corp.) and ^{14}C-uracil (50 mCi/mmol; Schwarz). The ^{14}C-amino acid mix lacks tryptophan and contains all other amino acids at a concentration of about 0.004 mg/ml each. To the samples for the pulse incorporation experiments was added 1 μCi of radioactive material per ml, i.e., 670 ng of amino acid mix per ml or 2 nmol of uracil per ml. The samples for continuous incorporation contained 0.1 μCi of radioactive material per ml. The incorporation was stopped by adding 10 volumes of ice-cold 5% trichloroacetic acid containing 0.5% casein hydrolysate or 0.02% uracil. For co-precipitation, 0.05 ml of 0.2% bovine serum albumin was added per sample. When ^{14}C-proteins were measured, the trichloroacetic acid-treated, samples were heated at 85 to 90 C for 30 min. For ^{14}C-RNA determination, the heating was omitted. The trichloroacetic acid precipitate was finally collected on membrane filters and counted in a low-background gas-flow counter (Nuclear-Chicago Corp.). In pulse experiments, trichloroacetic acid was added to the entire sample (1 ml) at the end of the pulse. In continuous incorporation experiments, 0.1-ml samples of the incubation mix were removed at given time intervals and added to the trichloroacetic acid.

RESULTS

Influence of Ala or GLC, or both, on phase-darkening. Spores suspended in buffer and observed in a phase-contrast microscope appear refractile as long as specific factors required for their germination are absent. Among a number of substances known to produce phase-darkening and germination of *B. subtilis*, Ala and GLC are commonly used (8, 10, 12, 13, 23, 37, 38, 40). In the following experiments we studied the effect of these substances on the rate of phase-darkening measured as the percentage of the spore population which becomes phase-dark at any given time. Heat-activated spores of *B. subtilis* 168 were suspended in TTK, pH 7.4, and incubated at 41 C. In the absence of any germinating agent no phase-darkening occurred. With Ala and GLC or Ala alone, phase-darkening occurred at a rapid, constant rate until, after 1 h, practically all spores ($\simeq$90%) were phase-dark (Fig. 1). By contrast, with GLC alone phase-

darkening was very slow and came to a stop in about 1 h with only 10% of the spore population phase-dark. If, however, Ala was added at any time during incubation in GLC, the number of phase-dark spores increased rapidly and reached 90 to 100% of the population at a rate similar to that measured initially in Ala + GLC.

Clearly, the presence of Ala is sufficient for phase-darkening while that of GLC is dispensable. The Ala analogues, ABU and AIBU, were found as effective as Ala in producing darkening, whereas the enantiomorph D-Ala was neither a darkening agent nor an inhibitor when added together with Ala at the same concentration (Table 1). A mixture of all other amino acids mimicking the composition of casein hydrolysate (including tryptophan but excluding Ala) did not replace or stimulate the effect of Ala.

Since some phase-darkening occurred in the presence of GLC alone, the question arose whether GLC could replace Ala, albeit with less efficiency. By testing incubation in media adjusted at different pH, we have found that the plateau of phase-dark spores reached after 3 h in GLC alone was relatively high at low pH (about 40% at pH 6.5; Table 2) and became almost zero by increasing the pH (about 2% at pH 8.6). By contrast, the rate of phase-darkening in Ala was practically independent of the pH: the optimal pH of phase-darkening in Ala or in Ala + GLC was 7.4 to 7.6 and the rate decreased slightly (no more than 10%) on either side of the pH scale. Thus, the phase-darkening observed with GLC alone cannot be due to traces of Ala. We have not analyzed further the mechanism of phase-darkening, but we took advantage of these observations for analyzing the effect of Ala and GLC individually on early macromolecular syntheses as described below.

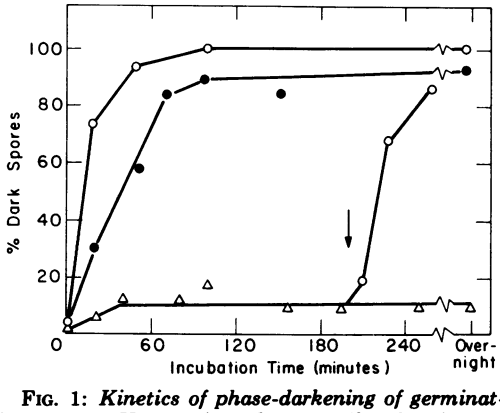

FIG. 1: *Kinetics of phase-darkening of germinating spores. Heat-activated spores (batch 19) were suspended in TTK containing: GLC + Ala, O—O; Ala, ●—●; or GLC, Δ—Δ. At the arrow, Ala was added to a sample of the suspension of spores incubated in GLC alone. The percent of the spore population becoming dark during incubation at 41 C was determined by examination of withdrawn samples in a phase-contrast microscope.*

TABLE 1. *Efficiency in spore-darkening of compounds related to Ala*

Compound	Initial rate[a] of darkening	Dark spores at 150 min (%)
None	0	3
Ala + GLC[b]	21	81
Ala[b]	12	78
ABU	10	88
AIBU	10	88
D-Ala	0	1
Ala + D-Ala (1:1)	11	79
Amino acid mix:[c]		
less Ala	0	3
plus Ala	10	80

[a] Time 0 of the experiment is the time of addition of the supplements to the heat-activated spores suspended in TTK. The numbers represent the increase in percentage of phase-dark spores between 10 and 20 min of incubation at 41 C.

[b] The values are the mean of 8 independent experiments.

[c] Mixture of all the amino acids mimicking the composition of casein hydrolysate, except Ala. Spores from batch 12 were used.

Conditions of initiation of macromolecular syntheses. As said in the introduction, it has been found that, at germination of spores of *B. cereus* and of other bacteria, the synthesis of a limited number of proteins takes place in media deficient for supporting vegetative growth. By using a Trp^- mutant of *B. subtilis* 168, we inquired specifically whether exogenous Trp was needed for synthesis of these early proteins. In the experiments presented in Fig. 2, ^{14}C-amino acid mix or ^{14}C-uracil was added to suspensions of activated spores in TTK at pH 7.4 containing either Ala alone or Ala + GLC. A control without germinating agents was also run in parallel. The samples were incubated at 42 C. Although Trp was absent, amino acids or uracil incorporation was found in the samples containing Ala + GLC, but not in the control (not shown) nor in Ala alone, although in this latter, phase-darkening was optimal. Thus, sporal protein synthesis occurred without exogenous addition of an amino acid not synthesized by the cell.

From Fig. 1 and 2 it appeared furthermore

TABLE 2. *Inhibition by Ala of amino acid incorporation*

pH of preincubation medium[a]	Dark spores at time of pulse (%)[b]	Pulse counts per min per ml		Inhibition by Ala of incorporation (%)[e]	Counts/min incorporated per phase-dark spores without Ala[f]
		Without Ala[c]	With Ala[d]		
6.5	36	2,397	858	64	6,650
7.6	11	747	339	55	6,600
8.6	2	119			6,000

[a] Preincubation: activated spores incubated for 3 h in Tris-Tween + K_2HPO_4 (0.19 M) + GLC; *p*H adjusted with HCl.

[b] The percentage of phase-dark spores does not increase during the 10-min pulse in GLC alone while it increases substantially during the pulse in GLC + Ala. For this reason, valid estimates of counts/min incorporated per phase-dark spores (reported in column 6) can only be calculated in the case of pulses without Ala.

[c, d] Pulse: at the end of the preincubation samples are diluted five times in TTK buffer *p*H 7.6 containing GLC (column 3) or GLC + Ala (column 4) and incubated for 10 min at 42 C with ^{14}C-amino acid mix. The spore concentration during the pulse is 0.2 mg per ml. The figures are counts of ^{14}C-amino acids incorporated into trichloracetic acid-precipitable material.

[e] The percentage of inhibition due to Ala is the difference between column (3) and (4) divided by column (3).

[f] Counts/min incorporated per phase-dark spores are calculated from column (3) × 100, divided by column (4).

that Ala and GLC had almost opposite effects. Ala provoked the best phase-darkening but did not support incorporation, and GLC was poorly efficient in promoting phase-darkening but was essential for incorporation. We took advantage of these contrasting effects of Ala and GLC to verify directly whether or not phase-darkening should precede macromolecular syntheses.

Activated spores were suspended in TTK buffer adjusted at different pH values, added with GLC alone, and incubated at 42 C for 3 h. At this time phase-darkening reached different plateaus ranging from 2 to 36% of the spore population (Table 2). The spores were then spun down, suspended in TTK at pH 7.4, and pulse-labeled for 10 min in the presence of either GLC alone or in GLC + Ala. The results indicated that: (i) incorporation was fastest in GLC alone; (ii) addition of Ala to GLC reduced the incorporation rate by about 60%, showing that Ala not only failed to support, but strongly inhibited incorporation; and (iii) incorporation was strictly proportional to the amount of dark spores present in the sample.

It was apparent that only the phase-dark spores were able to incorporate. This conclusion was confirmed by the following observations. During phase-darkening (for instance in a sample in GLC at pH 6.5), the rate of incorporation increased more than proportionally with time, until phase-darkening reached a plateau (in our example at about 40% darkening). However, if the incorporation was calculated as the percentage of phase-dark spores present at any time, then the incorporation appeared to increase linearly. Similarly, in a

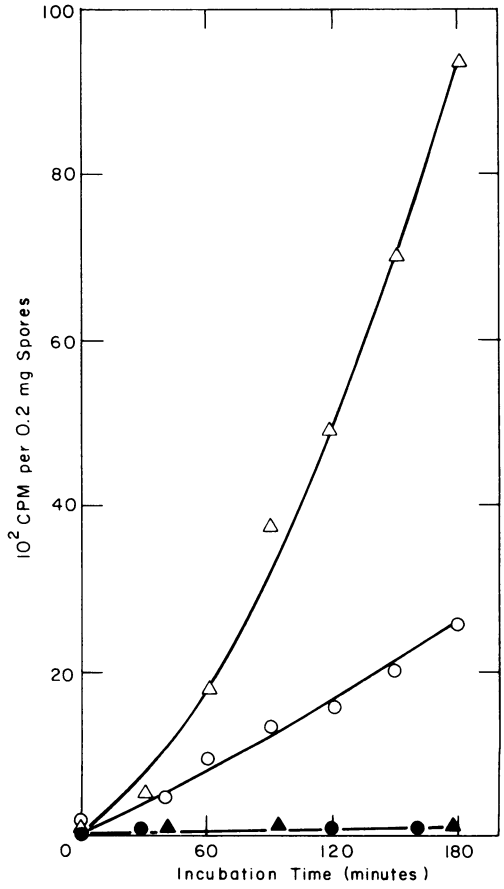

FIG. 2: *Kinetics of protein and RNA synthesis at spore germination. At time 0, spores suspended in TTK + Ala + GLC received ^{14}C-amino acids Δ—Δ or ^{14}C-uracil O—O. Spores suspended in TTK + Ala alone received ^{14}C-amino acids ▲—▲ or ^{14}C-uracil ●—●. At different times of incubation at 42 C, samples were withdrawn and counted.*

sample of spores already darkened (for instance, by preincubation in Ala + GLC), when suspended in GLC alone at pH 6.5, the incorporation increased linearly. Furthermore, since we find it possible to separate the phase-dark from the refractile spores by density gradient centrifugation using Renografin, we used this method to analyze a population of spores 40% phase-dark (in GLC + Ala at pH 6.5) and pulsed with ^{14}C-amino acids (1-h incubation). The ^{14}C counts were found entirely related to the phase-dark spores.

Effect of incubation in Ala alone. Tryptophan is not produced by the strain we use nor is the extent of amino acid pool found (20, 21) in dormant spores sufficient to account for the protein synthesis we observe at phase-darkening. It seems reasonable to suggest that the Trp needed for protein synthesis is provided by proteolytic degradation. We have seen, on the other hand, that protein synthesis occurs only in phase-darkened spores and that Ala promotes darkening, but inhibits synthesis. It might therefore be anticipated that, during incubation in Ala alone, the supposed proteolytic degradation should produce in the spore a piling up of Trp and (forcibly) of the other amino acids as well. Consequently, the preincubation in Ala alone is longer and the effect of the sporal pool in diluting the radioactive label is larger when protein synthesis is allowed by GLC addition (32).

We compared the effect of Ala or ABU or AIBU on the level of ^{14}C-amino acid incorporation when GLC was added after different times of incubation. Table 3, which gives the experimental details, shows that increasing times of incubation gradually reduced the rate of ^{14}C label incorporation. The figures obtained with equally efficient phase-darkening agents like Ala, ABU, AIBU (Table 1) were in close quantitative agreement, while incubation with substances related to Ala but unable to produce darkening, like D-Ala or all amino acids other than Ala, were ineffective. This result suggests that the amino acid pool increases during incubation in phase-darkening agents and is consistent with the assumption that proteolytic degradation occurs at phase-darkening and is the origin of building blocks needed for early protein syntheses. If, however, the decrease in radioactive amino acid incorporation were due solely to a dilution effect, the Ala preincubated spores should promptly complete their development into vegetative cells when transferred to a rich medium. Yet it could be shown, by placing spores treated or untreated with Ala on rich medium, that several additional hours of lag (about four) in developing a visible colony accompanied the Ala incubation.

Amino acid requirement during postgerminative development (outgrowth). The ability to synthesize proteins solely by turnover is limited to a relatively short period of time even under optimal conditions, i.e., in the simultaneous presence of Ala and GLC. We have determined the length of this time by incubating at 42 C two identical suspensions of activated spores in medium TTK (Ala and GLC added) with or without Trp. Samples were withdrawn during the incubation and the synthetic potential of the phase-dark spores present at that time was measured by determining the amount of radioactive label incorporated in a 5-min pulse. It was found (Fig. 3) that protein synthesizing activity in the phase-dark spores increases for about 1 h and then rapidly decreases.

Practically no difference was observed in the presence or in the absence of Trp, clearly indicating that Trp alone is not sufficient for further development of the germinated spores into outgrowth. In the following experiment we tested the effect of amino acids of casein hydrolysate on the synthetic ability of phase-darkened spores. Activated spores were germinated in TTK + Ala + GLC for 70 min, at which time the spore population was 90% phase-dark and the synthesis by turnover had reached a peak (Fig. 3). The spores were then centrifuged and transferred to medium TG20 containing 0.5 mg of casein hydrolysate per ml and further incubated at 42 C. During this incubation, samples were withdrawn and the synthetic activity was measured by giving a

TABLE 3. *Effect of preincubation in Ala or its analogues on the ^{14}C-amino acid incorporation*[a]

Time of preincubation (min)	Germinating agent		
	Ala	ABU	AIBU
0	7,400		
60	4,100	3,700	3,800
120	900	1,100	1,600
180	600	550	700

[a] At time 0 the germinating agent was added to the activated spores suspended in TTK, and the preincubation started. The preincubated spores were centrifuged and were resuspended in TTK with GLC and ^{14}C-amino acid mix at the times indicated. The numbers are counts/min incorporated in 1 h by 0.2 mg of dark spores.

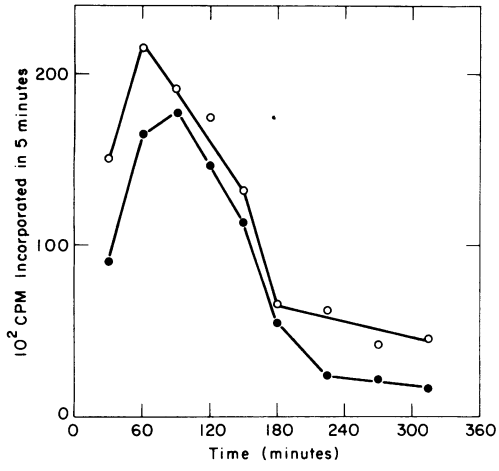

FIG. 3: *Variation in the rate of protein synthesis during germination. Activated spores were incubated at 42 C in TTK + GLC + Ala* (○—○) *or in TTK + GLC + Ala + Trp* (●—●). *At the incubation times given in the abscissa samples were withdrawn and pulsed with* ^{14}*C-amino acid mix for 5 min.*

5-min pulse of radioactive label. Two conditions were examined, i.e., the presence or the absence of Trp. The counts per minute and the optical density were determined simultaneously. The results are shown in Fig. 4. It is clear that protein synthesis steadily increased upon addition of casein hydrolysate and became dependent on the presence of Trp. Thus, it appears that during outgrowth protein synthesis is a constant function of the increase in cell mass, although the number of cells does not increase. In a separate experiment we compared the rates of protein synthesis during the self-sustaining synthesis in germinating spores and during exponential growth of vegetative cells. A 5-min pulse of radioactive label was given to a suspension of activated spores in medium TTK + Ala + GLC incubated at 42 C for 60 min, at which time the rate of protein synthesis by turnover reached its peak (Fig. 3), or to a culture exponentially growing in minimal medium TG20 (Trp added). The counts incorporated referred to the number of viable dark-spores or cells present per milliliter at the time of pulse. It was found that the rate of synthesis by turnover is 1 to 3% that of vegetative cells.

While the need for Trp during outgrowth was predictable in a Trp-defective mutant, the need for other amino acids deserved closer analysis, since they are not required later during vegetative growth of *B. subtilis* 168. In a preliminary experiment, activated spores were suspended in TG20 (GLC and Ala present), and four different conditions were tested: (i) no other additions; (ii) addition of casein hydrolysate free of Trp; (iii) addition of Trp; (iv) addition of casein hydrolysate and Trp. During incubation at 42 C it was found that in all four suspensions the optical density initially decreased (indicating initiation of germination). The subsequent increase in cellular mass, characteristic of growth, occurred after about 100 min in suspension (iv), after 6 to 7 h in suspension (iii), and did not occur at all even overnight (18 h) in suspension (i) and (ii).

The effect of each amino acid on the duration of outgrowth was tested by measuring the lag before increase in optical density of a suspension of spores germinated by Ala + GLC and then transferred into media TG20 + Trp to which the other amino acids were added singly. The effect of the same media on vegetative growth was tested by determining the doubling time of cells growing exponentially. The results are given in Table 4. It is seen that the complete mixture of amino acids contained in the casein hydrolysate and some amino acid added singly (arginine, proline, or glutamate) stimulated the onset of outgrowth and increased the rate of the vegetative growth. By contrast, serine, threonine, and cysteine were strong inhibitors of vegetative growth (doubling time) while isoleucine, lysine, valine, glycine, and aspartate completely inhibited outgrowth. Ala inhibited the vegetative growth by 40% but speeded up outgrowth. Notice that in the absence of all amino acids (except tryptophan which is always present), outgrowth was delayed indefinitely, while vegetative growth was only moderately affected. The doubling time is 70 min with casein hydrolysate and 90 min without.

DISCUSSION

Two phenomena occurring at initiation of germination were followed: (i) the darkening of the spore was observed in a phase-contrast microscope, and (ii) the synthesis of RNA and proteins demonstrable by the incorporation of a labeled amino acid or nucleic base used as tracer into sporal material precipitable with trichloroacetic acid. Of the factors known to be required for germination of *B. subtilis* 168, we studied the association of two, Ala and GLC, which are known (8, 10, 12–14, 25, 37, 38, 40) to be necessary and sufficient for promoting spore darkening and early macromolecular syntheses.

When Ala + GLC were present together a sequential order in the occurrence of phase-

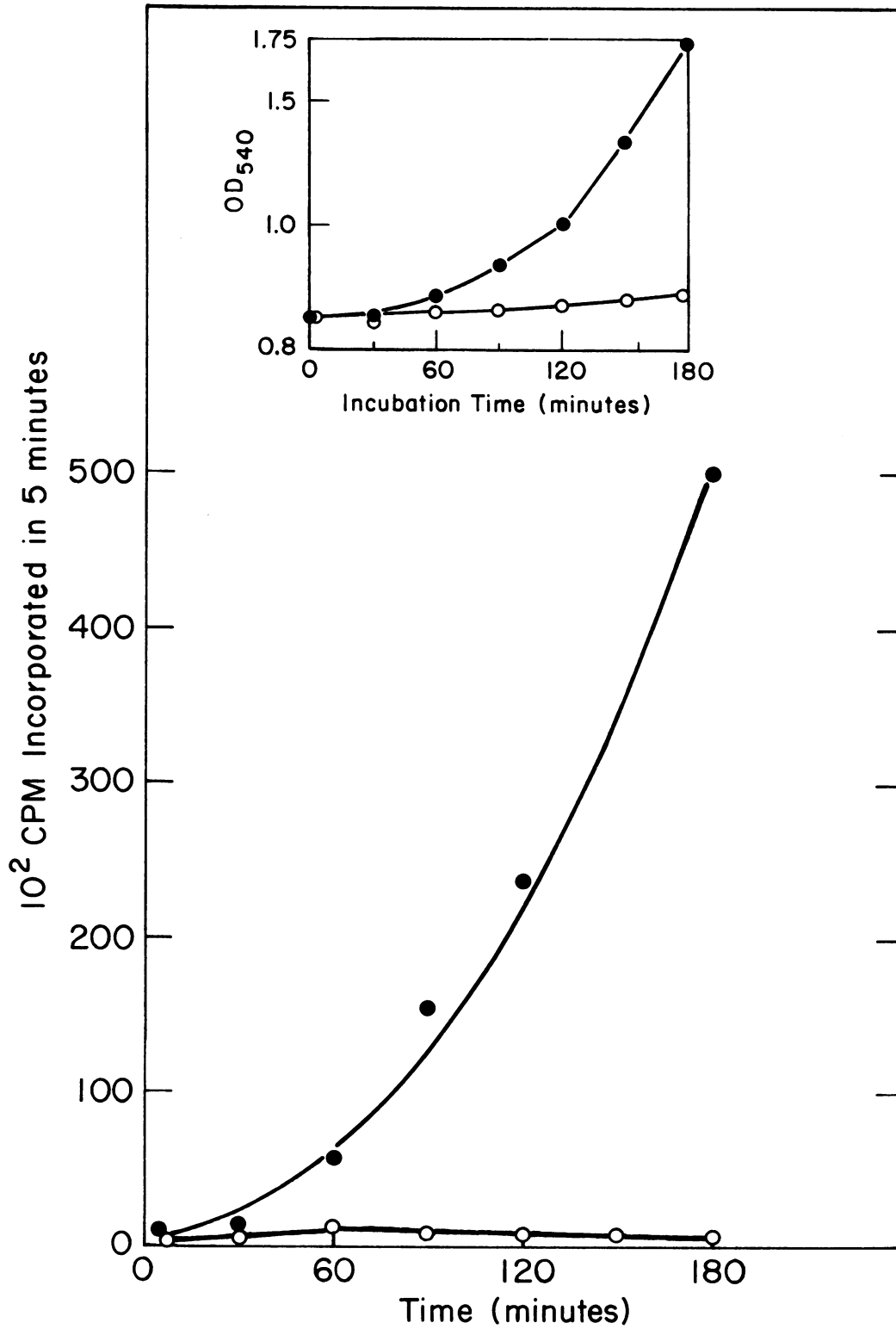

FIG. 4: *Variation in the rate of protein synthesis during outgrowth. Germinated spores were suspended in basal medium TG20 with addition of casein hydrolysate and with (●—●) or without (○—○) Trp. Samples were withdrawn at different times during the incubation at 42 C. After the determination of their optical density at 540 nm they were pulse-labeled for 5 min with ^{14}C-amino acid mix. The variation of the rate of protein synthesis (10^2 counts per min per ml incorporated in 5 min) can be compared with the rate of increase of optical density at 540 nm given in the insert.*

TABLE 4. *Influence of some amino acids on outgrowth and on vegetative growth*

Amino acids[a]	Duration of outgrowth (h)	Vegetative cells division time (min)
CH	4–12	70–90
Arg	4–12	70–90
Pro	4–12	70–90
Glu	4–12	70–90
Ala	4–12	
Minimal	Ind[b]	70–90
Ser	Ind	Ind
Thr	Ind	Ind
Ile	Ind	
Lys	Ind	
Val	Ind	
Gly	Ind	
Asp	Ind	
Cys	Ind	Ind

[a] Minimal, TG20 + Trp; CH, casein hydrolysate. The effect of other amino acids not listed is intermediate. Spores: germinated in TTK + Ala + GLC, centrifuged, and suspended in minimal alone or minimal with the supplements listed.

[b] Ind, Indefinite.

darkening and early protein syntheses could not be distinguished. However, the incorporation was strictly proportional to the amount of spores which were phase-dark and the radioactive label was found associated only with the phase-dark fraction of the population. The rate of macromolecular syntheses was studied separately from that of phase-darkening by labeling for short pulses, during which phase-darkening was practically stationary, and by expressing the results in percentage of phase-dark spores. We have established in this way that GLC is required and sufficient for incorporation, while Ala alone, although sufficient for optimal phase-darkening, does not support any incorporation and inhibits by 60% when added to GLC.

The failure to find incorporation by the refractile spores could be due to impermeability to the radioactive tracer of the spores before darkening and not to their inability to perform early syntheses. However, after hours of incubation without Ala, the spores which remained refractile were found still able, upon phase-darkening, to incorporate the same amount of label and at the same rate as the spores not incubated at all. This indicates that the potential for early syntheses has not been used up by the refractile spores and confirms the idea that phase-darkening must precede macromolecular syntheses.

The mechanism of action of Ala and GLC at germination is still a matter of discussion (9, 10, 15, 24, 25, 37, 38). An obvious, although remote possibility, is that Ala may function as a building block and GLC as a source of energy. Concerning Ala, we found that its structural analogs ABU and AIBU replaced Ala in its role of germination factor with equal efficiency; yet they cannot replace Ala as building block for proteins and AIBU cannot even be a substrate for alanine dehydrogenase (23, 38, 40). GLC could be replaced by ribose which is not an energy source for *B. subtilis* 168 (A. Torriani and G. Bergler, unpublished data). On the other hand, we have found in our system that the unnatural enantiomorphs, D-Ala and L-GLC, do not replace the natural ones, indicating that only the forms normally found in the cells are metabolically recognizable by the spore. Whatever the paths of "sporal" metabolism of Ala and GLC are, they are not the ones normally functioning in the vegative cell (41).

The syntheses of RNA and proteins detectable at initiation of germination occur in the spore as a closed system, i.e., without exogenous supply of building blocks. Evidence for this has been obtained previously (2, 3, 5, 18, 27, 32, 33, 36) by showing that the addition of only one radioactive amino acid or nucleic base was sufficient for obtaining incorporation of the label into sporal protein or RNA material. In the experiments we describe here, where the organism is a Trp-deficient mutant, we routinely used the commercially available ^{14}C-amino acid mix which lacks Trp; and also in a few experiments not presented here, we reproduced similar results by using ^{14}C-tryptophan or ^{14}C-phenylalanine alone. Such self-sustaining syntheses must be necessarily limited. In our system, when outgrowth was delayed, synthetic activity rapidly declined after 1 h. The maximal rate of synthesis was 1 to 3% that of normal protein synthesis by vegetative cells. The distinction between the early macromolecular syntheses and those occurring late, but still before the first cell division (i.e., during outgrowth), was very sharp; the first did not require exogenous tryptophan; the second were strictly dependent on it. Without Trp, the spores were unable to germinate past the initiation step, even upon addition of an excess of all other amino acids in a rich medium.

The requirements for outgrowth were determined by measuring the duration of the lag before the occurrence of the first cellular division. It was found that the presence of a complete mixture of amino acids, or at least

some of them, in addition to Trp reduces the lag before the first division from several days to a few minutes, while for vegetative growth no exogenous amino acid (except Trp) is required. This confirms the indication obtained by determining the stage at which Trp becomes an indispensable facor, i.e., protein synthesis ceases to be supported by turnover of sporal material and requires building blocks synthesized de novo or provided exogenously. The beneficial effect of external supply of amino acids could be explained by a slow start of the endogenous synthesis of the various amino acids due to sequential reading of the genome (16, 22, 28–30, 33).

All the preceding information can be organized in the following scheme. Protein synthesis starts very early in the spore, but not before phase-darkening, since it cannot be demonstrated in refractile spores. At this stage, only turnover of building blocks contained in preexisting sporal material can support protein synthesis, since no external supply is required, not even of Trp for spores of a Trp^- mutant. Moreover, under conditions (darkening by Ala alone) in which we observe no macromolecular syntheses, the ability to incorporate radioactive label gradually decreases. This decrease may be interpreted as dilution of the label by an expanded and unused amino acid pool, which suggests that proteolytic degradation occurs at phase-darkening. In addition we have observed that the appearance of visible colony on rich agar plates from spores incubated in Ala alone is delayed for several hours compared to spores germinated in Ala + GLC. Presumably, if the degradative processes are not closely followed by synthetic activity a metabolic imbalance is produced. The stage of self sustaining macromolecular synthesis ends long before the first division, since Trp becomes an indispensable factor during the active syntheses which are commonly designated as outgrowth. Finally, we have found that some amino acids inhibit outgrowth while they have no effect on vegetative growth. This specific inhibitory effect is not understood as yet, but in any case underlines the distinction, physiological and not only operational, between outgrowth and vegetative growth.

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LITERATURE CITED

1. Armstrong, R. L., and N. Sueoka. 1968. Phase transition in ribonucleic acid synthesis during germination of *Bacillus subtilis* spores. Proc. Nat. Acad. Sci. U.S.A. **59**:153–160.
2. Balassa, G., and G. Contesse. 1965. Syntheses macromoleculaires au cours de la germination des spores de *B. subtilis*. I. Cinetique. Ann. Inst. Pasteur **109**:683–705.
3. Balassa, G., and G. Contesse. 1966. Syntheses macromoleculaires au cours de la germination des spores de *B. subtilis*. II. Regulation. Ann. Inst. Pasteur **110**:25–48.
4. Bleyman, M., and C. Woese. 1969. Ribosomal ribonucleic acid maturation during bacterial spore germination. J. Bacteriol. **97**:27–31.
5. Cohen, A., and A. Keynan. Synthesis of a factor stimulating transcription in outgrowing Bacillus cereus spores. Biochem. Biophys. Res. Commun. **38**:744–749.
6. Demain, A. L., and J. E. Newkirk. 1960. Dissociation of spore germination from outgrowth by use of auxotrophic mutants of *Bacillus subtilis*. J. Bacteriol. **79**:783–788.
7. Echols, H., A. Garen, S. Garen, and A. Torriani. 1961. Genetic control of repression of alkaline phosphatase in *E. coli*. J. Mol. Biol. **3**:425–438.
8. Foerster, H. F., and J. W. Foster. 1966. Response of *Bacillus* spores to combination of germinative compounds. J. Bacteriol. **91**:1168–1177.
9. Freese, E., S. W. Park, and M. Cashel. 1964. The developmental significance of alanine dehydrogenase in *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **51**:1164–1172.
10. Gould, G. W. 1969. Germination, p. 397–438. *In* G. W. Gould and A. Hurst (ed.), The bacterial spore. Academic Press Inc., New York.
11. Halvorson, H. O., J. C. Vary, and W. Steinberg. 1966. Developmental changes during the formation and breaking of the dormant state in bacteria. Annu. Rev. Microbiol. **20**:169–188.
12. Hermier, J. 1962. La germination de la spore de *Bacillus subtilis*. I. Action des sucres et des acides amines sur la phase initiale de la germination. Ann. Inst. Pasteur **102**:629–643.
13. Hermier, J., and M. Rousseau. 1967. La germination de la spore de *Bacillus subtilis*. IV. Role des acides amines dans la perte de la refringence de la spore. Ann. Inst. Pasteur **113**:327–340.
14. Hermier, J., M. Rousseau, and C. Zevaco. 1970. Role des deshydrogenases a nicotinamide adenine dinucleotide dans la phase initiale de la germination chez la spore de *Bacillus subtilis*. Ann. Inst. Pasteur **118**:611–625.
15. Hyatt, M. T., and H. S. Levinson. 1964. Effects of sugars and other carbon compounds on germination and postgerminative development of *Bacillus megaterium* spores. J. Bacteriol. **88**:1403–1415.
16. Kennett, R. H., and N. Sueoka. 1971. Gene expression during outgrowth of *Bacillus subtilis* spores. The relationship between gene order on the chromosome and temporal sequence of enzyme synthesis. J. Mol. Biol. **60**:31–44.
17. Keynan, A., and H. O. Halvorson. 1965. Transformation of a dormant spore into vegetative cell, p. 174–187. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Bethesda, Md.
18. Kobayashi, Y., W. Steinberg, A. Higa, H. O. Halvorson, and C. Levinthal. 1965. Sequential synthesis of macromolecules during outgrowth of bacterial spores, p.

200–212. *In* L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Bethesda, Md.

19. Krask, B. J. 1961. The role of alanine dehydrogenase in the control of germination of aerobic spores initiated by L-alanine, p. 89–100. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis, Minn.
20. Nelson, D. L., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XVIII. Free amino acids in spores. J. Biol. Chem. **245**:1128–1136.
21. Nelson, D. L., J. A. Spudich, P. P. M. Bonsen, L. L. Bertsch, and A. Kornberg. 1969. Biochemical studies of bacterial sporulation and germination. XVI. Small molecules in spores, p. 59–71. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
22. Nukushina, J., and Y. Ikeda. 1969. Genetic analysis of the developmental processes during germination and outgrowth of *Bacillus subtilis* spores with temperature-sensitive mutants. Genetics **63**:63–74.
23. O'Connor, R. J., and H. O. Halvorson. 1961. L-Alanine dehydrogenase: a mechanism controlling the specificity of amino acid-induced germination of *Bacillus cereus* spores. J. Bacteriol. **82**:706–713.
24. O'Connor, R. J., and H. O. Halvorson. 1961. The role of alanine dehydrogenase in the control of germination of aerobic spores initiated by L-alanine, p. 73–88. *In* H. O. Halvorson (ed.), Spores II. Burgess Publishing Co., Minneapolis, Minn.
25. Prasad, C., M. Diesterhaft, and E. Freese. 1972. Initiation of spore germination in glycolytic mutants of *Bacillus subtilis*. J. Bacteriol. **110**:321–328.
26. Schaeffer, P., H. Ionesco, A. Ryter, and G. Balassa. 1965. La sporulation de Bacillus subtilis: étude génétique et physiologique, p. 553–563. *In* Mecanismes de regulation des activities cellulaires chez les microorganismes. Colloque International Centre National Recherche Scientifique, Paris.
27. Setlow, P., and A. Kornberg. 1970. Biochemical studies of bacterial sporulation and germination. XXIII. Nucleotide metabolism during spore germination. J. Biol. Chem. **245**:3645–3652.
28. Spiegelman, G., E. Dickinson, J. Idriss, W. Steinberg, S. Rodenberg, and H. O. Halvorson. 1969. Classes of ribonucleic acid and protein synthesized during outgrowth of spores of *Bacillus cereus*, 235–245. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
29. Steinberg, W., and H. O. Halvorson. 1968. Timing of enzyme synthesis during outgrowth of spores of *Bacillus cereus*. I. Ordered enzyme synthesis. J. Bacteriol. **95**:469–478.
30. Steinberg, W., H. O. Halvorson, A. Keynan, and E. Weinberg. 1965. Timing of protein synthesis during germination and outgrowth of spores of *Bacillus cereus* strain T. Nature (London) **208**:710–711.
31. Tamir, H., and C. Gilvarg. 1966. Density gradient centrifugation for the separation of sporulating forms of bacteria. J. Biol. Chem. **241**:1085–1090.
32. Torriani, A., L. Garrick, and Z. Silberstein. 1969. Onset of macromolecular syntheses at germination of bacterial spores, p. 247–261. *In* L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Bethesda, Md.
33. Torriani, A., and C. Levinthal. 1967. Ordered synthesis of proteins during outgrowth of spores of *Bacillus cereus*. J. Bacteriol. **94**:176–183.
34. Vinter, V. 1965. Commencement of synthetic activities of germinating bacterial spores and changes in vulnerability of cells during outgrowth, p. 25–37. *In* L. L. Campbell and H. O. Halvorson (ed.), Spores III. American Society for Microbiology, Bethesda, Md.
35. Vinter, V. 1966. Spores of micro-organisms. XX. Changes in the rate of incorporation of precursors of macromolecules during postgerminative development of bacterial spores. Folia Microbiol. (Prague) **11**:392–398.
36. Vinter, V., J. Chaloupka, J. Šťastná, and J. Čáslavská. 1972. Possibilities of cellular differentiation of bacilli into different hypometabolic forms, p. 390–397. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Bethesda, Md.
37. Wax, H., and E. Freese. 1968. Initiation of the germination of *Bacillus subtilis* spores by a combination of compounds in place of L-alanine. J. Bacteriol. **95**:433–438.
38. Wax, R., E. Freese, and M. Cashel. 1967. Separation of two functional roles of L-alanine in the initiation of *Bacillus subtilis* spore germination. J. Bacteriol. **94**:522–529.
39. Woese, C. R., and J. R. Forro. 1960. Correlations between ribonucleic acid and deoxyribonucleic acid metabolism during spore germination. J. Bacteriol. **80**:811–817.
40. Woese, C. R., H. J. Morowitz, and C. A. Hutchinson III. 1958. Analysis of action of L-alanine analogues in spore germination. J. Bacteriol. **16**:578–587.
41. Woese, C. R., J. C. Vary, and H. O. Halvorson. 1968. A kinetic model for bacterial spore germination. Proc. Nat. Acad. Sci. U.S.A. **59**:869–875.