EFFECT OF WITHHOLDING GLUTAMIC ACID AND ASPARAGINE ON THE GERMINATION OF SPORES OF *BACILLUS SUBTILIS*

CARL R. WOESE

Biophysics Department, Yale University, New Haven, Connecticut

Received for publication October 30, 1958

The initial stage in the germination of bacterial spores, which consists of a release to the medium of dipicolinic acid, a peptide, some amino acids, and glucosamine (Powell, 1953), can occur in very simple media, containing L-alanine only in the case of *Bacillus subtilis* Marburg strain (Woese and Morowitz, 1958). However, subsequent stages in spore germination require additional metabolites for their development. Hyatt and Levinson (1957) have shown that a germinating culture of *Bacillus megaterium* requires a source of sulfate and phosphate in order to increase its rate of oxygen consumption. Demain (1958) has shown that spores of *B. subtilis* require glutamic acid and asparagine in order to develop into colonies. The present work investigates the kinetics of growth and phosphorus uptake in germinating cultures of *B. subtilis* deprived of these two amino acids.

MATERIALS AND METHODS

The strain of *B. subtilis* and the particular spore stock used have been described previously (Woese and Morowitz, 1958). Germination media consisted of a 0.2 M Tris(hydroxymethyl)aminomethane buffer, pH 7.4, to which was added per L: Na_2HPO_4, 10 mg; Na_2SO_4 100 mg,; NH_4Cl, 500 mg; glucose, 10 g; and L-alanine, 100 mg. When used in the experiments described below, L-glutamic acid and L-asparagine were added at concentrations of 2.9 g per L and 2.6 g per L, respectively. Germination occurred under previously described conditions (Woese and Morowitz, 1958).

Measurements of optical density of the culture (Woese and Morowitz, 1958) and of the uptake of $P^{32}O_4$ were made. Phosphate uptake experiments were performed by adding a distilled water suspension of spores to 40 ml of the germination medium (containing alanine as the only amino acid) to which had also been added carrier-free $P^{32}O_4$. At the times indicated below, 8-ml portions of this culture are removed to tubes containing the two amino acids, glutamic acid and asparagine. Two-tenths-ml samples were removed from all cultures at times indicated and immediately put into 3 ml of cold 10 per cent trichloracetic acid. The P^{32} content of acid insoluble portions of each sample were then determined by the method of Britten *et al.* (1955).

RESULTS

Effect of time of addition of L-glutamic acid and L-asparagine on optical density of a germinating culture. The results of Hyatt and Levinson (1957) show that sulfate can be added to a sulfate-less growth inhibited culture of germinating *B. megaterium* at any time up to 130 to 150 min without affecting the normal course of the culture (elongation and rise in oxygen uptake rate begin at 150 min); further, the effect of adding sulfate to these cultures later than 150 min is immediate, as there is a negligible lag before the respiration increases at the normal rate. However, when L-glutamic acid and L-asparagine are withheld during germination of *B. subtilis*, the results are strikingly different from those obtained when sulfate is withheld from *B. megaterium*. A lag in the optical density rise results when the amino acids are withheld in germination of *B. subtilis* for more than 20 to 30 min, as compared to 130 to 150 min in the case of *B. megaterium*. Further, the addition of the amino acids does not manifest itself in an immediate change in optical density, but there is a lag of approximately 2 to 2½ hr before the effect is noted. These facts can be seen in figures 1 and 2, the former showing optical density of germinating cultures when the amino acids are added at various times in germination, the latter showing time delay in the rise in optical density as a function of time of amino acid addition. The line in figure 2 has a slope of 1.0.

If only the L-glutamic acid is deleted from the medium and then added as a function of time, the pattern observed above is not affected. However, if only L-asparagine is deleted, the lag

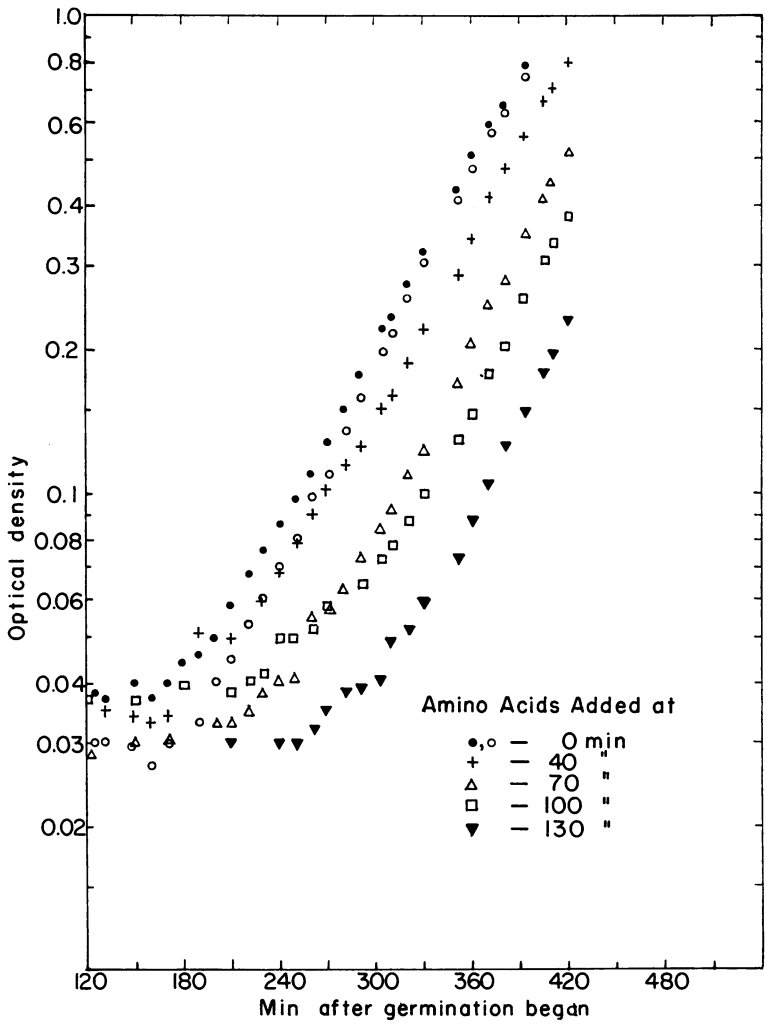

Figure 1. Optical density of cultures of germinating spores of *Bacillus subtilis* as a function of time of addition of L-glutamic acid and L-asparagine.

period upon its addition is considerably shortened. Although L-glutamic acid alone can cause some germinative development, it does not cause a rise in optical density.

Effect of time of addition of L-glutamic acid and L-asparagine on uptake of $P^{32}O_4$ into germinating cultures. There is a question as to what metabolic processes are occurring in the interval before addition of the amino acids. Is there synthesis of new material but no net synthesis; is there synthesis on so low a level that it is not detected by the assay; is there no new synthesis whatever? Further, what is the cause of the long lag before rise in optical density after addition of the amino acids? Studies on the uptake of labeled compounds can help to elucidate this problem.

In figure 3, data are presented to compare the optical density changes of a germinating culture with the amount of $P^{32}O_4$ incorporated into the acid insoluble fraction of the cells, when L-glutamic acid and L-asparagine are added to the system at various times. In the figure, the data for the 70 and 130 min subcultures are plotted as $C_t - C_a$, where C_t is cpm at time t, and C_a is cpm of the resulting culture immediately after transfer to the amino acids (70 min in one case, 130 min in the other). No data are included for optical density measurements on the "no amino

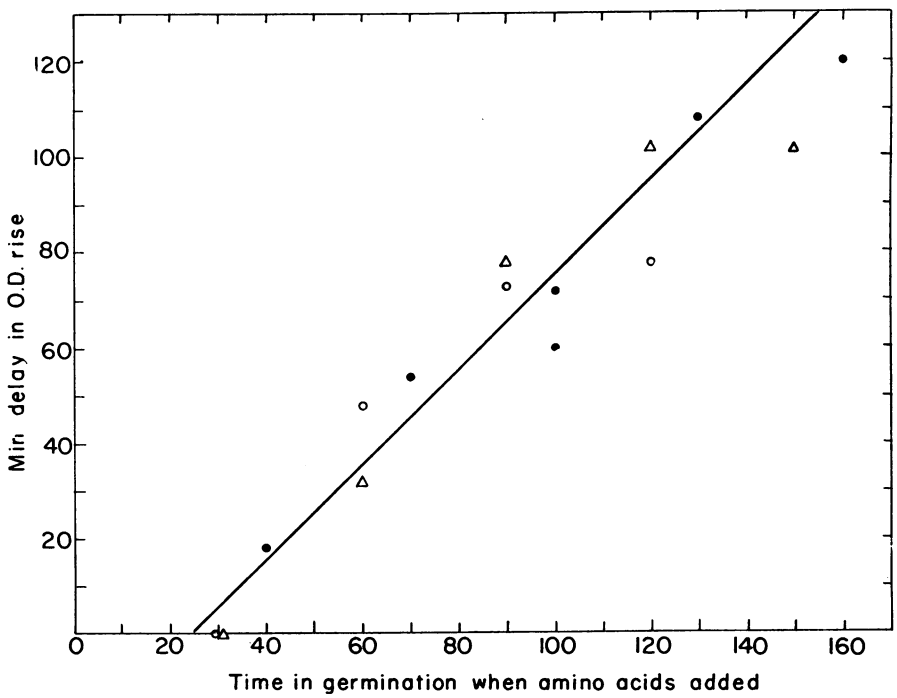

Figure 2. Minutes delay in germinative development (measured by optical density rise) as a function of time of addition of L-glutamic acid and L-asparagine.

acid" control; this optical density never increases. Though not apparent from the plotting, the limiting straight line slope of the uptake curves is the same as the final slope of the optical density curves (plotted in the same manner). It is seen that while there is some incorporation of phosphate into the acid insoluble fraction in the absence of the two amino acids, this incorporation approaches a limit of about 1000 cpm. Further, the effect of adding the amino acids on incorporation of phosphate is rapid. The kinetics of phosphate incorporation after addition of the amino acids are the same as those of the control culture if about 15 to 20 min are subtracted from the control times. In other words, when the two amino acids are added at 70 (or 130) min, the subsequent uptake curve is identical to the control uptake curve except that it lags, about 50 to 55 (or 110 to 115) min behind the control.

The specific activity of the $P^{32}O_4$ used is such that an incorporation of 1000 cpm is equivalent to the incorporation of 3.2×10^{-2} μg of P. In the particular experiment reported, the concentration of spores used was 8.4 μg dry weight per 0.2 ml (the sample size). If we assume a P content of the spore to be 0.8 per cent of its dry weight (which figure is that given for *B. cereus* spore P content (Fitz-James, 1954)), we find 6.7×10^{-2} μg P per 0.2 ml as the spore P content of the sample. Further, by 110 min of germination, the amount of P taken up by the germinating spore from the medium is essentially equal to the amount of P contained in the resting spore.

DISCUSSION

The block in germinative development caused by the deletion of L-glutamic acid and L-asparagine occurs early in germination. If these amino acids are withheld for more than about 20 min, a delay of $t - 20$ in development results after their addition (t = time of addition of the amino acids). By 20 min of germination, the spores have undergone the initial release stage, but have not yet begun to swell or emerge (Levinson and Hyatt, 1956; Woese and Morowitz, 1958). The block in germination of *B. megaterium* caused by withholding sulfate occurs at a much later stage, about the time of emergence, i. e., a delay of

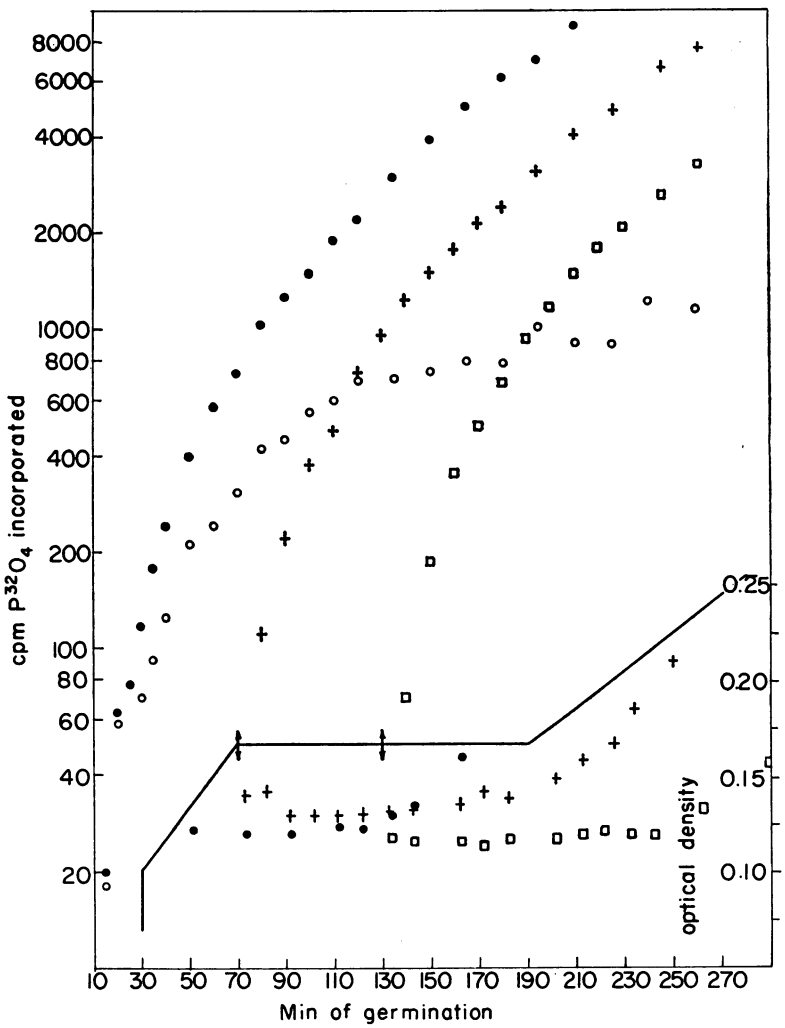

Figure 3. Comparison of $P^{32}O_4$ incorporation to optical density changes for cultures of germinating spores of *Bacillus subtilis* deprived of L-glutamic acid and L-asparagine for varying lengths of time. ○, No glutamic acid or asparagine added; ●, glutamic acid and asparagine added at t_0; +, glutamic acid and asparagine added at 70 min; □, glutamic acid and asparagine added at 130 min. Arrows indicate time of addition of amino acids.

$t - 150$. The author has been unable to obtain a sulfate deletion block in *B. subtilis*, due presumably to endogenous sources of sulfur. However, we have observed that a phosphate deletion effects a block at a later stage, at or near the emergence time.

As can be seen from figure 3, there is considerable uptake of phosphorus in the absence of glutamic acid and asparagine. However, this uptake seems to play no part in germinative development. If this were not the case, there would not be the constant difference of 20 min between the time of addition of the amino acids and the delay in germinative development produced by withholding them for that time. Another way of observing this is that only if the $P^{32}O_4$ incorporated into the germinating spore before addition of amino acids is neglected, i. e., uptake in figure 3 is plotted as $C_t - C_a$ (see above), do the uptake curves subsequent to addition of amino acids

become exactly parallel. The phosphorus compounds formed before the addition of L-glutamic acid and L-asparagine appear not only to be of no use to the spore in germinative development, but, further, to be of no use as internal sources of metabolites (containing phosphorus).

It is interesting to compare the changes in optical density to the amount of phosphorus incorporated at various stages in germination. Figure 3 shows that there is no appreciable change in optical density for any of the cultures until the P^{32} incorporation level reaches 2000 to 3000 cpm. This corresponds to 6 to 9 $\times 10^{-2}$ μg of phosphorus incorporated. As stated above, these spores probably contain about 7 $\times 10^{-2}$ μg of phosphorus. About 75 per cent of the spore phosphorus is in nucleic acid (Fitz-James, 1954), so that the spores contain about 5 $\times 10^{-2}$ μg of nucleic acid phosphorus. At least 75 per cent of the newly incorporated phosphorus should be in nucleic acid, so that before increasing appreciably in optical density, the germinating spores have synthesized at least as much nucleic acid as is present originally in the resting spore. This amounts to about 50 $\times 10^{-2}$ μg, or 6 per cent of the total dry weight of the spore.

Two facts emerge from this study. One is that although there is uptake of phosphate into germinating cultures deprived of L-glutamic acid and L-asparagine, the compounds thus synthesized probably have nothing to do with germinative development. The other is that germinative development is halted by this amino acid deficiency at about 20 min or perhaps slightly earlier. These facts together with the fact that the "1/*e* time" for completion of the initial stage of *B. subtilis* germination (i. e., release of dipicolinic acid, etc.) is 16 min (Woese and Morowitz, 1958) indicate that the glutamic-asparagine block occurs at the germination stage immediately following the initial one.

SUMMARY

When L-glutamic acid and L-asparagine are deleted from the germination medium, spores of *Bacillus subtilis* Marburg strain are prevented from developing to the point that they increase in optical density. The block caused by this deletion occurs at an early stage in germination, immediately succeeding the initial stage. Although there is synthesis of material (as measured by incorporation of labeled phosphate into the acid insoluble portion of the germinating spore), in the absence of these amino acids, the material plays no part in germinative development.

REFERENCES

BRITTEN, R., ROBERTS, R., AND FRENCH, E. 1955 Amino acid adsorption and protein synthesis in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S., **41**, 863–870.

DEMAIN, A. 1958 Minimal media for quantitative studies with *Bacillus subtilis*. J. Bacteriol., **75**, 517–522.

FITZ-JAMES, P. 1954 The phosphorus fraction of *Bacillus cereus* and *Bacillus megaterium*. I. A comparison of spores and vegetative cells. Can. J. Microbiol., **1**, 502–519.

HYATT, M. AND LEVINSON, H. 1957 Sulfur requirement for postgerminative development of *Bacillus megaterium* spores. J. Bacteriol., **74**, 176–183.

LEVINSON, H. AND HYATT, M. 1956 Correlation of respiratory activity with phases of spore germination and growth in *Bacillus megaterium* as influenced by manganese and L-alanine. J. Bacteriol., **72**, 176–183.

POWELL, J. F. 1953 Isolation of dipicolinic acid from spores of *Bacillus megaterium*. Biochem. J., **54**, 210–211.

WOESE, C. AND MOROWITZ, H. 1958 Kinetics of the release of dipicolinic acid from spores of *Bacillus subtilis*. J. Bacteriol., **76**, 81–83.