CORRELATIONS BETWEEN RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID METABOLISM DURING SPORE GERMINATION

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Studies carried out on synchronously growing bacterial cultures may greatly enhance our knowledge of the bacterial cell. Fitz-James (1955) has demonstrated that a high degree of synchrony exists in a germinating spore culture under certain conditions. Synchrony can be achieved by adding L-alanine at zero time to a suspension of spores in an appropriate medium; alanine starts germination simultaneously in at least 90 per cent of cells.

In addition to being phased a germinating culture possesses other qualities that make it useful in studies of the bacterial cell. The spore differs in many respects from the vegetative cell into which it develops. Examples are differences in ribonucleic acid-deoxyribonucleic acid (RNA-DNA) ratios, synthetic capacities, presence or lack of certain enzymes and other constituents, and state of the RNA (Fitz-James, 1955; Halvorson and Church, 1957; Woese, Langridge, and Morowitz, 1960). In view of these differences, it is likely that relatively profound changes accompany the transition from spore state to vegetative state.

The work reported here was prompted by the original observations of Fitz-James (1955) on synchronously germinating cultures of *Bacillus cereus* and *Bacillus megaterium*. He found that RNA increase preceded DNA increase, and that the rate of RNA increase varied at different stages of germination. We have carried out a similar study on germinating cultures of *Bacillus subtilis*. The net changes in DNA and RNA as a function of time during germination and the kinetics of incorporation of phosphate into the two nucleic acid fractions are reported below.

There is no a priori reason to assume that the correlations between DNA and RNA synthesis one observes during spore germination are the same as those existing in the vegetative state of the cell. However, the synchrony existing in a germinating culture can extend beyond the first cell division (Fitz-James, 1955). Therefore, if there are two modes of RNA and DNA metabolism (i.e., germinating and vegetative) it should be possible to ascertain them both using a germinating culture, and the subsequent comparison would prove more informative than the study of either one alone.

MATERIALS AND METHODS

The spores of *B. subtilis* (Marburg strain) used here are derived from a stock described previously (Woese and Morowitz, 1958). Procedures for preparation of the spore stock are as follows: potato agar plates are seeded with a very dilute suspension of the original spore stock and incubated for about 3 days at 35 C. At the end of this time sporulation is essentially complete. The bacterial growth is then scraped from the plates and suspended in distilled water. The cells are sedimented and resuspended in a buffer of pH about 7 to which is added lysozyme. The whole is heated at 50 C for 2 hr. At this temperature the action of the enzyme is facilitated, nonspore forms are killed, and there is little or no tendency for spores of *B. subtilis* to germinate (Woese, *unpublished data*). After heating, the cells are repeatedly centrifuged from and resuspended in distilled water to remove most of the buffer and lysozyme. The preparation is then lyophilized and the dried spore powder stored at room temperature in a desiccator. The spore stocks so produced are free from vegetative cells, but contain a low percentage of what appear to be either immature spores or early germinating forms.

Germination procedure is as follows. A weighed amount of dried spore stock is suspended in and centrifuged from distilled water twice. At zero time the spores (suspended in a few milliliters of distilled water) are added to aerated germination medium at 38 C. The final concentration of spores is 100 mg/liter. The medium consists of a 0.02 M tris(hydroxymethyl)aminomethane buffer, pH 7.3, containing: L-glutamic acid, 20 mmoles; L-asparagine, 20 mmoles; L-alanine, 0.5 mmole; glucose, 50 mmoles; $MgCl_2$, 5 mmoles;

NH_4Cl, 10 mmoles; Na_2SO_4, 1 mmole; Na_2HPO_4, 0.3 mmole; $MnCl_2$, 0.1 mmole; and KCl, 1 mmole. This medium is essentially that used in previous experiments (Woese, 1959). At various times samples are removed from the culture, sedimented in a Servall centrifuge kept at 0 to 5 C, resuspended in ice cold distilled water and made 5 per cent in cold trichloroacetic acid; the trichloroacetic acid suspension is then kept at 0 to 5 C, and after 30 min the acid insoluble portion of the sample is recovered by centrifugation.

Subsequent treatment of the sample depends upon whether DNA and RNA are to be fractionated from one another. If not, the sediment from the previous step is extracted with hot trichloroacetic acid to solubilize the nucleic acid components (Fitz-James, 1955; Schneider, 1945). The RNA is then determined by the orcinol reaction (Schneider, 1945), and the DNA by the Keck modification of the Cerriotti indole method (Keck, 1956). The probable error on an individual determination of RNA or DNA by these methods has been found to be 5 per cent and 7 per cent, respectively.

If DNA and RNA are to be fractionated, the method of overnight extraction with 2 N KOH is used (Schmidt and Thannhauser, 1945; Fitz-James, 1955). Upon subsequent neutralization, the RNA components are soluble, whereas DNA is contained in the insoluble residue. As in the previous method, RNA is then determined by orcinol reaction on the soluble portion and the DNA by the indole reaction on the hot trichloroacetic acid soluble portion of the KOH insoluble residue.

To measure phosphate incorporation into the nucleic acid fractions, $P^{32}O_4$ is used. Carrier-free $P^{32}O_4$ is added to a germinating culture, either at zero time or when indicated in the text below. A portion of the fractions upon which RNA and DNA analyses are made is dried on a planchet and its P^{32} content determined by standard Geiger counting methods.

RESULTS

The following morphological changes are observable (using a phase microscope) during germination of a culture of *B. subtilis*. At 0 to 20 min, spores appear unchanged, over 95 per cent are bright objects. At 20 to 60 min, 90 per cent of the bright objects become dark ones of the same size and shape; this constitutes the initial stage of germination. At 60 to 85 min, there is little change except for a swelling of the cells. At 80 min, the culture appears as a rather uniform population of fat egg-shaped dark objects; less than 5 per cent of cells are ungerminated at this point. Between 85 and 110 min, the majority of the cells have emerged. Subsequently, elongation proceeds until division occurs. Signs of motility first appear at approximately 130 min. The first divisions appear, in about 15 per cent of the cells, at 190 min, but the majority have not divided until 220 min.

The above observations show that although synchrony exists to some degree in the culture, there is a considerable spread in the distribution of cell types at any given time. Optical density measurements in addition to the microscopic observations give a more quantitative picture of the degree of synchrony existing during germination. It has been shown (Woese and Morowitz, 1958) that optical density drop can be used to measure the initial stages of spore germination. By this criterion the proportions of spores completing the initial stage of germination increased from 30 to 70 per cent in 17 min, 20 to 80 per cent in 30 min, and from 10 to 90 per cent in 42 min. This is to be compared to the results of Fitz-James (1955), in which the initial stage of *B. cereus* germination goes from 10 to 90 per cent completion in 10 min or less. In the present case we find that practically all of the asynchrony is introduced at the initial stage; microscopic observations of emergence and cell division show little or no increase in the spread of the distribution of cell types over that existing after the initial stage of germination.

It should be recognized that as germination proceeds, metabolism (as measured by phosphate incorporation, Woese, 1959) is increasing at an exponential rate. Therefore, the most advanced fraction of the culture accounts for a disproportionately high fraction of the culture's metabolism. With regard to some of the measurements reported below then, the effective degree of phasing in the culture is greater than is indicated by the microscopic and optical density measurements.

Net synthesis of nucleic acid during germination. The resting spore of *B. subtilis* has an RNA content of approximately 4 per cent on a dry weight basis. Its DNA content is 0.7 per cent. These measurements are in approximate agree-

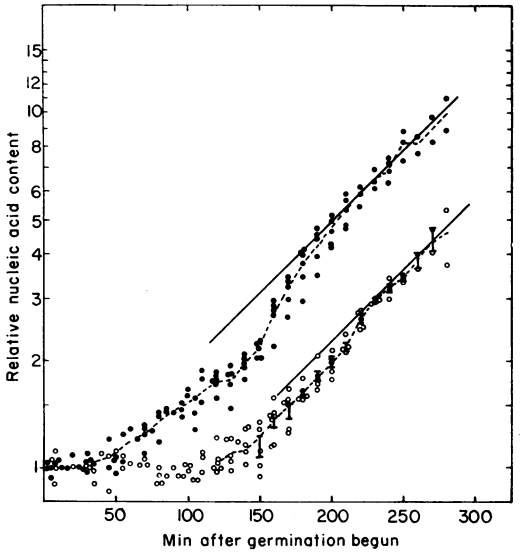

Figure 1. Total RNA (*closed circles*) and DNA (*open circles*) of a culture of germinating *Bacillus subtilis* spores as a function of time in germination. The RNA or DNA content of the resting spore is considered to be one unit. Dashed lines are drawn through average values; solid lines are parallel to (therefore, proportional to) the line describing logarithmic growth of the culture. The probable error of the mean is shown for the DNA determinations.

ment with those found in *B. cereus* and *B. megaterium* (Fitz-James, 1955). Figure 1 shows the net RNA and DNA of a germinating spore culture as a function of time in germination. The values of RNA and DNA in the figure are relative to their respective values in the spore state. The following facts are illustrated. a) There is no increase in DNA before approximately 125 min of germination. b) RNA has increased almost from the beginning of germination (after the first stage). c) At the time DNA of the germinating culture begins to increase the RNA content has almost doubled. d) There is an accelerated rate of increase in the RNA fraction in the interval 150 to 200 min, which precedes a similar accelerated rate of increase in the DNA fraction in the interval 200 to 230 min. Not shown in the figure is the fact that the initial DNA increase occurs at the same time as does the initial increase in optical density of the culture.

Turnover of nucleic acid during germination. To interpret the above results it is necessary to determine whether the increases are solely due to synthesis or whether turnover of RNA or DNA is also involved. This was done by adding $P^{32}O_4$ to a germinating culture at zero time and determining the P^{32} content of the nucleic acid fractions as a function of time in germination. It can be seen in figure 2 that although consider-

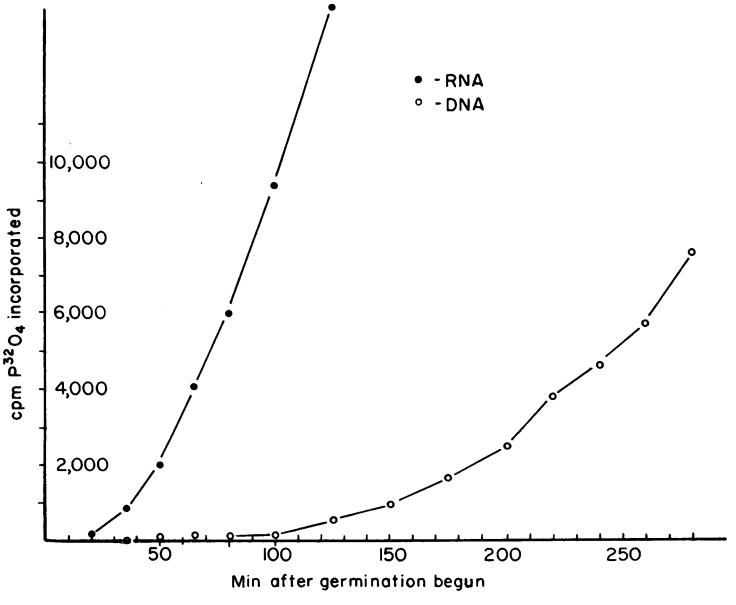

Figure 2. The uptake of $P^{32}O_4$ into the RNA or DNA fractions of *Bacillus subtilis* spores as a function of time in germination.

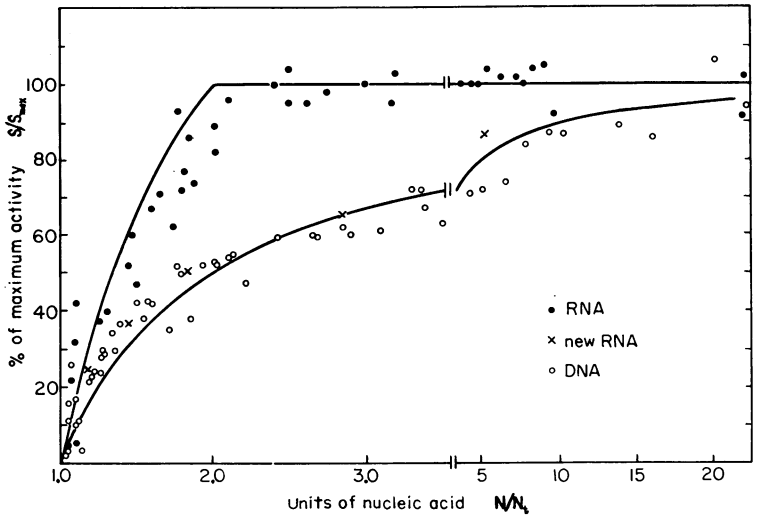

Figure 3. The per cent of maximal P^{32} activity in RNA and DNA fractions as a function of total RNA or DNA content of a germinating culture. N_t is the amount of either nucleic acid in the resting spore state. $P^{32}O_4$ added to the culture at zero time (see text).

able incorporation into RNA occurs during early germination, there is very little incorporation of isotope into the DNA fraction before 120 min, which is essentially the time when DNA increase is first observed.

Figure 3 illustrates the presence or absence of turnover in the nucleic acid fractions. P^{32} incorporation data are analyzed in terms of per cent maximal P^{32} activity; i.e., observed P^{32} per unit RNA or DNA at any given time in germination, divided by P^{32} per unit RNA or DNA for maximal (i.e., total) labeling of nucleic acid under the given experimental conditions. In the figure S/S_{max} is the per cent maximal P^{32} activity, and N/N_t is the RNA or DNA content of the culture at any time in germination divided by the RNA or DNA content, respectively, of the culture at zero time.

The curve that approximates the DNA data of figure 3 is derived on the assumption that the spore DNA phosphorus does not turn over; i.e., $S/S_{max} = (N - N_0)/N$. The curve approximating the RNA results is derived on the assumption that one unit of spore RNA phosphorus is lost from the RNA fraction for every two units of new RNA synthesized; i.e., $S/S_{max} = 2(N - N_0)/N$ for $N/N_0 < 2$, $S/S_{max} = 1$ for $N/N_0 > 2$. The simplest interpretation of the data in figure 3 is that spore DNA phosphorus is conserved, whereas spore RNA phosphorus turns over as new RNA is being formed. Turnover of spore RNA can also be demonstrated using a C^{14} uracil label instead of $P^{32}O_4$. In other words, the RNA curve of figure 3 is the same whether labeled uracil or phosphate is used. It seems likely then, that the entire RNA, and not merely the phosphate component, is labile during germination.

There is a question as to whether, at the time that the spore RNA is turning over, the newly formed RNA also turns over. This cannot be determined by the data of figure 3, since whether it turns over or not, the newly formed RNA would be expected to have maximal P^{32} activity. To demonstrate the presence or absence of turnover in the newly formed RNA, labeled phosphate was added initially to a germinating culture, subsequently large amounts (greater than 1,000 ×) of unlabeled phosphate were added so that negligible uptake then occurred. It was found that the amount of labeled phosphate in the RNA fraction remained constant after addition of the large amount of unlabeled phosphate, thus demonstrating lack of turnover in the newly formed RNA.

Another way of demonstrating the lack of turnover in the newly formed RNA during times when spore RNA is turning over is to add the $P^{32}O_4$ not at t_0 but at some later time, t, in germination. If the newly formed RNA phosphorus

turns over as does the spore RNA phosphorus, the curve of S/S_{max} vs N/N_t will be the same as that for total RNA turnover (closed circles in figure 3), but if new RNA phosphorus is conserved, such a plot will approach (depending upon how much residual spore RNA is still present at time t) that which describes DNA phosphorus activity in figure 3. The data represented by crosses in figure 3 again show that newly formed RNA does not turn over. In this case the label was added when total RNA was about 1.5 times its value in the spore state.

DISCUSSION

The time from onset of spore germination of *B. subtilis* to the first cell division under the germination conditions described above is slightly over 3 hr. During all but the initial 20 to 30 min of this time RNA synthesis occurs. On the other hand, DNA is synthesized during the last hour only. Comparable RNA-DNA time relations have been observed in spore germination of *B. cereus* and *B. megaterium*, although only net RNA and DNA were measured in these cases (Fitz-James, 1955). This long interval of growth in the absence of DNA synthesis seems to have no counterpart during the normal vegetative cell growth cycle. Fitz-James (1955), Schaechter, Bentzon, and Maaløe (1959), and van Tubergen (1960) have shown that DNA is synthesized throughout most if not all of the life cycle of a vegetative bacterial cell. The data presented above are consistent with this idea for the postgerminative vegetative cell of *B. subtilis*. In view of all these findings, it appears that further study of germinating systems will be most useful in elucidating the events leading to the synthesis of cell DNA.

Very little is known at present about the relationship of DNA synthesis to other cellular processes. The above data suggest that prior synthesis of RNA or protein is essential to the initiation of DNA synthesis. It should be noticed that the RNA of the germinating culture has almost doubled at the time its DNA synthesis begins; this means at least a doubling of the RNA in the more advanced cells, which are the only ones contributing to the initial DNA increase.

Unfortunately, the degree of synchrony in our

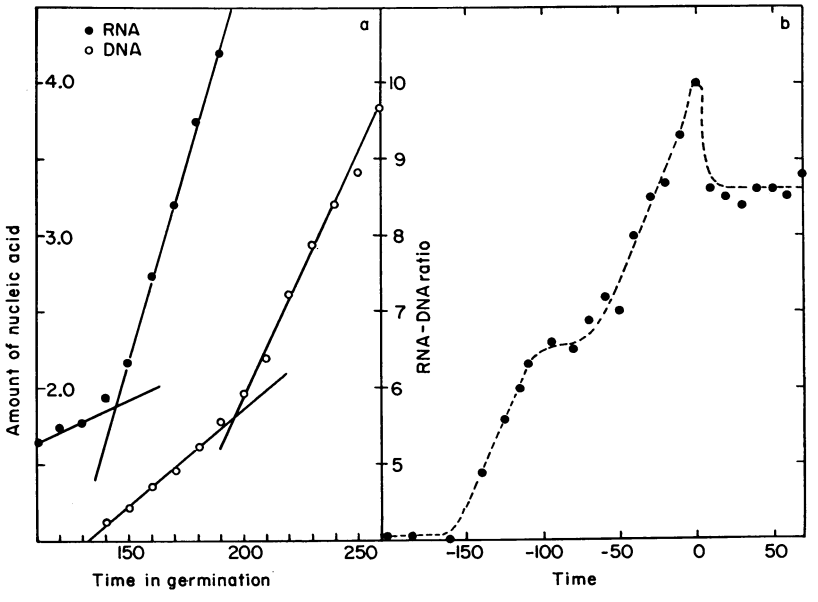

Figure 4a. Average value of RNA or DNA as a function of time in germination. Same units as in figure 1.

Figure 4b. Average RNA-DNA ratio, in arbitrary units, as a function of time in germination. The data are normalized with respect to time; i.e., the time at which the maximal RNA-DNA ratio occurs, which is between 190 and 210 min of germination in all cases, is called zero time; times before this are, consequently, negative.

cultures is not sufficient to determine accurate RNA-DNA correlations at the time of the first cell division and beyond, so nothing definitive can be said about correlations existing in the vegetative cell. The data at this time do show the following to occur at the approximate time of the first division: a) the rate of DNA synthesis increases sharply, as shown in figure 4*a*, and b) there is a drop in the RNA to DNA ratio, as shown in figure 4*b*. When the synchrony of the culture is improved, more can be said about this important period.

The results reported here indicate a possible effect of DNA synthesis on RNA synthesis. While the DNA is quiescent during early germination, the RNA synthesis rate is relatively low. Shortly after the onset of DNA synthesis the rate of RNA synthesis increases sharply. This can be seen best in figure 4*a*. The RNA synthesis rate in the interval 150 to 160 min of germination is over four times that existing at 120 to 130 min of germination. Such a rapid change in rate is unequaled at any other time in germination. An increase in the rate of RNA synthesis at or shortly after the onset of DNA synthesis has also been observed in germination of *B. megaterium* and *B. cereus* (Fitz-James, 1955; Woese, *unpublished data*).

The germinating spore and the vegetative cell differ profoundly in that spore RNA is labile whereas vegetative cell RNA (Hershey, 1954) is not. The above results are consistent with one unit of spore RNA turning over per two units of vegetative cell RNA formed. Though our data indicate that the major fraction of spore RNA turns over during germination, they do not answer the important question of what small percentage of spore RNA does not turn over. Preliminary experiments using $P^{32}O_4$-labeled spores indicate that of the order of 10 per cent of spore RNA is not labile.

Although there may be no common relationship, various labile RNA fractions in bacterial cells seem to be assuming more importance. Fifty per cent of the RNA produced in the presence of chloramphenicol is labile on removal of the drug (Neidhart and Gros, 1957); a labile RNA fraction is associated with the establishment of T_2 phage infection (Volkin and Astrachan, 1956); the RNA of uracil requiring mutants is labile in the absence of uracil (Cohen and Barner, 1958); furthermore, labile RNA may well be involved in synthesis of adaptive enzymes (Gale, 1956). An explanation for the labile nature of spore RNA is suggested in studies on the microsome fraction during germination of spores of *B. subtilis* (Woese et al., 1960). In the spore the major portion of the RNA is not contained in the microsomes, whereas in the vegetative cell it is. It is conceivable, therefore, that the spore RNA exists in a form that is sensitive to enzymatic breakdown during germination. This explanation, however, does not explain why the rate of destruction of spore RNA is related to the rate of synthesis of vegetative cell RNA.

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SUMMARY

Nucleic acid metabolism during the germination of spores of *Bacillus subtilis* differs from that found in the vegetative cell. During the initial 2 hr of germination, ribonucleic acid (RNA) only is synthesized. By the end of this time the total cell RNA has approximately doubled. Deoxyribonucleic acid (DNA) synthesis begins at 2 hr of germination, and the onset of optical density increase in the culture is coincident with this. Spore RNA is labile during germination. It is essentially completely turned over at the time DNA synthesis begins

There is a dramatic increase in the rate of ribonucleic acid synthesis at, or shortly after, the onset of DNA synthesis. There is a similar increase in the rate of DNA synthesis at the time of the first cell division.

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