Ribonucleic Acid and Protein Synthesis in a Mutant of *Bacillus subtilis* Defective in Potassium Retention[1]

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A mutant of *Bacillus subtilis* 168 (strain 168 KL), which had lost its normal capacity to accumulate K^+, was used to explore the interrelationship between protein and ribonucleic acid (RNA) synthesis. In contrast to the wild type, the growth rate of strain 168 KL was markedly dependent on the K^+ concentration in the medium. K^+ uptake in the mutant strain was identical to that in the parent, but the mutant was unable to retain and accumulate K^+. Protein synthesis was markedly dependent on the K^+ concentration in the medium, whereas RNA synthesis was relatively unaffected by changes in the level of K^+. Most of the RNA synthesized during K^+ depletion was ribosomal RNA; it appeared in crude extracts in the form of ribonucleoproteins particles with sedimentation values between $4S$ and $30S$. These particles were converted into mature ribosomes when growth was allowed to resume by the addition of K^+. Simultaneous synthesis of RNA and protein was necessary for the quantitative conversion of the ribonucleoprotein particles into ribosomes. During recovery from K^+ depletion, ribosomal protein was synthesized in preference to the other proteins of the cell.

The rates of ribonucleic acid (RNA) and protein synthesis in bacteria are rigidly coupled during balanced growth (19, 22). One method of uncovering the mechanisms responsible for the closely regulated synthesis of macromolecules is to examine the events that occur when the normal controls are disturbed. A number of systems exist in which RNA synthesis can be dissociated from protein synthesis. These include the addition of antibiotic inhibitors of protein synthesis (10, 26), starvation of a "relaxed control" amino acid auxotroph of *Escherichia coli* (1), and depletion of intracellular K^+ in certain mutants of *E. coli* (7).

A mutant of *Bacillus subtilis* 168 (strain 168 KL), which cannot maintain the normal high intracellular concentrations of K^+, synthesizes

RNA when protein synthesis is inhibited by depleting the cells of K^+ (14). The studies reported here further characterize some of the properties of this mutant, particularly the nature of the RNA formed in the absence of K^+ (KD-RNA) and the fate of the KD-RNA when growth is restored to K^+-depleted cells.

MATERIALS AND METHODS

Organisms and culture media. B. subtilis 168 KL requires tryptophan, thymine, and high levels of external K^+ for growth. Cells were cultured routinely in medium A, a glucose-salts medium (6) that contains 100 mM K^+. The basal medium was supplemented with 100 µg of L-tryptophan per ml, 40 µg of thymine per ml, 0.5% glucose, and 0.1% Casamino Acids (Difco). Medium NaA is an analogous medium containing Na^+ substituted for K^+ on a molar basis. All cultures were grown at 37 C with vigorous aeration and were harvested during exponential growth. The turbidity of the cultures was measured with a Klett-Summerson photoelectric colorimeter (no. 42 filter). One-hundred Klett units corresponds to 150 µg (dry weight) per ml of culture or 5×10^7 colony-forming units per ml. Cells were depleted of K^+ as previously described (7).

Chemicals. L-Leucine-*1*-[14]C (25 mc/mmole), uracil-*2*-[14]C (30 mc/mmole), and a ^{14}C-amino acid mixture (40 mc/m atom C) were products of New England

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Nuclear Corp., Boston, Mass. The Isoserve Corp., Cambridge, Mass., supplied ⁴²KCl (25 mc/mmole of K⁺). Macaloid was a product of the Baroid Division, National Lead Co., Houston, Tex., and was purified according to the method of Stanley (W. M. Stanley, Jr., Ph.D. Thesis, Univ. of Wisconsin, Madison, 1963). All other chemicals used were commercially available products.

Chemical determinations. Samples of cell suspensions were precipitated with equal volumes of cold 10% trichloroacetic acid. The precipitate was centrifuged at 4 C and washed once with cold 5% trichloroacetic acid. The pellet was then dissolved in 1 N NaOH, and was analyzed for protein by the method of Lowry et al. (13) or for RNA by the orcinol method (24).

Incorporation of radioactivity into RNA and protein. These techniques have been described previously (2).

Preparation of cell extracts. Samples (50 ml) from the experimental cultures were taken and centrifuged at 4 C. The packed cells were washed with 0.01 M tris(hydroxymethyl)aminomethane-0.005 M magnesium acetate buffer (pH 7.2) and were frozen as a pellet. The same buffer was used throughout the process of sucrose density gradient analysis. The frozen pellet was thawed in 5 ml of cold buffer, and the cells were broken in a French pressure cell at 4,000 psi. The cellular debris was removed by low-speed centrifugation at 4 C, and the supernatant extract was treated with 10 µg of deoxyribonuclease per ml (Worthington Biochemical Corp., Freehold, N.J.; ribonuclease free) before dialyzing against 200 volumes of cold buffer for 5 hr. A 1-ml amount of extract containing approximately 10 optical density units (at 260 nm) was gently layered on top of a 24-ml 5 to 20% (w/v) sucrose gradient. Centrifugation was carried out for 18 hr at 16,500 rev/min at 4 C in a Spinco Model L-2 ultracentrifuge with an SW-25.1 rotor. Samples were collected and processed as described previously (2, 8).

Extraction of purified RNA. These techniques have been described (2, 8).

Uptake of radioactive potassium. Immediately upon arrival, the ⁴²KCl was diluted 1:10,000, and an 0.1-ml sample was dried on a planchet for counting in a Nuclear-Chicago gas-flow counter. Enough of the radioactive material was added to a solution of medium A to give 10,000 counts per min per µmole of K⁺. Duplicate 1-ml samples of cells containing ⁴²K were collected by rapid filtration on membrane filters (Millipore Corp., Bedford, Mass.; pore size, 0.45 µm) and were washed with 20 ml of 2 mM MgCl₂, which does not cause a loss of K⁺ from the cells (12). Specific activities of ⁴²K in the medium were determined at the same time the cell samples were counted. In most instances, a series of samples was counted within 15 min, but corrections were applied when the radioactive decay amounted to more than 5%.

RESULTS

Growth of B. subtilis as a function of potassium concentration. The growth characteristics of *B. subtilis* 168 and the mutant strain 168 KL in media containing various concentrations of K⁺ were determined. Flasks containing 100, 10, 1, and 0 mM K⁺ were prepared by appropriate dilutions of medium A with medium NaA and were inoculated with washed logarithmically growing cells. The mutant 168 KL grew slowly in media with low concentrations of K⁺ but grew rapidly in high concentrations of K⁺. The parent strain grew rapidly in high or low K⁺, although the final yield of cells was dependent on the K⁺ content of the medium (Fig. 1).

Nature of the potassium transport defect. The existence of two types of K⁺ transport mutants has been reported for *E. coli* (5, 15) and for
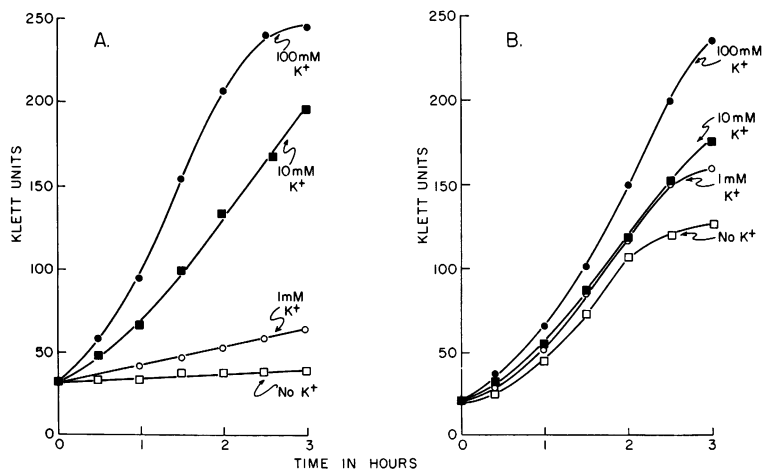

FIG. 1. *Growth of parent B. subtilis 168 and mutant 168 KL in media containing various concentrations of potassium. (A) Mutant; (B) parent.*

Streptococcus faecalis (11, 12). Mutant cells of the first type take up K⁺ as well as the wild type, but are unable to retain their K⁺ when placed in a Na⁺ salts medium. In the second type of mutant, the K⁺ entry mechanism is damaged. Two experiments were performed to determine which class the *B. subtilis* mutant 168 KL falls within. In the first experiment, we compared the kinetics of uptake of radioactive K⁺ in the parent and mutant strains. The uptake of ^{42}K was approximately the same in both strains, and the external K⁺ equilibrated with the internal K⁺ pools by the end of 20 min (Fig. 2).

Next, we examined the exit of ^{42}K from cells that were transferred to medium NaA after 2 hr of exponential growth in medium A containing K⁺ (10,000 counts per min per μmole). The parent 168 lost only 25% of its labeled K⁺ after 30 min, in contrast to mutant 168 KL, which lost 90% of its internal K⁺ (Fig. 3).

Synthesis of macromolecules in B. subtilis 168 KL. Lubin (14) reported that depleting strain 168 KL of K⁺ resulted in the continued accumulation of RNA, even though protein synthesis had ceased. We confirmed these results and, in addition, studied the effect of altering the K⁺ concentration on the rates of RNA and protein
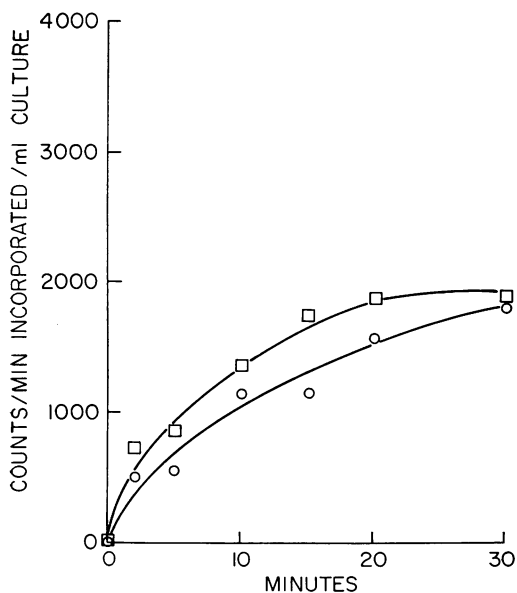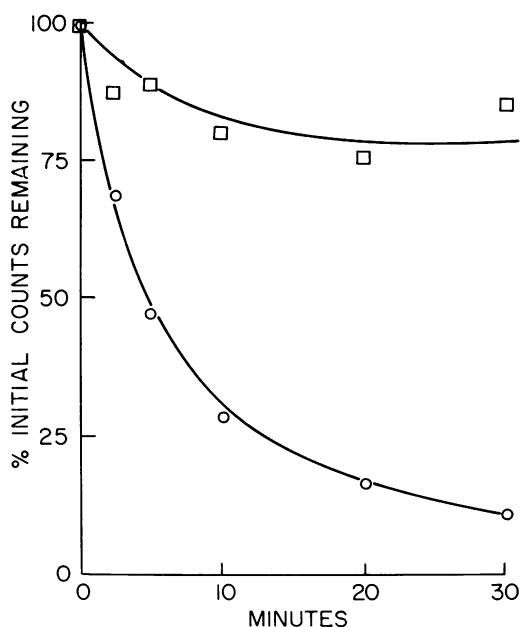

FIG. 3. *Loss of ^{42}K from B. subtilis suspended in medium lacking potassium. Cells were grown for 2 hr in medium A supplemented with 10,000 counts per min of ^{42}K per μmole of K⁺. The cells were then filtered, washed with 2 mM MgCl₂, and resuspended in medium NaA lacking tryptophan. Symbols: parent 168, □; mutant 168 KL, ○.*

synthesis. There was a definite dependence of ^{14}C-leucine incorporation on the external K⁺ concentration (Fig. 4). The relationship between the rate of RNA synthesis and the K⁺ concentration was less critical. The rate of RNA synthesis was about 45% of the maximum in the complete absence of K⁺ and rose steadily with increased K⁺ concentration.

Analysis of potassium-depletion RNA. The characteristics of the RNA synthesized in the absence of K⁺ (KD-RNA) were examined. As illustrated in Fig. 5, the bulk of the RNA formed during 30 min of K⁺ depletion sedimented in a broad region between 4S and 30S. The large peak of absorbance seen in the 4S region is probably due to oligodeoxyribonucleotides, because treatment with deoxyribonuclease and overnight dialysis resulted in the disappearance of this peak. However, since overnight dialysis allowed considerable breakdown of KD-RNA, short dialysis periods of 5 hr were routinely used. Control experiments showed that there was no breakdown of preexisting ribosomes during K⁺ depletion. Sucrose density gradient analysis of purified RNA from identical K⁺-depleted extracts demonstrated that most of the

FIG. 2. *Kinetics of uptake of ^{42}K in B. subtilis. Exponentially growing cells were suspended in medium A lacking tryptophan (to prevent uptake of ^{42}K as a result of increased cell mass). At time zero, sufficient ^{42}KCl was added to give a specific activity of 10,000 counts per min per μmole of K⁺. Symbols: parent 168, □; mutant 168 KL, ○.*
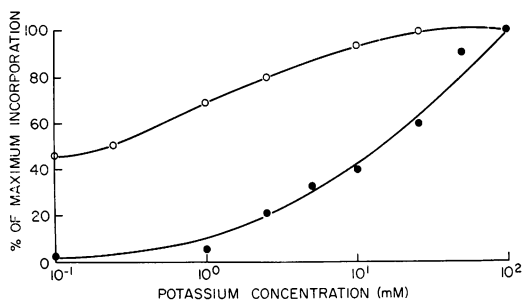
FIG. 4. *Incorporation of* ^{14}C*-uracil into RNA and* ^{14}C*-leucine into protein during growth in media containing various concentrations of potassium. Exponentially growing cells were washed twice with medium NaA and were resuspended in flasks containing appropriate mixtures of medium A and medium NaA. The rates of RNA and protein synthesis were determined after 15 min of incubation in fresh medium, and the results are plotted as the per cent of the rate observed in the highest K^+ concentration (100 mM). Symbols:* ^{14}C*-uracil incorporation,* ○; ^{14}C*-leucine incorporation,* ●.
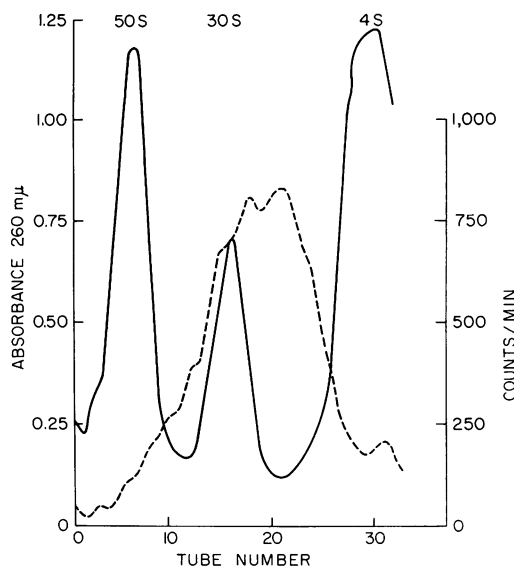

FIG. 5. *Sucrose density gradient analysis of crude extracts from potassium-depleted cells. A culture of strain 168 KL was allowed to incorporate* ^{14}C*-uracil into RNA for 30 min in the absence of* K^+. *A crude extract was prepared and mixed with a 10-fold excess of an extract taken from cells growing normally in medium A. Absorbance at 260 nm, solid line; counts/min, dashed line.*

RNA synthesized during 30 min of K^+ depletion sedimented at the same rate as normal ribosomal RNA (Fig. 6).

Synthesis of macromolecules during recovery

from potassium depletion. Previous reports (4, 9, 19) indicated that restoring protein synthesis to cells in which the synthesis of this macromolecule had been inhibited resulted in a decreased rate of RNA synthesis and a higher than normal rate of protein synthesis. Therefore, we performed a series of experiments to determine the rate of RNA and protein synthesis during recovery from K^+ depletion. Figure 7 illustrates the net accumulation of RNA and protein during 90 min of K^+ starvation and 90 min of recovery. The total amount of RNA nearly doubled after 90 min in K^+-free media, whereas the protein content remained invariant. Consequently, the RNA to protein ratio increased steadily over this time period from 0.38 to 0.68. When K^+ was added to the K^+-depleted cultures, the rate of total RNA synthesis declined as protein synthesis resumed. The result was a gradual return of the RNA to protein ratio to the value observed during balanced growth. The same results were obtained when synthesis of macromolecules was measured by uptake of radioactive precursors of RNA and protein.

Nakada (17) reported that the rate of protein synthesis in *B. subtilis* cells recovering from puromycin treatment was greater than that in normally growing cells. Therefore, the rate of protein synthesis after adding back K^+ was compared to the rate of protein synthesis in exponentially growing cells of the same optical
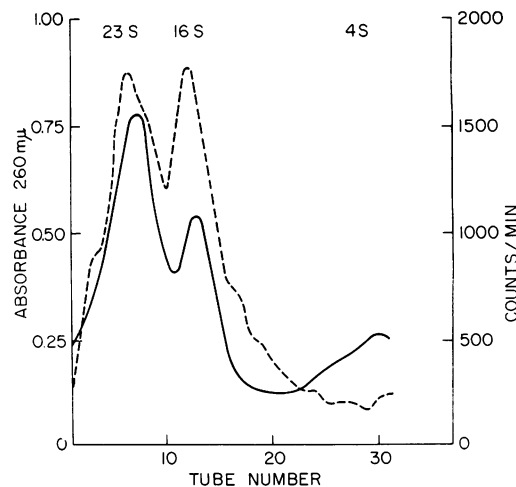

FIG. 6. *Sucrose density gradient analysis of purified RNA from potassium-depleted cells. Cultures of strain 168 KL were allowed to incorporate* ^{14}C*-uracil into RNA for 30 min in the absence of* K^+. *The purified RNA from these extracts was mixed with a 10-fold excess of RNA from normally growing cells. Absorbance at 260 nm, solid line; counts/min, dashed line.*
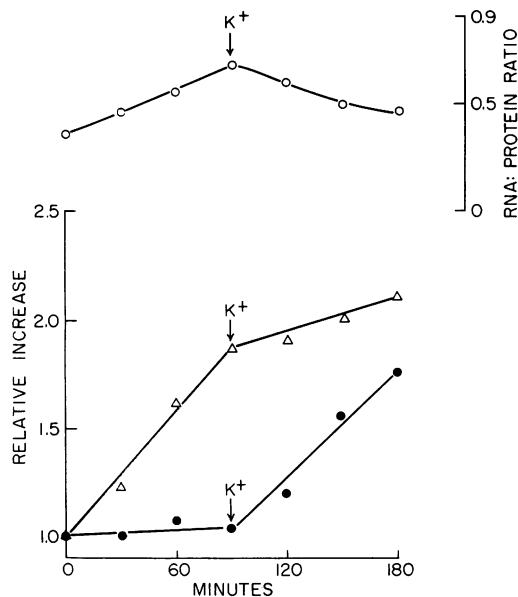
FIG. 7. *RNA and protein synthesis during potassium depletion and during resumption of growth in B. subtilis 168 KL. Exponentially growing cells were washed with and resuspended in medium NaA. Samples were taken at 30-min intervals for chemical determinations of RNA and protein. At 90 min, 4 M KCl was added to give a final concentration of 100 mM K+, and samples were withdrawn at intervals for an additional 90 min. Symbols: protein, ●; RNA, △; RNA to protein ratio, ○.*

density. The rate of protein synthesis in recovering cells took about 30 min to attain the rate observed in normally growing cells (Fig. 8).

Further investigation into the fate of KD-RNA after cell growth had resumed showed that, when protein synthesis was restored to cells containing labeled KD-RNA, a rapid distribution of much of the labeled RNA into stable cell components occurred. The labeled KD-RNA first appeared in the 30S ribosomal subunit and subsequently was seen in the region of the 50S subunit (Fig. 9). A large amount of 4S RNA was also evident. During this time, there was little or no loss of radioactivity from the cells.

Addition of actinomycin D to cells containing KD-RNA (Fig. 10) resulted in the loss of over half of the ^{14}C-uracil-labeled KD-RNA. When K+ was present in the medium, the fraction of radioactivity which was stable was found to be in 50S and 30S ribosomal subunits and in 4S RNA. As can be seen, there was a synthesis of 50S ribosomal subunits under these conditions. It is not possible, however, to say that new 30S subunits were formed. On the other hand, when K+ was absent during treatment with actino-

mycin, no stable RNA was found in 50S and 30S subunits. However, the RNA that was stable under these conditions sedimented slightly slower than 50S and 30S, which are perhaps ribosome precursors (16). The small amount of protein synthesized after actinomycin addition in the presence of K+ was perhaps sufficient to convert any ribosome precursors present into normal ribosomes.

Because of the many reports of the preferential synthesis of ribosomal protein when cells are recovering from a period of RNA synthesis in the absence of protein synthesis (9, 17, 18, 25), we analyzed the proteins formed in *B. subtilis* 168 KL upon recovery from K+ depletion. Actinomycin D (10 µg/ml) was added to the recovery medium to find out whether the messenger RNA (mRNA) for ribosomal protein had accumulated in the depleted cells. The relative amount of ribosomal protein to soluble protein was much greater in the recovering cells (Fig. 11). Other experiments in which actinomycin was not employed gave the same results. The use of actinomycin, however, serves to
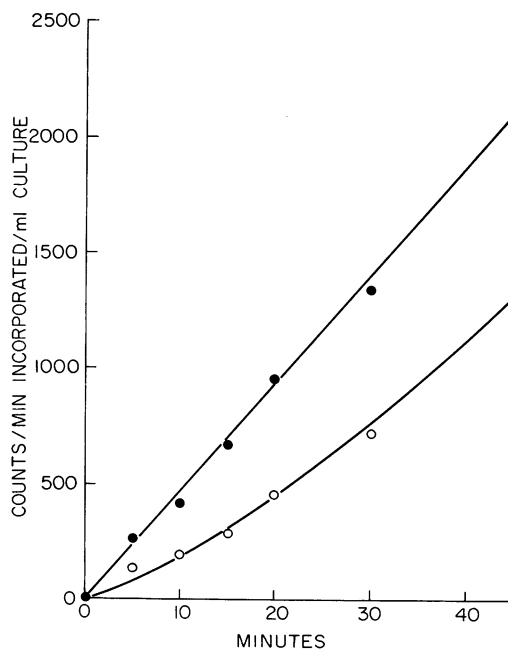

FIG. 8. *^{14}C-leucine incorporation by normally growing cells and by cells recovering from potassium depletion. K+ (100 mM) was added to cells that had been permitted to accumulate KD-RNA for 30 min; the rate of uptake of ^{14}C-leucine into hot trichloroacetic acid-insoluble material was determined and compared to that of normally growing cells. Symbols: normal cells, ●; recovering cells, ○.*
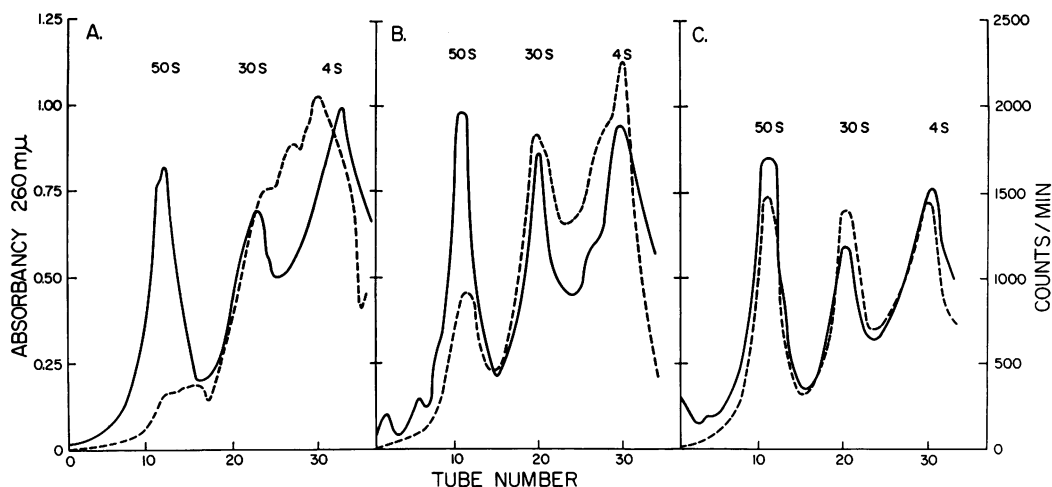
FIG. 9. *Synthesis of ribosomes from potassium-depletion RNA. Cells of strain 168 KL were permitted to accumulate KD-RNA for 1 hr in medium NaA containing* ^{14}C-*uracil. The cells were washed free from radioactive uracil and were resuspended in medium A containing 100 μg/ml each of cold uracil and cytosine. For the preparation of crude extracts, samples were taken after (A) 5 min, (B) 15 min, and (C) 30 min of growth. Extracts were mixed with a 10-fold excess of extract from normally growing cells and were subjected to sucrose density gradient analysis. Absorbance at 260 nm, solid line; counts/min, dashed line.*
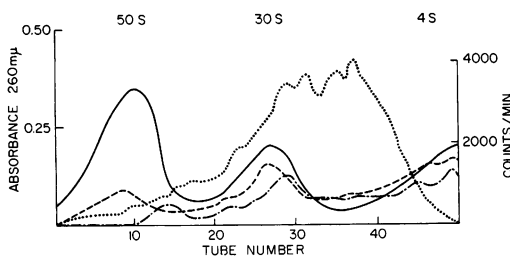

FIG. 10. *Fate of potassium-depletion RNA in the presence of actinomycin D. A culture of cells containing* ^{14}C-*uracil-labeled KD-RNA was divided into two equal parts. One part was centrifuged and frozen immediately. To the second part, we added 10 μg of actinomycin D per ml, along with enough KCl to give 100 mM* K^+. *A third culture was the same as the second except that the KCl was omitted. After 30 min, these two cultures were collected, and crude extracts were prepared from both. Each extract was mixed with a 10-fold excess of unlabeled normal extract and was analyzed by sucrose density gradient centrifugation. Absorbance at 260 nm, solid line; counts/min of* ^{14}C-*uracil in control KD-RNA, dotted line; counts/min of* ^{14}C-*uracil after 30 min in* K^+ *and actinomycin D, dashed line; counts/min of* ^{14}C-*uracil after 30 min in medium lacking* K^+, *but containing actinomycin D (line with alternating dots and dashes).*

emphasize any accumulation of mRNA for ribosomal protein during K^+ depletion.

DISCUSSION

Our major intent in this paper was to document another simple method which may be useful in studying the coupling of the synthesis of macromolecules in bacterial cells.

We described some of the properties of a mutant of *B. subtilis* that is deficient in its ability to accumulate and retain K^+. This mutant possessed many of the properties of a similar mutant of *E. coli* (7–9). Its growth and ability to synthesize protein were markedly dependent on the presence of K^+ in the growth medium, whereas RNA synthesis was less affected. Since RNA polymerase and phosphorylation enzymes must be functioning in these K^+-depleted cells (because RNA synthesis continues in the absence of the cation), our results strongly suggested that K^+ depletion specifically suppresses protein synthesis.

During inhibition of growth of this *B. subtilis* mutant by K^+ depletion, the RNA that was synthesized appeared in particles that sedimented in a sucrose density gradient in the region between 4S and 30S. These particles are similar to those that appear during inhibition of protein synthesis by a variety of other methods (3, 8, 20, 25). When growth was restored by the addition of K^+ to the cells containing these particles, the RNA was stabilized. This stabilization appears to be the result of the addition of new protein to the particles with the direct conversion of the particles into complete ribosomes. However, when RNA synthesis was blocked by actinomycin D during the recovery from K^+ depletion, much of the RNA was degraded, with a small,
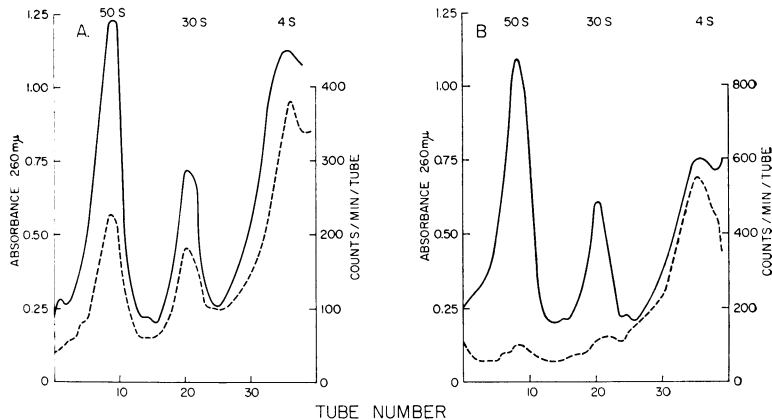
FIG. 11. *Incorporation of* ^{14}C-*amino acids into ribosomal and supernatant protein fractions in the presence of actinomycin D. Cells were depleted of* K^+ *for 45 min; then* K^+ *was added simultaneously with 10 μg of actinomycin D per ml and 0.1 μc of* ^{14}C-*amino acid mixture per ml. After 15 min, Casamino Acids (Difco) was added (1% final concentration), and incubation was continued for another 15 min before preparation of extract (A). A similar pulse-chase experiment was performed in normally growing cells (B). The extracts were prepared and subjected to sucrose density gradient analysis. The percentage of radioactivity in the 4S, 30S, and 50S regions were: for cells recovering from* K^+ *depletion (A), 49, 24, and 27, respectively; for normally growing cells (B), 75, 14, 11, respectively.*

but significant amount appearing in mature ribosomes.

During the early recovery from K+ depletion, two interesting control mechanisms were uncovered. First, although protein synthesis accelerated rapidly, net RNA synthesis was initially depressed. When the RNA to protein ratio reached the level observed during balanced growth in the medium, RNA synthesis also proceeded rapidly. Therefore, we demonstrated a mechanism that is present in growing cells controlling the protein and RNA content of the cells. This is in agreement with previous work with exponentially growing cells (19, 22).

Second, the protein synthesized by cells during recovery from K+ depletion was predominantly ribosomal, even when new RNA synthesis was prevented. This indicated that some messenger RNA coding for ribosomal proteins accumulated during K+ depletion, in agreement with previous studies (9, 17, 25). It is equally possible that the preferential synthesis of ribosomal proteins observed during recovery was due to preferential translation of mRNA for ribosomal protein.

Both puromycin treatment and K+ depletion of *E. coli* result in the loss of polysomes (Ennis and Sells, *in press*). Nakada (17) reported that cells recovering from puromycin treatment synthesized protein at a more rapid rate than normally growing cells. It is difficult to reconcile this finding with the fact that it takes about 20 min for the polysomes of recovering cells to reach their former level (Ennis and Sells, *in press*). Protein synthesis in *B. subtilis* 168 KL recovering

from K+ depletion was, in contrast to the finding of Nakada (17) with cells recovering from puromycin inhibition, less, than that observed in normally growing cells. However, as we have already discussed, there was proportionately more ribosomal protein synthesized during recovery from K+ depletion than in normally growing cells.

The precise requirement for K+ in protein synthesis is unknown. It has been shown that K+ is necessary for the integrity of the polysome structures (Ennis and Sells, *in press*) and for the attachment of the amino-acyl transfer RNA (tRNA) that holds the 50S and 30S ribosomal subunits together to form a 70S ribosome (21, 23). K+ also seems to be important in the formation of certain types of amino-acyl tRNA (27), although the requirement for K+ is not absolute. The availability of a K+-requiring mutant of *B. subtilis* provides another system in which to study the role of K+ in protein synthesis in vivo and the mechanisms involved in the fine control of the synthesis of macromolecules. In another investigation, we discuss the control of bacteriophage functions during inhibition of protein synthesis with this system (Willis and Ennis, *in preparation*).

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