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Potassium Requirement for Synthesis of Macromolecules in *Bacillus subtilis* Infected with Bacteriophage 2C[1]

DAWN B. WILLIS[2] AND HERBERT L. ENNIS

Laboratory of Pharmacology, St. Jude Children's Research Hospital, and Departments of Microbiology and Biochemistry, University of Tennessee Medical Units, Memphis, Tennessee 38101

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A mutant of *Bacillus subtilis* 168 (strain 168 KW), defective in its ability to concentrate K^+ from low levels in the growth medium, was used to study the role of K^+ in the development of phage 2C. Both the final burst size and the duration of the rise period depended on the K^+ concentration in the medium. During normal infection (in the presence of K^+), host deoxyribonucleic acid (DNA) synthesis stopped. The synthesis of host messenger ribonucleic acid (RNA) continued throughout infection, albeit at a steadily decreasing rate. The synthesis of ribosomal RNA and its subsequent incorporation into mature ribosomes also proceeded. In contrast to these findings, host DNA and messenger RNA synthesis were not inhibited in cells infected in the absence of K^+. Only "early" phage messenger RNA was synthesized under these conditions of infection. Phage DNA synthesis was dependent on K^+ irrespective of the requirement for this cation in protein synthesis.

Bacillus subtilis 168 KW is a mutant of *B. subtilis* 168 that is defective in its ability to concentrate K^+ from the growth medium. Cells of strain 168 KW do not synthesize protein, but continue to synthesize ribonucleic acid (RNA) at a nearly normal rate when depleted of this cation (13, 21). Previous reports from this laboratory (2, 3; Ennis and Cohen, *submitted for publication*) demonstrated that K^+ is required for the synthesis of both deoxyribonucleic acid (DNA) and protein in T4-infected *Escherichia coli*, as well as for the transcription of "late" phage messenger RNA (mRNA). The *B. subtilis* mutant was used to examine the role of K^+ in the synthesis of host and viral macromolecules in cells infected with phage 2C.

MATERIALS AND METHODS

Chemicals. *4,5-^{3}H*-L-leucine, 40 c/mmole; *^{14}C-1*-L-leucine, 25 mc/mmole; *^{3}H-6*-uracil, 9 c/mmole; *^{14}C-2*-uracil, 30 mc/mmole; and *^{3}H-6*-thymine, 3.2 c/mmole were purchased from New England Nuclear Corp., Boston, Mass. Macaloid was a product of the Baroid Division of the National Lead Co., Houston, Tex., and was purified according to the method of Stanley (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1963). Chloramphenicol was a gift of Parke, Davis & Co., Detroit, Mich. All other chemicals were commercially available products.

Bacteria and phage. *B. subtilis* 168 KW, a mutant requiring high levels of K^+ for growth, was isolated by ultraviolet irradiation and selection with penicillin (9) from a parent strain donated by Farmer and Rothman (5). This mutant is similar to a K^+-requiring strain of *B. subtilis* isolated by Lubin (13) and described by us in a recent report (21). It differs from Lubin's strain in its ability to support the growth of phage 2C.

Phage 2C, a virulent phage whose DNA contains 5-hydroxymethyl uracil in place of thymine (17), was a gift from Julius Marmur.

Media. The medium used for propagation of phage 2C in infected cells was TY broth (18) modified by the substitution of KCl for NaCl and the addition of 40 μg of thymine per ml. This same broth solidified with agar was used to assay phage plaques by the method of Adams (1). The minimal medium used in phage experiments, medium SP-A, is a glucose-salts medium (4; containing 100 mM K^+) supplemented with 100 μg of L-tryptophan per ml, 40 μg of thymine per ml, 0.5% glucose, 0.1% Casamino Acids (Difco), 0.1 mM $CaCl_2$, and 0.01 mM $MnCl_2$. Medium SP-NaA is the analogous medium containing Na^+ substituted mole for mole for K^+.

[1] This paper was taken in part from a dissertation by Dawn B. Willis submitted to the faculty of the University of Tennessee in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of this work was presented at the Annual Meeting of the American Society for Microbiology in Detroit, Mich., 5-10 May 1968.

[2] Present address: Laboratory of Molecular Biology, Department of Medicine, University of Tennessee Medical Units, Memphis, Tenn. 38103.

Infection of B. subtilis 168 KW. Bacteria were grown to an optical density of 75 Klett units (42 filter; 3.8×10^7 colony-forming units per ml) in medium SP-A, washed twice in medium SP-NaA, and resuspended in the same volume of medium SP-NaA. The initial infection was carried out in the absence of K^+ for 5 min, and the infected cells were then filtered and resuspended in the appropriate medium. Adsorption of phage proceeded equally well in either the presence or absence of K^+. At a multiplicity of infection of 5 to 10 plaque-forming units, over 98% of the cells were infected at the end of 5 min. Further phage adsorption was prevented either by the addition of a 1:100 dilution of phage antisera (K_{vel} = 152 ml/min) or by filtration of infected cells through a membrane filter (0.65 μm pore size; Millipore Corp., Bedford, Mass.). The recovery of infectious centers was generally 65 to 85% of the total cell count.

Measurement of incorporation of radioactivity into RNA, DNA, and protein were described by Cohen and Ennis (2).

Preparation of cell extracts and sucrose density gradients were described in a previous report (21).

Purification of RNA and DNA. RNA was purified by a modification of the hot phenol-SDS procedure (3). DNA was extracted from bacteria according to the method of Marmur (14) and from phage suspensions according to the technique of Young and Sinsheimer (22).

DNA-RNA hybridization assays. DNA was denatured by boiling for 10 min and cooling rapidly, then immobilized on nitrocellulose membrane filters. The procedure of Friesen (6) was used to determine the percentage of ^{3}H-RNA hydbridizable with bacterial or phage DNA.

RESULTS

Growth of phage 2C in the presence or absence of K^+. Single-step growth curves at various concentrations of K^+ were performed to assess the effect of K^+ concentration on both the kinetics of phage growth and the final burst size. As shown in Fig. 1, maximal phage production occurred at 100 mM K^+. At this concentration of K^+, the latent period was about 50 min, and the burst size after 90 min was between 90 and 100 phage per infected cell. Below this level of K^+, both the rate of phage release and the final burst size were markedly dependent on the K^+ concentration. A small loss of infective centers was observed after prolonged incubation of infected cells in media lacking K^+. This was probably due to the increased fragility of infected cells in the absence of K^+, a phenomenon also noted in T4 infection of *E. coli* B207 (2).

The dependence of average burst size of phage 2C on the concentration of K^+ in the medium was analyzed in detail. This relationship was shown to be linear (Fig. 2).

Synthesis of macromolecules in infected cells. The incorporation of radioactive precursors into RNA, DNA, and protein was used to study the role of K^+ in the synthesis of macromolecules in infected cells. As was the case in uninfected cells, infected cells did not incorporate ^{14}C-leucine into protein in the absence of K^+. On the other hand, the incorporation of ^{14}C-uracil into RNA was greater in the absence of K^+ than in its presence (Fig. 3). That the increased incorporation was not due to increased stability of mRNA in the absence of K^+ was shown by adding 10 μg of actinomycin

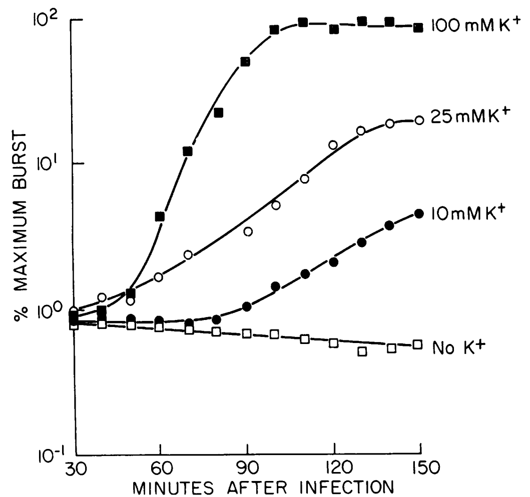

FIG. 1. *Single-step growth curves of phage 2C grown in B. subtilis 168 KW at various K^+ concentrations. Cells were infected for 5 min in the absence of K^+. Phage antiserum was then added for an additional 5 min, and the cultures were diluted into flasks containing various concentrations of K^+. The results are plotted as per cent of the maximum burst size observed in the optimal K^+ concentration (100 mM), which was about 90–100 per cell.*

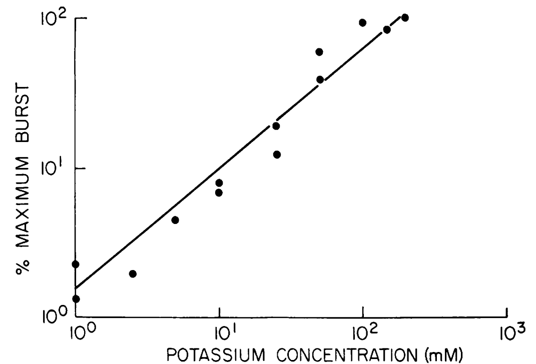

FIG. 2. *Dependence of average burst size of phage 2C on the concentration of K^+ in the medium. The cells were infected as described in the legend to Fig. 1, and incubated with shaking for an additional 3 hr in media containing the indicated amounts of K^+.*

D per ml to infected cultures that had been labeled with radioactive uracil for 3 min. The rate of degradation of this pulse-labeled RNA was the same (50% of initial counts remaining after 20 min in actinomycin D) regardless of the presence or absence of K^+. This would indicate that probably the overall rate of RNA synthesis is actually increased in the absence of K^+.

We next examined the synthesis of host DNA during infection. Since the phage DNA contains no thymine, the incorporation of ^{3}H-thymine into DNA was used as an index of host DNA synthesis in infected cells. It was necessary to supplement the medium with 20 μg of deoxyuridine per ml to obtain sufficient uptake of exogenous thymine (15). Within 5 to 15 min after infection, no thymine was incorporated into DNA (Fig. 4). The slight rise in labeled DNA noted after 20 min was probably due to conversion of some thymine to hydroxymethyl uracil or to hydrogen exchange. This finding indicated that host cell DNA synthesis was inhibited by phage infection. There was no observable difference between the thymine incorporated in uninfected or infected cells in the absence of K^+. Since K^+ is necessary for protein synthesis, host DNA synthesis may continue after phage infection in the absence of K^+, because a new protein is needed to cause inhibition.

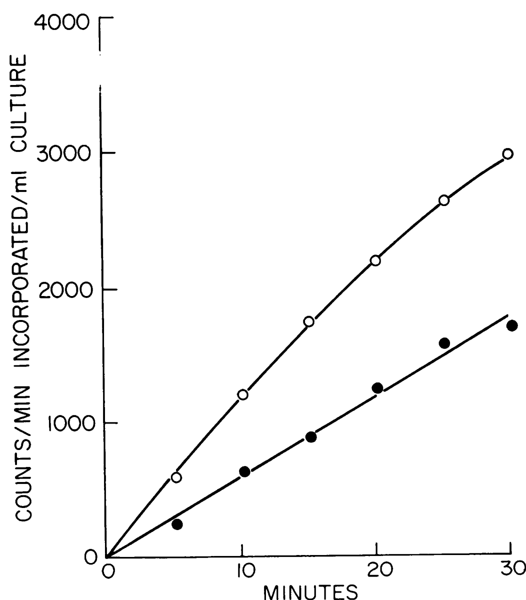

FIG. 3. *Incorporation of ^{14}C-uracil into RNA by B. subtilis infected in the presence and absence of K^+. Cells were infected with phage 2C, filtered, and suspended in the appropriate media containing ^{14}C-uracil. Samples were withdrawn at intervals for determination of trichloroacetic acid-precipitable radioactivity. No added K^+ (○); 100 mM K^+ (●).*

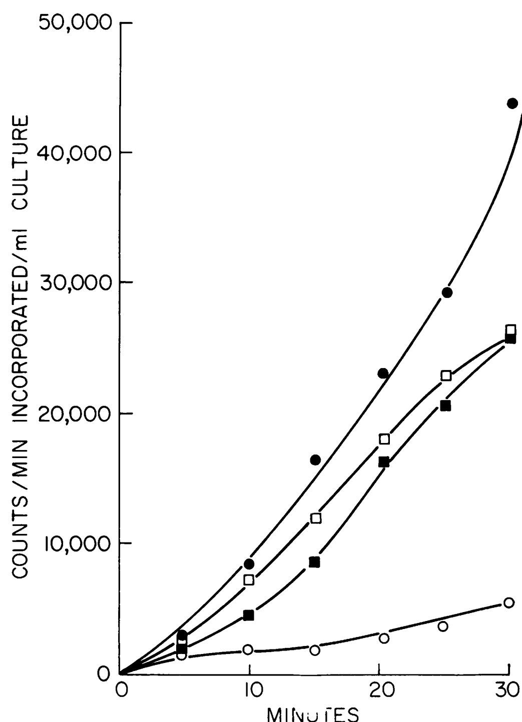

FIG. 4. *Incorporation of ^{3}H-thymine into DNA by infected and uninfected B. subtilis 168 KW in the presence and absence of K^+. Cells were infected, filtered, and suspended in the appropriate media containing ^{3}H-thymine. Samples were removed at intervals for analysis of alkali-stable, cold trichloroacetic acid-precipitable material. Infected in K^+ (○); infected in no K^+ (□); uninfected in K^+ (●); uninfected in no K^+ (■).*

Other reports from this laboratory (2) have shown that K^+ is necessary for the synthesis of phage T4 DNA irrespective of the requirement for this cation in protein synthesis. Therefore, we performed an additional experiment to determine whether this was also the case during *B. subtilis* phage infection. Cells were infected in K^+-containing medium for 10 min to allow the formation of the enzymes necessary for phage DNA synthesis. The infected bacteria were then filtered and suspended in (i) medium SP-A (containing K^+), (ii) medium SP-NaA (no K^+), and (iii) medium SP-A plus 200 μg of chloramphenicol per ml. Phage 2C DNA synthesis was completely blocked in the absence of K^+, even though the enzymes necessary for DNA synthesis were present (Fig. 5). In the presence of K^+ and chloramphenicol, phage DNA synthesis continued at a normal rate for 20 min, after which it was stopped. We therefore conclude that K^+ is needed for DNA synthesis per se, independent of the requirement for K^+ in protein synthesis.

mRNA synthesized during infection. One measure of the ability of an infecting phage to take over the host metabolic machinery is the rate at which host functions are terminated. Therefore, we next examined the relative amounts of host and phage mRNA formed at various times after infection with 2C. Cells were infected with phage at a multiplicity of five phage per cell in the presence or absence of K^+. After 5 min, a 1:100 dilution of phage antiserum was added, and the infected culture was divided into three equal portions. ^{3}H-uracil was added for 2 min to one culture at 5 min, to the second culture at 15 min, and to the third at 30 min after infection. The RNA was isolated from the cells in each culture and 5 μg of purified RNA from each sample was hybridized with an excess (150 μg) of DNA extracted from uninfected cells or from purified phage suspensions. RNA was also isolated from uninfected cells and similarly assayed (Table 1). The percentage of hybridizable RNA present at nonsaturating level of RNA is taken as an index of the amount of messenger-like RNA in the preparation (6, 19). The data indicate that after 15 min of infection in the presence of K^+, host cell transcription had been reduced by 90%, whereas the amount of RNA hybridizable with phage DNA increased with the duration of infection. In the absence of K^+, host cell transcription was not inhibited, and the per cent hybridizable phage RNA never attained the level observed during normal infection.

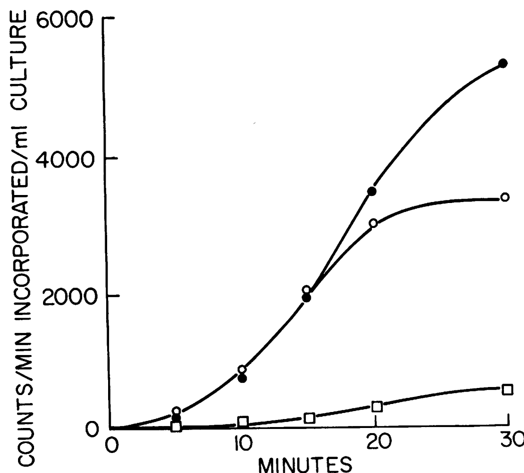

FIG. 5. *Requirement for K^+ for phage 2C DNA synthesis. Cells were infected for 10 min in medium SP-A, filtered, washed with medium Sp-NaA, and suspended in appropriate media containing ^{14}C-uracil. Samples were withdrawn at intervals and analyzed for alkali-stable cold trichloroacetic acid-precipitable material. Symbols: 100 mM K^+ (●); 100 mM K^+ plus 200 µg of chloramphenicol. (○); no K^+ (□).*

TABLE 1. *Hybridization of RNA synthesized at various times after infection*[a]

Time after infection ^{3}H-uracil added	Ribonuclease-resistant hybrid formed during infection			
	RNA made in the presence of K^+ hybridized with		RNA made in the absence of K^+ hybridized with	
	Cell DNA	Phage DNA	Cell DNA	Phage DNA
min	%	%	%	%
Uninfected cells	22.8	0.82	24.3	0.76
5	10.4	10.0	19.2	10.2
15	2.9	25.6	21.6	9.6
30	1.3	41.0	20.1	13.5

[a] RNA was isolated from cells labeled with ^{3}H-uracil (0.5 μc/ml; 0.06 μg/ml) for 2 min at the indicated intervals during infection in the presence or absence of K^+. A 5-μg amount of each RNA was hybridized with 150 μg of either *B. subtilis* or phage 2C DNA. In this reaction, DNA is in excess. The data are averages of three separate experiments.

Cells infected in the absence of K^+ did not synthesize phage DNA, and by analogy with coliphage T4, might be expected not to synthesize "late" phage mRNA (Ennis and Cohen, *in press*). We performed a series of hybrid competition studies to examine this point. The cells were labeled with ^{3}H-uracil for 2 min during infection in the presence or absence of K^+ at 32 or 37 C. The RNA labeled from 3 to 5 min postinfection (called 5-min RNA) was considered "early" RNA; that labeled from 30 to 32 min after infection (called 32-min RNA) was termed "late" RNA. Filters bearing 20 μg of phage DNA were prepared, and reciprocal hybridization competition studies were carried out by using 5 μg of ^{3}H-RNA to 100 μg of unlabeled RNA (Table 2). This experiment showed that, although there was a class of RNA synthesized late after infection that was not present at early times, the species of RNA synthesized at 3 to 5 min after infection was still being made at 30 to 32 min. Furthermore, there did not appear to be detectable late mRNA synthesized during infection in the absence of K^+, because unlabeled RNA extracted after 30 min of infection in the absence of K^+ was comparable in degree of competition to 5-min unlabeled RNA taken from cells infected in the presence or absence of K^+.

Synthesis of ribosomes in infected cells. Levinthal et al. (12) reported that ribosomal RNA (rRNA) and ribosomal proteins continued to be synthesized in *B. subtilis* infected with the related phage SP01. To determine whether this is the

TABLE 2. *Hybridization competition between RNA synthesized in infected cells in the presence and absence of potassium*[a]

Source of competing unlabeled RNA	Source of ^{3}H-RNA	Control radioactivity in ribonuclease-resistant hybrid
		%
5-min, K^+	5-min, K^+	20.0
32-min, K^+	5-min, K^+	28.5
5-min, K^+	32-min, K^+	56.9
32-min, K^+	32-min, K^+	21.0
5-min, no K^+	5-min, K^+	23.7
32-min, no K^+	5-min, K^+	17.5
5-min, no K^+	32-min, K^+	58.5
32-min, no K^+	32-min, K^+	53.0
Uninfected, K^+	5-min, K^+	98.7
Uninfected, no K^+	5-min, K^+	98.0
Uninfected, K^+	32-min, K^+	98.1
Uninfected, no K^+	32-min, K^+	99.2

[a] RNA was isolated from cells pulse-labeled with ^{3}H-uracil (0.5 μc/ml; 0.06 μg/ml; middle column) and from unlabeled cells (left column). Conditions of infection and growth are indicated in these two columns. The data are averages of three separate experiments. The reaction contained 20 μg of phage DNA and 5 μg of ^{3}H-RNA. Nonradioactive RNA (100 μg/ml) was added as indicated at the same time as the ^{3}H-RNA to the complete reaction mixture. The average per cent hybrid in the control reactions (no unlabeled competing RNA added) was 12.5% for 3- to 5-min RNA and 32% for 30- to 32-min RNA. This was set as 100% for purposes of this comparison.

same in 2C infection, we performed sucrose density gradient analyses on crude extracts taken from infected and uninfected cells (Fig. 6). After 10 min of infection, rRNA was being synthesized and incorporated into ribosomal subunits at about one-third the rate observed in uninfected cells. The amount of ribosomal protein labeled was almost as great in infected cells as in uninfected ones. Similar gradients (not shown) of extracts of cells infected in the absence of K^+ showed that most of the RNA synthesized under these conditions was rRNA in the form of "pre-ribosomal" particles identical to the ones seen in uninfected cells (21).

Purified RNA prepared from extracts of cells infected in the presence or absence of K^+ showed that the majority of the RNA made under either of these circumstances was rRNA.

DISCUSSION

Our data show that no mature phage were produced in media lacking K^+. Both the average burst size and the duration of the rise period depended on the concentration of K^+ in the medium.

During 2C infection of *B. subtilis* in the presence of K^+, considerable host mRNA was synthesized for about 10 min. The synthesis of host mRNA declined during subsequent minutes, so that late in infection (30 min), the majority of the mRNA manufactured hybridized with phage DNA. The synthesis of host rRNA and ribosomal proteins continued at a measurable rate, even at a time when host DNA synthesis had stopped and host mRNA synthesis was severely inhibited. Levinthal et al. (12) also observed that ribosomes were made during SP01 infection of *B. subtilis*. This situation is in decided contrast to that observed in T4-infected *E. coli*, in which the vast majority of the RNA made is phage messenger (16).

In contrast to these observations, during infection in the absence of K^+, when no protein synthesis occurs, host DNA and mRNA synthesis are not inhibited, but phage mRNA is also synthesized. Although host rRNA is being made, no mature ribosomes are formed, since no protein is synthesized during infection in the absence of K^+.

In general, the proteins made after phage infection have been classified temporally as either "early" or "late" (20). It is, therefore, necessary to assume that a mechanism exists which controls the switch from early to late protein synthesis. One quesion that can be asked is whether the control is at the level of transcription of RNA to DNA, or whether it is at the level of translation of RNA to protein. Characterization of RNA synthesized after T4 infection of *E. coli* (10, 11) has shown that there is a class of mRNA found late in infection that is not present at early times. Nevertheless, the "early" RNA continued to be synthesized throughout infection and was found associated with polysomes (7), even when it appeared to be no longer utilized. The present examination of transcription during the development of bacteriophage 2C also revealed the existence of "early" and "late" classes of RNA, with "early" RNA being synthesized late in infection. However, in contrast to T4 infection, at least one "early" enzyme, in 2C infection, deoxycytidylate deaminase, is still being synthesized after 30 min (17). Therefore, the presence of "early" phage messenger would be expected.

Other investigators, working with the subtilis phage SP01, showed that "early" mRNA transcription ceased late in infection (8). In this respect, SP01 differs from 2C, a closely related phage, as well as from the more distantly related coliphages.

The inhibition of both phage protein and DNA

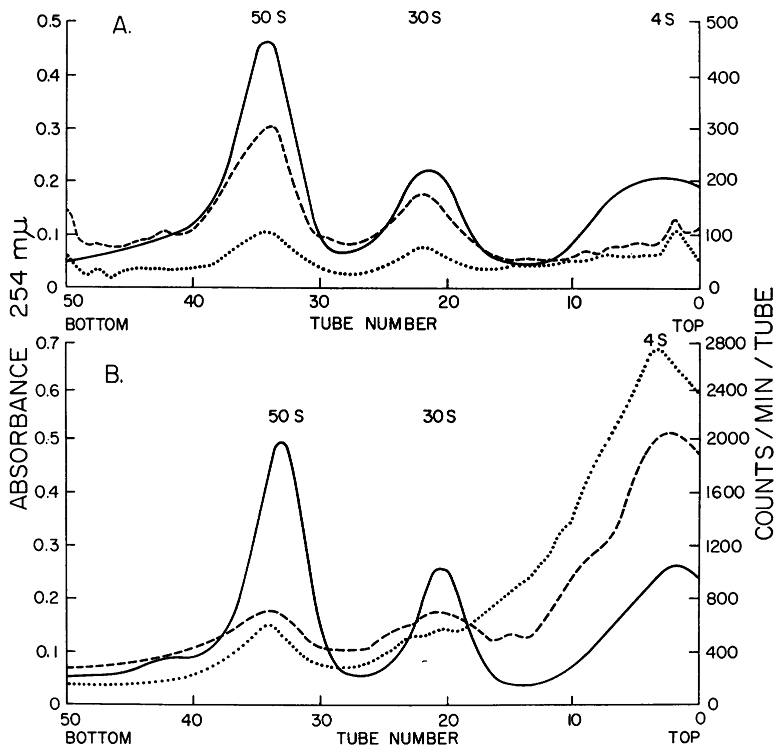

FIG. 6. *Synthesis of RNA and protein in infected and uninfected cells. Cells were infected in the presence of K^+ for 10 min at a multiplicity of 7 to 10 phage per cell. Cultures were then labeled with either ^{14}C-uracil (A) or ^{3}H-leucine (B) for 15 min, followed by a 15-min chase with 1 mg of the appropriate nonradioactive compound per ml. Extracts were prepared from the cultures of infected cells and from uninfected cultures that received identical treatment. The extracts were dialyzed against buffer containing 5×10^{-4} M Mg^{2+} to dissociate ribosomes into subunits. Equivalent amounts of each extract were mixed with a 10-fold excess of extract prepared from normally growing uninfected cells, and layered on a 5 to 20% sucrose density gradient. Samples were collected and analyzed with the aid of an ISCO density gradient fractionator. Absorbance at 254 nm (——); infected cells (····); uninfected cells (- - - -).*

synthesis by K^+-depletion of *E. coli* infected with T4 resulted in the inhibition of "late" phage messenger formation (Ennis and Cohen, *in press*). K^+ was also required for production of "late" classes of phage RNA in 2C-infected *B. subtilis*. Our data indicate that the RNA extracted from cells infected for 30 min in the absence of K^+ did not contain those classes of RNA present after 30 min of infection in the presence of K^+. Pène and Marmur (17) showed that phage DNA replication and RNA synthesis were necessary for formation of the "late" enzyme lysozyme. The results reported here support the hypothesis that the synthesis of "late" enzymes is controlled at the level of transcription. In 2C infection, there is no observed shut-off of "early" enzyme synthesis (17) and, therefore, there is no necessity for postulating a translational control to account for the synthesis of nonfunctional mRNA. However, as others have indicated (8, 12), the possibility exists that there is a spectrum of phage mRNA types each synthesized only during a defined interval of the infectious cycle. This has, so far, been demonstrated only for phage SP01 (8), but further refinements of technique may demonstrate its existence in other systems also.

The system we have described has the advantage that it can be used to control the rate of both phage DNA and protein synthesis by the manipulation of the external K^+ concentration. We believe that K^+-depletion is a useful tool for studying the regulation of phage development. There are many ways to inhibit the synthesis of macromolecules, but it is difficult to obtain with antibiotics the precision of control over the rates of protein and DNA synthesis that is possible by the simple adjustment of the external K^+ concentrations in K^+-requiring bacterial mutants. At present, the most satisfactory methods for inhibiting DNA synthesis, e.g., ultraviolet irradia-

tion, amber mutations, and treatment with mitomycin, are irreversible. On the contrary, the blocking of phage DNA synthesis by K^+-depletion is readily reversible. In addition, low rates of both protein and DNA synthesis can be easily maintained over long intervals.

With this system, it should be possible to obtain detailed information concerning the ordered sequence of phage maturation, and also to determine whether slowing down the metabolic processes by lowering the available K^+ reduces the synthesis of every class of RNA and of protein to the same degree.

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